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GENOME-WIDE VIEW ON THE PHYSIOLOGY OF VITAMIN D

Topic Editor
Carsten Carlberg

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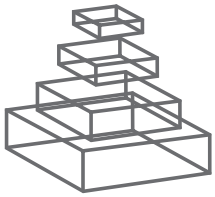

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GENOME-WIDE VIEW ON THE PHYSIOLOGY OF VITAMIN D

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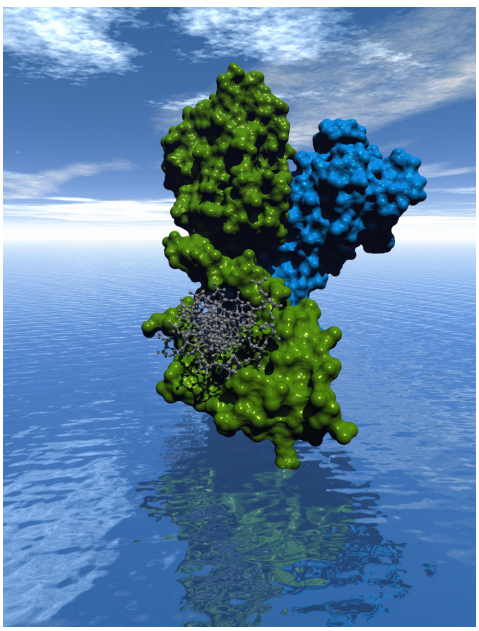

Structural model of the full length RXR-VDR heterodimer. A surface representation of the RXR (blue) - VDR (green) complex on a DR3-type DNA binding site.
Cover by Ferdinand Molnar, modified from: Molnár F (2014) Structural considerations of vitamin D signaling. Front. Physiol. 5:191. doi: 10.3389/fphys.2014.00191

The main physiological actions of the biologically most active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), are calcium and phosphorus uptake and transport and thereby controlling bone formation. Other emergent areas of $1\alpha,25(OH)_2D_3$ action are in the control of immune functions, cellular growth and differentiation. This fits both with the widespread expression of the VDR and the above described consequences of vitamin D deficiency. Transcriptome-wide analysis indicated that per cell type between 200 and 600 genes are primary targets of vitamin D. Since most of these genes respond to vitamin D in a cell-specific fashion, the total number of vitamin D targets in the human genome is far higher than 1,000. This is supported by the genome-wide view on VDR binding sites in human lymphocytes, monocytes, colon and hepatic cells. All genomic actions of $1\alpha,25(OH)_2D_3$ are mediated by the transcription factor vitamin D receptor (VDR) that has been the subject of intense study since the 1980's. Thus, vitamin D signaling primarily implies the molecular actions of the VDR. In this research topic, we present in 15 chapters different perspectives on the action of vitamin D and its receptor, such as the impact of the genome-wide distribution of VDR binding loci, ii) the transcriptome- and proteome-wide effects of vitamin D, iii) the role of vitamin D in health, iv) tissue-specific functions of vitamin D and v) the involvement of vitamin D in different diseases, such as infections, autoimmune diseases, diabetes and different types of cancer.

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EDITORIAL
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The physiology of vitamin D—far more than calcium and bone

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Keywords: vitamin D, vitamin D receptor, genomics, physiology, immune system

Vitamin D is a molecule displaying an important physiological impact. Average human diet is neither rich in vitamin D_2 (of plant origin) nor in vitamin D_3 (of animal origin). Therefore, humans have to rely on the endogenous production of vitamin D_3 in UVB exposed skin. This process was an import evolutionary driver for skin lightening after our ancestors decided some 100,000 years ago to move North out of Africa toward Asia and Europe (Juzeniene et al., 2009). Did this happen only to extract calcium efficiently from our diet and to keep our bones strong?

Vitamin D_3 exerts most, if not all, of its physiological effects via its metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which acts as a nuclear hormone, since it is the only high affinity ligand for the transcription factor vitamin D receptor (VDR). VDR is expressed in the majority of human tissues and cell types, i.e., at far more places than needed for calcium homeostasis and proper bone formation. The aim of this Research Topic is to explore the physiology of vitamin D from the perspective of the genome-wide distribution of VDR-binding sites in cells as different as B lymphocytes, monocytes, macrophages, colon cancer cells, and hepatic stellate cells (Tuoresmäki et al., 2014). The choice of these cell types as experimental models is already a clear indication that the physiology of vitamin D involves also actions on the adaptive and innate immune system and on cancer cells.

This Research Topic starts with an overview on the recently explored genome-wide locations of VDR and their link to the accessibility of chromatin and its 3-dimensional organization (Carlberg, 2014). This genomic view is extended by a structural view on the interaction of the VDR with DNA, natural and synthetic ligands and co-regulatory proteins (Molnar, 2014). The VDR-mediated genome-wide actions of vitamin D result in a change of the transcriptome in all VDR expressing tissues and cell types. Taking all human tissues together this does not only affect thousands of protein-coding mRNAs but also a comparable number of non-coding RNAs (Campbell, 2014). The signal transduction of the lipophilic molecule $1,25(OH)_2D_3$ is straightforward, since it reaches the VDR directly in the nucleus. Nevertheless, vitamin D signaling functionally interacts with a number of other signal transduction pathways, many of which start with receptors at the cell membrane (Larriba et al., 2014). The introductory section of this Research Topic is complemented by a view on the epigenome-wide effects of vitamin D, such as DNA methylation and histone modifications (Fetahu et al., 2014).

The general physiological function of vitamin D is to keep us healthy by promoting strong bones, properly functioning muscles and a potent immune system. When weather and season allows, we can keep our vitamin D levels up through endogenous production during carefully dosed exposure to sunlight (Reichrath et al., 2014). However, at winter above latitudes of 40° North or below 40° South insufficient amounts of UVB radiation pass the atmosphere. This implies that at least during winter we have to consider vitamin D as an essential micronutrient that we should supplement via fortified food compounds, such as milk and margarine, or appropriately dosed pills (Bendik et al., 2014). Both sun exposure in summer and supplementation during winter should keep our vitamin D status on an optimal level, which most likely is individual for each of us (Carlberg et al., 2013). Under these conditions cells of our innate and our adaptive immune system, such as monocytes and macrophage as well as B and T lymphocytes, can take maximal benefit from the gene regulatory potential of vitamin D (Chun et al., 2014).

In addition to the cells of the immune system VDR is expressed in most other tissues that origin from mesenchymal cells, such as bone (Van De Peppel and Van Leeuwen, 2014), myocytes (Polly and Tan, 2014), and adipose tissue (Mutt et al., 2014). This demonstrates that the well-known role of vitamin D in bone extrapolates to skeletal muscle and fat.

Most common diseases, such as type 2 diabetes, cancer and autoimmune diseases, are associated with chronic inflammation. Inflammation is mediated by tissue-associated macrophages, dendritic cells, and T lymphocytes, in which vitamin D has important gene regulatory functions (Wöbke et al., 2014). This may also be a key mechanism for the beneficial effects of vitamin D in cancers of breast (Narvaez et al., 2014) and prostate (Wang and Tenniswood, 2014). Furthermore, the pleiotropy of vitamin D suggests additional mechanisms for its anti-cancer effects, such as the modulation of intracellular metabolism. However, in case when supra-physiological concentrations of $1,25(OH)_2D_3$ would be required, in order to obtain a therapeutic effect, the application of synthetic vitamin D analogs is suggested (Leyssens et al., 2014).

Taken together, the 15 chapters of this Research Topic present the wide physiological impact of vitamin D and link it to its molecular basis, which is the genome-wide action of the transcription factor VDR in most human tissues and cell types.

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REFERENCES

Bendik, I., Friedel, A., Roos, F. F., Weber, P., and Eggersdorfer, M. (2014). Vitamin D: a critical and essential micronutrient for human health. *Front. Physiol.* 5:248. doi: 10.3389/fphys.2014.00248

Campbell, M. J. (2014). Vitamin D and the RNA transcriptome: more than mRNA regulation. *Front. Physiol.* 5:181. doi: 10.3389/fphys.2014.00181

Carlberg, C. (2014). Genome-wide (over)view on the actions of vitamin D. *Front. Physiol.* 5:167. doi: 10.3389/fphys.2014.00167

Carlberg, C., Seuter, S., De Mello, V. D., Schwab, U., Voutilainen, S., Pulkki, K., et al. (2013). Primary vitamin D target genes allow a categorization of possible benefits of vitamin D_3 supplementation. *PLoS ONE* 8:e71042. doi: 10.1371/journal.pone.0071042

Chun, R. F., Liu, P. T., Modlin, R. L., Adams, J. S., and Hewison, M. (2014). Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front. Physiol.* 5:151. doi: 10.3389/fphys.2014.00151

Fetahu, I. S., Hobaus, J., and Kallay, E. (2014). Vitamin D and the epigenome. *Front. Physiol.* 5:164. doi: 10.3389/fphys.2014.00164

Juzeniene, A., Setlow, R., Porojnicu, A., Steindal, A. H., and Moan, J. (2009). Development of different human skin colors: a review highlighting photobiological and photobiophysical aspects. *J. Photochem. Photobiol. B* 96, 93–100. doi: 10.1016/j.jphotobiol.2009.04.009

Larriba, M. J., Gonzalez-Sancho, J. M., Bonilla, F., and Munoz, A. (2014). Interaction of vitamin D with membrane-based signaling pathways. *Front. Physiol.* 5:60. doi: 10.3389/fphys.2014.00060

Leyssens, C., Verlinden, L., and Verstuyf, A. (2014). The future of vitamin D analogs. *Front. Physiol.* 5:122. doi: 10.3389/fphys.2014.00122

Molnar, F. (2014). Structural considerations of vitamin D signaling. *Front. Physiol.* 5:191. doi: 10.3389/fphys.2014.00191

Mutt, S. J., Hyppönen, E., Saarnio, J., Järvelin, M. R., and Herzig, K. H. (2014). Vitamin D and adipose tissue-more than storage. *Front. Physiol.* 5:228. doi: 10.3389/fphys.2014.00228

Narvaez, C. J., Matthews, D., Laporta, E., Simmons, K. M., Beaudin, S., and Welsh, J. (2014). The impact of vitamin D in breast cancer: genomics, pathways, metabolism. *Front. Physiol.* 5:213. doi: 10.3389/fphys.2014.00213

Polly, P., and Tan, T. C. (2014). The role of vitamin D in skeletal and cardiac muscle function. *Front. Physiol.* 5:145. doi: 10.3389/fphys.2014.00145

Reichrath, J., Reichrath, S., Heyne, K., Vogt, T., and Roemer, K. (2014). Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling. *Front. Physiol.* 5:166. doi: 10.3389/fphys.2014.00166

Tuoresmäki, P., Väisänen, S., Neme, A., Heikkinen, S., and Carlberg, C. (2014). Patterns of genome-wide VDR locations. *PLoS ONE* 9:e96105. doi: 10.1371/journal.pone.0096105

Van De Peppel, J., and Van Leeuwen, J. P. (2014). Vitamin D and gene networks in human osteoblasts. *Front. Physiol.* 5:137. doi: 10.3389/fphys.2014.00137

Wang, W.-L. W., and Tenniswood, M. (2014). Vitamin D, intermediary metabolism and prostate cancer tumor progression. *Front. Physiol.* 5:183. doi: 10.3389/fphys.2014.00183

Wöbke, T. K., Sorg, B. L., and Steinhilber, D. (2014). Vitamin D in inflammatory diseases. *Front. Physiol.* 5:244. doi: 10.3389/fphys.2014.00244

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Genome-wide (over)view on the actions of vitamin D

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For a global understanding of the physiological impact of the nuclear hormone 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) the analysis of the genome-wide locations of its high affinity receptor, the transcription factor vitamin D receptor (VDR), is essential. Chromatin immunoprecipitation sequencing (ChIP-seq) in GM10855 and GM10861 lymphoblastoid cells, undifferentiated and lipopolysaccharide-differentiated THP-1 monocytes, LS180 colorectal cancer cells and LX2 hepatic stellate cells revealed between 1000 and 13,000 VDR-specific genomic binding sites. The harmonized analysis of these ChIP-seq datasets indicates that the mechanistic basis for the action of the VDR is independent of the cell type. Formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) data highlight accessible chromatin regions, which are under control of $1,25(OH)_2D_3$. In addition, public data, such as from the ENCODE project, allow to relate the genome-wide actions of VDR and $1,25(OH)_2D_3$ to those of other proteins within the nucleus. For example, locations of the insulator protein CTCF suggest a segregation of the human genome into chromatin domains, of which more than 1000 contain at least one VDR binding site. The integration of all these genome-wide data facilitates the identification of the most important VDR binding sites and associated primary $1,25(OH)_2D_3$ target genes. Expression changes of these key genes can serve as biomarkers for the actions of vitamin D_3 and its metabolites in different tissues and cell types of human individuals. Analysis of primary tissues obtained from vitamin D_3 intervention studies using such markers indicated a large inter-individual variation for the efficiency of vitamin D_3 supplementation. In conclusion, a genome-wide (over)view on the genomic locations of VDR provides a broader basis for addressing vitamin D's role in health and disease.

Keywords: vitamin D, vitamin D receptor, chromatin, gene regulation, epigenomics, genomics

INTRODUCTION

During evolution the secosteroid vitamin D_3 became a pleiotropic signaling molecule (Jones et al., 1998). Initially, the molecule was used by early unicellular organisms to protect their DNA against UV-B irradiation (Holick, 2011). Far later, when the first fish with bones evolved, the endocrinology of vitamin D_3 was established, and still is very conserved in all higher organisms, including humans (Bouillon and Suda, 2014). In this system, the energy of UV-B is used to convert 7-dehydrocholesterol into pre-vitamin D_3, i.e., UV-B became essential for the synthesis of vitamin D_3 (Holick, 2004) (more details in the article by Reichrath et al. in this issue). The central importance of this step is emphasized by the step-wise depigmentation of human skin, when modern humans started to move out of Africa some 100,000 years ago (Hochberg and Templeton, 2010). Two hydroxylation steps are necessary for the conversion of vitamin D_3 via 25-hydroxyvitamin D_3 ($25(OH)D_3$) into the biologically active vitamin D_3 metabolite, $1,25(OH)_2D_3$ (Norman, 2008). The latter molecule participates in a large number of physiological processes, such as bone formation, immune function and cellular growth and differentiation (Deluca, 2004) (more details in the articles by van Leeuwen et al., Hewison et al. and Munoz et al. in this issue).

The transcription factor VDR is the only high-affinity target for $1,25(OH)_2D_3$ within the cell nucleus (Haussler et al., 1997). VDR is one of approximately 1900 transcription factors, which are encoded by the human genome (Vaquerizas et al., 2009). In addition, VDR is a member of the superfamily of nuclear receptors, most of which are specifically activated by lipophilic molecules in the size of cholesterol (Carlberg and Molnár, 2012). Its lipophilic allows $1,25(OH)_2D_3$ to pass through all biological membranes, i.e., gene regulation by vitamin D

Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; ALOX5, arachidonate 5-lipoxygenase; CBS, cystathionine β-synthase; CCNC, cyclin C; CDKN1A, cyclin-dependent kinase inhibitor 1A; CHD7, chromodomain helicase DNA binding protein 7; ChIA-PET, chromatin interaction analysis by paired-end tag sequencing; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP coupled with massive parallel sequencing; CTCF, CCCTC-binding factor; CYP, cytochrome P450; DNase-seq, DNase I hypersensitivity sites sequencing; DR3, direct repeat spaced by 3 nucleotides; FAIRE-seq, formaldehyde-assisted isolation of regulatory elements sequencing; IGV, Integrative Genomics Viewer; LPS, lipopolysaccharide; LRP5, low density lipoprotein receptor-related protein 5; MYC, v-myc avian myelocytomatosis viral oncogene homolog; PBMC, peripheral blood mononuclear cell; RXR, retinoid X receptor; THBD, thrombomodulin; TNFSF11, tumor necrosis factor (ligand) superfamily, member 11; TRPV6, transient receptor potential cation channel, subfamily V, member 6; TSS, transcription start site; VDR, vitamin D receptor; ZMIZ1, zinc finger, MIZ-type containing 1.

does not involve additional signal transduction steps, as they are known for hydrophilic signaling molecules, such as peptide hormones, growth factors and cytokines. Moreover, VDR is rather ubiquitously expressed, i.e., most human tissues and cell types are responsive to $1,25(OH)_2D_3$ (Wang et al., 2012).

VDR shares the main structural characteristics of nuclear receptors, which is a highly conserved DNA-binding domain and a structurally conserved ligand-binding domain (Mangelsdorf et al., 1995). VDR's DNA-binding domain specifically contacts the hexameric consensus sequence RGKTSA (R = A or G, K = G or T, S = C or G) within the major groove of genomic DNA (Shaffer and Gewirth, 2002). However, like most other transcription factors, VDR uses a partner DNA-binding protein, in order to bind efficiently to its target sites. More than 20 years ago, this heterodimeric partner turned out to be the nuclear receptor retinoid X receptor (RXR) (Sone et al., 1991; Carlberg et al., 1993). Steric constraints of the dimerizing DNA-binding domains of VDR and RXR determine the optimal binding site of the VDR-RXR complex as a direct repeat of two hexameric nuclear receptor binding motifs spaced by three nucleotides (DR3) (Umesono et al., 1991; Shaffer and Gewirth, 2004). Within VDR's ligand-binding domain, a network of some 40 mostly non-polar amino acids forms a ligand-binding pocket, in which $1,25(OH)_2D_3$ and its synthetic analogs are specifically fixed with high affinity (Molnár et al., 2006). This ligand binding process induces a conformational change to the surface of VDR's ligand-binding domain, which results in a significant change of VDR's protein-protein interaction profile: it transforms from a repressor to an activator (Moras and Gronemeyer, 1998; Carlberg and Campbell, 2013) (more details on VDR structure in the article by Molnar in this issue).

Taken together, vitamin D signaling primarily comprises the molecular actions of the VDR, i.e., the physiological effects of $1,25(OH)_2D_3$ are largely identical to those of its receptor. This reduces vitamin D signaling to one central question: which are the most important genomic targets of VDR in a given tissue and which genes are controlled via these sites? Thus, this review focuses on the description of the genome-wide binding of VDR and its mechanistic implications. This analysis will be in the context of genome-wide information on chromatin accessibility and the presence of other nuclear proteins, such as provided by the ENCODE consortium.

GENOME-WIDE VDR BINDING

The method chromatin immunoprecipitation (ChIP) was developed, in order to monitor the binding of transcription factors to their genomic targets (Orlando, 2000). The core of the method is (i) mild chemical cross-linking of living cells or tissues, e.g., with 1% formaldehyde, in order to fix nuclear proteins to genomic DNA, (ii) sonication of chromatin into small (200–400 bp) fragments, and (iii) immunoprecipitation with an antibody specific for the chosen nuclear protein (Maston et al., 2012). In this way, chromatin regions, which, at the moment of cross-linking, had been in contact with the protein of choice, are specifically enriched. A specific ChIP signal, in reference to a control (often unspecific IgGs), is a strong indication that the protein of choice had been in contact with the selected genomic region at the moment of cross-linking.

At earlier times, the isolated chromatin template was analyzed by site-specific quantitative PCR (ChIP-qPCR). This approach had been used to study, for example, the extended promoter regions of the primary VDR target genes *CYP24A1* (Väisänen et al., 2005), *CYP27B1* (Turunen et al., 2007), *CCNC* (Sinkkonen et al., 2005), and *CDKN1A* (Saramäki et al., 2006, 2009). Alternatively, the abundance of immunoprecipitated chromatin fragments had been detected by tiled microarrays (so-called "chips,") which covered a selection of promoter and enhancer regions or any other subset of the genome (ChIP-chip). The group of Pike et al. had extensively used ChIP-chip, in order to locate VDR binding sites within the regulatory regions of the mouse genes *Vdr* (Zella et al., 2006), *Trpv6* (Meyer et al., 2006), *Lrp5* (Fretz et al., 2007), *Tnfsf11* (also known as *Rankl)* (Kim et al., 2006), *Cyp24a1* (Meyer et al., 2010), and *Cbs* (Kriebitzsch et al., 2011). The latest development of the ChIP method is the unbiased analysis of the precipitated chromatin by massively parallel DNA sequencing (ChIP-seq), i.e., the detection of the binding sites of the transcription factor of choice in the complete genome. To date, ChIP-qPCR is primarily used for the confirmation of ChIP-seq results, while ChIP-chip got outdated shortly after its introduction. This leaves, at present, ChIP-seq as the method of choice for analyzing VDR's genomic binding loci.

At present, the readouts of massive parallel sequencing are small sequence tags (35–50 nucleotides), but in the future there will be in majority longer reads used, which will lead to improved significance of the results. These sequence tags are aligned to a reference genome (for human samples this is, at present, hg19) and specifically represent the enriched chromatin fragments. Then "peak calling" software is used to identify genomic regions, in which significantly more sequence tags are detected than in control reactions. Therefore, tags that accumulate as "peaks" at specific genomic loci mark the presence of the investigated nuclear protein (Park, 2009; Furey, 2012). At present, ChIP is still performed with millions of cells; in case of a prominent binding site, most of these cells contribute to the ChIP signal, i.e., it can be assumed that in the majority of cells the locus is occupied by VDR. However, when only in some cells a site is bound by VDR, the respective peak is far less prominent, i.e., most likely of less impact for the regulation of $1,25(OH)_2D_3$ target genes.

To date, VDR ChIP-seq data are available from (i) the immortalized lymphoblastoid cell lines GM10855 and GM10861 (Ramagopalan et al., 2010), (ii) undifferentiated THP-1 monocyte-like cells (Heikkinen et al., 2011), (iii) lipopolysaccharide (LPS)-polarized THP-1 macrophage-like cells (Tuoresmäki et al., 2014), (iv) LS180 colorectal cancer cells (Meyer et al., 2012), and (v) LX2 hepatic stellate cells (Ding et al., 2013). The original publications reported between 1600 and 6200 VDR binding sites (in ligand-stimulated samples) within the human genome. However, these numbers are not directly comparable, since different peak calling software, alternative threshold settings and even an older version of the reference genome (hg18) were used. A harmonized re-analysis of all six VDR ChIP-seq datasets with identical peak calling settings (MACS, version 2) resulted for $1,25(OH)_2D_3$-stimulated and unstimulated cells, respectively,

in following number of binding sites: 6172 and 3144 (GM10855), 12,353 and 4072 (GM10861), 774 and 609 (undifferentiated THP-1), 953 and 529 (LPS-differentiated THP-1), 3777 and 165 (LS180) and 1532 and 1474 (LX2) (Tuoresmäki et al., 2014).

In total, the six VDR ChIP-seq datasets indicated 21,776 non-overlapping VDR binding sites when allowing a distance of up to 250 bp between the peak summits (Tuoresmäki et al., 2014). However, the vast majority of these VDR loci (67%) are unique for one of the analyzed cellular models. In contrast, under the above mentioned conditions only 54 sites are common within all six datasets. In general, this indicates that VDR displays a very individual pattern of cell-specific genomic locations, which overlaps between multiple tissues only at key sites. The VDR binding site of the $1,25(OH)_2D_3$ target gene *ZMIZ1*, which is located 15.3 kb downstream of the transcription start site (TSS), represents an example of such a locus (**Figure 1**). In general, the rates of overlaps between the cell types follow roughly their developmental and functional relatedness, i.e., the two lymphoblastoid cell lines, GM10855 and GM10861, or LPS-differentiated and undifferentiated THP-1 cells show more overlapping VDR binding sites than all other comparisons between the VDR ChIP-seq datasets. Moreover, the VDR binding profiles of ligand-stimulated cells matched better than those of unstimulated cells (Tuoresmäki et al., 2014).

Genome-wide studies on VDR binding have changed the view on vitamin D signaling. The few dozens rather well characterized VDR binding sites in less than 10 kb distance to the TSS of $1,25(OH)_2D_3$ target genes (Haussler et al., 2013), which were known before, were complemented by thousands of additional VDR loci spread over the whole genome. However, the very most of the loci, which were highlighted by ChIP-seq, have not yet been validated by ChIP-qPCR or similar methods (and many will never be confirmed). Some previously known VDR binding sites, such as those controlling the genes *MYC* (Toropainen et al., 2010), *VDR* (Zella et al., 2010), *CCNC* (Sinkkonen et al., 2005), and *ALOX5* (Seuter et al., 2007), could be confirmed by the VDR ChIP-seq datasets. However, for many known $1,25(OH)_2D_3$ target genes the ChIP-seq data suggest additional or alternative VDR binding sites, many of these being far more distant to the gene's TSS region than previously foreseen. In the past, many of these VDR binding sites had been overlooked due to a focus to only a few kb upstream of the TSS of $1,25(OH)_2D_3$ target genes. However, in accordance with the results of the ENCODE project (ENCODE-Project-Consortium et al., 2012), VDR binding sites are found with equal probability upstream and downstream of the TSS region of $1,25(OH)_2D_3$ target genes. In addition, VDR loci in distance of even more than 1 Mb from the gene's TSS are accepted as regulatory sites (more details below).

In summary, there seem to be 1000–10,000 genomic VDR binding sites per cell type. This is far more than the number of primary $1,25(OH)_2D_3$ target genes, which is in the order of 100-500 per tissue. This even holds true for undifferentiated THP-1 cells, where 774 VDR loci in ligand-stimulated cells are facing 408 statistically significantly up-regulated early $1,25(OH)_2D_3$ responding genes (Heikkinen et al., 2011). The indicates that some genes are controlled by more than one VDR binding site, i.e., they may have a higher potential to be regulated by $1,25(OH)_2D_3$ than target genes with only one active VDR locus (more details on the transcriptome-wide response to $1,25(OH)_2D_3$ in the article by Campbell et al. in this issue).

MECHANISTIC INSIGHT FROM VDR ChIP-SEQ STUDIES

The close to 22,000 non-overlapping VDR peaks, which are indicated by the public ChIP-seq datasets (Tuoresmäki et al., 2014), show rather different characteristics. Despite the rather different total number of reported VDR peaks per cellular model, each of the six ChIP-seq datasets contains an in part overlapping subset of less than 200 sites, where a stimulation with $1,25(OH)_2D_3$ resulted in a significant increase of VDR binding compared to unstimulated samples. These VDR loci are far more prominent than most of the other sites, for which ligand treatment was either repressive, had no effect or was only minor stimulatory.

Another important parameter for the characterization of a VDR binding site is the presence or absence of a high confidence DR3-type binding site below the summit ($\pm$100 bp) of the respective ChIP-seq peak. This can be investigated with the help of binding site screening algorithms, such as provided by HOMER (Heinz et al., 2010). Depending on the threshold settings the software detects binding sites that deviate more or less from the consensus sequence. For example, for a moderate setting of a HOMER score of 7, from the total of 21,776 non-overlapping VDR sites in all six ChIP-seq datasets only 3801 (17.5%) contain a DR3-type sequence. Interestingly, the percentage of DR3-type motifs differs significantly between the datasets and ranges from 38.2% (483 of 1264 sites) in LPS-polarized THP-1 cells via 36.4% (373 of 1023) in undifferentiated THP-1 cells, 28.6% (1062 of 3706) in LS180 cells, 27.8% (611 of 2194) in LX2 cells, 13.0% (909 of 6975) in GM10855 cells to 9.0% (1118 of 12,438) in GM10861 cells. This indicates that the total number of identified VDR binding sites in each cell line inversely correlates with the percentage of peak summits with DR3-type sites. However, when the analysis is restricted to the top 200 VDR sites (based on fold enrichment scoring), for all six ChIP-seq datasets a DR3-like sequence rate of more than 60% is observed, i.e., DR3 motifs are found preferentially at highly ligand responsive VDR loci. In this way, the different VDR ChIP-seq datasets show a very similar relationship between VDR occupancy and DR3 percentage. This suggests that the mechanistic basis for the action of the VDR is independent of the cell type and the total number of identified binding sites.

Transcription factor binding site screening software, such as HOMER, suggests that DR3-type binding sequences are the most abundant sites below the summits of VDR ChIP-seq peaks. However, a significant number of the genomic VDR loci (depending on the dataset 60-90% of all, see above) do not associate with a DR3-type site. This indicates that at these loci VDR uses a different mode of interaction with genomic DNA. This could be either the use of a different heterodimeric binding partner or an indirect binding "backpack" of a DNA-binding transcription factor (Carlberg and Campbell, 2013). In both scenarios the specific DNA binding site would be different to a DR3-type sequence. Interestingly, for the VDR ChIP-seq datasets originating from hematopoietic cells, HOMER indicated binding sites for the transcription factors PU.1 (also called SPI1), ESRRB (also called NR3B2) and GABPA as significantly enriched (Tuoresmäki

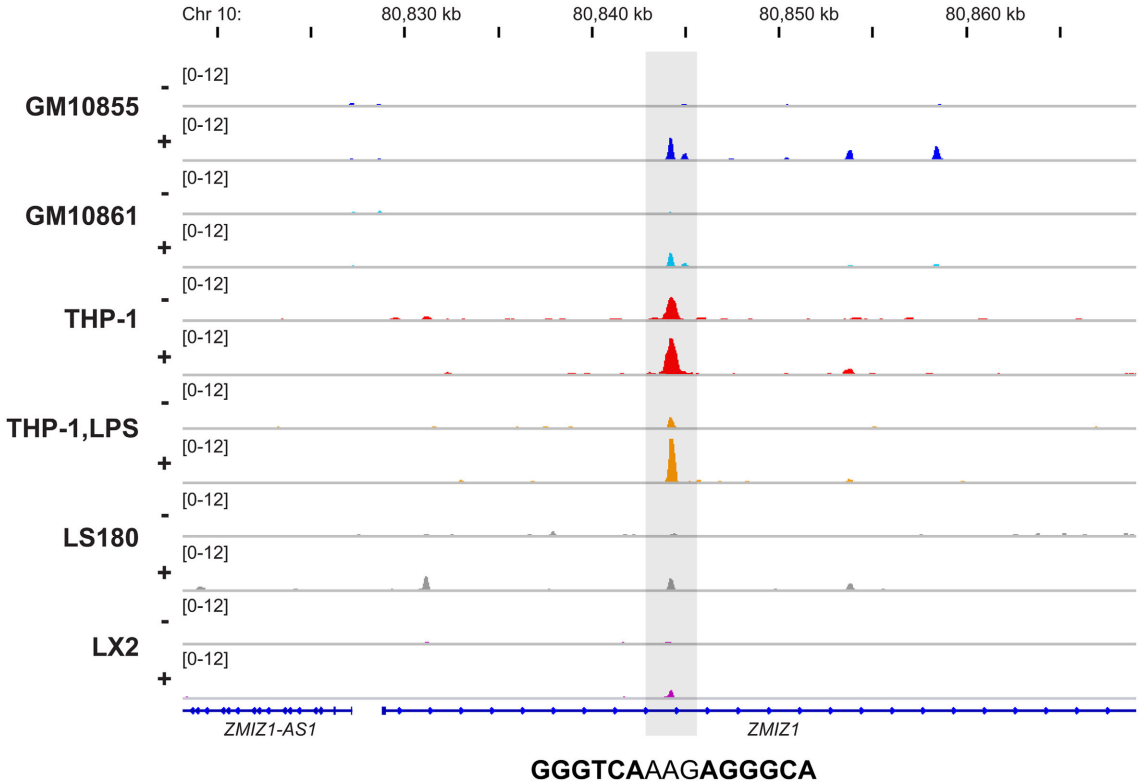

FIGURE 1 | Conserved genomic VDR binding in six cellular models. The Integrative Genomics Viewer (IGV) browser (Robinson et al., 2011) was used to visualize the VDR binding site 15.3 kb downstream of the *ZMIZ1* TSS. The peak tracks display data from VDR ChIP-seq datasets from two B cell-like cells (dark and light blue), monocyte-like cells (red), macrophage-like cells (orange), colon cells (gray) and liver cells (violet). The cells were either unstimulated (–) or treated with VDR ligand (+). The gene structures are shown in blue and the sequence of the DR3-type element below the summit of the VDR peak is indicated.

et al., 2014). PU.1 is well-known as a pioneer factor (Zaret and Carroll, 2011), i.e., as a transcription factor with (i) a high number of genomic binding sites, (ii) a greater binding promiscuity and (iii) higher diversity of interactions. Pioneer factors are the first that bind regulatory genomic regions, such as promoters and enhancers, and interact with chromatin modifying enzymes, in order make the chromatin more accessible for regular transcription factors, such as VDR. At present, a direct protein-protein interaction of VDR with PU.1, ESRRB or GABPA has not been demonstrated, but for the Ets family, to which PU.1 belongs, there were indications for an interaction (Tolon et al., 2000). However, for the pure function as a pioneer factor a direct protein-protein interaction with the "settler factor" is not needed. Moreover, there is older evidence from single gene studies that DNA binding of VDR is modulated by the transcription factors AP1 (Schüle et al., 1990) and RUNX2 (Sierra et al., 2003). In contrast, a genome-wide study on the interaction of VDR with the transcription factor TCF7L2 did not provide any evidence that the latter acts as a pioneer factor for VDR (Meyer et al., 2012).

Below VDR peak summits no dominant non-DR3 binding sequence could be identified. Moreover, the six VDR ChIP-seq datasets differ in the ranking and identity of the non-DR3 sites found below the peaks (Tuoresmäki et al., 2014). This suggests that in total there must be a larger number of VDR partnering proteins. Most likely, these proteins have a cell-specific expression pattern and may explain in part the cell-specific actions of VDR and its natural ligand $1,25(OH)_2D_3$. Moreover, ChIP-seq datasets have indicated that, in contrast to steroid receptors, VDR binds a number of its genomic targets already in the absence of ligand. These ligand-independent genomic VDR loci have a clearly lower rate of DR3-type sequences than ligand-dependent sites (Heikkinen et al., 2011). In contrast, they associate preferentially with proteins related to gene repression, such as demonstrated for the example of the *CYP27B1* gene (Turunen et al., 2007). This implies that the functional profile of VDR is larger than that of its ligand (Polly et al., 2000) as previously shown for other members of the nuclear receptor superfamily, such as thyroid hormone receptor or liver X receptor (Perissi et al., 2010).

Taken together, all VDR ChIP-seq studies confirm the preferential binding of VDR to DR3-type sequences. However, only one in six of some 22,000 presently known VDR loci within the human genome carry a DR3 site. Thus, there have to be additional mechanisms for the association of VDR with its genomic loci, which may include partnering with presently undefined partner proteins or the tethering to other DNA-binding transcription factors, such as pioneer factors. These should explain some of the cell-specific actions as well as repressive functions of $1,25(OH)_2D_3$ and the VDR.

RESPONSES OF CHROMATIN TO $1,25(OH)_2D_3$

Histone proteins forming the nucleosome core are DNA-binding proteins but do not show any sequence specificity. Therefore, the complex of nucleosomes and genomic DNA, which is referred to as chromatin, has an intrinsic repressive potential: it prevents access of transcription factors to their genomic targets (Razin, 1998). This provides essential stability to the epigenetic landscape for long-lasting regulatory decisions, such as gene expression in terminally differentiated cells (Mohn and Schubeler, 2009). In contrast, some regions of the epigenome show highly dynamic changes in response to extra- and intracellular signals, such as the activation of VDR by $1,25(OH)_2D_3$ binding (Talbert and Henikoff, 2006). These changes involve the methylation of genomic DNA and/or reversible post-translational modifications of histone proteins, such as acetylation or deacetylation at exposed lysine residues (Narlikar et al., 2002). Dynamic chromatin modifications change the access to regulatory genomic regions, such as promoter and enhancers, for the binding of transcription factors, i.e., they determine whether at these regions chromatin is open or closed. This can be monitored genome-wide by using the method DNase I hypersensitive sites sequencing (DNase-seq), which highlights genomic regions being most sensitive to cleavage by the enzyme DNase I (Crawford et al., 2006). A very similar technique is Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-seq), which identifies genome-wide accessible DNA regions (Giresi et al., 2007) (more details on the relation of the epigenome and $1,25(OH)_2D_3$ in the article by Kallay et al. in this issue).

At present, the only publically available dataset describing genome-wide effects of $1,25(OH)_2D_3$ on the epigenome, is a detailed FAIRE-seq time course in THP-1 cells (Seuter et al., 2013). These data demonstrate that some 87% of the more than 1000 VDR binding sites in this cellular model co-localize with open chromatin. Interestingly, at 165 of these VDR loci a strong $1,25(OH)_2D_3$-dependent increase of chromatin accessibility is found. Importantly, at 66% of these chromatin regions a DR3-type sequence is found, i.e., they overlap with loci, at which VDR binding is enhanced most by $1,25(OH)_2D_3$ stimulation (Seuter et al., 2013). Moreover, the binding of VDR to its genomic loci is a dynamic process, which takes at least some 2 h to saturate the sites. One example is a site located 225 kb downstream of the TSS of the chromodomain helicase DNA binding protein 7 (*CHD7*) gene (**Figure 2**). It demonstrates that at the same locus, where a strong ligand-dependent increase of VDR binding is observed, the rate of open chromatin more than doubled already 40 min after incubation of THP-1 cells with $1,25(OH)_2D_3$. At some 200 additional VDR binding loci the chromatin shows detectable but less prominent response to $1,25(OH)_2D_3$ treatment, while at the remaining 500 sites the VDR ligand did not affect chromatin accessibility. Accordingly, only at less than 20% of the latter sites DR3-type sequences are found. At many of these sites, VDR binds already in the absence of ligand and may have a different mode of DNA recognition and action (see above).

In summary, at approximately a third of its genome-wide binding loci VDR dynamically controls the epigenetic state of chromatin. At these sites, VDR binding and chromatin opening are tightly interconnected and provide indications for primary $1,25(OH)_2D_3$ target genes. This allows a better understanding of the $1,25(OH)_2D_3$ signaling cascade.

THE USE OF ENCODE DATA FOR UNDERSTANDING $1,25(OH)_2D_3$ SIGNALING

In addition to the above described $1,25(OH)_2D_3$-triggered chromatin open, in the future there will be much more data available on the interaction of VDR and $1,25(OH)_2D_3$ with the epigenome. This will include FAIRE-seq and DNase-seq studies in further $1,25(OH)_2D_3$-responsive tissues as well as investigations on changes of histone modifications and/or co-factor binding in $1,25(OH)_2D_3$-responsive genomic regions. However, already at present existing genome-wide data on the annotation of the genomes of human, mouse and other species can be used. The best example is the large range of data collected by the ENCODE consortium (ENCODE-Project-Consortium et al., 2012). The core of the ENCODE datasets are publically available ChIP-seq results on approximately 100 transcription factors and 20 histone modifications from more than 100 human cellular systems. From the latter, the human monocytic leukemia cell line K562 is represented with highest number of datasets, while the majority of the other cells has not been studied with the same intensity. At present, ENCODE data describe primarily the basal status of cells, i.e., only in a very few cases a stimulation with hormones, growth factor, cytokines or similar molecules had been performed. Neither data on $1,25(OH)_2D_3$ stimulations nor VDR ChIP-seq data are comprised in the ENCODE dataset. Nevertheless, the examples shown below will illustrate, how already on this stage ENCODE data are useful for a more detailed understanding of $1,25(OH)_2D_3$ signaling.

All six VDR ChIP-seq datasets agree with observations of the ENCODE project that (i) transcription factors bind equally likely both up- and downstream of their target gene TSSs and (ii) the likelihood of detecting functional transcription factor binding sites for a given gene decreases by distance from its TSS region (ENCODE-Project-Consortium et al., 2012). This means that, in relation to the TSS of primary $1,25(OH)_2D_3$ target genes, the distribution of the VDR binding sites has a Gaussian shape. In turn, this suggest that on the same chromosome there would be no threshold distance for the interaction between a VDR binding locus and the TSS of a primary $1,25(OH)_2D_3$ target gene. However, there are limitations provided by higher-order structures of chromatin.

Chromatin forms loops (Kadauke and Blobel, 2009), which contribute to many nuclear functions, such as the control of gene expression (Misteli, 2007). Chromatin loops segregate each chromosome into domains, which are separated by an insulator region (Van Bortle and Corces, 2013). Most insulator regions are associated with the highly conserved transcription factor CCCTC-binding factor (CTCF) (Schmidt et al., 2012). Therefore, ChIP-seq data for CTCF binding from multiple human cell lines, such as provided by ENCODE (ENCODE-Project-Consortium et al., 2012), allow a first estimation of the chromatin domain borders (**Figure 3**). However, only 15–20% of all genomic CTCF binding sites are involved in insulator function. The method chromatin interaction analysis by paired-end tag sequencing (ChIA-PET)

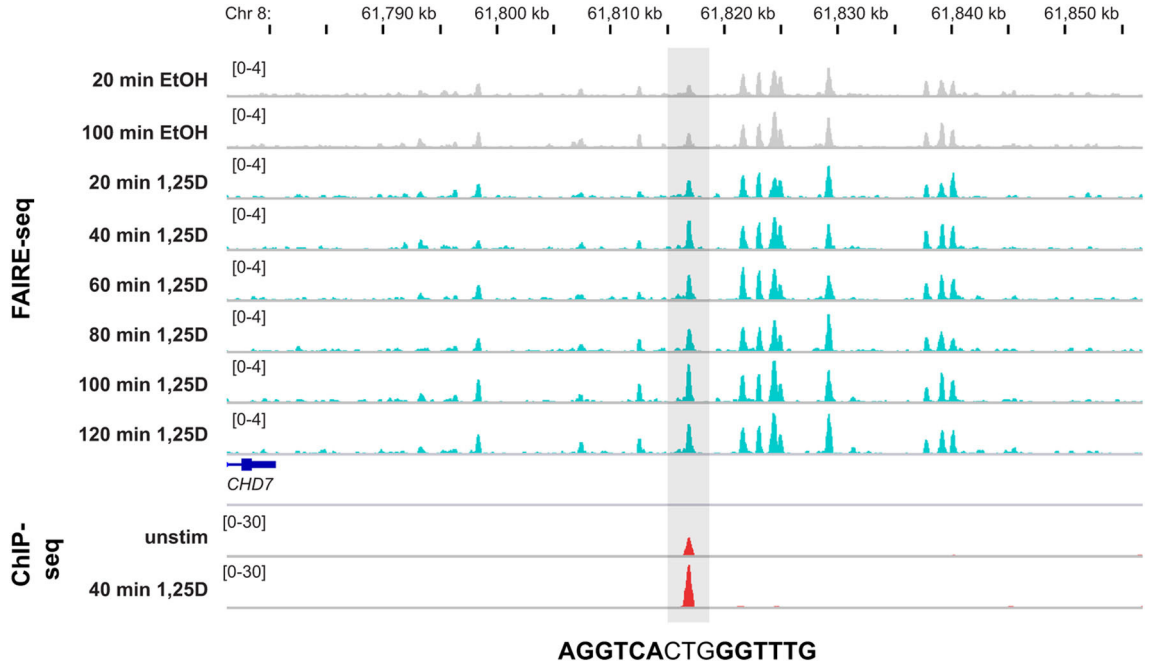

FIGURE 2 | Genomic view of 1,25(OH)$_2$D$_3$-dependent chromatin opening. The IGV browser visualizes the loci of a VDR locus 225 kb downstream of the *CHD7* gene (±40 kb of the peak summit). The peak tracks display data from THP-1 cells: a time course of FAIRE-seq data [gray for EtOH-treated controls and turquoise for 1,25(OH)$_2$D$_3$ (1,25D) treatments for the indicated time periods] and a VDR ChIP-seq data [red, from unstimulated cells and after 40 min 1,25(OH)$_2$D$_3$ treatment]. The gene structures are shown in blue and the sequence of the DR3-type element below the summit of the VDR peak is indicated.

(Fullwood et al., 2009) allows an assessment of the 3-dimensional structure of chromatin. When applied for CTCF in K562 cells it mapped more than 120,000 intra-chromosomal, CTCF-mediated chromatin interactions (ENCODE-Project-Consortium et al., 2012). The high conservation of CTCF binding sites allows a reliable extrapolation of the CTCF ChIA-PET data from K562 cells to THP-1 cells, for which VDR ChIP-seq data is available. The combination of both datasets suggests that in THP-1 cells there are some 1600 chromatin domains, which contain at least one VDR binding site (Seuter et al., 2014). When the TSS region of a gene is within one of these chromatin regions, it may be a primary 1,25(OH)$_2$D$_3$ target. In case of the *CD14* gene, CTCF ChIA-PET data from K562 cells defined a chromatin domain spanning from 1.5 kb upstream to 57 kb downstream of the gene's TSS (**Figure 3**). This domain spans over the whole *CD14* gene and comprises two VDR binding sites 24 and 26 kb downstream of the gene's TSS. This provides a straightforward gene regulatory scenario explaining (i) the primary response of *CD14* to 1,25(OH)$_2$D$_3$ and (ii) why the neighboring genes of *CD14* do not respond to VDR ligand treatment.

The chromatin domain of the *CD14* gene is with a size of less than 60 kb rather small (**Figure 3**). In contrast, one of the largest VDR-containing chromatin domains spans over 2.3 Mb of chromosome 8 and combines the *MYC* gene with four VDR binding sites, of which the most prominent is 1215 kb of the gene's TSS (Ryynänen et al., 2013). This suggests that under the condition of sufficiently large chromatin domains, gene regulation by VDR can be over a distance of more than 1 Mb.

At present, there is no VDR ChIA-PET data available but will come in the future. However, via the ENCODE experiment matrix (http://encodeproject.org/ENCODE/dataMatrix/encodeDataMatrixHuman.html) there is access to ChIA-PET data for RNA polymerase II in HeLa human cervix carcinoma and MCF-7 human breast carcinoma and for estrogen receptor α in MCF-7 cells. The latter may be of special interests for the breast cancer field.

Taken together, public ENCODE data are important tools, which can be used in combination with genome-wide data on VDR for an extrapolation on the 3-dimensional organization of gene regulation by 1,25(OH)$_2$D$_3$.

1,25(OH)$_2$D$_3$ TARGET GENES AS BIOMARKERS FOR THE VITAMIN D STATUS OF HUMAN INDIVIDUALS

In contrast to a number of other nuclear receptor ligands, such as cortisol or estrogen, the endocrinology of 1,25(OH)$_2$D$_3$ does not imply any fast changes (Deluca, 2004; Norman, 2008). Under normal circumstances, either the production in UV-B exposed skin or the intake of from diet or supplements should provide sufficient amounts of vitamin D$_3$, in order to achieve optimal serum 25(OH)D$_3$ concentrations. The latter vitamin D metabolite is the widely accepted indicator of the vitamin D$_3$ status of the human body (Hollis, 2005). The serum 25(OH)D$_3$ concentrations change only in the order of weeks and months, such as the result of seasonal variations in sun exposure (Virtanen et al., 2011). This indicates that stimulation experiments with 1,25(OH)$_2$D$_3$ over a few hours, as performed in *in vitro* experiments, do not represent

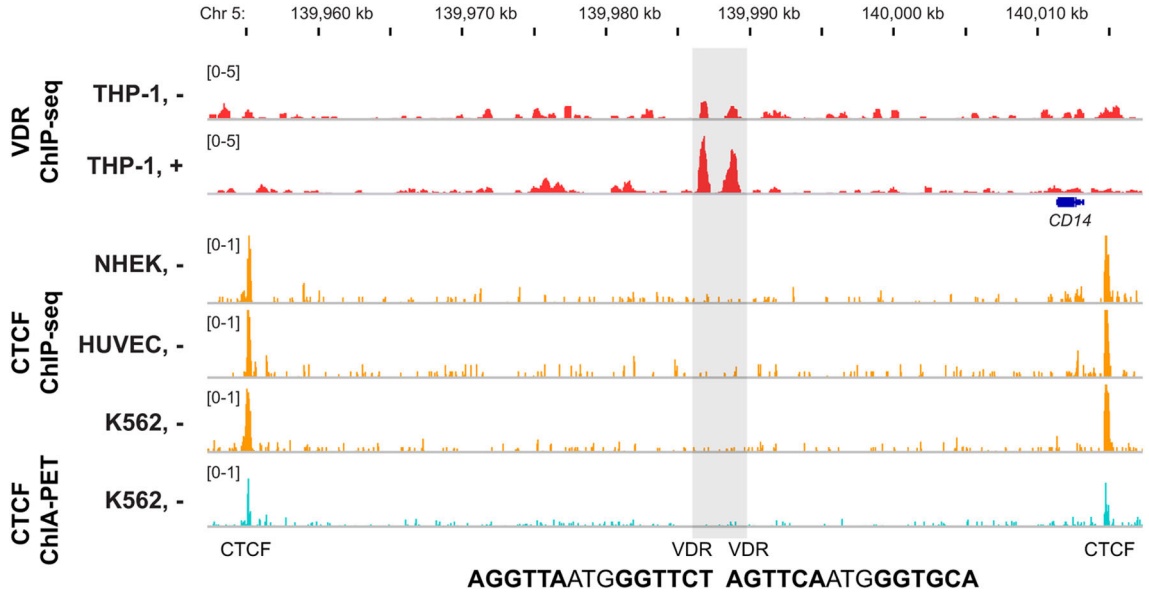

FIGURE 3 | Chromatin domain containing VDR binding sites. The IGV browser was used to display the chromatin domain around the *CD14* gene. VDR ChIP-seq data from THP-1 cells [unstimulated (–) and treated for 40 min with 1,25(OH)$_2$D$_3$ (+), red] are shown in comparison with CTCF ChIP-seq data from the ENCODE cell lines NHEK, HUVEC and K562 (orange) and CTCF ChIA-PET data from K562 cells in the track view (light blue). The gene structures are shown in blue and the sequence of the DR3-type elements below the summits of the VDR peaks are indicated.

the physiological reality. In contrast, the effects of more long-lasting changes of serum 25(OH)D$_3$ concentrations should be considered. On a genome-wide level, this was investigated first with primary T cells isolated from nine human individuals with variant serum 25(OH)D$_3$ concentrations (Handel et al., 2013). The number of the observed VDR ChIP-seq peaks, which varied between 200 and more than 7000, correlated with the 25(OH)D$_3$ levels of the individuals, i.e., the higher the circulating 25(OH)D$_3$ concentrations, the more VDR loci were identified in T cells. Unfortunately, the raw data of this study is not available, i.e., a harmonized re-analysis in comparison with other published VDR ChIP-seq data cannot be performed. However, from the 14,044 unique VDR peaks reported for the sum of the nine individuals, only 442 (3.1%) associated with a DR3-type sequence (based on HOMER score 7 settings).

Serum 25(OH)D$_3$ concentrations vary widely from person to person based on (i) varied diet and sun exposure, (ii) different age and/or level of adiposity and (iii) genetic and epigenetic variations (Engelman et al., 2008; Orton et al., 2008; Snellman et al., 2009). The Institute of Medicine recommends a serum 25(OH)D$_3$ level of 50 nM (Institute-of-Medicine, 2011), but it is under debate, whether this is sufficient for every individual (Holick, 2007). In fact, a substantial proportion of the world's population could be considered as vitamin D deficient. This condition may accelerate age-related bone loss and morbidity from falls and fractures. In addition, vitamin D insufficiency is associated with a number of diseases, such as cancer, autoimmune disorders and all components of the metabolic syndrome (more details in the article by Bendik et al. in this issue).

This important medical problem guided to the question, whether an insight into the genome- and transcriptome-wide actions of VDR and 1,25(OH)$_2$D$_3$ can help in a more accurate evaluation of the human individual's responsiveness to, and needs for, vitamin D. A first approach in this direction was done by studying peripheral blood mononuclear cells (PBMCs) and adipose tissue biopsies from 71 elderly, pre-diabetic individuals, which participated in a 5-month vitamin D$_3$ intervention trial (VitDmet) during Finnish winter (Carlberg et al., 2013). The changes in the mRNA expression of the primary 1,25(OH)$_2$D$_3$ target genes *CD14* and thrombomodulin (*THBD*), which had been identified in a recent comparative study as most reliable biomarkers (Standahl Olsen et al., 2013), in both PBMCs and fat samples were correlated with the alterations in the serum 25(OH)D$_3$ levels of the 71 individuals. Interestingly, only for a subset of individuals significant correlations between the up-regulation of both genes and the intervention-induced raise in serum 25(OH)D$_3$ concentrations were obtained. This suggests that, on a molecular level, not all study participants benefited from the vitamin D$_3$ supplementation, because (i) they had already reached their individual optimal vitamin D status before the start of the intervention, (ii) they carry a genetic polymorphism making them less responsive to vitamin D$_3$ or (iii) other undefined reasons (Carlberg et al., 2013). Interestingly, the categorization of the human individuals by their vitamin D responsiveness unmasked a negative correlation between changes in serum concentrations of 25(OH)D$_3$ and the inflammation marker interleukin 6, i.e., the more responsive the study participants were to vitamin D$_3$ supplementation, the lower was their inflammatory status. At present, a number of other primary 1,25(OH)$_2$D$_3$ target genes, which were highlighted in the comparison of VDR ChIP-seq data, are evaluated for their potential to serve as even better biomarkers

for the vitamin D status of human individuals than *CD14* and *THBD*.

In summary, vitamin D deficiency may negatively contribute to a number of diseases. Genome-wide insight led to the use of mRNA expression changes of the genes *CD14* and *THBD* as biomarkers for a molecular evaluation of vitamin D_3 supplementation studies. The results allow a classification of human individuals based on their responsiveness to vitamin D_3.

CONCLUSIONS

Genome-wide data on (i) the location of transcription factor binding sites in living cells, (ii) histone modifications and (iii) accessible chromatin, such as provided by ChIP-seq, DNase-seq and FAIRE-seq studies, have significantly changed the view on, and the understanding of the regulation of the entirety of the genes of our genome. This applies also to the transcription factor VDR, for which at present ChIP-seq data from six human cell lines and the T cells of nine human individuals are available. The abovementioned modern genome-wide techniques allow a more unbiased identification of transcription factor binding sites compared to previous studies, which were mostly focused on regions a few kb upstream of a primary $1,25(OH)_2D_3$ target gene. VDR binding loci have now been shown to be localized equally likely up- and downstream of TSS regions in distances of even more than 1 Mb.

Genome-wide studies have confirmed DR3-type sequences as the preferential binding sites for VDR (most likely as a heterodimer with RXR), but only one in seven of the close to 20,000 known VDR binding loci carry such a motif. This is unanimously observed in all investigated cellular models. Therefore, there must be other types of binding motifs and partnering proteins that attract VDR to its genomic targets. These presently poorly understood alternative binding modes may explain some of VDR's function in the (trans)repression of its target genes. Moreover, the VDR cistrome seems to be largely cell-specific with only some 50 loci overlapping in all investigated models. However, these conserved sites could be fundamental entry ports of VDR to the human genome, which may serve as the unified core of the various pleiotropic functions of $1,25(OH)_2D_3$.

The 22,000 detected VDR binding loci within six cell lines as well as the 14,000 peaks found in primary T cells from nine human individuals may be far more than what is needed to control the physiological actions of $1,25(OH)_2D_3$, i.e., many sites may represent rather "noise" than having a specific function. Therefore, different approaches to categorize VDR loci are useful. It turned out that VDR binding sites that (i) carry a DR3-type sequence, (ii) show ligand-stimulated VDR association, (iii) co-locate with ligand-induced chromatin opening and (iv) are conserved between several cellular systems may play a more important role in mediating the functions of $1,25(OH)_2D_3$ than the vast majority of other VDR sites that lack most of these properties. Therefore, for a cellular system of interest, the combination of (i) a genome-wide assessment of open chromatin by DNase-seq or FAIRE-seq, (ii) the monitoring of genomic VDR loci by ChIP-seq and (iii) a screening for DR3-type sequences below the peak summits is an efficient tool for the prediction and identification of primary $1,25(OH)_2D_3$ target genes.

FUTURE PERSPECTIVES

Although historically $1,25(OH)_2D_3$ was understood to be a hormone controlling calcium homeostasis and bone formation, to date the genome-wide the actions of VDR are best understood and monitored in cells of the hematopoietic system. This emphasizes the impact of $1,25(OH)_2D_3$ on the function of innate and adaptive immunity. There are first indications that the core actions of $1,25(OH)_2D_3$ and its receptor VDR can be extrapolated from hematopoietic cells to other tissues and cell types of the human body (Carlberg et al., 2013). If this holds true, the vitamin D status and responsiveness of a human individual can be derived from the response of, for example, PBMCs. Technically, various types of leukocytes can be collected far easier from blood samples than any other tissue biopsy. Like a glucose tolerance test is used to monitor the functionality of the carbohydrate metabolism of an individual, there may be in the future a higher dose vitamin D_3 challenge test, where the (epi)genomic and transcriptomic profiles of leukocytes before and after supplementation are measured. Routine measurements of healthy individuals may also in the future more likely be based on a few selected biomarkers, such as *CD14* and *THBD*, while more complex scenarios in disease settings, such as cancer or autoimmune diseases, will be assessed genome- or transcriptome-wide. In the same way, basic research on $1,25(OH)_2D_3$ and VDR will shift more and more from cell culture models to primary tissues and cell types and will eventually reach the single cell level.

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REFERENCES

Bouillon, R., and Suda, T. (2014). Vitamin D: calcium and bone homeostasis during evolution. *Bonekey Rep.* 3:480. doi: 10.1038/bonekey.2013.214

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L. J., Grippo, J. F., and Hunziker, W. (1993). Two nuclear signalling pathways for vitamin D. *Nature* 361, 657–660.

Carlberg, C., and Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78, 127–136. doi: 10.1016/j.steroids.2012.10.019

Carlberg, C., and Molnár, F. (2012). Current status of vitamin D signaling and its therapeutic applications. *Curr. Top. Med. Chem.* 12, 528–547. doi: 10.2174/156802612799436623

Carlberg, C., Seuter, S., De Mello, V. D., Schwab, U., Voutilainen, S., Pulkki, K., et al. (2013). Primary vitamin D target genes allow a categorization of possible benefits of vitamin D_3 supplementation. *PLoS ONE* 8:e71042. doi: 10.1371/journal.pone.0071042

Crawford, G. E., Holt, I. E., Whittle, J., Webb, B. D., Tai, D., Davis, S., et al. (2006). Genome-wide mapping of DNase hypersensitive sites using massively parallel signature sequencing (MPSS). *Genome Res.* 16, 123–131. doi: 10.1101/gr.4074106

Deluca, H. F. (2004). Overview of general physiologic features and functions of vitamin D. *Am. J. Clin. Nutr.* 80, 1689S–1696S.

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Encode-Project-Consortium, Bernstein, B. E., Birney, E., Dunham, I., Green, E. D., Gunter, C., and Snyder, M. (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74. doi: 10.1038/nature11247

Engelman, C. D., Fingerlin, T. E., Langefeld, C. D., Hicks, P. J., Rich, S. S., Wagenknecht, L. E., et al. (2008). Genetic and environmental determinants

of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels in Hispanic and African Americans. *J. Clin. Endocrinol. Metab.* 93, 3381–3388. doi: 10.1210/jc.2007-2702

Fretz, J. A., Zella, L. A., Kim, S., Shevde, N. K., and Pike, J. W. (2007). 1,25-Dihydroxyvitamin D_3 induces expression of the Wnt signaling co-regulator LRP5 via regulatory elements located significantly downstream of the gene's transcriptional start site. *J. Steroid Biochem. Mol. Biol.* 103, 440–445. doi: 10.1016/j.jsbmb.2006.11.018

Fullwood, M. J., Liu, M. H., Pan, Y. F., Liu, J., Xu, H., Mohamed, Y. B., et al. (2009). An oestrogen-receptor-alpha-bound human chromatin interactome. *Nature* 462, 58–64. doi: 10.1038/nature08497

Furey, T. S. (2012). ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions. *Nat. Rev. Genet.* 13, 840–852. doi: 10.1038/nrg3306

Giresi, P. G., Kim, J., McDaniell, R. M., Iyer, V. R., and Lieb, J. D. (2007). FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.* 17, 877–885. doi: 10.1101/gr.5533506

Handel, A. E., Sandve, G. K., Disanto, G., Berlanga-Taylor, A. J., Gallone, G., Hanwell, H., et al. (2013). Vitamin D receptor ChIP-seq in primary $CD4^+$ cells: relationship to serum 25-hydroxyvitamin D levels and autoimmune disease. *BMC Med* 11:163. doi: 10.1186/1741-7015-11-163

Haussler, M. R., Haussler, C. A., Jurutka, P. W., Thompson, P. D., Hsieh, J. C., Remus, L. S., et al. (1997). The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *J. Endocrinol.* 154(Suppl.), S57–S73.

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J.-C., and Jurutka, P. W. (2013). Molecular mechanisms of vitamin D action. *Calcif. Tissue Int.* 92, 77–98. doi: 10.1007/s00223-012-9619-0

Heikkinen, S., Väisänen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1α,25-dihydroxyvitamin D_3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Hochberg, Z., and Templeton, A. R. (2010). Evolutionary perspective in skin color, vitamin D and its receptor. *Hormones (Athens)* 9, 307–311. doi: 10.14310/horm.2002.1281

Holick, M. F. (2004). Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *Am. J. Clin. Nutr.* 80, 1678S–1688S.

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Holick, M. F. (2011). Vitamin D: evolutionary, physiological and health perspectives. *Curr. Drug Targets* 12, 4–18. doi: 10.2174/138945011793591635

Hollis, B. W. (2005). Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J. Nutr.* 135, 317–322.

Institute-of-Medicine. (2011). *Dietary Reference Intakes for Calcium and Vitamin D*. Washington, DC: National Academies Press.

Jones, G., Strugnell, S. A., and Deluca, H. F. (1998). Current understanding of the molecular actions of vitamin D. *Physiol. Rev.* 78, 1193–1231.

Kadauke, S., and Blobel, G. A. (2009). Chromatin loops in gene regulation. *Biochim. Biophys. Acta* 1789, 17–25. doi: 10.1016/j.bbagrm.2008.07.002

Kim, S., Yamazaki, M., Zella, L. A., Shevde, N. K., and Pike, J. W. (2006). Activation of receptor activator of NF-kappaB ligand gene expression by 1,25-dihydroxyvitamin D_3 is mediated through multiple long-range enhancers. *Mol. Cell Biol.* 26, 6469–6486. doi: 10.1128/MCB.00353-06

Kriebitzsch, C., Verlinden, L., Eelen, G., Van Schoor, N. M., Swart, K., Lips, P., et al. (2011). 1,25-dihydroxyvitamin D_3 influences cellular homocysteine levels in murine preosteoblastic MC3T3-E1 cells by direct regulation of cystathionine beta-synthase. *J. Bone Miner. Res.* 26, 2991–3000. doi: 10.1002/jbmr.493

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., et al. (1995). The nuclear receptor superfamily: the second decade. *Cell* 83, 835–839.

Maston, G. A., Landt, S. G., Snyder, M., and Green, M. R. (2012). Characterization of enhancer function from genome-wide analyses. *Annu. Rev. Genomics Hum. Genet.* 13, 29–57. doi: 10.1146/annurev-genom-090711-163723

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2010). A downstream intergenic cluster of regulatory enhancers contributes to the induction of CYP24A1 expression by 1α,25-dihydroxyvitamin D_3. *J. Biol. Chem.* 285, 15599–15610. doi: 10.1074/jbc.M110.119958

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2012). VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol. Endocrinol.* 26, 37–51. doi: 10.1210/me.2011-1109

Meyer, M. B., Watanuki, M., Kim, S., Shevde, N. K., and Pike, J. W. (2006). The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D_3 in intestinal cells. *Mol. Endocrinol.* 20, 1447–1461. doi: 10.1210/me.2006-0031

Misteli, T. (2007). Beyond the sequence: cellular organization of genome function. *Cell* 128, 787–800. doi: 10.1016/j.cell.2007.01.028

Mohn, F., and Schubeler, D. (2009). Genetics and epigenetics: stability and plasticity during cellular differentiation. *Trends Genet.* 25, 129–136. doi: 10.1016/j.tig.2008.12.005

Molnár, F., Peräkylä, M., and Carlberg, C. (2006). Vitamin D receptor agonists specifically modulate the volume of the ligand-binding pocket. *J. Biol. Chem.* 281, 10516–10526. doi: 10.1074/jbc.M513609200

Moras, D., and Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: structure and function. *Curr. Opin. Cell Biol.* 10, 384–391.

Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 108, 475–487. doi: 10.1016/S0092-8674(02)00654-2

Norman, A. W. (2008). From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am. J. Clin. Nutr.* 88, 491S–499S.

Orlando, V. (2000). Mapping chromosomal proteins *in vivo* by formaldehyde-crosslinked-chromatin immunoprecipitation. *Trends Biochem. Sci.* 25, 99–104. doi: 10.1016/S0968-0004(99)01535-2

Orton, S. M., Morris, A. P., Herrera, B. M., Ramagopalan, S. V., Lincoln, M. R., Chao, M. J., et al. (2008). Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis. *Am. J. Clin. Nutr.* 88, 441–447.

Park, P. J. (2009). ChIP-seq: advantages and challenges of a maturing technology. *Nat. Rev. Genet.* 10, 669–680. doi: 10.1038/nrg2641

Perissi, V., Jepsen, K., Glass, C. K., and Rosenfeld, M. G. (2010). Deconstructing repression: evolving models of co-repressor action. *Nat. Rev. Genet.* 11, 109–123. doi: 10.1038/nrg2736

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000). VDR-Alien: a novel, DNA-selective vitamin D_3 receptor-corepressor partnership. *FASEB J.* 14, 1455–1463. doi: 10.1096/fj.14.10.1455

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Razin, A. (1998). CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J.* 17, 4905–4908.

Robinson, J. T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., and Mesirov, J. P. (2011). Integrative genomics viewer. *Nat. Biotechnol.* 29, 24–26. doi: 10.1038/nbt.1754

Ryynänen, J., Seuter, S., Campbell, M. J., and Carlberg, C. (2013). Gene regulatory scenarios of primary 1,25-dihydroxyvitamin D_3 target genes in a human myeloid leukemia cell line. *Cancers* 5, 1221–1241. doi: 10.3390/cancers5041221

Saramäki, A., Banwell, C. M., Campbell, M. J., and Carlberg, C. (2006). Regulation of the human $p21^{(waf1/cip1)}$ gene promoter via multiple binding sites for p53 and the vitamin D_3 receptor. *Nucleic Acids Res.* 34, 543–554. doi: 10.1093/nar/gkj460

Saramäki, A., Diermeier, S., Kellner, R., Laitinen, H., Väisänen, S., and Carlberg, C. (2009). Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1α,25-dihydroxyvitamin D_3. *J. Biol. Chem.* 284, 8073–8082. doi: 10.1074/jbc.M808090200

Schmidt, D., Schwalie, P. C., Wilson, M. D., Ballester, B., Gonçalves, A., Kutter, C., et al. (2012). Waves of retrotransposon expansion remodel genome organization and CTCF binding in multiple mammalian lineages. *Cell* 148, 335–348. doi: 10.1016/j.cell.2011.11.058

Schüle, R., Umesono, K., Mangelsdorf, D. J., Bolado, J., Pike, J. W., and Evans, R. M. (1990). Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell* 61, 497–504.

Seuter, S., Neme, A., and Carlberg, C. (2014). Characterization of genomic vitamin D receptor binding sites through chromatin looping and opening. *PLoS ONE* 9. doi: 10.1371/journal.pone.0096184

Seuter, S., Pehkonen, P., Heikkinen, S., and Carlberg, C. (2013). Dynamics of 1α,25-dihydroxyvitamin D-dependent chromatin accessibility of early vitamin D receptor target genes. *Biochim. Biophys. Acta* 1829, 1266–1275. doi: 10.1016/j.bbagrm.2013.10.003

Seuter, S., Väisänen, S., Radmark, O., Carlberg, C., and Steinhilber, D. (2007). Functional characterization of vitamin D responding regions in the human 5-lipoxygenase gene. *Biochim. Biophys. Acta* 1771, 864–872. doi: 10.1016/j.bbalip.2007.04.007

Shaffer, P. L., and Gewirth, D. T. (2002). Structural basis of VDR-DNA interactions on direct repeat response elements. *EMBO J.* 21, 2242–2252. doi: 10.1093/emboj/21.9.2242

Shaffer, P. L., and Gewirth, D. T. (2004). Structural analysis of RXR-VDR interactions on DR3 DNA. *J. Steroid Biochem. Mol. Biol.* 89–90, 215–219. doi: 10.1016/j.jsbmb.2004.03.084

Sierra, J., Villagra, A., Paredes, R., Cruzat, F., Gutierrez, S., Javed, A., et al. (2003). Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D_3 receptor but not p300 intrinsic histone acetyltransferase activity. *Mol. Cell Biol.* 23, 3339–3351. doi: 10.1128/MCB.23.9.3339-3351.2003

Sinkkonen, L., Malinen, M., Saavalainen, K., Väisänen, S., and Carlberg, C. (2005). Regulation of the human cyclin C gene via multiple vitamin D_3-responsive regions in its promoter. *Nucleic Acids Res.* 33, 2440–2451. doi: 10.1093/nar/gki502

Snellman, G., Melhus, H., Gedeborg, R., Olofsson, S., Wolk, A., Pedersen, N. L., et al. (2009). Seasonal genetic influence on serum 25-hydroxyvitamin D levels: a twin study. *PLoS ONE* 4:e7747. doi: 10.1371/journal.pone.0007747

Sone, T., Ozono, K., and Pike, J. W. (1991). A 55-kilodalton accessory factor facilitates vitamin D receptor DNA binding. *Mol Endocrinol* 5, 1578–1586.

Standahl Olsen, K., Rylander, C., Brustad, M., Aksnes, L., and Lund, E. (2013). Plasma 25 hydroxyvitamin D level and blood gene expression profiles: a cross-sectional study of the Norwegian women and cancer post-genome cohort. *Eur. J. Clin. Nutr.* 67, 773–778. doi: 10.1038/ejcn.2013.53

Talbert, P. B., and Henikoff, S. (2006). Spreading of silent chromatin: inaction at a distance. *Nat. Rev. Genet.* 7, 793–803. doi: 10.1038/nrg1920

Tolon, R. M., Castillo, A. I., Jimenez-Lara, A. M., and Aranda, A. (2000). Association with ets-1 causes ligand- and AF2-independent activation of nuclear receptors. *Mol. Cell Biol.* 20, 8793–8802. doi: 10.1128/MCB.20.23.8793-8802.2000

Toropainen, S., Väisänen, S., Heikkinen, S., and Carlberg, C. (2010). The down-regulation of the human MYC gene by the nuclear hormone 1α,25-dihydroxyvitamin D_3 is associated with cycling of corepressors and histone deacetylases. *J. Mol. Biol.* 400, 284–294. doi: 10.1016/j.jmb.2010.05.031

Tuoresmäki, P., Väisänen, S., Neme, A., Heikkinen, S., and Carlberg, C. (2014). Patterns of genome-wide VDR locations. *PLoS ONE* 9. doi: 10.1371/journal.pone.0096105

Turunen, M. M., Dunlop, T. W., Carlberg, C., and Väisänen, S. (2007). Selective use of multiple vitamin D response elements underlies the 1α,25-dihydroxyvitamin D_3-mediated negative regulation of the human CYP27B1 gene. *Nucleic Acids Res* 35, 2734–2747. doi: 10.1093/nar/gkm179

Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* 65, 1255–1266.

Väisänen, S., Dunlop, T. W., Sinkkonen, L., Frank, C., and Carlberg, C. (2005). Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1α,25-dihydroxyvitamin D_3. *J. Mol. Biol.* 350, 65–77. doi: 10.1016/j.jmb.2005.04.057

Van Bortle, K., and Corces, V. G. (2013). The role of chromatin insulators in nuclear architecture and genome function. *Curr. Opin. Genet. Dev.* 23, 212–218. doi: 10.1016/j.gde.2012.11.003

Vaquerizas, J. M., Kummerfeld, S. K., Teichmann, S. A., and Luscombe, N. M. (2009). A census of human transcription factors: function, expression and evolution. *Nat. Rev. Genet.* 10, 252–263. doi: 10.1038/nrg2538

Virtanen, J. K., Nurmi, T., Voutilainen, S., Mursu, J., and Tuomainen, T. P. (2011). Association of serum 25-hydroxyvitamin D with the risk of death in a general older population in Finland. *Eur. J. Nutr.* 50, 305–312. doi: 10.1007/s00394-010-0138-3

Wang, Y., Zhu, J., and Deluca, H. F. (2012). Where is the vitamin D receptor? *Arch. Biochem. Biophys.* 523, 123–133. doi: 10.1016/j.abb.2012.04.001

Zaret, K. S., and Carroll, J. S. (2011). Pioneer transcription factors: establishing competence for gene expression. *Gen. Dev.* 25, 2227–2241. doi: 10.1101/gad.176826.111

Zella, L. A., Kim, S., Shevde, N. K., and Pike, J. W. (2006). Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D_3. *Mol. Endocrinol.* 20, 1231–1247. doi: 10.1210/me.2006-0015

Zella, L. A., Meyer, M. B., Nerenz, R. D., Lee, S. M., Martowicz, M. L., and Pike, J. W. (2010). Multifunctional enhancers regulate mouse and human vitamin D receptor gene transcription. *Mol. Endocrinol.* 24, 128–147. doi: 10.1210/me.2009-0140

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Structural considerations of vitamin D signaling

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Crystal structures represent the static picture in the life of a molecule giving a sneak preview what it might be in reality. Hence, it is very hard to extrapolate from these photos toward dynamic processes such as transcriptional regulation. Mechanistically VDR may be considered as molecular machine able to perform ligand-, DNA- and protein recognition, and interaction in a multi-task manner. Taking this into account the functional net effect will be the combination of all these processes. The long awaited answer to explain the differences in physiological effects for various ligands was one of the biggest disappointment that crystal structures provided since no substantial distinction could be made for the conformation of the active VDR-ligand complexes. This may have come from the limitation on the complexity of the available ligand-VDR structures. The recent studies with full length VDR-RXRα showed somewhat more comprehensive perspective for the 3D organization and possible function of the VDR-RXRα-cofactor complex. In addition to *in vitro* approaches, also computational tools had been introduced with the aim to get understanding on the mechanic and dynamic properties of the VDR complexes with some success. Using these methods and based on measurable descriptors such as pocket size and positions of side chains it is possible to note subtle differences between the structures. The meaning of these differences has not been fully understood yet but the possibility of a "butterfly effect" may have more extreme consequences in terms of VDR signaling. In this review, the three functional aspects (ligand-, DNA- and protein recognition, and binding) will be discussed with respect to available data as well as possible implication and questions that may be important to address in the future.

Keywords: VDR, crystal structure, molecular dynamics, molecular mechanism, cofactors, response elements, ligand-binding

INTRODUCTION

One way of understanding life at molecular level is to obtain the three-dimensional (3D) structures of the molecules. Such structural views represent a static picture in the life of a molecule giving a sneak preview what it might be in reality. For the understanding of the functional implication of vitamin D (VD) signaling it is also important to look at various structural complexes of the vitamin D receptor (VDR), which may outline its possible dynamics and mechanics. VDR is able to perform the ligand-, DNA- and protein recognition, and interaction in a multi-task manner thus can be viewed as molecular machine which will regulate gene expression with the combination/sum of all these particular functions.

Before the year 2000 people in the VD field were only guessing how may the 3D structure of VDR look like. Some implications were coming from already known crystal structures e.g., receptors for retinoids such as retinoid X receptor (RXR) (Bourguet et al., 1995) and retinoic acid receptor (RAR) (Renaud et al., 1995). Not until exactly 14 years ago the structure of VDR-1α,25-dihydroxyvitamin D_3 ($1,25D_3$) complex has been solved (Rochel et al., 2000) and a long journey started in understanding the binding of various VDR analogs and the structure-based analog design. Within the first 5 years more structures have been solved (Tocchini-Valentini et al., 2001, 2004; Eelen et al., 2005) but the long awaited answer to explain the differences in physiological effects for various ligands was one of the biggest disappointment that crystal structures provided since no substantial distinction could be made for the conformation of the active VDR-ligand complexes.

Compared to the beginning of the last decade a huge number of X-ray crystal structures are available for VDR. In detail, there are VDR LBDs from three different species *H. sapiens* (34), *R. norvegicus* (40) and *D. rerio* (13) and four DBD-DNA complexes from *H. sapiens*. The basic information about these complexes is summarized in **Table 1**. This data makes also possible to analyze orthologous molecules with reflection to functional and structural differences. However, to understand this aspect well it would be beneficial to have more data from numerous organisms. In this review evolutionary aspects and species-specific difference will be not discussed in depth. More space will be given to

Abbreviations: $1,25D_3$, 1α, 25-dihydroxyvitamin D_3; 3D, three-dimensional; 3Klca, 3-keto lithocholic acid; 9-*cis* RA, 9-*cis* retinoic acid; aa, amino acids; cryo-EM, cryo-electron microscope; *Cyp27b1*, 25-hydroxyvitamin D_3 1α-hydroxylase; *D.*rerio, Danio rerio; DBD, DNA-binding domain; DNA, deoxyribonucleic acid; DR, direct repeat; DRIP, vitamin-D-receptor interacting protein; h, human; *H. sapiens, Homo sapiens;* HDX, H/D exchange; LBD, ligand-binding domain; LBP, ligand-binding pocket; LCA, lithocholic acid; MD, molecular dynamics; OH, hydroxyl; *P. marinus*, Petromyzon marinus; PXR, pregnane X recptor; RAR, Retinoic acid receptor; r, rat; *R. norvegicus, Rattus norvegicus;* RE, response element; RID, receptor interaction domain; RXR, retinoid X receptor; SAXS, short angle X-ray scattering; TR, thyroid hormone receptor; VD, vitamin D; VDR, vitamin D receptor; VDRE, VD response element; z, zebrafish.

Table 1 | List of crystal structure data available for LBD and DBD of VDR (source: www.pdb.org).

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
H. sapiens LBDs (34)	1DB1	Rochel et al., 2000	1,25D_3		3CS6	Hourai et al., 2008	AMCR277B	
	1IE8	Tocchini-Valentini et al., 2001	KH1060		3M7R	Rochel et al., 2010	1,25D_3	H305Q mutant
	1IE9		MC1288		3B0T	[b]	Maxacalcitol	
	1S0Z	Tocchini-Valentini et al., 2004	EB1089		3OGT	[b]	(1S,3R,5Z,7E,14beta,17alpha, 20S)-20-[5-(1-hydroxy-1-methylethyl) furan-2-yl]-9,10-secopregna-5,7,10-triene-1,3-diol	
	1S19		MC903		3P8X	Verlinden et al., 2011	20(17→18)-abeo-1α,25-dihydroxy-22-homo-21-norvitamin D_3	
	1TXI	Eelen et al., 2005	TX522		3AUQ	Sawada et al., 2011	14-epi-2α-methyl-19-norvitamin D_3	
	2HAM	Hourai et al., 2006	2α-propyl-1,25D_3		3AUR		14-epi-2β-methyl-19-norvitamin D_3	
	2HAR		2α-(3-hydroxy-1-propoxy)-1,25D_3		3AX8	Shindo et al., 2011	15α-methoxy-1,25D_3	
	2HAS		2α-(1-propoxy)-1,25D_3		3KPZ	Rochel and Moras, 2012	ZK203278	
	2HB7		2α(3-hydroxy-1-propyl) -1,25D_3		3AZ1	Kashiwagi et al., 2011	{4-[3-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}-3-methylphenyl)pentan-3-yl]-2-methylphenoxy}acetic acid	
	2HB8		2α-methyl-1,25D_3		3AZ2		5-{4-[3-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}-3-methylphenyl)pentan-3-yl]-2-methylphenoxy}pentanoic acid	

(Continued)

Table 1 | Continued

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
	3A2I	Kakuda et al., 2010	TEI-9647 hVDR_H305F		3AZ3		(4S)-4-hydroxy-5-[4-(3-{4-[(3S)-3-hydroxy-4,4-dimethylpentyl]-3-methylphenyl}pentan-3-yl)-2-methylphenoxy]pentanoic acid	
	3A2J		TEI-9647 hVDR_H305F/H397F		3TKC	Fraga et al., 2012	(1S,3R,5Z,7E,14beta,17alpha,20S)-20-[5-(1-hydroxy-1-methylethyl)furan-2-yl]-9,10-secopregna-5,7,10-triene-1,3-diol	
	3A3Z	Antony et al., 2010	2α-methyl-AMCR277A(C23S)		3VHW	Saito et al., 2013	4MP	
	3A40		2α-methyl-AMCR277B(C23R)		3W0A	[b]	(4S)-4-hydroxy-5-[2-methyl-4-(3-{3-methyl-4-[4,4,4-trifluoro-3-hydroxy-3-(trifluoromethyl)but-1-yn-1-yl]phenyl}pentan-3-yl)phenoxy]pentanoic acid	
	3A78	Molnár et al., 2011	1,25-3-epi-D_3		3W0C		(4S)-4-hydroxy-5-[2-methyl-4-(3-{3-methyl-4-[(1E)-4,4,4-trifluoro-3-hydroxy-3-(trifluoromethyl)but-1-en-1-yl]phenyl}pentan-3-yl)phenoxy]pentanoic acid	
	3CS4	Hourai et al., 2008	AMCR277A		3W0Y		[3-fluoro-2′-methyl-4′-(3-{3-methyl-4-[(1E)-4,4,4-trifluoro-3-hydroxy-3-(trifluoromethyl)but-1-en-1-yl]phenyl}pentan-3-yl)biphenyl-4-yl]acetic acid	

(Continued)

Table 1 | Continued

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
H. sapiens DBDs (4)	1KB2	Shaffer and Gewirth, 2002	mouse osteopontin DR3		1KB6	Shaffer and Gewirth, 2002	rat osteocalcin DR3	
	1KB4	Shaffer and Gewirth, 2002	Canonical DR3		1YNW	Shaffer and Gewirth, 2004	Canonical DR3	+ RXRα DBD
R. norvegicus LBDs (40)	1RJK	Vanhooke et al., 2004	2-methylene-19-nor-(20S)-1,25D_3	+ DRIP205 (NR2)[c]	3VRU	Yoshimoto et al., 2012	2-Methylidene-19,24-dinor-1α,25D_3	+ DRIP205 (NR2)[c]
	1RK3		1,25D_3	+ DRIP205 (NR2)[c]	3VRV		2-Methylidene-26,27-dimethyl-19,24-dinor-1α,25D_3	+ DRIP205 (NR2)[c]
	1RKG		1α-hydroxy-2-methylene-19-nor-(20S)-bishomopregnacalciferol	+ DRIP205 (NR2)[c]	3VRW		22S-Butyl-2-methylidene-26,27-dimethyl-19,24-dinor-1α,25D_3	+ DRIP205 (NR2)[c]
	1RKH		2α-methylene-19-nor-1,25D_3	+ DRIP205 (NR2)[c]	3VT3	Nakabayashi et al., 2013	1,25D_3	+ DRIP205 (NR2)[c] R270L mutant
	2O4J	Vanhooke et al., 2007	17Z-1,25-17(20)-dehydro-2-methylene-19-nor-D_3	+ DRIP205 (NR2)[c]	3VT4		(1R,2Z,3R,5E,7E)-17-{(1S)-1-[(2-ethyl-2-hydroxybutyl)sulfanyl]ethyl}-2-(2-hydroxyethylidene)-9,10-secoestra-5,7,16-triene-1,3-diol	+ DRIP205 (NR2)[c] R270L mutant
	2O4R		17E-1,25-17(20)-dehydro-2-methylene-19-nor-D_3	+ DRIP205 (NR2)[c]	3VT5		(1R,2E,3R,5Z,7E)-17-{(1S)-1-[(2-ethyl-2-hydroxybutyl)sulfanyl]ethyl}-2-(2-hydroxyethylidene)-9,10-secoestra-5,7,16-triene-1,3-diol	+ DRIP205 (NR2)[c] R270L mutant
	2ZFX	Kakuda et al., 2008	YR301	+ DRIP205 (NR2)[c]	3VT7		1,25D_3	+ DRIP205 (NR2)[c] W282R mutant
	2ZL9	Shimizu et al., 2008	(20S)-1,25-2β-(2-hydroxy ethoxy)-16-ene-22-thia -26,27-dimethyl-19,24-dinor-D_3	+DRIP205 (NR2)[c]	3VT8		(1R,3R,7E,9beta,17beta)-9-butyl-17-[(2R)-6-hydroxy-6-methylheptan-2-yl]-9,10-secoestra-5,7-diene-1,3-diol	+ DRIP205 (NR2)[c] W282R mutant

(Continued)

Table 1 | Continued

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
	2ZLA		(20R)-1,25-2β-(2-hydroxy ethoxy)-16-ene-22-thia -26,27-dimethyl-19,24-dinor-D_3	+ DRIP205 (NR2)[c]	3VT9		(1R,2Z,3R,5E,7E,9beta,17beta)-2-(2-hydroxyethylidene)-17-[(2R)-6-hydroxy-6-methylheptan-2-yl]-9-(prop-2-en-1-yl)-9,10-secoestra-5,7-diene-1,3-diol	+ DRIP205 (NR2)[c] W282R mutant
	2ZLC	Shimizu et al., 2008	1,25D_3	+ DRIP205 (NR2)[c]	3VTB	[b]	(1R,3R,7E,17beta)-17-{(2R,6S)-6-hydroxy-6-[(3S,5S,7S)-tricyclo[3.3.1.1∼3,7∼]dec-1-yl]hex-4-yn-2-yl}-2-methylidene-9,10-secoestra-5,7-diene-1,3-diol	+ DRIP205 (NR2)[c]
	2ZMH	Nakabayashi et al., 2008	(25R)-25-adamantyl-1,25-2-methylene-22,23-didehydro-19,26,27-trinor-20-epi-D_3	+ DRIP205 (NR2)[c]	3VTC		(1R,3R,7E,17beta)-17-{(2R,6R)-6-hydroxy-7-[(3S,5S,7S)-tricyclo[3.3.1.1∼3,7∼]dec-1-yl]hept-4-yn-2-yl}-2-methylidene-9,10-secoestra-5,7-diene-1,3-diol	+ DRIP205 (NR2)[c]
	2ZMI		(24R)-24-adamantyl-1,24-2-methylene-22,23-didehydro-19,25,26,27-tetranor-20-epi-D_3	+ DRIP205 (NR2)[c]	3VTD		(1R,3R,7E,17beta)-17-{(2R,6S)-6-hydroxy-7-[(3S,5S,7S)-tricyclo[3.3.1.1∼3,7∼]dec-1-yl]hept- 4-yn-2-yl}-2-methylidene-9,10-secoestra-5,7- diene-1,3- diol	+ DRIP205 (NR2)[c]
	2ZMJ		26-adamantyl-1,25R-2-methylene-22,23-didehydro-19,27-dinor-20-epi-D_3	+ DRIP205 (NR2)[c]	3W5P	Masuno et al., 2013	lithocholic acid	+ DRIP205 (NR2)[c]
	2ZXM	Inaba et al., 2009	(22S)-butyl-1,24-24,25,26-trinor-D_3	+ DRIP205 (NR2)[c]	3W5Q		3-keto litocholic acid	+ DRIP205 (NR2)[c]

(Continued)

Table 1 | Continued

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
	2ZXN		20S(22S)-butyl-1,24-24,25,26-trinor-D_3	+ DRIP205 (NR2)[c]	3W5R		lithocholic acid acetate	+ DRIP205 (NR2)[c]
	3A2H	Kakuda et al., 2010	TEI-9647	+ DRIP205 (NR2)[c]	3W5T		lithocholic acid propionate	+ DRIP205 (NR2)[c]
	3AFR	Inaba et al., 2010	22S-Butyl-1a,24R-dihydroxyvitamin D_3	+ DRIP205 (NR2)[c]	3W0G	Asano et al., 2013	(2S)-3-{4-[2-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}phenyl)propan-2-yl]phenoxy}propane-1,2-diol	+ DRIP205 (NR2)[c]
	3VJS	Fujii et al., 2011	1-(2-[(S)-2,4-Dihydroxybutoxy]ethyl)-12-(5-ethyl-5-hydroxyheptyl)-1,12-dicarba-closo-dodecaborane	+ DRIP205 (NR2)[c]	3W0H		(2S)-3-{4-[4-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}phenyl)heptan-4-yl]phenoxy}propane-1,2-diol	+ DRIP205 (NR2)[c]
	3VJT		1-(2-[(R)-2,4-Dihydroxybutoxy]ethyl)-12-(5-ethyl-5-hydroxyheptyl)-1,12-dicarba-closo-dodecaborane	+ DRIP205 (NR2)[c]	3W0I		(2S)-3-{4-[3-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}phenyl)pentan-3-yl]phenoxy}propane-1,2-diol	+ DRIP205 (NR2)[c]
	3VRT	Yoshimoto et al., 2012	2-Mehylidene-19,25,26,27-tetranor-1α,24-D_3	+ DRIP205 (NR2)[c]	3W0J		(2S)-3-{4-[2-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}-3-methylphenyl)propan-2-yl]-2-methylphenoxy}propane-1,2-diol	+ DRIP205 (NR2)[c]
D. rerio LBDs (19)	2HBH	Rochel et al., 2007	21-nor-20(22),23-diyne-1,25D_3	+ SRC1 (NR2)[d]	4G20	Ciesielski et al., 2012	CD4849	+ SRC1 (NR2)[d]
	2HC4	Ciesielski et al., 2007	1,25D_3	+ SRC1 (NR2)[d]	4G21		CD4742	+ SRC1 (NR2)[d]
	2HCD		Gemini	+ SRC1 (NR2)[d]	4G2H		CD4528	+ SRC1 (NR2)[d]

(Continued)

Table 1 | Continued

Ortholog	PDBID[a]	References	Ligand name	Notes	PDBID[a]	References	Ligand name	Notes
	3DR1	Eelen et al., 2008	CD578	+ SRC1 (NR2)[d]	4FHH	Fischer et al., 2012	N-hydroxy-2-{4-[3-(4-{[(2S)-2-hydroxy-3,3-dimethylbutyl]oxy}-3-methylphenyl)pentan-3-yl]-2-methylphenoxy}acetamide	+ SRC1 (NR2)[d]
	3O1D	Huet et al., 2011	Gemini-0072	+ SRC1 (NR2)[d]	4FHI		N-hydroxy-2-{4-[3-(4-{[(2R)-2-hydroxy-3,3-dimethylbutyl]oxy}-3-methylphenyl)pentan-3-yl]-2-methylphenoxy}acetamide	+ SRC1 (NR2)[d]
	3O1E		Gemini-0097	+ SRC1 (NR2)[d]	4IA1	Maehr et al., 2013	1,25-Dihydroxy-21-(3-hydroxy-3-trideuteriomethyl-4,4,4-tri-deuteriobut-1-yl)-19-nor-cholecalciferol	+ SRC1 (NR2)[d]
	4G1D	Ciesielski et al., 2012	CD4720	+ SRC1 (NR2)[d]	4IA2		1,25-Dihydroxy-21-(3-hydroxy-3-trideuteriomethyl-4,4,4-tri-deuteriobut-1-yl)-cholecalciferol	+ SRC1 (NR2)[d]
	4G1Y		CD3938	+ SRC1 (NR2)[d]	4IA3		1α,25-Dihydroxy-21-(3-hydroxy-3-methyl-but-1-yl)-26,27-hexadeuterio-cholecalciferol	+ SRC1 (NR2)[d]
	4G2I		CD4528	+ SRC1 (NR2)[d]	4IA7		21-nor-9,10-secocholesta-5,7,10(19)-triene- 1,3,25-triol, 20-(4-hydroxy-4-methylpentyl)-, (1A,3B,5Z,7E)	+SRC1 (NR2)[d]
	4G1Z		CD4802	+ SRC1 (NR2)[d]				

[a] *Protein Data Bank identifier.*

[b] *Unpublished structure.*

[c] *Synthetic peptide corresponding to NR2 Box of DRIP205/TRAP220/MED1.*

[d] *Synthetic peptide corresponding to NR2 Box of SRC1.*

general domain organization, binding mode of natural ligands and recognition of DNA by VDR. Some data coming from molecular dynamics (MD) simulations will be also discussed since this approach represents a compromise in obtaining 3D structural models and have been proven to be well aligned with the wet lab data. At last, recent data from small-angle X-ray scattering (SAXS), cryo-electron microscopy (cryo-EM) and H/D exchange (HDX) experiments will be discussed with some perspectives highlighted.

WHAT DO VDR STRUCTURES TELL US?

From functional and structural organization point of view VDR is formed by DNA-binding domain (DBD:24–89 aa; domain C), ligand-binding domain (LBD:126–427 aa; domain E), a connective hinge (domain D) between them and a short A/B domain located at the N-terminus. Compare to RXR it completely lacks the F domain, which is the very last part of the LBD after helix 12. The LBD is formed by a three layer anti-parallel α-helical sandwich (**Figures 1A,B** highlighted in green, blue and red). Based of the particular structure it contains all together 11–13 α-helices (Li et al., 2003) (**Figure 2A**). The internal structure of the LBDs of the respective nuclear receptors (NRs) shows a high similarity with specialized diversity based on functional properties of the particular receptor. In VDR, the LBD is responsible for active ligand recognition and interaction with partnering proteins such as coregulators, and RXR to form the functionally active RXR-VDR heterodimer. In particular, helices 3, 4, and 10–12 are involve in the interaction with protein partners. Interestingly, to date all solved VDR crystal structures show very ubiquitous and conserved organization of the of overall structural fold not reflecting the divergent nature of the bound natural or synthetic ligands. What are in fact the differences in structures that reflect various physiological effects of the particular ligands? Allegedly there will not be a simple answer to this question since we may face the limitation on the complexity of the available ligand-VDR structures or have to allow the possibility that the subtle differences between the structures may cause a "butterfly effect" that have more extreme consequences in terms of VDR signaling than initially thought. By all means there are important differences in the metabolism of various synthetic ligands and a possible unique coactivator recruitment may also play its role. However, none of these possibilities can be fully explored using the available VDR crystal structures. Nevertheless, what we may agree on is that all VDR crystal structures show agonistic conformation, surprisingly even in case of antagonists, that is canonically represented by a closed conformation of the helix 12 providing a docking platform for the recruitment of coactivators. This may be due to the shifted equilibrium that drives VDR for closed helix 12 with minimal energy conformation. In addition, the VDR structures do tell us the binding mode, anchoring points and subtle changes

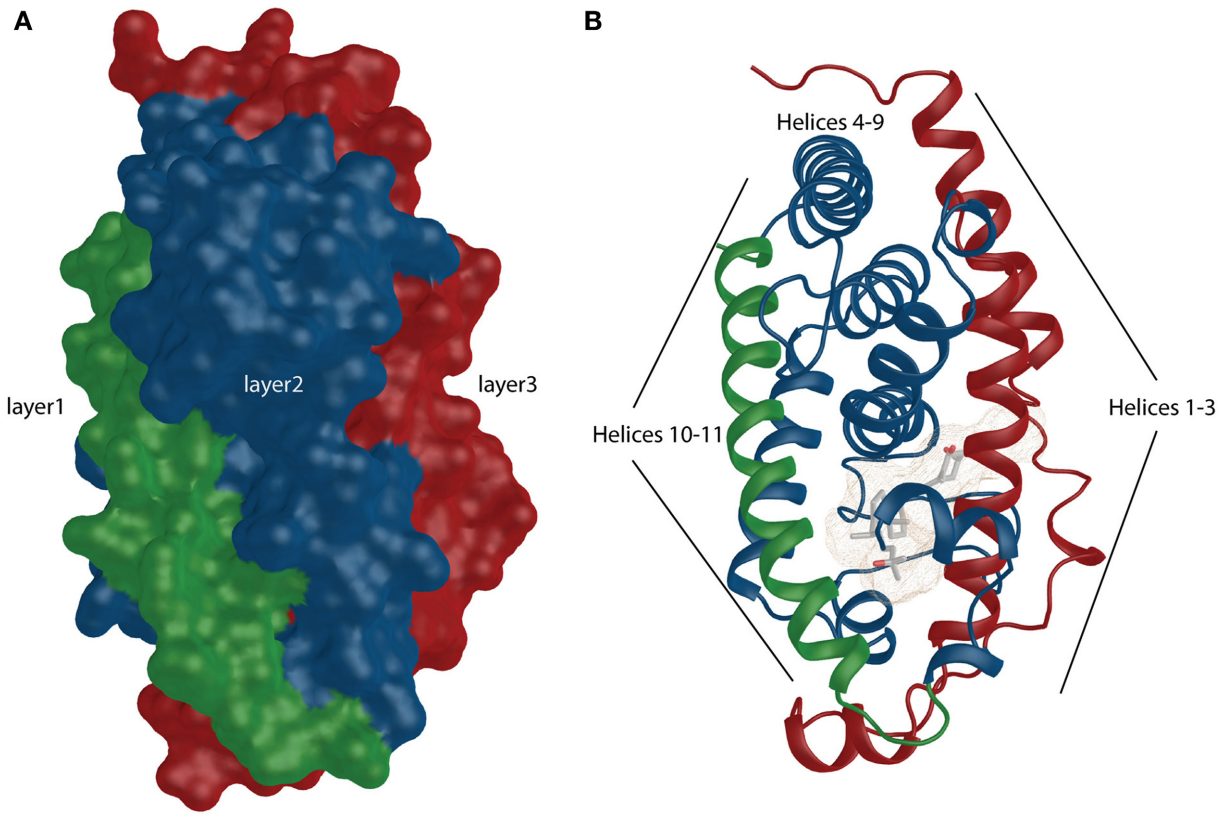

FIGURE 1 | VDR shows similarity to canonical NR structural organization. (A) The overall surface depiction of the VDR showing the three layers sandwich-like molecule where the layers are highlighted in green, blue and red. **(B)** Numbered helices belonging to different layers are shown and they are highlighted similarly as in surface representation.

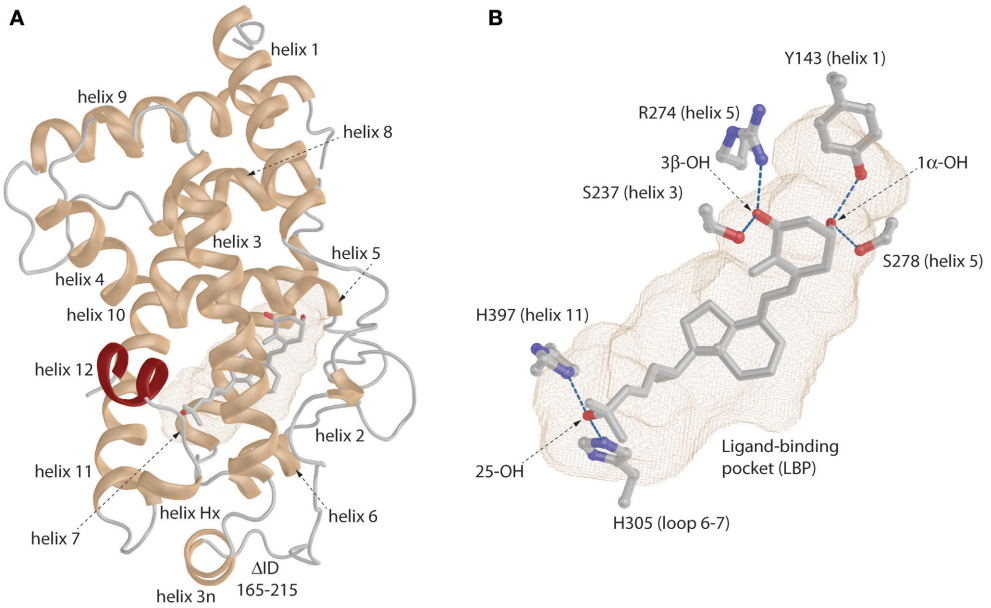

FIGURE 2 | General view of the VDR. (A) Ribbon representation of the VDR with annotated helices. The very last helix (helix 12) is highlighted in red color. **(B)** The binding mode of the 1,25D in the ligand-binding pocket (LBP). The important anchoring residues and their location in the VDR structure is depicted. Hydrogen bonds are shown in blue (for details see the text).

in the position of residues that may be effectively used for *de novo* design of superagonist such as AMCR277A (PDBID:3CS4) (Hourai et al., 2008). The frequently mentioned subtle changes that are characteristic for the ligand-binding pocket (LBP) may be further analyzed and can explain some of the binding differences between various ligands in correlation to their functional and biological properties.

THE INNER CIRCLE: LOOKING INTO THE VDR POCKETS

The "lower part" of the LBDs of all ligand-activated NRs contains a LBP which volume size range between 400 and 1400 Å^3 (**Figures 2A,B** light-brown mesh) (Nagy and Schwabe, 2004). It is not quite different in case of VDR where LBP primarily serves for effective recognition of various natural ligands such as 1,25D$_3$ and its metabolites or bile acids. In addition, this is one of the most important parts to modulate VDR's activity via various synthetic compounds. The VDR pocket can be placed in the middle range of the volume scale showing rather high dynamic plasticity toward various ligands.

The first VDR crystal structure confirmed the conserved contact or anchoring points for the interaction of VDR with 1,25D$_3$ (Rochel et al., 2000) (**Figure 2B**). The residues involved in the positioning of the 1,25D$_3$ in the LBP are Y143 (helix 1) and S278 (helix 5) that contact the 1,25D$_3$1α-hydroxyl (OH) group, S237 (helix 3) and R274 (helix 5) contact 3β-OH, and H305 (loop 6–7) and H397 (helix 11) interact with 25-OH. In most of the cases if these anchoring point are disrupted a decrease in the activation potential of the ligand can be observed. Besides these residues the interior surface of the VDR LBP is formed of about 40 mostly non-polar amino acids. An interesting approach is to look how the LBP is changed upon ligand-binding especially its volume or how much volume (%) is occupied by the ligand. A rather straightforward example is the comparison of the 1,25D$_3$ and MC1288 LBPs. Although the volumes of these ligands are highly comparable 434 Å^3 and 427 Å^3 (probe radius 1.9 Å), respectively, the volumes of the LBP show slight differences 776 Å^3 and 643 Å^3 (same probe radius) leading to ratio of 56 and 66% of ligand occupancy of the LBP volume (Molnár et al., 2006). When a ligand occupancy is higher compared to 1,25D$_3$ then it increases the stability of VDR. This factor holds true for ligands that show high structural similarity and binding mode to 1,25D$_3$. It seems to some extend that the ratio of the ligand to LBP volume can be a good descriptor of the ligands' activation potential. In addition, the actual shape of the cavity also reflects differences in the binding of various ligands which is illustrated in **Figure 3**. There are slight differences in the shapes of the LBP depending on the bound ligand. The red arrow shows the part where the shape is conserved well. Topologically this is the place where the 1,25D$_3$ A-ring is located e.g., helices 1, 3, and 5. The more plastic part is the one where the 1,25D$_3$ side chain is found with helices 6, 7, and 11. This region shows the highest variation between the ligand-bound structures and is indicated with green arrow **Figure 3**. In addition, small changes can be also detected for instance the part highlighted with green circle. Very interesting in the difference between the two Gemini structures 2HCD and 4IA1, where the largest difference for the two side chains is that "C17–20 threo 20S—Gemini" has the hydrogens in its methyl groups substituted with deuterium. Interestingly, in this case the pocket shows more compact shape in the region with the double side chains and the volume of the location highlighted with green circle is decreased, which may be due to modification of the A-ring. Another interesting question is the maximum volume to which the VDR LBP can be stretched. MD simulations showed that by docking a Gemini with fluorinated methyls groups, (CF3)$_2$-Gemini, the LBP could

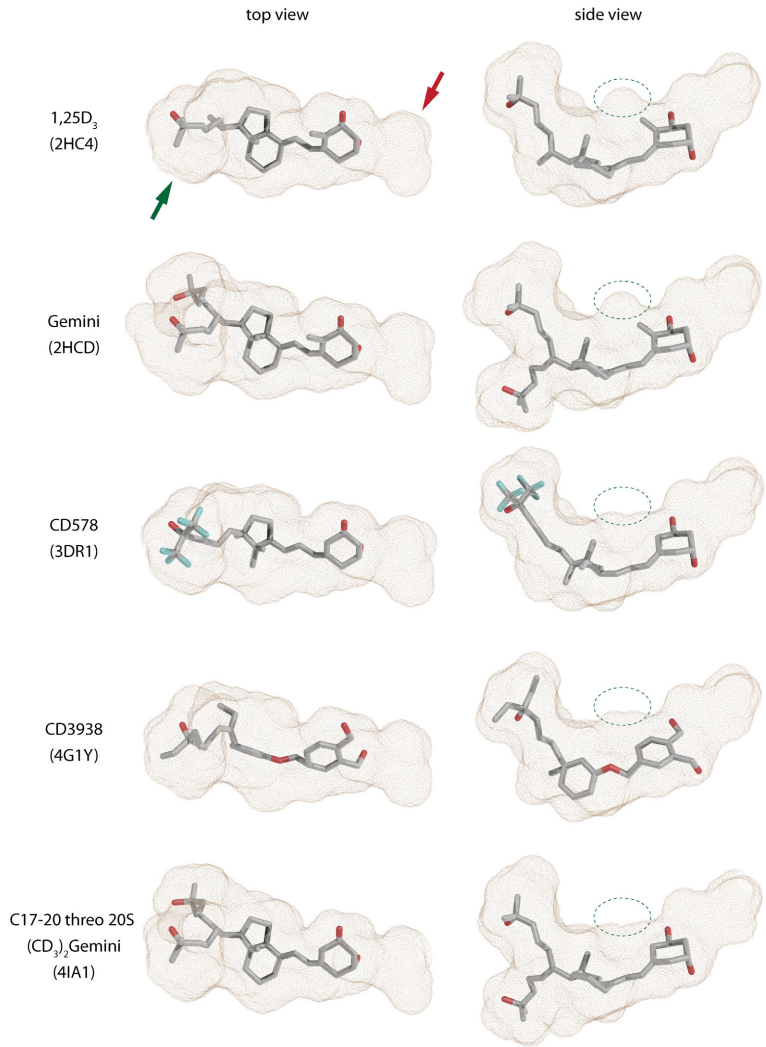

FIGURE 3 | Shapes of the zebrafish VDR ligand-binding pockets (LBPs) with various compounds (source PDBID: 2HC4, 2HCD, 3DR1, 4G1Y, and 4IA1).

be expanded by 1/3 of the $1,25D_3$ LBP volume. In proportion to this, the compound's volume in the pocket is also increased about 30%.

An appealing view opens up when a comparison of the VDR structure with one of its closest relative pregnane X receptor (PXR) is made (Watkins et al., 2001). As for VDR there are plenty of PXR crystal structures available and it can extend its LBP to very large volume (∼1400 Å^3) to accommodate various compounds. The published VDR structures lack the insertion domain (Δ166–216 aa), but even without it the LBP as discussed earlier is as large as 700–800 Å^3 with MD simulations showing that it can expand beyond 1000 Å^3, which is already comparable to PXR's LBP. This may suggest that VDR is able to accommodate variety of other compounds in addition to $1,25D_3$ such as LCA/3-keto-LCA (Makishima et al., 2002). The later have been crystallized with rVDR and will be discussed in this review. The indications for binding additional compounds besides $1,25D_3$ are coming also from *P. marinus* (sea lamprey) where despite of lack of the calcified skeleton and teeth it may serve as a xenobiotic activator for detoxification by regulating P450 enzymes (Whitfield et al., 2003; Krasowski et al., 2005). However, it is yet to be determined whether there are existing other ligands that bind to VDR. One part of the LBD, which may allow the binding of these compounds is the insertion domain. Although its clear functional role has not been identified and it seems that it is not directly required for the binding of the $1,25D_3$ (Rochel et al., 2001), it may play some other roles. A mutation C190W was reported in patients that results in loss of $1,25D_3$ binding (Malloy et al., 1999), though this may be due to the disruption of the VDR structure by introducing a large bulky tryptophan residue. Secondly, the homologous part of PXR (142–431 aa) especially the occurrence of the two β-strands and the associated coiled regions are responsible for the expandability of the PXR's LBP thus an analogous role cannot be out ruled in case of VDR as well. There has been also proposed that an alternative pocket, which can be considered as an enlargement of the original pocket, is formed in the VDR that

extends toward the helix 2/β-sheet region of the LBD (Mizwicki et al., 2004). Especially this can be observed with covalently locked $1,25D_3$-derived compounds such as $1,25(OH)_2$-lumisterol, which has been showed by *in silico* docking studies.

Due to the space limitation this review cannot address and discuss all the VDR-ligand complexes, for some more details see text below or recent reviews (Carlberg and Molnár, 2012; Carlberg et al., 2012), but some of the structures with natural ligands will be discussed in more details. One of them is 3-epi-1α,25-dihydroxyvitamin D_3 (3-epi-$1,25D_3$) a $1,25D_3$ metabolite that has been shown to exhibit tissue specific activities comparable to $1,25D_3$ (Norman et al., 1993; Reddy et al., 2000). The structural analysis showed a binding mode very similar to that of $1,25D_3$ with interesting compensation for the lacking S278-3α-OH hydrogen bond for the epimer using water mediated contacts **Figure 4** (Molnár et al., 2011). Interestingly, the same water channel is present in the $1,25D_3$ complex and was observed with other complexes as well (Tocchini-Valentini et al., 2001; Hourai et al., 2006).

A widely accepted fact is that precursor 25-hydroxyvitamin D_3 ($25D_3$) and its metabolite 24R,25-hydroxyvitamin D_3 ($24,25D_3$) does not posses significant biological activities. However, it has been shown that in *Cyp27b1*$^{-/-}$ cells, that are unable to produce actively $1,25D_3$, the VD signaling may be primary mediated via $25D_3$ (Lou et al., 2010). It stays a matter of discussion that under physiological condition how much of the VD signaling is mediated via $1,25D_3$ vs. $25D_3$. $24,25D_3$ shows only weak potency of influencing VD signaling at concentration 500nM although an enhancement for human osteoblast differentiation has been shown at concentration 1 μM (van Driel et al., 2006). Docking and subsequent MD simulations have been done to see the binding mode of these compounds and it has been confirmed that the position of residues mediating the anchoring hydrogen bonds are conserved with the exception of R274 which is located further than 3.5 Å from $24,25D_3$ **Figure 5**. Another residue that is unable to make a binding contribution is S237, but its position in the pocket is maintained. The void created by the missing R274 increases the LBP and lowers the occupancy factor for this compound. The analysis of the simple binding mode confers the activity range from $1,25D_3 > 25D_3 > 24,25D_3$. This also shows in general the importance of the 1α-OH group for the potency of the VDR agonist.

Recently, the crystal structures for another group of natural ligands have been solved. From the identification of secondary bile acids as VDR agonist (Makishima et al., 2002) the interesting question remained how these compounds bind to VDR. From the structural data it is evident that the litocholic acid (LCA) and 3-keto litocholic acid (3kLCA) are located in the opposite orientation than $1,25D_3$ (**Figure 6**). The 24-carboxyl group faces the β-turns of VDR, the β-region of the steroid backbone the helix 6–7/11 region and the A-ring is in the direction of helix 12. The S274 (hVDR S278) and Y143 hydrogen bonds are conserved

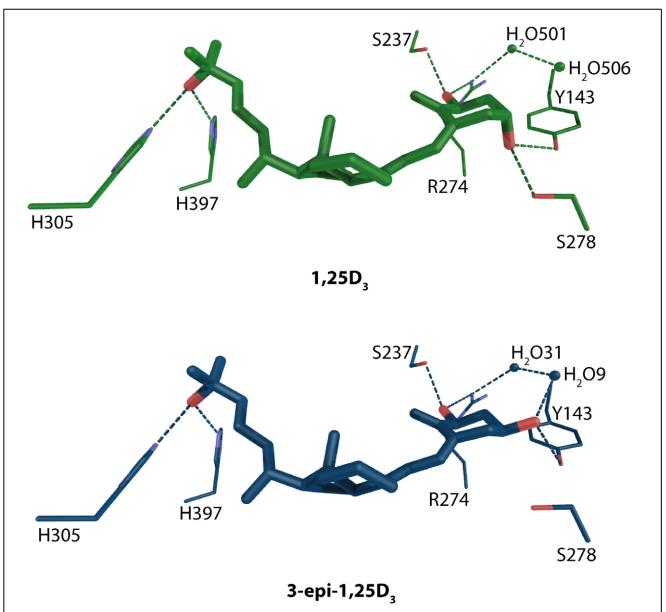

FIGURE 4 | Binding mode of 1α, 25-dihydroxyvitamin D_3 and 3-epi-1α,25-dihydroxyvitamin D_3 in the LBP of human VDR (source PDBID: 1DB1 and 3A78). $1,25D_3$ and 3-epi-$1,25D_3$ are highlighted in green and blue color, respectively. The hydrogen bonds are in green and blue dashed lines.

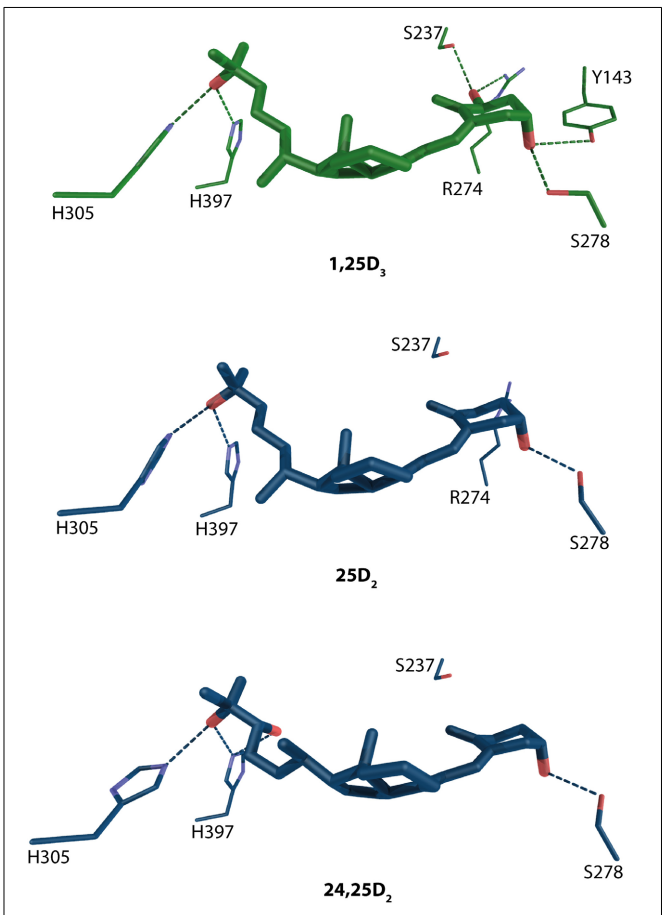

FIGURE 5 | Binding mode of 1α, 25-dihydroxyvitamin D_3, 25-hydroxyvitamin D_3 and its metabolite 24R,25-hydroxyvitamin D_3 in the LBP of human VDR (source MD simulation Lou et al., 2010). $1,25D_3$ and $25D_3$/$24,25D_3$ and are highlighted in green and blue color, respectively. The hydrogen bonds are in green and blue dashed lines.

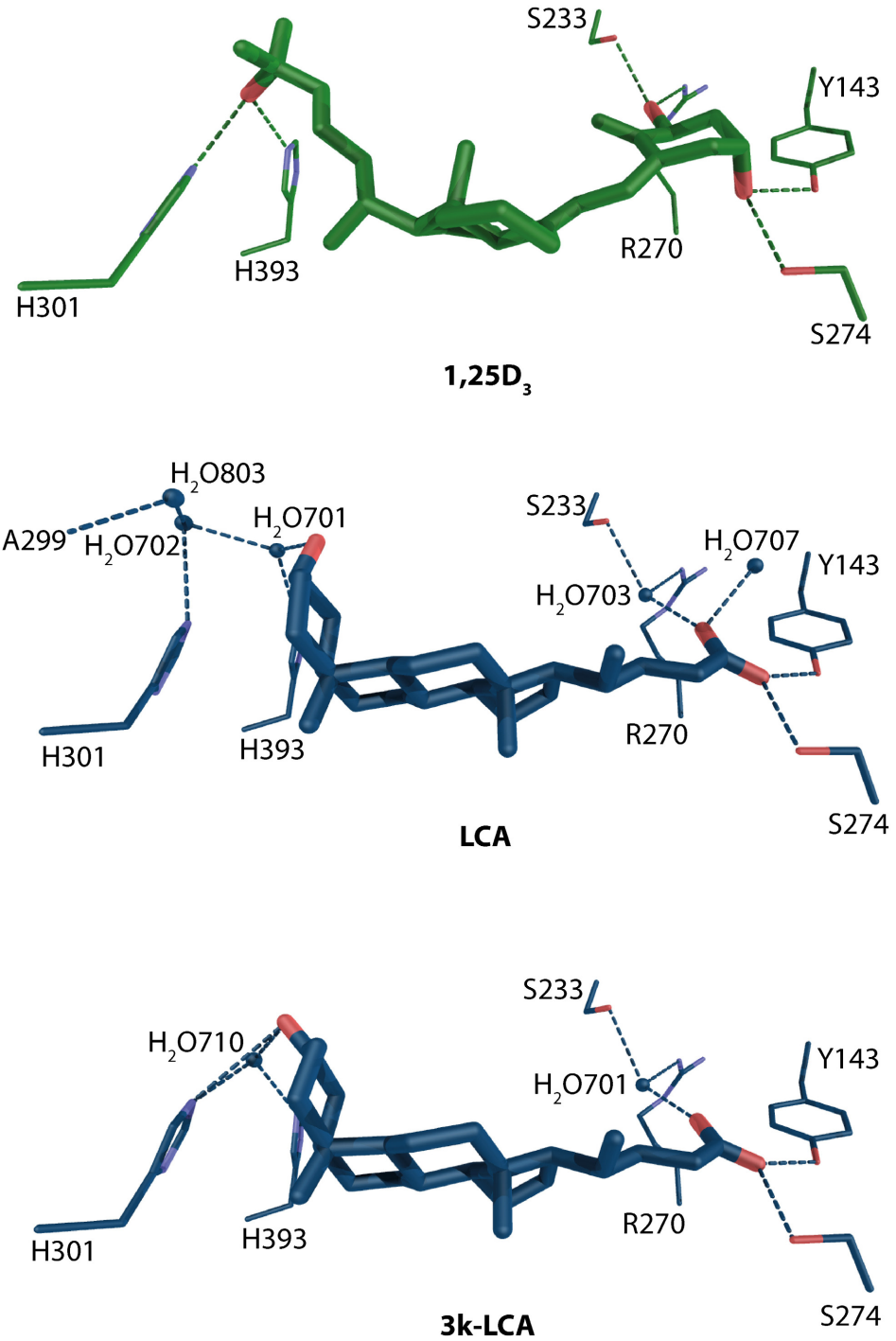

FIGURE 6 | Binding mode of 1α, 25-dihydroxyvitamin D_3, lithocholic acid and 3-ketolitocholic acid in the LBP of rat VDR (source PDBID: 1RK3, 3W5P, and 3W5Q). 1,25D_3 and LCA/3kLCA and are highlighted in green and blue color, respectively. The hydrogen bonds are in green and blue dashed lines.

in all structures (**Figure 6**). The difference for this part of the ligand-binding comes from the water mediated contacts with both R270 (hVDR R274) and S233 (hVDR S237). These contacts seem to be weaker compare to 1,25D_3 but not so weak as in case of 25D_3 and 24,25D_3, where in fact they are missing due to lack of 1α-OH group. The opposite part of the bile acids shows also weaker hydrogen bonding network than for the previously discussed VD metabolites including 1,25D_3. The structural comparison between the two bile acids shows a less complex, more straightforward binding mode for 3kLCA with only one water molecule involved. Whereas, for LCA there are no direct contacts with H301 (hVDR H305) and H393 (hVDR H397). All these

contacts are mediated thorough waters. This also may explain the lower VDR activation potential of 3kLCA compared to LCA.

I would seems that the evolutionary design of VDR reached by recognizing $1,25D_3$ its perfection. All the anchoring points show effective hydrogen bonding and by looking at the limited data for natural ligands any deviation from $1,25D_3$ will result in the use of alternative bridging contacts such water molecules. This shows a rather limited adaptability of VDR in effectively recognizing its natural ligands, yet larger than we envisioned a decade ago. One must keep in mind that the activation of VDR with one or another metabolite will largely depend on the local cellular concentration of this compounds leading for instance for favored activation via bile acids instead of classical $1,25D_3$ binding.

DNA-RECOGNITION AND BINDING

VDR belongs to the class of zinc finger transcriptional factors with DBD that consists of a highly conserved 66 aa residue core (Khorasanizadeh and Rastinejad, 2001) and an adjacent C-terminal extension. The conserved core has two zinc fingers where one contains fours cystein residues per atom of zinc (**Figure 7**). This feature allows VDR to effectively recognize and bind hormone response elements (REs) - termed VDREs. VDREs are

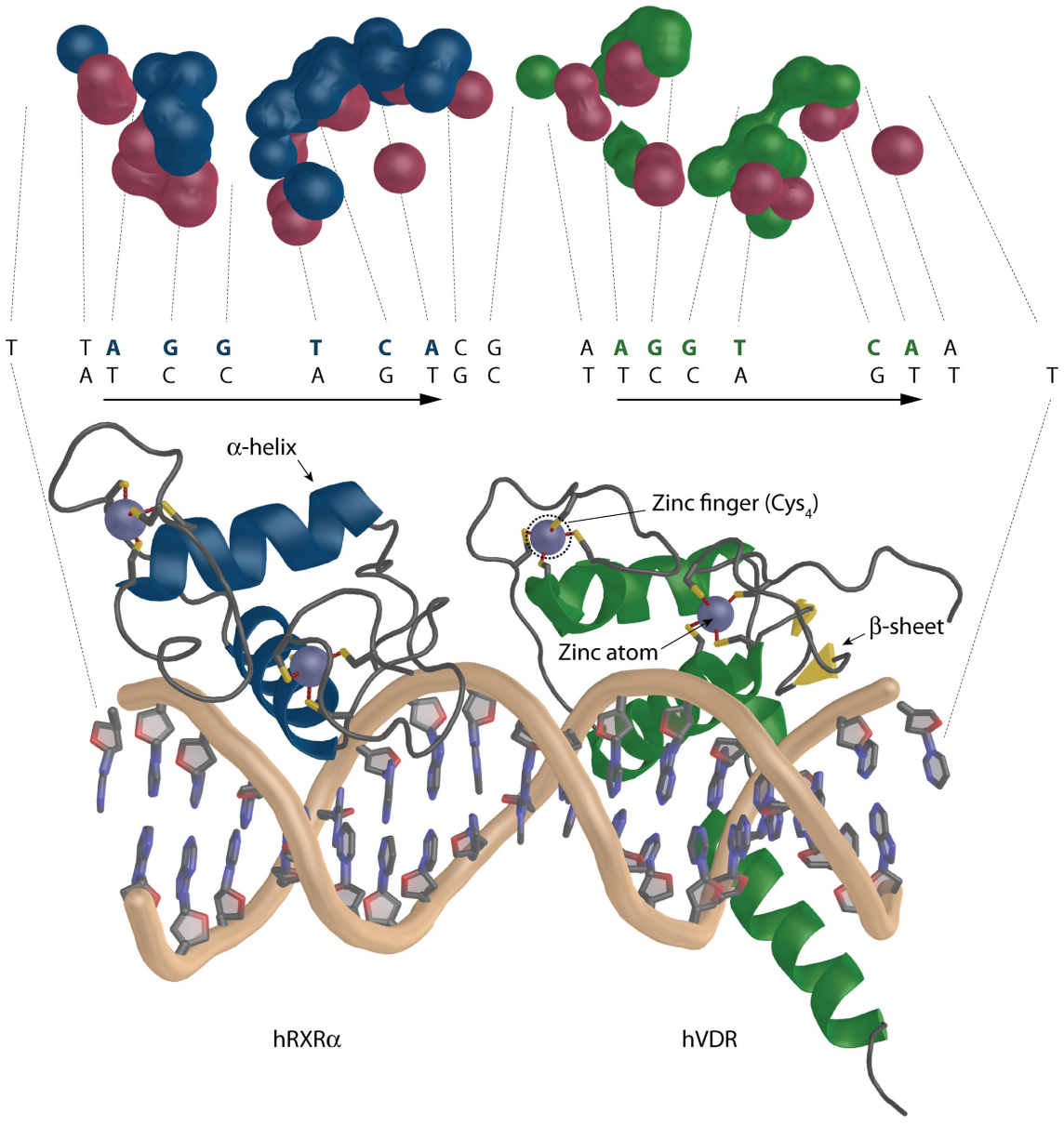

FIGURE 7 | The overall architecture of the DBD complex of RXR-VDR on canonical DR3 element (PDBID: 1YNW). The two zinc atoms (light blue spheres) with the respective cysteins are shown (bottom). RXR is shown in blue and VDR in green. The coiled protein regions are in gray and β-sheets in yellow. The surface representation of the contact atoms interacting between DNA and the heterodimer is shown (top). The proteins and DNA are visualized in different color DNA (red), RXR (blue), and VDR (green).

typically made up of two hexameric half-sites whose consensus sequence is 5′-RGKTCA-3′, where R = A or G and K = G or T. The half-sites may be arranged in various orientation most commonly forming a direct repeat with three neutral base pairs separating the half-sites (DR3) (Umesono et al., 1991). The unliganded VDR can occupy its REs also as a homodimer (Carlberg et al., 1993). Upon binding of ligand, VDR interacts with RXR and forms a heterodimeric complex that binds to VDREs with 5′-prime bound RXR.

The data for structural view on VDR-DNA recognition is scarce. To date we have only four structures published where three represent VDR homodimers on direct repeat 3 (DR3) from mouse osteopontin (mSPP) (CAC*GGTTCA*CGA*GGTTCA*), rat osteocalcin promoter (rOC)(CAC*GGGTGA*ATG*AGGACA*) and a canonical DR3 element (cDR3) (CAC*AGGTCA*CGA*AGGTCA*) (**Table 1**) (Shaffer and Gewirth, 2002). The last structure represents the DBDs of the heterodimeric RXR-VDR on canonical DR3 element (cDR3) (TT*AGGTCA*CGA*AGGTCA*A) (**Table 1**) (Shaffer and Gewirth, 2004). The 66 to 70 aa of the DBD are structurally coordinated by two zinc atoms that create a structure (**Figure 7**), in which one short α-helix is interacting directly with the major groove of the DNA (Härd et al., 1990; Lee et al., 1993). The VDR homodimers show asymmetric head to tail arrangements. The experimentally determined range of affinities of DR3s used for crystallographic studies are mSPP > cDR3 > rOC with mSPP supporting both VDR homodimer and RXR-VDR heterodimer binding, cDR3 requiring 10x higher VDR homodimer protein levels and weak heterodimer binding, whereas rOC is unable to bind VDR homodimers and has a very weak heterodimer binding ability (Freedman and Towers, 1991; Nishikawa et al., 1993; Toell et al., 2000). The change at third position, a purine to pyrimidine, of the consensus half sites AG*G*TCA allows the additional water mediated hydrogen contact of E42 with the DNA, which increase the stability of the mSPP-VDR complex. In case of rOC the reason for diminished VDR homodimer binding is because there is a G at position five of the upstream half-site GGGTGA, where supposedly RXR is bound in case of heterodimer binding. In high affinity half-sites in this location there is a C in the first strand but a complementary G in the second DNA strand. The interaction, which involves hydrogen bonds is between the R50 of VDR and the G from the second complementary strand. In rOC upstream half-site instead of G there is a C in the second strand, which is not a hydrogen bond acceptor thus R50 cannot interact with it. There is also some agreement between the strength of the homodimer binding and a sum of all existing DNA-VDR contacts within 3.5 Å calculated with the *ncont* program of the CCP4 suite (Winn et al., 2011). The results show 88, 85, and 83 contacts for rOC, cDR3, and mSPP, respectively. Interesting is also the interacting surface ratio between the two VDR homodimer molecules in percentage (5′upstream: 3′downstream) 38.52:61.48% for rOC, 46.21: 53.8% for the cDR3 and a reversed ratio of 57.3:42.7% for mSPP. This would suggest that for strong VDR homodimer binding there is a more contribution from the upstream half-site, but a clear conclusion cannot be reached based on this limited data set. In the RXR-VDR crystal structure there are two asymmetric units where the full complex shows an orientation RXR to VDR for unit 2 and a reversed orientation VDR to RXR for unit 1 with RXR bound on the downstream part of the VDRE. This may be due to the stabilization contacts between the adjacent VDR molecules where the hinge of one VDR molecule is stabilized with the DBD of the second VDR molecule. The general organization of the unit 2 is depicted also on the **Figure 7** bottom. The two zinc atoms (light blue color) with the respective cysteins are visualized (the second heterodimer unit is missing from the representation). The overall number of contacts is only 65, which is much lower than in case of VDR homodimers discussed earlier. This indicates that the binding of the RXR-DBD heterodimer to cDR3s is not optimal and for an effective binding a certain point of VDRE degeneration is needed. Moreover, the contribution of the monomers to the binding is rather interesting. The ratio between interacting monomeric surfaces is 56.22:44.26% (RXR-VDR) suggesting a higher contribution of RXR to the binding **Figure 7** top (see also the H/D exchange experiments discussed below). This is most likely due to the reverse orientation of the RXR-VDR on the cDR3. The surfaces of the interacting atoms are visualized in different color DNA (red), RXR (blue) and VDR (green). It is to be noted that compared to DNA-protein interaction there are hardly any interactions between the protein monomers. None for RXR-VDR, for VDR homodimers there are two for mSPP and rOC, and five on cDR3. This is quite in agreement with full length receptor studies which suggest that most of the heterodimerisation is contributed from hinges and LBDs.

THE OUTSIDE SHELL: PARTNERING AND THE COMPLEX VIEW

VDR INTERACTING PROTEIN PARTNERS

For VDR to function effectively as a regulator of transcription it is inevitable to interact with various protein partners. They show high structural and functional diversities ranging from enzymes, co-integrators and cofactors to components of distinct transduction signaling pathways. A comprehensive list of these partners with the accompanying citations are listed in **Table 2**.

One of the first complex identified using co-immunoprecipitation from mammalian cells was the VDR interacting protein DRIP complex, which is recruited to VDR in a completely ligand-dependent manner (Rachez et al., 1999). Many of the its components were shared with the earlier identified thyroid hormone receptor (TR) interacting protein complex TRAP (Fondell et al., 1996). It has been no surprise that majority of the interacting proteins can be related directly to transcriptional regulation such as subunits of the mediator complex MED1, 4, 6, 7, 12, 16, 17, and 23 (Rachez et al., 1999) or cofactors such as coactivators NCOA1-3 (Hong et al., 1997; Castillo et al., 1999; Molnár et al., 2005), NCOA6 (Mahajan and Samuels, 2000), ARA54 (Ting et al., 2005), SKIP (Baudino et al., 1998), RBP2 (Chan and Hong, 2001), SRB7 (Ito et al., 1999) and corepressors Alien (Polly et al., 2000), NCOR1 and 2 (Tagami et al., 1998; Kim et al., 2009), SIN3A (Fujiki et al., 2005), LCOR (Fernandes et al., 2003). Others show more selective properties functioning as coactivators and corepressors depending on the particular conditions such as RIP140 (Albers et al., 2005) or TIF1α (Thénot et al., 1997). Some are implicated in cellular processes such as cell cycle regulation CDK7 (Nevado et al., 2004), RAP46 (Guzey et al., 2000), DNA repair

Table 2 | List of VDR interacting proteins.

Name	Gene symbol/alternative name	Role	References
Alien	ALIEN	Transcriptional corepressor	Polly et al., 2000
Androgen receptor-associated protein 54	ARA54*	Transcriptional coactivator	Ting et al., 2005
Androgen receptor-associated protein 70	ARA70	Transcriptional coactivator implicated in cancer	Ting et al., 2005
Brahma-related gene 1	BRG1/SMARCA4	ATPase subunit of the SWI/SNF complex	Fujiki et al., 2005
CREB-binding protein	CBP	Transcriptional cointegrator	Castillo et al., 1999
Cyclin D3	CCD3	Subunits of the cyclin-dependent kinases	Jian et al., 2005
Cyclin-dependent kinase 7	CDK7/hMo15	Component of the TFIIH transcription complex	Nevado et al., 2004
CXXC finger 5	CXXC5	Cell cycle regulation	Marshall et al., 2012
E1A binding protein p300	p300	Transcriptional cointegrator	Kim et al., 2005
Fas-activated serine/threonine kinase	FASTK	Involvement in splicing	Marshall et al., 2012
Feline Gardner-Rasheed sarcoma viral oncogene homolog	FRG	Signal transduction (protein tyrosine kinase)	Ellison et al., 2005
General transcription factor IIB	TFIIB	Subunit of the basal transcription machinery	Nevado et al., 2004
Hairless	HR	Transcriptional corepressor	Hsieh et al., 2003
High mobility group nucleosomal binding domain 3	HMGN3/TRIP7	Possible chromatin modifier	Albers et al., 2005
Histone deacetylase 2	HDAC2	Histone modifier	Fujiki et al., 2005
Ligand-dependent NR corepressor	LCOR	Transcriptional corepressor	Fernandes et al., 2003
Mediator complex subunit 1	MED1/TRAP220/RIP205/PPARBP	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 4	MED4/DRIP36/p34	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 6	MED6/DRIP33	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 7	MED7/DRIP34	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 12	MED12/DRIP240/ARC240/TRAP230	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 16	MED16/DRIP92/TRAP95	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 17	MED16/DRIP77/TRAP80	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mediator complex subunit 23	MED23/DRIP130/CRSP130	Transcriptional regulation/part of the mediator	Rachez et al., 1999
Mothers against decapentaplegic homolog 3	SMAD3	Transcriptional coactivator	Yanagisawa et al., 1999
Myosin light chain 3	MYL3	Regulatory light chain of myosin	Marshall et al., 2012
NR coactivator 1	NCOA1/SRC1	p160 family coactivator	Castillo et al., 1999
NR coactivator 2	NCOA2/TIF2/GRIP1	p160 family coactivator	Hong et al., 1997)
NR coactivator 3	NCOA3/RAC3/SRC3/AIB1	p160 family coactivator	Molnár et al., 2005
NR coactivator 6	NCOA6/PRIP/ASC2	Transcriptional coactivator	Mahajan and Samuels, 2000
NR corepressor 1	NCOR1	Transcriptional corepressor	Tagami et al., 1998
NR corepressor 2	NCOR2/SMRT/TRAC2	Transcriptional corepressor	Kim et al., 2009
NR subfamily 0, group B, member 2	NR0B2 (SHP)	Negative transcriptional regulator	Albers et al., 2005
NR subfamily 4, group A, member 1	NR4A1 (NGFIB)	Expression genes during liver regeneration	Marshall et al., 2012
p53	PT53	Tumor supression	Stambolsky et al., 2010

(Continued)

Table 2 | Continued

Name	Gene symbol/alternative name	Role	References
Receptor-associated protein 46	RAP46/BAG1	Regulation of cell growth in response to stress	Guzey et al., 2000
Receptor-interacting protein 140	RIP140/NRIP1	Coregulator with selective properties	Albers et al., 2005
Retinoblastoma 1	RB	NR coregulator/tumor suppressor	Chan and Hong, 2001
Retinoblastoma-binding protein 2	RBP2	Transcriptional coactivator	Chan and Hong, 2001
Retinoid X receptor α	RXRα	Heterodimeric VDR partner	Liu et al., 2000
Retinoid X receptor β	RXRβ	Heterodimeric VDR partner	Rachez et al., 1999
Retinoid X receptor γ	RXRγ	Heterodimeric VDR partner	Albers et al., 2005
Protooncogene c jun	JUN	Transcriptional factor	Towers et al., 1999
SIN3 homolog A, transcriptional regulator (yeast)	SIN3A	Transcriptional corepressor/cointergrator	Fujiki et al., 2005
SKI interacting protein	SKIP/SNW1/NCoA-62	Transcriptional coactivator	Baudino et al., 1998
Suppressor of RNA polymerase B 7	SRB7	Transcriptional coactivator	Ito et al., 1999
Thymine-DNA glycosylase	TDG	Coregulator/base excision repair	Chen et al., 2003
Thyroid receptor-interacting protein 1	TRIP1/SUG1/PSMC5	CAD (Conserved ATPase domain) protein	Masuyama and Hiramatsu, 2004
Transcriptional intermediary factor 1	TIF1α/CCCP	Coregulator with selective properties	Thénot et al., 1997
Tropomyosin	TPM2	Possible role in receptor Internalization	Marshall et al., 2012
Vitamin D receptor-interacting protein (100kD)	DRIP100/ARC100/TRAP100	VDR associated DRIP complex	Rachez et al., 1999
Vitamin D receptor-interacting protein (150kD)	DRIP150/ARC150/TRAP170	VDR associated DRIP complex	Rachez et al., 1999
Vitamin D receptor-interacting repressor	VDIR/TCF3/ITF1	Negative regulator of the CYP27B1	Kim et al., 2007
Williams syndrome transcription factor	WSTF/BAZ1B	Recruitment of unliganded VDR to target promoters	Fujiki et al., 2005
Xin actin-binding repeat containing protein 1	XIRP1	Protects actin filaments from depolymerization	Marshall et al., 2012

**no direct physical interaction but has positive effect on VDR transactivation.*

TDG (Chen et al., 2003) or signaling cascade FRG (Ellison et al., 2005). Interesting is the interaction and thus possible crosstalk with other NRs such as SHP (Albers et al., 2005), which lacks DBD and has corepressor-like behavior, and NGFIB, which has been shown to have a role during liver regeneration (Marshall et al., 2012). Some of the newly identified protein partners that may implicate VDR's involvements in new processes are XIRP1 that protects actin filaments from depolymerization or MYL3 a regulatory light chain of myosin (Marshall et al., 2012).

STRUCTURAL DATA OF SRC1 AND MED1/DRIP205 INTERACTION WITH VDR

There is a big limitation in obtaining large transcriptional complexes, which is firstly due to the transient nature of the complex where VDR serves as a docking and acquiring platform bringing other proteins that either act as chromatin modifiers, parts of the mediator, of various cofactors and bridging factors to the close proximity of the functional VDREs. The complex may be assembled for a short moment to initiate and/or repress the transcription thereafter fulfilling this action it falls apart. The second reason might be that many of the interacting proteins such as cofactors show a high degree of disorder. The crystallization of unfolded proteins is very tricky and many times even impossible. The intrinsic disorder of a VDR interacting proteins is an expected structural property since e.g., members of the p160 general coactivator family have to adopt and interact with many various transcription factors. Thus structural data from only short interacting peptides derived from steroid receptor coactivator 1 (SRC1) with zVDR (**Figure 8A**) and mediator complex subunit 1 (MED1/DRIP205) with rVDR are available showing only the core interaction between VDR and the LXXLL motif of coactivators (**Figure 8B**). Both peptides interact in a very similar fashion. The α-helix of the peptide is oriented with its N-terminus toward helix 12. The two peptides interact through their LXXLL motifs, LHRLL in SRC1 and LMNLL in MED1, and most of the interaction is contributed from hydrophobic contacts of coactivator's leucine residues with the hydrophobic core from VDR helices 3, 4, and 12. The anchoring points of the short α-helix are based on the interaction with the "charge clamp" consisting of the conserved glutamate in helix 12 and lysine in helix 3, and the backbone amides of the coactivator peptide. The similar interaction of the two LXXLL motifs raises the question on how the

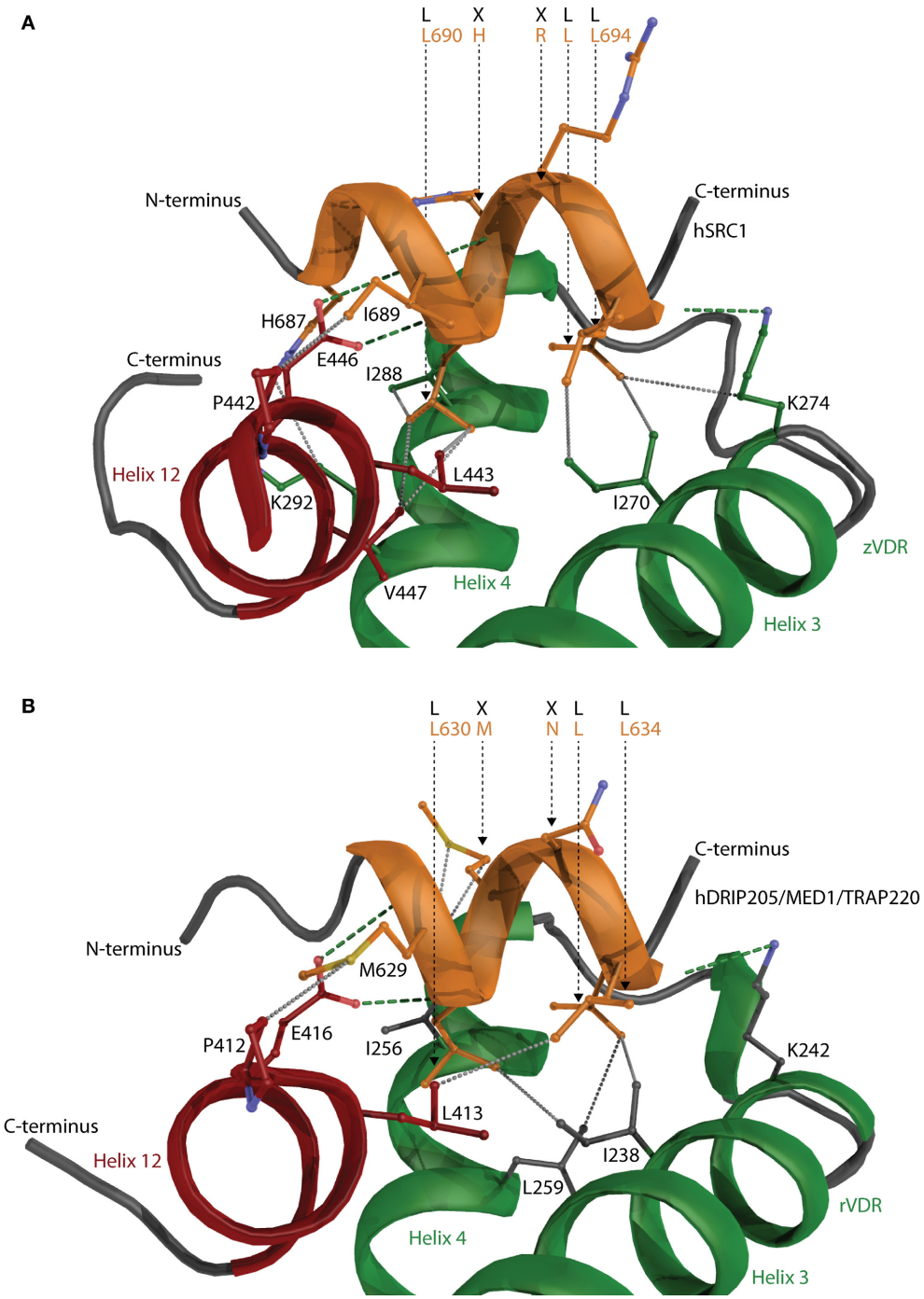

FIGURE 8 | The interaction of coactivator peptides with VDR. Peptides derived from **(A)** steroid receptor coactivator 1 (SRC1) with zVDR and **(B)** mediator complex subunit 1 (MED1/DRIP205) with rVDR shown. SRC1 and MED1 is shown in orange and VDR in green. Helix 12 is highlighted in red. The hydrogen bonds and hydrophobic interactions are visualized with green and gray dashed lines, respectively. The important residues such as the conserved "charge clamp" glutamate from helix 12 and lysine in helix 3 contributing to the CoA-VDR interaction are also shown.

specificity is achieved in the interaction. The situation is complicated with the fact that some of the coactivators have more than one interaction motif such as SRC1 has five of them that are similar or related to LXXLL motifs, but so far only three of them were reported/studied in detail.

SAXS, CRYO-EM, AND H/D EXCHANGE STUDIES WITH FULL LENGTH RXR-VDR COMPLEXES

The recent studies with VDR complexes in solution that employed the use of modern techniques such as SAXS, cryo-EM, HDX with full length VDR-RXRα showed somewhat more comprehensive

perspective for the 3D organization and possible function of the VDR-RXRα-cofactor complex. The SAXS (Rochel et al., 2011) and cryo-EM (Orlov et al., 2012) derived model is shown in **Figure 9A**.

Upon binding to DNA from osteocalcin VDRE, the SAXS data derived RXR-VDR shows an elongated asymmetric open conformation with separate DBDs and LBDs and a well structured VDR hinge with VDR located downstream and RXR on upstream half sites (Rochel et al., 2011) (**Figure 9B**). On contrary the coiled structure of the RXR hinge allows its adaptability to different REs. The hinges play an important role in an open conformation. The hinges also underly one of the very important feature of the RXR-VDR complex, the dynamic character. The DBD binding to DR3 results in a rotation of the LBD dimers take around 90° with respect to the DNA (**Figure 9A**). The same study showed that coactivators MED1/DRIP205 and SRC1 have higher affinity to VDR compared to RXR. This points to the binding of only one molecule of coactivator through VDR, which is not supporting the "deck model" of binding (one LXXLL motif to VDR and the other to RXR) for these coactivators. Studies using mutants show the preferential binding of VDR to the second LXXLL motif of MED1/DRIP205 compared the weak recruitment of the first motif to RXR (Ren et al., 2000). However, both motifs are crucial for the effectivity of the NR activation complex *in vivo* (Malik et al., 2004). RXR may play a role in coactivator recruitment as well by associating to some other factors.

The cryo-EM studies of the RXR-VDR have a higher resolution than SAXS data and it is possible to obtain more precise electron densities for VDR A/B and hinge domains for both receptors (Orlov et al., 2012). The heterodimer takes an L-shape form on the DR3 with a proper orientation of RXR on the upstream and VDR on the downstream half site (similar to SAXS model). The complex supports also the asymmetric open architecture from SAXS data. Both LBDs are positioned above the 5′ upstream half site as shown on **Figure 9A**. This result also emphasizes the importance of the hinges for the correct function of the complex. The main nature of the flexible hinge of RXR allows to contact the CTE helix with helix 1 and makes it possible to adopt differential spacing in DR REs. In addition, the coiled RXR hinge has to be long to reach DBD to its LBD which is located on the opposite side of the DR3 VDRE. The cryo-EM data points to one interesting feature, the potential in modulation of the DNA-binding using the 17 aa long A/B domain of VDR, which in fact interacts with the major groove of the DNA.

The H/D exchange (HDX) is a great tools to address the dynamic properties of the RXR-VDR-SRC1 complex (Zhang et al., 2011). The addition of RXR to VDR stabilizes region with helices 6–7, very similar to what is seen when some of agonists bind to VDR. As expected, upon 1,25D_3 binding in the VDR LBD the helices 1, 3, 5–7, and 11 (the actual region that forms the LBP) have been stabilized, but binding efficiency of RXR to VDR is not enhanced. For RXR the helices 7 and 10 are stabilized (increase of the heterodimerisation) and an allosteric communication has been shown for the helix 3 of RXR. The 9-*cis* RA binding in general stabilized RXR, but in contrast to the crystal structure, it increased the fluctuation in helix 12. This observation may also indicate that the crystal structure take the minimized energy conformation of the complex, but in reality it is more dynamic. The allosteric communication in VDR upon 9-*cis* RA binding was seen in the helices 3, 5, and 7 that are adjacent from the heterodimerisation interface pointing to modulation of the complex upon only 9-*cis* RA binding. Surprisingly, the concurrent binding of 1,25D_3 and 9-*cis* RA has a destabilizing affect

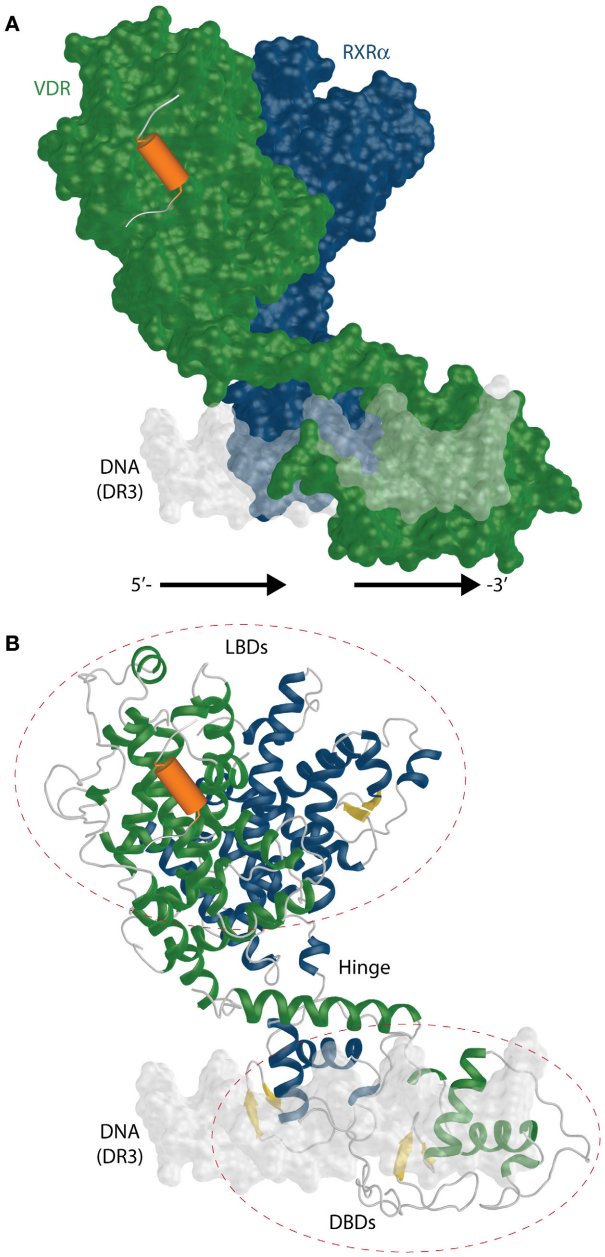

FIGURE 9 | The full length RXR-VDR structural model derived from SAXS and cryo-EM experiments. (A) A surface representation of the RXR(blue)-VDR(green) heterodimer is shown on DR3 VDRE. The possible location of the coactivator peptide (orange) is highlighted as well. The 5′- and 3′-prime orientation of the DR3 is annotated. **(B)** Ribbon representation of the same complex shows the relative organization and fold of LBD, DBD and the connective hinge between them. The β-sheets are shown in yellow color.

on the VDR DBD. A stronger increase of the VDR binding has been observed compared to RXR DBD in the presence of cDR3 element and in the absence of ligands, pointing to the higher contribution of the VDR for DNA interaction. In addition, interesting allosteric stabilization were observed for the VDR hinge and for helices 7–8 and 9–10. Moreover, unexpectedly the helix 12 of VDR showed increased fluctuation. However, it should be noted that in this experimental setup a coactivator has been missing from the protein complex (see below). The binding to the natural VDRE from *CYP24A1* gene showed similar result except it seemed that 5′-AGGTCA-3′ half-site was occupied by VDR and helix 12 of RXR was more stable. Unexpectedly, the stability of the heterodimer on *CYP24A1* VDRE was reduced although the binding affinities of the two VDREs are in the same magnitude. The interaction of the coactivator SRC1, that contained three LXXLL motifs, with the heterodimer bound to both $1,25D_3$ and 9-*cis* RA increased the stability of VDR's helix 12 and helices 3 and 10–11 of RXR. Helices 3 and 4 of VDR cannot be further stabilized since they achieve maximal stabilization upon $1,25D_3$ binding. For RXR the loop between helices 10 and 11 is important in the formation of the hydrophobic groove facilitating coactivator binding. Besides the classical charge clamp RXR contains the so called "aromatic clamp" consisting of residues in helices 3 (F437, F277) and 12 (F450) that is important for coactivator binding. As expected in the absence of both ligand no coactivator interaction was observed and the separate addition of the $1,25D_3$ or 9-*cis* RA recruited the coactivator in a ligand-specific manner. Further HDX and cell based experiments showed that a simultaneous binding of the coactivator to both receptors is important and in the interaction with the RXR-VDR heterodimer only one SRC1 molecule is required (Zhang et al., 2011). This is in contrast to the SAXS derived model (Rochel et al., 2011). For the RXR-VDR-SRC1 complex the helix 12 of VDR has been stabilized upon addition of the DNA. In addition, HDX shows that the DNA-binding enhances the recruitment of SRC1 to RXR, thus the binding of the DNA stabilizes the recruitment of SRC1 to the whole heterodimer not just to the partner such as VDR.

Both, the SAXS and cryo-EM, studies highlighted the open architecture conformation in solution unlike it has been shown for the full length PPAR-RXR (Chandra et al., 2008). The recent crystal structure of the full length RXRα–LXRβ on DR4 RE provides also a support for the open conformation of the NR heterodimer complex (Lou et al., 2014). It will have to be seen whether the closed PPAR-RXR complex on DR1 is and exception, although more plausible is the open conformation giving a rather high dynamic freedom for the NR heterodimer in the large transcriptional complex.

CONCLUSIONS

The main aim of this review was to collect and discuss structural data that is related to vitamin D signaling. The structural data for various isolated domains (LBD and DBD) show their organization on atomic level. This data is sufficient for understanding the particular ligand- and DNA-protein interactions but fails to provide spatial information on the mutual orientation of the domains of RXR-VDR on its natural promoters. It also fails to highlight inter-domain communication after DNA-, ligand- or cofactor-binding. However, they are irreplaceable tools for structure-based drug design and mechanistic view of the VDR action. The dissemination of the information derived from structural data and *in silico* models may help to understand how VDR works in its natural settings and provides a space for the intervention in various diseases. The recent SAXS, Cryo-EM and H/D exchange studies with full length RXR-VDR complexes show more complex views on VDR function and provide first tools for the integration of structural information with genomic, epi-genomic, transcriptional and functional data.

FUTURE PERSPECTIVE

There are numerous scientific questions connected to VDR that may be interesting to answer on structural level. The list is not complete but here are some of them:

- Do the subtle differences between the various ligand-bound VDR complexes indeed represent the differences in the biological specificities and activities *in vivo*?
- Can the LBP related measurements and other descriptors be related to the potency of VDR ligands?
- Are there some additional natural compounds that are able to bind VDR and thus influence the metabolism and detoxification?
- What is the real role of the insertion domain in VDR?
- Is it possible to relate structurally the binding affinities of the RXR-VDR complexes with DNA?
- Are there other *in vivo* significant binding modes of VDR to the DNA such as monomers?
- What is the architecture of the RXR-VDR complex on non-DR3 VDREs and in indirect DNA interactions?
- How the recruitment specificity of the LXXLL containing proteins is regulated in the cell?
- How is the inter-domain communication precisely mediated upon ligand- DNA- and protein-VDR interaction?

REFERENCES

Albers, M., Kranz, H., Kober, I., Kaiser, C., Klink, M., Suckow, J., et al. (2005). Automated yeast two-hybrid screening for nuclear receptor-interacting proteins. *Mol. Cell. Proteomics* 4, 205–213. doi: 10.1074/mcp.M400169-MCP200

Antony, P., Sigüeiro, R., Huet, T., Sato, Y., Ramalanjaona, N., Rodrigues, L. C., et al. (2010). Structure-function relationships and crystal structures of the vitamin D receptor bound 2 α-methyl-(20S,23S)- and 2 α-methyl-(20S,23R)-epoxymethano-1 α,25-dihydroxyvitamin D_3. *J. Med. Chem.* 53, 1159–1171. doi: 10.1021/jm9014636

Asano, L., Ito, I., Kuwabara, N., Waku, T., Yanagisawa, J., Miyachi, H., et al. (2013). Structural basis for vitamin D receptor agonism by novel non-secosteroidal ligands. *FEBS Lett.* 587, 957–963. doi: 10.1016/j.febslet.2013.02.028

Baudino, T. A., Kraichely, D. M., Jefcoat, S. C., Winchester, S. K., Partridge, N. C., and MacDonald, P. N. (1998). Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *J. Biol. Chem.* 273, 16434–16441. doi: 10.1074/jbc.273.26.16434

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the ligand-binding domain of the human nuclear receptor RXRα. *Nature* 375, 377–82. doi: 10.1038/375377a0

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L. J., Grippo, J. F., et al. (1993). Two nuclear signalling pathways for vitamin D. *Nature* 361, 657–660. doi: 10.1038/361657a0

Carlberg, C., and Molnár, F. (2012). Current status of vitamin D signaling and its therapeutic applications. *Curr. Top. Med. Chem.* 12, 528–547. doi: 10.2174/156802612799436623

Carlberg, C., Molnár, F., and Mouriño, A. (2012). Vitamin D receptor ligands: the impact of crystal structures. *Exp. Opin. Ther. Pat.* 22, 417–435. doi: 10.1517/13543776.2012.673590

Castillo, A. I., Jiménez-Lara, A. M., Tolón, R. M., and Aranda, A. (1999). Synergistic activation of the prolactin promoter by vitamin D receptor and GHF-1: role of the coactivators, CREB-binding protein and steroid hormone receptor coactivator-1 (SRC-1). *Mol. Endocrinol.* 13, 1141–1154. doi: 10.1210/mend.13.7.0320

Chan, S. W., and Hong, W. (2001). Retinoblastoma-binding protein 2 (Rbp2) potentiates nuclear hormone receptor-mediated transcription. *J. Biol. Chem.* 276, 28402–28412. doi: 10.1074/jbc.M100313200

Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T., et al. (2008). Structure of the intact PPAR-γ-RXR-α nuclear receptor complex on DNA. *Nature* 456, 350–356. doi: 10.1038/nature07413

Chen, D., Lucey, M. J., Phoenix, F., Lopez-Garcia, J., Hart, S. M., Losson, R., et al. (2003). T:G mismatch-specific thymine-DNA glycosylase potentiates transcription of estrogen-regulated genes through direct interaction with estrogen receptor α. *J. Biol. Chem.* 278, 38586–38592. doi: 10.1074/jbc.M304286200

Ciesielski, F., Rochel, N., and Moras, D. (2007). Adaptability of the Vitamin D nuclear receptor to the synthetic ligand Gemini: remodelling the LBP with one side chain rotation. *J. Steroid Biochem. Mol. Biol.* 103, 235–242. doi: 10.1016/j.jsbmb.2006.12.003

Ciesielski, F., Sato, Y., Chebaro, Y., Moras, D., Dejaegere, A., and Rochel, N. (2012). Structural basis for the accommodation of bis- and tris-aromatic derivatives in vitamin D nuclear receptor. *J. Med. Chem.* 55, 8440–8449. doi: 10.1021/jm300858s

Eelen, G., Valle, N., Sato, Y., Rochel, N., Verlinden, L., de Clercq, P., et al. (2008). Superagonistic fluorinated vitamin D_3 analogs stabilize helix 12 of the vitamin D receptor. *Chem. Biol.* 15, 1029–1034. doi: 10.1016/j.chembiol.2008.08.008

Eelen, G., Verlinden, L., Rochel, N., Claessens, F., de Clercq, P., Vandewalle, M., et al. (2005). Superagonistic action of 14-epi-analogs of 1,25-dihydroxyvitamin D explained by vitamin D receptor-coactivator interaction. *Mol. Pharmacol.* 67, 1566–1573. doi: 10.1124/mol.104.008730

Ellison, T. I., Dowd, D. R., and MacDonald, P. N. (2005). Calmodulin-dependent kinase IV stimulates vitamin D receptor-mediated transcription. *Mol. Endocrinol.* 19, 2309–2319. doi: 10.1210/me.2004-0382

Fernandes, I., Bastien, Y., Wai, T., Nygard, K., Lin, R., Cormier, O., et al. (2003). Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. *Mol. Cell.* 11, 139–150. doi: 10.1016/S1097-2765(03)00014-5

Fischer, J., Wang, T.-T., Kaldre, D., Rochel, N., Moras, D., White, J. H., et al. (2012). Synthetically accessible non-secosteroidal hybrid molecules combining vitamin D receptor agonism and histone deacetylase inhibition. *Chem. Biol.* 19, 963–971. doi: 10.1016/j.chembiol.2012.05.024

Fondell, J. D., Ge, H., and Roeder, R. G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8329–8333. doi: 10.1073/pnas.93.16.8329

Fraga, R., Zacconi, F., Sussman, F., Ordóñez-Morán, P., Muñoz, A., Huet, T., et al. (2012). Design, synthesis, evaluation, and structure of vitamin D analogues with furan side chains. *Chemistry* 18, 603–612. doi: 10.1002/chem.201102695

Freedman, L. P., and Towers, T. L. (1991). DNA binding properties of the vitamin D_3 receptor zinc finger region. *Mol. Endocrinol.* 5, 1815–1826. doi: 10.1210/mend-5-12-1815

Fujii, S., Masuno, H., Taoda, Y., Kano, A., Wongmayura, A., Nakabayashi, M., et al. (2011). Boron cluster-based development of potent nonsecosteroidal vitamin D receptor ligands: direct observation of hydrophobic interaction between protein surface and carborane. *J. Am. Chem. Soc.* 133, 20933–20941. doi: 10.1021/ja208797n

Fujiki, R., Kim, M.-S., Sasaki, Y., Yoshimura, K., Kitagawa, H., and Kato, S. (2005). Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. *EMBO J.* 24, 3881–3894. doi: 10.1038/sj.emboj.7600853

Guzey, M., Takayama, S., and Reed, J. C. (2000). BAG1L enhances trans-activation function of the vitamin D receptor. *J. Biol. Chem.* 275, 40749–40756. doi: 10.1074/jbc.M004977200

Härd, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., et al. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 249, 157–160. doi: 10.1126/science.2115209

Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol. Cell. Biol.* 17, 2735–2744.

Hourai, S., Fujishima, T., Kittaka, A., Suhara, Y., Takayama, H., Rochel, N., et al. (2006). Probing a water channel near the A-ring of receptor-bound 1 α,25-dihydroxyvitamin D_3 with selected 2 α-substituted analogues. *J. Med. Chem.* 49, 5199–5205. doi: 10.1021/jm0604070

Hourai, S., Rodrigues, L. C., Antony, P., Reina-San-Martin, B., Ciesielski, F., Magnier, B. C., et al. (2008). Structure-based design of a superagonist ligand for the vitamin D nuclear receptor. *Chem. Biol.* 15, 383–392. doi: 10.1016/j.chembiol.2008.03.016

Hsieh, J.-C., Sisk, J. M., Jurutka, P. W., Haussler, C. A., Slater, S. A., Haussler, M. R., et al. (2003). Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. *J. Biol. Chem.* 278, 38665–38674. doi: 10.1074/jbc.M304886200

Huet, T., Maehr, H., Lee, H. J., Uskokovic, M. R., Suh, N., Moras, D., et al. (2011). Structure–function study of gemini derivatives with two different side chains at C-20, Gemini-0072 and Gemini-0097. *Med. Chem. Commun.* 2:424. doi: 10.1039/c1md00059d

Inaba, Y., Nakabayashi, M., Itoh, T., Yoshimoto, N., Ikura, T., Ito, N., et al. (2010). 22S-butyl-1α,24R-dihydroxyvitamin D_3: recovery of vitamin D receptor agonistic activity. *J. Steroid Biochem. Mol. Biol.* 121, 146–150. doi: 10.1016/j.jsbmb.2010.02.033

Inaba, Y., Yoshimoto, N., Sakamaki, Y., Nakabayashi, M., Ikura, T., Tamamura, H., et al. (2009). A new class of vitamin D analogues that induce structural rearrangement of the ligand-binding pocket of the receptor. *J. Med. Chem.* 52, 1438–1449. doi: 10.1021/jm8014348

Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., et al. (1999). Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol. Cell.* 3, 361–370. doi: 10.1016/S1097-2765(00)80463-3

Jian, Y., Yan, J., Wang, H., Chen, C., Sun, M., Jiang, J., et al. (2005). Cyclin D_3 interacts with vitamin D receptor and regulates its transcription activity. *Biochem. Biophys. Res. Commun.* 335, 739–748. doi: 10.1016/j.bbrc.2005.07.141

Kakuda, S., Ishizuka, S., Eguchi, H., Mizwicki, M. T., Norman, A. W., and Takimoto-Kamimura, M. (2010). Structural basis of the histidine-mediated vitamin D receptor agonistic and antagonistic mechanisms of (23S)-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone. *Acta Crystallogr. D Biol. Crystallogr.* 66, 918–926. doi: 10.1107/S0907444910020810

Kakuda, S., Okada, K., Eguchi, H., Takenouchi, K., Hakamata, W., Kurihara, M., et al. (2008). Structure of the ligand-binding domain of rat VDR in complex with the nonsecosteroidal vitamin D_3 analogue YR301. *Acta Crystallogr. Sect F Struct. Biol. Cryst. Commun.* 4, 970–973. doi: 10.1107/S1744309108026754

Kashiwagi, H., Ono, Y., Shimizu, K., Haneishi, T., Ito, S., Iijima, S., et al. (2011). Novel nonsecosteroidal vitamin D_3 carboxylic acid analogs for osteoporosis, and SAR analysis. *Bioorg. Med. Chem.* 19, 4721–4729. doi: 10.1016/j.bmc.2011.07.001

Khorasanizadeh, S., and Rastinejad, F. (2001). Nuclear-receptor interactions on DNA-response elements. *Trends Biochem. Sci.* 26, 384–390. doi: 10.1016/S0968-0004(01)01800-X

Kim, J. Y., Son, Y. L., and Lee, Y. C. (2009). Involvement of SMRT corepressor in transcriptional repression by the vitamin D receptor. *Mol. Endocrinol.* 23, 251–264. doi: 10.1210/me.2008-0426

Kim, M.-S., Fujiki, R., Kitagawa, H., and Kato, S. (2007). 1α,25$(OH)_2D_3$-induced DNA methylation suppresses the human *CYP27B1* gene. *Mol. Cell. Endocrinol.* 265–266, 168–173. doi: 10.1016/j.mce.2006.12.014

Kim, S., Shevde, N. K., and Pike, J. W. (2005). 1,25-Dihydroxyvitamin D_3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J. Bone Miner. Res.* 20, 305–317. doi: 10.1359/JBMR.041112

Krasowski, M. D., Yasuda, K., Hagey, L. R., and Schuetz, E. G. (2005). Evolutionary selection across the nuclear hormone receptor superfamily with a focus on the NR1I subfamily (vitamin D, pregnane X, and constitutive androstane receptors). *Nucl. Recept.* 3:2. doi: 10.1186/1478-1336-3-2

Lee, M. S., Kliewer, S. A., Provencal, J., Wright, P. E., and Evans, R. M. (1993). Structure of the retinoid X receptor α DNA binding domain: a helix required for homodimeric DNA binding. *Science* 260, 1117–1121. doi: 10.1126/science.8388124

Li, Y., Lambert, M., and Xu, H. (2003). Activation of nuclear receptors: a perspective from structural genomics. *Structure (Camb.)* 11, 741–746. doi: 10.1016/S0969-2126(03)00133-3

Liu, Y. Y., Nguyen, C., and Peleg, S. (2000). Regulation of ligand-induced heterodimerization and coactivator interaction by the activation function-2 domain of the vitamin D receptor. *Mol. Endocrinol.* 14, 1776–1787. doi: 10.1210/mend.14.11.0560

Lou, X., Toresson, G., Benod, C., Suh, J. H., Philips, K. J., Webb, P., et al. (2014). Structure of the retinoid X receptor α-liver X receptor β (RXRα-LXRβ) heterodimer on DNA. *Nat. Struct. Mol. Biol.* 21, 277–281. doi: 10.1038/nsmb.2778

Lou, Y.-R., Molnár, F., Peräkylä, M., Qiao, S., Kalueff, A. V., St-Arnaud, R., et al. (2010). 25-Hydroxyvitamin D_3 is an agonistic vitamin D receptor ligand. *J. Steroid Biochem. Mol. Biol.* 118, 162–170. doi: 10.1016/j.jsbmb.2009.11.011

Maehr, H., Rochel, N., Lee, H. J., Suh, N., and Uskokovic, M. R. (2013). Diastereotopic and deuterium effects in gemini. *J. Med. Chem.* 56, 3878–3888. doi: 10.1021/jm400032t

Mahajan, M. A., and Samuels, H. H. (2000). A new family of nuclear receptor coregulators that integrate nuclear receptor signaling through CREB-binding protein. *Mol. Cell. Biol.* 20, 5048–5063. doi: 10.1128/MCB.20.14.5048-5063.2000

Makishima, M., Lu, T., Xie, W., Whitfield, G., Domoto, H., Evans, R., et al. (2002). Vitamin D receptor as an intestinal bile acid sensor. *Science* 296, 1313–1316. doi: 10.1126/science.1070477

Malik, S., Guermah, M., Yuan, C.-X., Wu, W., Yamamura, S., and Roeder, R. G. (2004). Structural and functional organization of TRAP220, the TRAP/mediator subunit that is targeted by nuclear receptors. *Mol. Cell. Biol.* 24, 8244–8254. doi: 10.1128/MCB.24.18.8244-8254.2004

Malloy, P. J., Pike, J. W., and Feldman, D. (1999). The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. *Endocr. Rev.* 20, 156–188.

Marshall, P. A., Hernandez, Z., Kaneko, I., Widener, T., Tabacaru, C., Aguayo, I., et al. (2012). Discovery of novel vitamin D receptor interacting proteins that modulate 1,25-dihydroxyvitamin D_3 signaling. *J. Steroid Biochem. Mol. Biol.* 132, 147–159. doi: 10.1016/j.jsbmb.2012.05.001

Masuno, H., Ikura, T., Morizono, D., Orita, I., Yamada, S., Shimizu, M., et al. (2013). Crystal structures of complexes of vitamin D receptor ligand-binding domain with lithocholic acid derivatives. *J. Lipid Res.* 54, 2206–2213. doi: 10.1194/jlr.M038307

Masuyama, H., and Hiramatsu, Y. (2004). Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors. *J. Biol. Chem.* 279, 12020–12026. doi: 10.1074/jbc.M312762200

Mizwicki, M. T., Keidel, D., Bula, C. M., Bishop, J. E., Zanello, L. P., Wurtz, J.-M., et al. (2004). Identification of an alternative ligand-binding pocket in the nuclear vitamin D receptor and its functional importance in $1\alpha,25(OH)_2$-vitamin D_3 signaling. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12876–12881. doi: 10.1073/pnas.0403606101

Molnár, F., Matilainen, M., and Carlberg, C. (2005). Structural determinants of the agonist-independent association of human peroxisome proliferator-activated receptors with coactivators. *J. Biol. Chem.* 280, 26543–26556. doi: 10.1074/jbc.M502463200

Molnár, F., Peräkylä, M., and Carlberg, C. (2006). Vitamin D receptor agonists specifically modulate the volume of the ligand-binding pocket. *J. Biol. Chem.* 281, 10516–10526. doi: 10.1074/jbc.M513609200

Molnár, F., Sigüeiro, R., Sato, Y., Araujo, C., Schuster, I., Antony, P., et al. (2011). 1α,25(OH)(2)-3-Epi-Vitamin D_3, a natural physiological metabolite of Vitamin D_3: its synthesis, biological activity and crystal structure with its receptor. *PLoS ONE* 6:e18124. doi: 10.1371/journal.pone.0018124

Nagy, L., and Schwabe, J. (2004). Mechanism of the nuclear receptor molecular switch. *Trends Biochem. Sci.* 29, 317–324. doi: 10.1016/j.tibs.2004.04.006

Nakabayashi, M., Tsukahara, Y., Iwasaki-Miyamoto, Y., Mihori-Shimazaki, M., Yamada, S., Inaba, S., et al. (2013). Crystal structures of hereditary vitamin D-resistant rickets-associated vitamin D receptor mutants R270L and W282R bound to 1,25-dihydroxyvitamin D_3 and synthetic ligands. *J. Med. Chem.* 56, 6745–6760. doi: 10.1021/jm400537h

Nakabayashi, M., Yamada, S., Yoshimoto, N., Tanaka, T., Igarashi, M., Ikura, T., et al. (2008). Crystal structures of rat vitamin D receptor bound to adamantyl vitamin D analogs: structural basis for vitamin D receptor antagonism and partial agonism. *J. Med. Chem.* 51, 5320–5329. doi: 10.1021/jm8004477

Nevado, J., Tenbaum, S. P., and Aranda, A. (2004). hSrb7, an essential human Mediator component, acts as a coactivator for the thyroid hormone receptor. *Mol. Cell. Endocrinol.* 222, 41–51. doi: 10.1016/j.mce.2004.05.003

Nishikawa, J., Matsumoto, M., Sakoda, K., Kitaura, M., Imagawa, M., and Nishihara, T. (1993). Vitamin D receptor zinc finger region binds to a direct repeat as a dimer and discriminates the spacing number between each half-site. *J. Biol. Chem.* 268, 19739–19743.

Norman, A. W., Bouillon, R., Farach-Carson, M. C., Bishop, J. E., Zhou, L. X., Nemere, I., et al. (1993). Demonstration that 1 β,25-dihydroxyvitamin D_3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of 1 α,25-dihydroxyvitamin D_3. *J. Biol. Chem.* 268, 20022–20030.

Orlov, I., Rochel, N., Moras, D., and Klaholz, B. P. (2012). Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. *EMBO J.* 31, 291–300. doi: 10.1038/emboj.2011.445

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000). VDR-Alien: a novel, DNA-selective vitamin D_3 receptor-corepressor partnership. *FASEB J.* 14, 1455–1463. doi: 10.1096/fj.14.10.1455

Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Näär, A. M., et al. (1999). Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 398, 824–828. doi: 10.1038/19783

Reddy, G. S., Rao, D. S., Siu-Caldera, M. L., Astecker, N., Weiskopf, A., Vouros, P., et al. (2000). 1α,25-dihydroxy-16-ene-23-yne-vitamin D_3 and 1α,25-dihydroxy-16-ene-23-yne-20-epi-vitamin D_3: analogs of 1α,25-dihydroxyvitamin D_3 that resist metabolism through the C-24 oxidation pathway are metabolized through the C-3 epimerization pathway. *Arch Biochem. Biophys.* 383, 197–205. doi: 10.1006/abbi.2000.2074

Ren, Y., Behre, E., Ren, Z., Zhang, J., Wang, Q., and Fondell, J. D. (2000). Specific structural motifs determine TRAP220 interactions with nuclear hormone receptors. *Mol. Cell. Biol.* 20, 5433–5446. doi: 10.1128/MCB.20.15.5433-5446.2000

Renaud, J., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., et al. (1995). Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. *Nature* 378, 681–689. doi: 10.1038/378681a0

Rochel, N., Ciesielski, F., Godet, J., Moman, E., Roessle, M., Peluso-Iltis, C., et al. (2011). Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacings. *Nat. Struct. Mol. Biol.* 18, 564–570. doi: 10.1038/nsmb.2054

Rochel, N., Hourai, S., and Moras, D. (2010). Crystal structure of hereditary vitamin D-resistant rickets-Associated mutant H305Q of vitamin D nuclear receptor bound to its natural ligand. *J. Steroid Biochem. Mol. Biol.* 121, 84–87. doi: 10.1016/j.jsbmb.2010.04.008

Rochel, N., Hourai, S., Pérez-García, X., Rumbo, A., Mouriño, A., and Moras, D. (2007). Crystal structure of the vitamin D nuclear receptor ligand binding domain in complex with a locked side chain analog of calcitriol. *Arch. Biochem. Biophys.* 460, 172–176. doi: 10.1016/j.abb.2007.01.031

Rochel, N., and Moras, D. (2012). Crystal structure of a vitamin D_3 analog, ZK203278, showing dissociated profile. *Anticancer Res.* 32, 335–339.

Rochel, N., Tocchini-Valentini, G., Egea, P. F., Juntunen, K., Garnier, J. M., Vihko, P., et al. (2001). Functional and structural characterization of the insertion region in the ligand binding domain of the vitamin D nuclear receptor. *Eur. J. Biochem.* 268, 971–979. doi: 10.1046/j.1432-1327.2001.01953.x

Rochel, N., Wurtz, J., Mitschler, A., Klaholz, B., and Moras, D. (2000). The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell.* 5, 173–179. doi: 10.1016/S1097-2765(00)80413-X

Saito, H., Takagi, K., Horie, K., Kakuda, S., Takimoto-Kamimura, M., Ochiai, E., et al. (2013). Synthesis of novel C-2 substituted vitamin D derivatives having ringed side chains and their biological evaluation on bone. *J. Steroid Biochem. Mol. Biol.* 136, 3–8. doi: 10.1016/j.jsbmb.2013.02.004

Sawada, D., Tsukuda, Y., Saito, H., Kakuda, S., Takimoto-Kamimura, M., Ochiai, E., et al. (2011). Development of 14-epi-19-nortachysterol and its unprecedented binding configuration for the human vitamin D receptor. *J. Am. Chem. Soc.* 133, 7215–7221. doi: 10.1021/ja201481j

Shaffer, P. L., and Gewirth, D. T. (2002). Structural basis of VDR-DNA interactions on direct repeat response elements. *EMBO J.* 21, 2242–2252. doi: 10.1093/emboj/21.9.2242

Shaffer, P. L., and Gewirth, D. T. (2004). Structural analysis of RXR-VDR interactions on DR3 DNA. *J. Steroid Biochem. Mol. Biol.* 89–90, 215–219. doi: 10.1016/j.jsbmb.2004.03.084

Shimizu, M., Miyamoto, Y., Takaku, H., Matsuo, M., Nakabayashi, M., Masuno, H., et al. (2008). 2-Substituted-16-ene-22-thia-1α,25-dihydroxy-26,27-dimethyl-19-norvitamin D_3 analogs: Synthesis, biological evaluation, and crystal structure. *Bioorg. Med. Chem.* 16, 6949–6964. doi: 10.1016/j.bmc.2008.05.043

Shindo, K., Kumagai, G., Takano, M., Sawada, D., Saito, N., Saito, H., et al. (2011). New C15-substituted active vitamin D_3. *Org. Lett.* 13, 2852–2855. doi: 10.1021/ol200828s

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D_3 response by cancer-associated mutant p53. *Cancer Cell* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Tagami, T., Lutz, W. H., Kumar, R., and Jameson, J. L. (1998). The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. *Biochem. Biophys. Res. Commun.* 253, 358–363. doi: 10.1006/bbrc.1998.9799

Thénot, S., Henriquet, C., Rochefort, H., and Cavailles, V. (1997). Differential interaction of nuclear receptors with the putative human transcriptional coactivator hTIF1. *J. Biol. Chem.* 272, 12062–12068. doi: 10.1074/jbc.272.18.12062

Ting, H.-J., Bao, B.-Y., Hsu, C.-L., and Lee, Y.-F. (2005). Androgen-receptor coregulators mediate the suppressive effect of androgen signals on vitamin D receptor activity. *Endocrine* 26, 1–9. doi: 10.1385/ENDO:26:1:001

Tocchini-Valentini, G., Rochel, N., Wurtz, J. M., Mitschler, A., and Moras, D. (2001). Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5491–5496. doi: 10.1073/pnas.091018698

Tocchini-Valentini, G., Rochel, N., Wurtz, J., and Moras, D. (2004). Crystal structures of the vitamin D nuclear receptor liganded with the vitamin D side chain analogues calcipotriol and seocalcitol, receptor agonists of clinical importance. Insights into a structural basis for the switching of calcipotriol to a receptor antagonist by further side chain modification. *J. Med. Chem.* 47, 1956–1961. doi: 10.1021/jm0310582

Toell, A., Polly, P., and Carlberg, C. (2000). All natural DR3-type vitamin D response elements show a similar functionality *in vitro*. *Biochem. J.* 352(Pt 2), 301–309. doi: 10.1042/0264-6021:3520301

Towers, T. L., Staeva, T. P., and Freedman, L. P. (1999). A two-hit mechanism for vitamin D_3-mediated transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-Jun. *Mol. Cell. Biol.* 19, 4191–4199.

Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* 65, 1255–1266. doi: 10.1016/0092-8674(91)90020-Y

van Driel, M., Koedam, M., Buurman, C. J., Roelse, M., Weyts, F., Chiba, H., et al. (2006). Evidence that both 1α,25-dihydroxyvitamin D_3 and 24-hydroxylated D_3 enhance human osteoblast differentiation and mineralization. *J. Cell. Biochem.* 99, 922–935. doi: 10.1002/jcb.20875

Vanhooke, J. L., Benning, M. M., Bauer, C. B., Pike, J. W., and DeLuca, H. F. (2004). Molecular structure of the rat vitamin D receptor ligand binding domain complexed with 2-carbon-substituted vitamin D_3 hormone analogues and a LXXLL-containing coactivator peptide. *Biochemistry* 43, 4101–4110. doi: 10.1021/bi036056y

Vanhooke, J. L., Tadi, B. P., Benning, M. M., Plum, L. A., and DeLuca, H. F. (2007). New analogs of 2-methylene-19-nor-(20S)-1,25-dihydroxyvitamin D_3 with conformationally restricted side chains: evaluation of biological activity and structural determination of VDR-bound conformations. *Arch. Biochem. Biophys.* 460, 161–165. doi: 10.1016/j.abb.2006.11.029

Verlinden, L., Verstuyf, A., Eelen, G., Bouillon, R., Ordóñez-Morán, P., Larriba, M. J., et al. (2011). Synthesis, structure, and biological activity of des-side chain analogues of 1α,25-dihydroxyvitamin D_3 with substituents at C18. *ChemMedChem* 6, 788–793. doi: 10.1002/cmdc.201100021

Watkins, R., Wisely, G., Moore, L., Collins, J., Lambert, M., Williams, S., et al. (2001). The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* 292, 2329–2333. doi: 10.1126/science.1060762

Whitfield, G. K., Dang, H. T. L., Schluter, S. F., Bernstein, R. M., Bunag, T., Manzon, L. A., et al. (2003). Cloning of a functional vitamin D receptor from the lamprey (Petromyzon marinus), an ancient vertebrate lacking a calcified skeleton and teeth. *Endocrinology* 144, 2704–2716. doi: 10.1210/en.2002-221101

Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., et al. (2011). Overview of the CCP4 suite and current developments. *Acta Crystallogr. D Biol. Crystallogr.* 67, 235–242. doi: 10.1107/S0907444910045749

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., et al. (1999). Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321. doi: 10.1126/science.283.5406.1317

Yoshimoto, N., Sakamaki, Y., Haeta, M., Kato, A., Inaba, Y., Itoh, T., et al. (2012). Butyl pocket formation in the vitamin D receptor strongly affects the agonistic or antagonistic behavior of ligands. *J. Med. Chem.* 55, 4373–4381. doi: 10.1021/jm300230a

Zhang, J., Chalmers, M. J., Stayrook, K. R., Burris, L. L., Wang, Y., Busby, S. A., et al. (2011). DNA binding alters coactivator interaction surfaces of the intact VDR-RXR complex. *Nat. Struct. Mol. Biol.* 18, 556–563. doi: 10.1038/nsmb.2046

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Vitamin D and the RNA transcriptome: more than mRNA regulation

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The GRCh37.p13 primary assembly of the human genome contains 20805 protein coding mRNA, and 37147 non-protein coding genes and pseudogenes that as a result of RNA processing and editing generate 196501 gene transcripts. Given the size and diversity of the human transcriptome, it is timely to revisit what is known of VDR function in the regulation and targeting of transcription. Early transcriptomic studies using microarray approaches focused on the protein coding mRNA that were regulated by the VDR, usually following treatment with ligand. These studies quickly established the approximate size, and surprising diversity of the VDR transcriptome, revealing it to be highly heterogenous and cell type and time dependent. With the discovery of microRNA, investigators also considered VDR regulation of these non-protein coding RNA. Again, cell and time dependency has emerged. Attempts to integrate mRNA and miRNA regulation patterns are beginning to reveal patterns of co-regulation and interaction that allow for greater control of mRNA expression, and the capacity to govern more complex cellular events. As the awareness of the diversity of non-coding RNA increases, it is increasingly likely it will be revealed that VDR actions are mediated through these molecules also. Key knowledge gaps remain over the VDR transcriptome. The causes for the cell and type dependent transcriptional heterogenetiy remain enigmatic. ChIP-Seq approaches have confirmed that VDR binding choices differ very significantly by cell type, but as yet the underlying causes distilling VDR binding choices are unclear. Similarly, it is clear that many of the VDR binding sites are non-canonical in nature but again the mechanisms underlying these interactions are unclear. Finally, although alternative splicing is clearly a very significant process in cellular transcriptional control, the lack of RNA-Seq data centered on VDR function are currently limiting the global assessment of the VDR transcriptome. VDR focused research that complements publically available data (e.g., ENCODE Birney et al., 2007; Birney, 2012), TCGA (Strausberg et al., 2002), GTEx (Consortium, 2013) will enable these questions to be addressed through large-scale data integration efforts.

Keywords: VDR, microRNA, transcriptome, epigenetic, microarray

THE TRANSCRIPTIONAL LANDSCAPE OF THE HUMAN GENOME

An appreciation of the diversity of transcription across the human genome has undergone a rapid expansion in recent years, in large part thanks to the efforts of integrative genomic approaches such as those of ENCODE consortium (Birney, 2012; Maher, 2012; Stamatoyannopoulos, 2012; Rosenbloom et al., 2013). From these studies it has become apparent that there is considerable variation and diversity in; the distribution of transcription factor binding across the human genome; the interplay between transcription factors and different co-regulating partners; the extent of the genome that is transcribed; the number and functionally different RNA-based molecules that are transcribed, the impact of mechanisms that process and edit RNA molecules that generate even greater diversity of gene expression.

In this context it is timely to review the functions of the vitamin D receptor (VDR/NR1I1) (Pike et al., 1980; Baker et al., 1988; Carlberg and Campbell, 2013), and consider how its actions contribute to this diversity of transcriptional and post-transcriptional events.

THE VDR ACTS IN MULTIMERIC PROTEIN COMPLEXES TO REGULATE TRANSCRIPTION

The VDR, like many other members of the nuclear receptor superfamily are relatively well-understood transcription factors. Their actions have been dissected and modeled, and have generated the concept of cyclical gene regulation in which transcription factors oscillate between on and off states (Metivier et al., 2003; Reid et al., 2003; Kim et al., 2005; Vaisanen et al., 2005; Carroll et al., 2006; Meyer et al., 2006; Saramaki et al., 2006; Yang et al., 2006; Zella et al., 2006; Malinen et al., 2008; Seo et al., 2007).

A direct consequence of VDR genomic interactions and gene regulation is the control of the epigenetic states at receptor binding regions, and more broadly across target gene loci. Epigenetic events play a central role for transcriptional complexes and the various components in these multimeric complexes are able to

initiate, sustain, and finally terminate transcription (Dobrzynski and Bruggeman, 2009). For example, different histone modifications can control the rate and magnitude of transcription (reviewed in Goldberg et al., 2007). These events are intertwined with levels of CpG methylation (Kangaspeska et al., 2008; Metivier et al., 2008; Le May et al., 2010). Thus the histone modifications regulated by VDR actions, and other epigenetic events including DNA methylation processes, combine during transcription to generate highly flexible chromatin states that are either transcriptionally receptive and resistant (Mohn and Schubeler, 2009). That is, the specific transcriptional potential of a gene is flexibly controlled by the combination of epigenetic events. These events are varied in space across the genomic loci, and in time through the course of the transcriptional cycle.

The diversity of histone modifications, and their association with different DNA functions formed the basis for the histone code hypothesis. This concept, first proposed in 1993, held that these modifications were governed in a coordinated manner and formed a code that mirrored the underlying DNA code to convey heritable information on transcription and expression (Turner, 1993). Given the rapid expansion of the understanding in the number of histone modifications, their genomic distribution and their combinatorial manner, it is only relatively recently that the true diversity of the range of histone states, and their functional outcomes, has become apparent (Goldberg et al., 2007). The strongest evidence that histone modifications at the level of meta-chromatin architecture form a stable and heritable "histone code," is perhaps seen with X chromosome inactivation (reviewed in Turner, 1998). The extent to which similar processes operate to govern the activity of micro-chromatin contexts at gene promoter regions, is an area of debate (Jenuwein and Allis, 2001; Turner, 2002).

The regulation of transcription and the patterns of mRNA expression have been related to the expression of these histone modifications through a wide range of correlative and functional studies. For example, histone H3 lysine 4 tri-methylation (H3K4me3) is found in the promoter regions of actively transcribed genes. This mark is mutually exclusive with H3K9me, which instead is associated with transcriptionally silent promoter regions. Acetylation of H3K9 is found along with methylation of H3K4 at active promoter regions. Similarly, H3K27 can be either acetylated or methylated, with acetylation associated with active gene transcription and methylation associated with gene silencing.

In many experimental cases it has been established that VDR activation by natural ligand 1α,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D_3$) or synthetic analogs, can lead to a highly dynamic exchange of co-factors by releasing co-repressors and inducing a receptor conformation that attracts binding of co-activator proteins (**Figure 1**). This exchange of associations induces a more relaxed, or open, chromatin status and the recruitment of linking factors and subsequently the basal transcriptional machinery. However, this is not an indefinite signal and the ligand, is rapidly metabolized. Also the VDR itself is limited in function by proteasome-mediated receptor degradation (Peleg and Nguyen, 2010). In the absence of ligand, some basal level of receptor remains in the nucleus associated with co-repressor complex and leads to silencing of transcription (Malinen et al., 2008; Saramaki et al., 2009; Thorne et al., 2011; Doig et al., 2013). The sequencing, or choreography of these actions, give rise to the periodicity of transcriptional activation and pulsatile mRNA and protein accumulation and reflect intrinsic control mechanisms required to tightly regulate the expression of important signaling molecules. At closer resolution, for example on shorter time-scales, the patterns of regulation show some degree of coordinated pulsatile regulation, and probably reflect aspects of specific VDR binding sites impacting gene regulation for example through emerged to support chromatin looping within the same VDR target gene loci (Vaisanen et al., 2005; Saramaki et al., 2006, 2009).

Whilst these scenarios are relatively well characterized for the positive regulation of gene expression, it is probably not a complete understanding as the distribution of VDR binding in the genome (Ramagopalan et al., 2010; Heikkinen et al., 2011; Meyer et al., 2012; Ding et al., 2013) and the patterns of associated gene regulation suggest that the VDR is actually associated broadly with gene activation and repression. The mechanisms that drive gene repression appear more diverse than gene activation and reflect differences in complex formation, and the choreography of binding.

The patterns of protein-protein interaction identified for the VDR allude to both positive and negative gene regulation (**Table 1**). The VDR commonly forms a heterodimer with RXRα (Quack and Carlberg, 2000). However, the identification of partners that interact in the same complex supports a broad role for the VDR complex to regulate other signal transduction events and RNA processing activities.

A number of proteins with transcriptional activator function have been identified in complex with VDR. For example, CBP/p300 is a transcriptional co-integrator with histone acetlyase activity (Wang et al., 2011a,b, 2013a) and is associated with the VDR. Other proteins such as SNW1/NCOA62 which has function as a transcriptional co-activator(Baudino et al., 1998), as well as other proteins that have more recently been characterized to have coativator function (CCND3) (Cenciarelli et al., 1999; Lazaro et al., 2002; Despouy et al., 2003; Sarruf et al., 2005). Similarly the corepressor HR is also identified by such protein-protein interactions (Hsieh et al., 2003).

Aside from these traditional roles to modulate transcriptional actions, there is evidence to support a wider range of actions for the VDR in the control of mRNA. The coactivator SNW1 also plays a role as a splicing factor. This latter function is also shared by another VDR interacting protein, namely SRPK1, which is a protein kinase that regulates the activity of various splicing factors (Hayes et al., 2006; Aubol et al., 2013). Other interactions allude to the cross-talk between the VDR and different signal transduction processes. For example, the VDR interacts with negative regulators of WNT signaling (NKD2) (Katoh and Katoh, 2007), substrates for PKC signaling (PRKCSH) (Gkika et al., 2004), p53 (Kommagani et al., 2006; Lambert et al., 2006; Maruyama et al., 2006; Saramaki et al., 2006; Ellison et al., 2008) and SMAD3 (Ding et al., 2013; Ito et al., 2013; Zerr et al., 2014). The VDR functionally interacts with a range of co-repressors such as NCOR1 (Saramaki et al., 2009; Doig et al., 2013), NCOR2/SMRT (Khanim

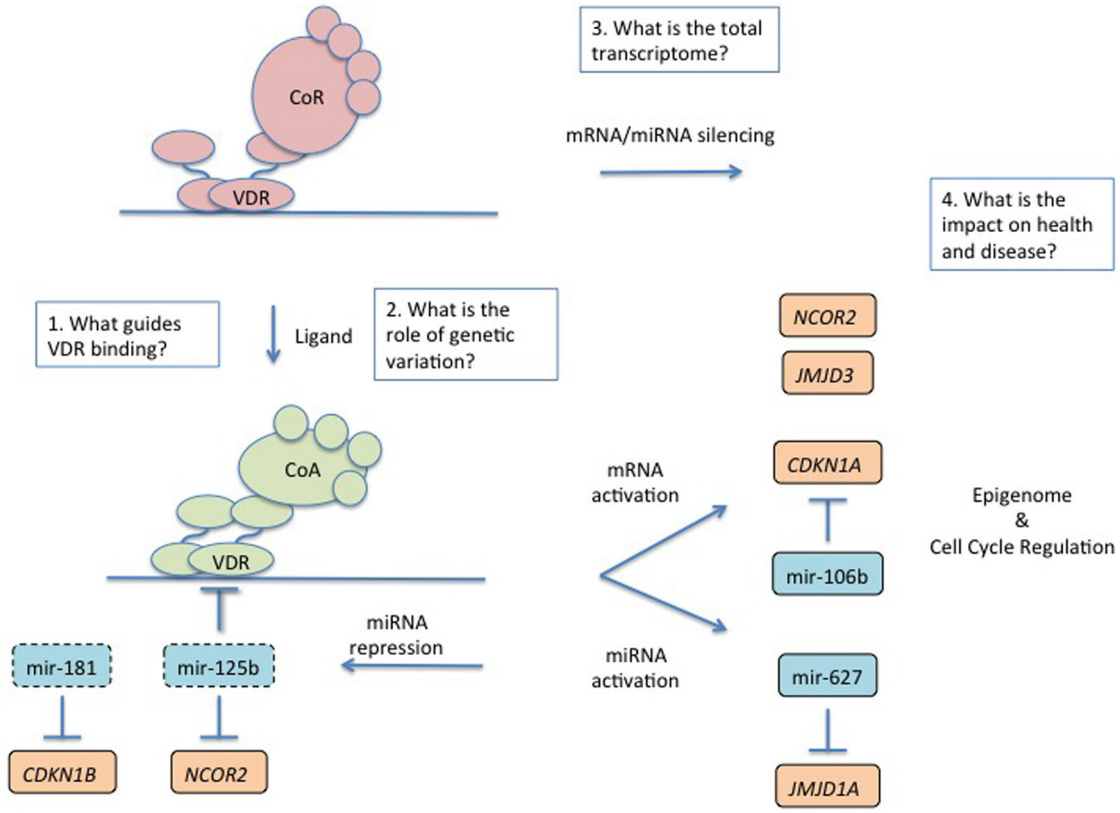

FIGURE 1 | An overview of regulation and impact of the transcriptome by the nuclear VDR and the future challenges. The VDR toggles between a gene repressive and activating complex depending on the presence of ligand. Ligand activated gene regulated scenarios are most well understood. In the presense of ligand the VDR complex is associated with both mRNA and miRNA activation and there is clear evidence for co-regulation of these transcriptomes to tightly control final protein coding gene expression and phenotype. Ligand activated VDR also represses a number of miRNA for example including those that control expression of the VDR and components of the repressive complex. The major phenotypes associated with VDR control include cell cycle regulation and an emerging theme is the control of the epigenome. Four major questions remain in understanding VDR transcription. (1) What guides where the VDR binds in the genome? (2) What role does genetic variation in VDR binding sites play in changing the VDR transriptome? (3) What is the total VDR transcriptome? (4) What is the combined effect of the VDR transcriptome on health and disease and how can this be monitored and exploited?

et al., 2004; Gynther et al., 2011), TRIP15/ALIEN (Polly et al., 2000; Cui et al., 2009) and DREAM (Scsucova et al., 2005) but, interestingly, agnostic protein-protein interaction tools such as INTACT (**Table 1**) have not identified direct VDR co-repressor interactions. It is unclear why these proteins are not identified in such protein-protein screens, but may reflect an experimental artifact as a result of investigators using ligand stimulated VDR to capture interacting proteins.

THE DIVERSITY OF VDR-PROTEIN INTERACTIONS IS REFLECTED BY THE DISTRIBUTIONS OF GENOMIC BINDING SITES

To identify VDR binding sites through the genome several groups have now applied ChIP-Seq approaches in different human cell types including immortalized lymphoblastoids (Ramagopalan et al., 2010), hepatic stellate cells (Ding et al., 2013) and cancer cell lines representing monocytic leukemia (Heikkinen et al., 2011) and colon cancer (Meyer et al., 2012). These studies also differed in the time of cell exposure to 1α,25 $(OH)_2D_3$ ranging from 40 min to 36 h identify binding sites, on the order of hundreds to several thousands of different binding sites depending on time of treatment with 1α25$(OH)_2D_3$ and cell background, with longer treatment time points tending to be associated with more binding sites. However, as yet there are not uniform standards for the analyses of NGS data, and therefore it is likely that different analytical approaches are influencing the number and significance of the VDR enriched peaks identified.

These differences in treatment and analytical approaches aside, these VDR binding data sets reveals that fewer than 20% of the VDR binding sites are in common between the different cell types. These finding perhaps offers strong support for the concept that VDR transcription is extremely tailored in different cell types, presumably through interactions with either equal or more dominant co-factors that combine to determine its binding. Another important finding from these studies is that the canonical binding site for the VDR, termed the direct repeat (DR) spaced by 3 nucleotides (DR-3), which was identified by traditional biochemical approaches in candidate gene studies, appears to be the minority genomic element that directly binds the receptor. Fewer that 30% of genomic VDR binding sites contain a DR-3, although this number is increased following ligand treatment

Table 1 | Proteins known to interact with the VDR.

Interacting protein	Function	Detection method	Interaction	Publication
Retinoid X receptor alpha (RXRα)	Transcription factor	Electron microscopy	Direct interaction	Orlov et al., 2012
Retinoid X receptor beta (RXRβ)	Transcription factor	Two hybrid	Physical association	Wang et al., 2011a
E1A-associated protein p300 (CBP/p300)	Histone acetyltransferase	Two hybrid	Physical association	Wang et al., 2011a
Mediator complex subunit (MED1)	Mediator complex that binds basal transcriptional machinery and drives transcriptional initiation	Pull down	Physical association	Yuan et al., 1998
Nuclear receptor coactivator 6 (NCOA6)	Transcriptional coactivator of multiple nuclear receptors and other transcription factors	Two hybrid	Physical association	Mahajan and Samuels, 2000
CXXC-type zinc finger protein 5 (CXXC5)	Transcription factor co-regulator of WNT signaling	Two hybrid	Physical association	Wang et al., 2011a
Tumor Protein P53 (p53)	Tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains	Fluorescence microscopy	Co-localization	Stambolsky et al., 2010
Protein naked cuticle homolog 2 (NKD2)	Antagonist of WNT via degradation DVL	Two hybrid	Physical association	Wang et al., 2011a
SMAD family member 3 (SMAD3)	Transcriptional effector of TGFβ	Pull down	Physical association	Leong et al., 2001
SNW Domain containing 1(SNW1)	Co-activator function with known roles as a splicing factor	Two hybrid	Physical association and co-localization	Baudino et al., 1998; Zhang et al., 2003
SFRS protein kinase 1 (SRPK1)	Serine/arginine protein kinase specific for the SR (serine/arginine-rich domain) family of splicing factors	Protein kinase assay	Phosphorylation reaction	Varjosalo et al., 2013
Protein kinase C substrate 80 K-H (PRKCSH)	Substrate for protein kinase C	Two hybrid	Physical association	Wang et al., 2011a
Protein-tyrosine phosphatase H1(PTPN3)	Protein tyrosine phosphatase that regulate a variety of cellular processes	Pull down	Physical association	Zhi et al., 2011
Complement Factor H (CFH)	Regulator of complement activation (RCA) gene cluster and plays a role in the defense mechanism to microbial infections	Two hybrid	Physical association	Wang et al., 2011a
β-catenin	Dual function protein, regulating the coordination of cell–cell adhesion and gene transcription.	Co-localization	Functional interaction	Pálmer et al., 2001
Prolylcarboxypeptidase (Angiotensinase C) (PRCP)	A lysosomal prolylcarboxypeptidase, which cleaves C-terminal amino acids linked to proline	Two hybrid	Physical association	Wang et al., 2011a
Cyclin D3 (CCND3)	Cyclin associated with control of cell cycle and known co-factor for several nuclear receptods	Two hybrid	Physical association	Wang et al., 2011a

(Continued)

Table 1 | Continued

Interacting protein	Function	Detection method	Interaction	Publication
Hair growth associated (HR)	Transcriptional corepressor of multiple nuclear receptors	Pull down	Direct interaction	Hsieh et al., 2003
Nuclear corepressor 1 (NCOR1)	Transcriptional corepressor	Two hybrid	Physical association	Tagami et al., 1998
Nuclear corepressor 2 (NCOR2)	Transcriptional corepressor	Immunoprecipitation	Physical association	Kim et al., 2009
COP9 signalosome subunit 2 (COPS2)	Transcriptional corepressor and component of the ubiquitin conjugation pathway	Two hybrid	Physical association	Polly et al., 2000

The INTACT database curated by EBI http://www.ebi.ac.uk/intact/ was interrogated for interactions with the VDR. Interactions of VDR with NCOR1, NCOR2, and COPS2 were curated from the literature.

when there is increased enrichment for VDR binding to DR-3 elements (reviewed in Carlberg and Campbell, 2013). Nonetheless, a range of other genomic elements were enriched in VDR binding peaks suggesting that the VDR co-operates closely with other factors to associate with the genome, both in the absence and presence of ligand. Indeed, the study of Evans and co-workers in the hepatic stellate cells (Ding et al., 2013) and the work of Pike and co-workers in colon cancer cells (Meyer et al., 2012) both address this significant cross-talk of the VDR. In the case of the hepatic cells this is considered in the context of TGF® and in the case of colon cancer cells this is with TCF4, downstream of ®-catenin. Both of these studies therefore reflect the finding of VDR interactions with SMAD3 specifically, and more generally with regulators of WNT signaling (**Table 1**).

THE HUNT FOR PIONEER FACTORS TO EXPLAIN THE DIVERSITY OF VDR FUNCTION

Together these ChIP-Seq studies suggest that the VDR combines with other proteins in a network of interactions, quite likely in a cell type specific manner, to participate in diverse gene regulatory networks. It remains to be established how targeted or stochastic this is. The variation observed in both the type and position of binding sites for the VDR, depending on cell phenotype and disease state, suggests it is directed, and at least will establish a paradigm for hypothesis testing concerning what directs the VDR to bind and participate in gene transcription. The specificity of VDR signaling may arise due to integration with other perhaps more dominant transcription factors. Again, for other nuclear receptors (e.g., AR and ER) the concept has emerged that receptor binding is guided by the actions of more dominant so-called pioneer factors including the Forkhead (FKH) family members (Lupien et al., 2008; Serandour et al., 2011; Sahu et al., 2013). Efforts to define the major pioneer factors for the VDR have proved to be less consistent between the different VDR ChIP-Seq studies and may reflect the biology of the VDR which, given that it exists in the nucleus both in the presence and absence of ligand, potentially is a more interactive protein such that a single dominant pioneer factor is not so deterministic.

Another approach to identify the interacting partners of the VDR has been to examine the gene networks it regulates and to cluster genes by known regulating transcription factors. Novershtern et al. (2011) measured the transcriptome profiles of a large number of hematopoietic stem cells, multiple progenitor states and terminally differentiated cell types. They found distinct regulatory circuits in both stem cells and differentiated cells, which implicated dozens of new regulators in hematopoiesis. They identified 80 distinct modules of tightly co-expressed genes in the hematopoietic system. One of these modules is expressed in granulocytes and monocytes and includes genes encoding enzymes and cytokine receptors that are essential for inflammatory responses. Major players in this module are VDR together with the factors CEBPα and SPI1/PU.1. This indicates that VDR works together with this small set of transcription factors, in order to regulate granulocyte and monocyte differentiation. It is reasonable to anticipate that such modules exist in multiple cell types but are guided by the tissue specific expression of such factors.

VDR REGULATION OF THE PROTEIN-CODING TRANSCRIPTOME

Anti-proliferative effects of $1\alpha,25(OH)_2D_3$ have been demonstrated in a wide variety of cancer cell lines, including those from prostate, breast, and colon (Colston et al., 1982, 1989; Peehl et al., 1994; Campbell et al., 1997; Koike et al., 1997; Elstner et al., 1999; Welsh et al., 2002; Pálmer et al., 2003). Following on from these, VDR transcriptional studies were initially undertaken at the candidate level to identify processes by which the VDR mediated its cellular effects. These approaches identified of the gene encoding the $1\alpha,25(OH)_2D_3$ metabolizing enzyme *CYP24A1* (Dwivedi et al., 2000; Anderson et al., 2003) and *CDKN1A* (encodes $p21^{(waf1/cip1)}$) (Schwaller et al., 1995) as VDR targets. Subsequently, with the emergence of differential expression and membrane array technology, workers applied these wider screening approaches to identify multiple genes regulated by the VDR. For example, Freedman and colleagues applied differential expression approaches in the context of $1\alpha,25(OH)_2D_3$ induced myeloid differentiation and identified a number of cyclin dependent kinase inhibitors including *CDKN1A* and undertook functional confirmation studies to suggest the importance of the regulation of these targets to trigger myeloid differentiation (Liu et al., 1996). Others undertook so-called focused array technology whereby cDNA probes for selected genes involved in key biological processes or disease states were arranged on macro scale membrane arrays. Such arrays contained anywhere from several

hundred to several thousand probes, and so were not genome-wide in terms of coverage but rather were candidate arrays often focused around specific pathways or disease states such as cancer. Despite the limitations, these approaches yielded important information supporting the links between VDR action and the regulation of growth and signaling (Savli et al., 2002). Similarly, first generation arrays chips, for example from Affymetrix which contained 4500 probes (Akutsu et al., 2001), also enabled sufficient genomic coverage to begin to define specific regulated gene networks. This particular study from White (Akutsu et al., 2001) and co-workers identified 38 genes that were responsive to $1\alpha,25(OH)_2D_3$ exposure, which represented approximately 1% of the transcriptome studied, and included *GADD45A*. These earlier studies already suggested at the footprint of the VDR dependent transcriptome (reviewed in Rid et al., 2013). In many ways these studies highlighted the heterogeneity of VDR actions that was to be identified subsequently by ChIP-Seq studies. This heterogeneity may in part reflect experimental conditions with very different cell line differences, and genuine tissue-specific differences of co-factor expression that alter the amplitude and periodicity of VDR transcriptional actions.

Even within this diversity there is some consistency on a certain targets and the biological actions they relate to, including cell cycle regulation (Akutsu et al., 2001; Pálmer et al., 2003; Eelen et al., 2004; Wang et al., 2005). A common anti-proliferative VDR function is associated with arrest at G_0/G_1 of the cell cycle, coupled with up-regulation of a number of cell cycle inhibitors. Candidate promoter characterization studies have demonstrated a series of VDR binding sites in the promoter/enhancer region of *CDKN1A* (Liu et al., 1996; Saramaki et al., 2006). By contrast the regulation of the related CDKI $p27^{(kip1)}$ is mechanistically enigmatic, and included translational regulation and enhanced mRNA translation, and attenuating degrading mechanisms (Hengst and Reed, 1996; Wang et al., 1996; Huang et al., 2004; Li et al., 2004). The up-regulation of $p21^{(waf1/cip1)}$ and $p27^{(kip1)}$ principally mediate G_1 cell cycle arrest, but $1\alpha,25(OH)_2D_3$ has been shown to mediate a G_2/M cell cycle arrest in a number of cancer cell lines via direct induction of *GADD45A* (Akutsu et al., 2001; Jiang et al., 2003; Khanim et al., 2004).

In the transition to genome wide understanding, workers applied more comprehensive array approaches to define VDR mRNA transcriptomes. For example, investigations of squamous cells (Lin et al., 2002; Wang et al., 2005) identified networks of genes that trigger the response to wounding, protease inhibition, secondary metabolite biosynthesis, cellular migration, and amine biosynthetic processes. Another approach has been to examine vitamin D sensitive and responsive isogenic cell pairs and undertake analyses to identify key networks that are critical for mediating antiproliferative sensitivity toward $1\alpha25(OH)_2D_3$. In this manner a critical role for TGF® signaling was again revealed, to associate with VDR antiproliferative sensitivity toward $1\alpha25(OH)_2D_3$ in breast cancer cell models (Towsend et al., 2006). Exploiting leukemia cell models with differential responsiveness toward $1\alpha25(OH)_2D_3$ triggered-differentiation (Tagliafico et al., 2006) identified that certain VDR transcriptional targets could distinguish the aggressiveness of the leukemia, again, focused around cell cycle and included *MS4A3* which can modulate the phosphorylation of CDK2 and therefore exert control over the cell cycle. This concept of VDR sensitive vs. resistant models was also exploited in prostate cancer to identify the critical VDR transcriptional targets that mediate antiproliferative sensitivity and again also identified cell cycle and signal transduction components including *GADD45A* and *MAPKAPK2* that were required to mediate the sensitivity of cells to $1\alpha,25(OH)_2D_3$. Furthermore, these studies examined the epigenetic basis for the transcriptionally inert state of these targets in resistant models (Rashid et al., 2001; Khanim et al., 2004).

Identification of the VDR-dependent transcriptome using microarray approaches is heavily dependent on a range of statistical and technical considerations (Do and Choi, 2006; Zhang et al., 2009) including hybridization variations and limitations, background effects, normalization procedures, the choice of statistical test to identify differentially expressed genes, which in turn relies on study design and the numbers of arrays and samples chosen for study. Many of these study components were only formally agreed upon with the establishment of the MIAME compliant protocols in 2001 (Brazma et al., 2001), and as these became accepted standards for journal publication these approaches became widespread through the biological community.

MIAME compliant array experiments are subsequently published in public archives, such as GEO (Barrett et al., 2005, 2013) and ArrayExpress at EMBL (Parkinson et al., 2007) and Stanford microarray database (Marinelli et al., 2008). These three repositories between them contain thousands of genome-wide microarray experiments, containing millions of individual microarrays. Mining these repositories reveals a range of experiments (not all published) where cells have been treated with $1\alpha,25(OH)_2D_3$ and other vitamin D compounds, and RNA effects studied between short time points (1–2 h) to several days (**Table 2**). Again, these studies have supported consistent themes in terms of the VDR-mediated control of cell cycle and signal transduction processes, the suppression of WNT and NF-κB, and the regulation of IGF1 signaling (Kovalenko et al., 2011), and integrated actions with TGF® signaling. A final area to emerge from these agnostic studies of the VDR transcriptome is the impact that $1\alpha25(OH)_2D_3$ exposure exerts on a range of chromatin remodeling components. Interestingly, NCOR2/SMRT appears to be a target of VDR signaling (Dunlop et al., 2004), and adds to the concept that VDR signaling is cyclical and based on the functions of various negative feedback loops. Similarly, KDM6B/JMJD3 is a histone H3 lysine demethylase and expression is induced by the activated VDR. In this manner, VDR action can feed-forward its own transcriptional program by promoting H3K9 acetylation and gene action (Pereira et al., 2011).

Given the number of arrays available, it is now timely to consider meta-analyses across the arrays to reveal common themes; this forward compatibility is one the key benefits of MIAME compliance. Meta-integration of array data has been shown to be surprisingly revealing in a range of studies. For example at the larger scale various workers have integrated multiple microarray data to reveal underlying patterns in the context of disease classification (Shah et al., 2009; Engreitz et al., 2011) but can also be applied to consider that specific phenotypes (Martinez-Climent et al., 2010; Rantala et al., 2010; Lai et al., 2014). It is

Table 2 | Publically available MIAME compliant microarray studies of VDR function.

Experimental title/design	GEO series accession number	Publication
PROTEIN CODING MRNA		
Vitamin D effect on bronchial smooth muscle cells	GSE5145	Bosse et al., 2007
Genome-wide analysis of vitamin D receptor (VDR) target genes in THP-1 monocytic leucemia cells	GSE27270	Heikkinen et al., 2011
Transcriptional effects of 1,25 dihydroxi-vitamin D3 physiological and supra-physiological concentrations in breast cancer organotypic culture	GSE27220	
Analysis of vitamin D response element binding protein target genes reveals a role for vitamin D in osteoblast mTOR signaling	GSE22523	Lisse et al., 2011
Expression profiling of androgen receptor and vitamin D receptor mediated signaling in prostate cancer cells	GSE17461	Wang et al., 2011b
Understanding vitamin D resistance using expression microarrays	GSE9867	Costa et al., 2009
Effects of TX527, a hypocalcemic vitamin D analog on human activated T lymphocytes	GSE23984	Baeke et al., 2011
Transcriptome profiling of genes regulated by RXR and its partners in monocyte-derived dendritic cells	GSE23073	Szeles et al., 2010
NON-PROTEIN CODING RNA		
MicroRNA-22 upregulation by vitamin D mediates its protective action against colon cancer.	GSE34564	Alvarez-Diaz et al., 2012
miRNA profiling of androgen receptor and vitamin D receptor mediated signaling in prostate cancer cells	GSE23814	Wang et al., 2013b
Identification of miRNAs regulated by vitamin D within primary human osteoblasts	GSE34144	
Vitamin D and microRNA expression	GSE20122	

therefore timely for these data to mined, and integrated with related nuclear receptor actions or other transcription factors that appear to co-operate with the VDR, for example SMADs.

VDR REGULATION OF NON-CODING RNA SPECIES

The human genome project in many ways was a race to define the protein coding genes within the human genome. Bacterial artificial chromosomes (BAC) clones enabled relatively large pieces of DNA, upto 300 kb to be inserted for sequencing (Osoegawa et al., 2000). However, a key step in the initial alignment process was to leverage cDNA and EST libraries and therefore naturally steered workers to protein coding genes, and the significance and extent of non-protein coding RNA remained largely unexplored.

Although non-coding RNA forms were well described in terms of ribosomal function it was little understood beyond this. The interpretation of the human genome, and other large scale approaches to investigating chromosomal function (Consortium et al., 2007; Tress et al., 2007) all led to a growing awareness of the extent of non-coding RNA and at least suggested that their was an unknown. This uncertainty has been reflected in the debate within the biological community over the extent and roles of so-called Junk DNA (Kapranov and St Laurent, 2012; Doolittle, 2013). As a result researchers have considered roles for non-coding RNA in the regulation of cell function and have begun to examine the interplay between the at least 20 different types of different non-coding RNA (reviewed in Ling et al., 2013). Many of these RNA species are gene regulatory RNA and include microRNA (miRNA), long non-coding RNA (long ncRNA), whereas others are involved in the post-transcriptional modification of RNA for example small nucleolar RNA (snoRNA).

Workers have now principally examined miRNA regulation by the VDR and evidence has emerged to support a role for the VDR to control regulation. For example Studzinski and co-workers revisited the mechanistically enigmatic VDR-mediated control $p27^{(kip1)}$. They elegantly demonstrated a role for VDR to down-regulate miR181a, which when left unchecked degrades $p27^{(kip1)}$(Wang et al., 2009) (**Figure 1**). Thus, indirectly, VDR activation elevates expression of $p27^{(kip1)}$, initiates cell cycle arrest and commits cells toward differentiation. These studies illuminated the earlier ones that suggested that $p27^{(kip1)}$ protein levels appeared to be regulated by a range of post-transcriptional mechanisms, such as enhanced mRNA translation, and attenuating degradative mechanisms (Hengst and Reed, 1996; Huang et al., 2004; Li et al., 2004). Similar integration of miRNA and mRNA was revealed to control the regulation of *CDKN1A*. Dynamic patterns of *CDKN1A* mRNA accumulation have been observed in various cell systems (Thorne et al., 2011). This is in part explained by the epigenetic state of different VDR binding sites on the *CDKN1A* promoter. However, VDR-dependent co-regulation of miR-106b also appears to modulate the precise timing of *CDKN1A* accumulation and expression of $p21^{(waf1/cip1)}$ in a feed-forward loop and determine the final extent of the cell cycle arrest. $1\alpha,25(OH)_2D_3$ regulates the DNA helicase *MCM7* (Khanim et al., 2004) that encodes the miR-106b, in intron 13 of the *MCM7* gene, and together these co-regulation processes control $p21^{(waf1/cip1)}$ through the balance of MCM7 and *CDKN1A* (Saramaki et al., 2006; Ivanovska et al., 2008) (**Figure 1**).

MicroRNA (miRNA) contribute negative regulatory aspects to normal gene regulation, for example as part of feed-forward loop motifs (Mangan and Alon, 2003; Mangan et al., 2006). The co-regulation of mRNA and miRNA in motifs that included

feed forward structures appears quite common (Song and Wang, 2008; Gatfield et al., 2009; Ribas et al., 2009; Sun et al., 2009; Wang et al., 2009). Other established miRNA targets of the VDR include miR-627 (Padi et al., 2013) that in turn targets *JMJD1A* (another histone H3 lysine demethylase) miR-98 (Ting et al., 2013) and let-7a-2 (Guan et al., 2013). However, one of the more explored relationships between VDR and miRNA is the relationship between VDR and miR-125b. MiR-125b inhibits VDR (Mohri et al., 2009; Zhang et al., 2011) and in turn VDR can down-regulate miR-125b (Iosue et al., 2013; Zhou et al., 2014), and the other targets of miR-125b include NCOR2/SMRT (Yang et al., 2012) (itself a VDR target gene) suggesting multiple levels of co-regulation and interdependent relationship between the VDR, and the mRNA and miRNA transcriptomes, and the epigenome. Finally, it is interesting to note that altered levels of miRNA are associated with cancer states and progression risks and indeed miR-125b is associated with aggressive prostate cancer (Shi et al., 2011; Amir et al., 2013; Singh et al., 2014).

Beyond these candidate studies, a number of investigators have undertaken miRNA microarray analyses (**Table 2**) and these approaches have identified various networks, including the control of lipid metabolism and PPAR© function (Wang et al., 2013b). It is likely that with the increased application of array approaches and next gen sequencing approaches will identify the key networks downstream of the VDR miRnome. This is unfortunately a more challenging research question owing to the many-to-many nature of miRNA; a given miRNA target many mRNA, and a given mRNA may have many miRNA targeting it. Thus, the computational challenges to resolve these relationships are not insignificant. Together these findings suggest that co-regulated miRNA may form an integral part of VDR signaling to control gene expression.

Of the other types of non-coding RNA, their regulation by VDR remains far less explored. Recently the group of Bickle have begun to dissect VDR regulation of lncRNA in keratinocytes and identified a number of target lncRNA and in doing so have raised the curtain on new avenues of exploration (Jiang and Bikle, 2014).

CONCLUSIONS AND FUTURE CHALLENGES FOR UNDERSTANDING THE VDR TRANSCRIPTOME

WHAT ARE THE PROTEINS, OR PROCESSES THAT GUIDE WHERE THE VDR BINDS IN THE GENOME?

It is unclear what key pioneer factors will be identified and if the VDR is in a strong relationship with a specific family of pioneer factors, in the same that the AR is profoundly influenced by the Forkhead family members. Indeed, the precise pioneer factor may even be tissue specific as also revealed for the AR (Pihlajamaa et al., 2014). This may reflect the fact the ligand activation of the VDR is more associated with re-distribution of the VDR through the genome, rather than triggering movement into the nucleus (as in the case of the classic steroid hormone receptors).

The specific epigenetic niche that characterizes the VDR binding may also be revealing of where and why the VDR binds to the genome. These analyses will require agnostic integration of multiple genomic data sets, for example histone modifications, transcription factor binding, chromatin conformation and transcriptomic data and application of machine learning approaches to reveal the significance of the underlying patterns, VDR binding and transcriptional activity. Whilst the VDR was not included in the ENCODE project, judicious choice of a cell line model for these studies, most likely a Tier 1 cell line from ENCODE, will enable leverage of a considerable volume of cistromic and epigenomic data to be combined with *de novo* VDR ChIP-Seq data. In this manner the question of how TGF® and/or WNT signaling interacts directly or indirectly with VDR binding can be addressed relatively easily.

Another major knowledge gap in VDR understanding concerns the spatial relationships between VDR binding and the control of transcription. It is clear that chromatin looping processes can transiently bring distal regulatory regions into physical proximity to the proximal regions of a gene and lead to dynamic gene expression (Saramaki et al., 2009). To transition from examination of looping of this process at a single locus using established binding sites to the genome wide investigation is technically and statistically very challenging. Again, ENCODE analyses may be useful here, as Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) data are available for specific cell lines; for example using RNA-PolII in K562 cells and therefore VDR ChIP-Seq in these cells would again be able to leverage this data to begin to understand how the VDR distributes and loops across the genome.

WHAT IS THE ROLE OF GENETIC VARIATION IN DETERMINING HOW AND WHERE THE VDR BINDS?

Genetic variation exists throughout the genome and by definition is predominately in non-RNA coding regions. This realization has been the catalyst for examining how genetic variation impacts transcription factor binding and activity. Perhaps the most comprehensive integration of these concepts has been the development of the RegulomeDB tool (Boyle et al., 2012) which considers the impact of genetic variation on the function of all transcription factors analyzed by ENCODE. To date this question has not been seriously considered in terms of the VDR. A major hurdle to addressing this question is very large potential for Type 1 error owing to the large-scale data sets that need to be integrated, namely; all SNPs and those in linkeage disequilibrium that are significantly associated with disease in replicated studies and all binding sites for a given transcription factor against the backdrop of the number of SNPs for a given trait, the platform used for identification and the total number of SNPs in the human genome. In the context of the VDR specifically, this challenge is compounded by the fact that the majority of VDR binding sites through the genome do not contain a canonical DR3 type binding element and therefore a critical question will remain around what protein is the VDR interacting with in the genomic context and how is this influenced by genetic variation. This challenge is clearly intertwined with developing a comprehensive knowledge of how the VDR binds to the genome.

WHAT IS THE COMPLETE VDR TRANSCRIPTOME AND HOW DOES IT DIFFER BY TISSUE AND BY DISEASE?

Surprisingly, no RNA-Seq data are yet available for the VDR. Therefore to capture all RNA regulated by the VDR will require RNA-Seq approaches applied to libraries that capture short and

long RNA species. The ENCODE consortium have undertaken over 400 RNA-Seq experiments focused on different RNA species in multiple cell models, and this makes a compelling case for exploiting these data, especially in Tier 1 or Tier 2 cell lines. For example undertaking VDR ChIP-Seq and a limited number of RNA-Seq approaches in K562 cells has the potential to leverage a remarkable volume of data. Predictions from such integrative analyses could then be tested in other ENCODE resources, in RNA-Seq from multiple tissues in normal healthy donors, through the GTEx consortium, or through the vast numbers of microarrays that are publically available. For example, in the case of K562, which is a CML cell line, there are many large-scale microarray analyses of patients with CML to examine how the VDR transcriptome relates to disease state and drug response. A parallel outcome of investigating VDR function will be to address the role it plays in regulating splice variation as suggested by the interactions with proteins such as SNW1.

HOW WILL THIS KNOWLEDGE BE EXPLOITED IN PERSONALIZED MEASURES OF VDR SYSTEM IN HEALTH AND DISEASE?

Many aspects of the relationships identified above can be interpreted by serum borne measurements, which are highly attractive owing to their ease of measurement. Serum levels of the prohormone, 25(OH) vitamin D3, are strongly correlated with the generation of the active hormone $1\alpha,25(OH)_2D_3$ and VDR function. For example, reduced serum levels of 25(OH) vitamin D3 levels are associated with increased risk of either cancer initiation and/or progression (Drake et al., 2010; Shanafelt et al., 2011). Therefore the serum level of 25(OH) vitamin D3can yield the "potential" of the VDR system to signal (Brader et al., 2014). This potential is impacted by the various cellular mechanisms outlined above. Of these, genetic variation that impacts VDR binding can obviously be measured in any cell in the body. The total transcriptome can be challenging to measure but perhaps small non-coding RNA represent a highly attractive marker of activity. Remarkably, miRNA are readily secreted into serum where they remain stable (El-Hefnawy et al., 2004; Goyal et al., 2006; Taylor and Gercel-Taylor, 2008; Valadi et al., 2007) and can be reliably extracted and measured (Chen et al., 2008; Liu et al., 2008; Mitchell et al., 2008; Rabinowits et al., 2009). Using serum-borne molecules as prognostic markers is highly attractive for several reasons. First, they can overcome the limitations of inaccurate sampling for example in the case of the presence of cancer within a tumor biopsy. Second, they can encapsulate the effects of heterotypic cell interactions, again, for example within the tumor microenvironment. Third, they form a non-invasive test procedure. From a biostatistical perspective, given there are fewer miRNA than protein coding mRNA, genome-wide coverage is more readily achieved and avoids the statistical penalties typically associated mRNA genome wide testing (Lussier et al., 2012).

This raises the very exciting possibility that generating integrated models of VDR binding, the impact of genetic variation, the tissue specific differences in the transcriptome and identifying miRNA contained within transcriptional circuits offers the opportunity of exploiting their serum expression levels of 25(OH) vitamin D3, genetic variation and miRNA expression will be able to be exploited to predict accurately the capacity of VDR function.

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REFERENCES

Akutsu, N., Lin, R., Bastien, Y., Bestawros, A., Enepekides, D. J., Black, M. J., et al. (2001). Regulation of gene Expression by 1alpha,25-dihydroxyvitamin D3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. *Mol. Endocrinol.* 15, 1127–1139. doi: 10.1210/mend.15.7.0655

Alvarez-Diaz, S., Valle, N., Ferrer-Mayorga, G., Lombardia, L., Herrera, M., Dominguez, O., et al. (2012). MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells. *Hum. Mol. Genet.* 21, 2157–2165. doi: 10.1093/hmg/dds031

Amir, S., Ma, A. H., Shi, X. B., Xue, L., Kung, H. J., and Devere White, R. W. (2013). Oncomir miR-125b suppresses p14(ARF) to modulate p53-dependent and p53-independent apoptosis in prostate cancer. *PLoS ONE* 8:e61064. doi: 10.1371/journal.pone.0061064

Anderson, P. H., O'Loughlin, P. D., May, B. K., and Morris, H. A. (2003). Quantification of mRNA for the vitamin D metabolizing enzymes CYP27B1 and CYP24 and vitamin D receptor in kidney using real-time reverse transcriptase- polymerase chain reaction. *J. Mol. Endocrinol.* 31, 123–132. doi: 10.1677/jme.0.0310123

Aubol, B. E., Jamros, M. A., McGlone, M. L., and Adams, J. A. (2013). Splicing kinase SRPK1 conforms to the landscape of its SR protein substrate. *Biochemistry* 52, 7595–7605. doi: 10.1021/bi4010864

Baeke, F., Korf, H., Overbergh, L., Verstuyf, A., Thorrez, L., Van Lommel, L., et al. (2011). The vitamin D analog, TX527, promotes a human CD4+CD25highCD127low regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation. *J. Immunol.* 186, 132–142. doi: 10.4049/jimmunol.1000695

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., et al. (1988). Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. U.S.A.* 85, 3294–3298. doi: 10.1073/pnas.85.10.3294

Barrett, T., Suzek, T. O., Troup, D. B., Wilhite, S. E., Ngau, W. C., Ledoux, P., et al. (2005). NCBI GEO: mining millions of expression profiles–database and tools. *Nucleic Acids Res.* 33, D562–D566. doi: 10.1093/nar/gki022

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., et al. (2013). NCBI GEO: archive for functional genomics data sets–update. *Nucleic Acids Res.* 41, D991–D995. doi: 10.1093/nar/gks1193

Baudino, T. A., Kraichely, D. M., Jefcoat, S. C. Jr., Winchester, S. K., Partridge, N. C., and MacDonald, P. N. (1998). Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *J. Biol. Chem.* 273, 16434–16441. doi: 10.1074/jbc.273.26.16434

Birney, E. (2012). The making of ENCODE: lessons for big-data projects. *Nature* 489, 49–51. doi: 10.1038/489049a

Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigo, R., Gingeras, T. R., Margulies, E. H., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799–816. doi: 10.1038/nature05874

Bosse, Y., Maghni, K., and Hudson, T. J. (2007). 1alpha,25-dihydroxy-vitamin D3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. *Physiol. Genomics* 29, 161–168. doi: 10.1152/physiolgenomics.00134.2006

Boyle, A. P., Hong, E. L., Hariharan, M., Cheng, Y., Schaub, M. A., Kasowski, M., et al. (2012). Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res.* 22, 1790–1797. doi: 10.1101/gr.137323.112

Brader, L., Rejnmark, L., Carlberg, C., Schwab, U., Kolehmainen, M., Rosqvist, F., et al. (2014). Effects of a healthy Nordic diet on plasma 25-hydroxyvitamin D concentration in subjects with metabolic syndrome: a randomized, placebo-controlled trial (SYSDIET). *Eur. J. Nutr.* doi: 10.1007/s00394-014-0674-3. [Epub ahead of print].

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., et al. (2001). Minimum information about a microarray experiment

(MIAME)-toward standards for microarray data. *Nat. Genet.* 29, 365–371. doi: 10.1038/ng1201-365

Campbell, M. J., Elstner, E., Holden, S., Uskokovic, M., and Koeffler, H. P. (1997). Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. *J. Mol. Endocrinol.* 19, 15–27. doi: 10.1677/jme.0.0190015

Carlberg, C., and Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78, 127–136. doi: 10.1016/j.steroids.2012.10.019

Carroll, J. S., Meyer, C. A., Song, J., Li, W., Geistlinger, T. R., Eeckhoute, J., et al. (2006). Genome-wide analysis of estrogen receptor binding sites. *Nat. Genet.* 38, 1289–1297. doi: 10.1038/ng1901

Cenciarelli, C., De Santa, F., Puri, P. L., Mattei, E., Ricci, L., Bucci, F., et al. (1999). Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. *Mol. Cell. Biol.* 19, 5203–5217.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., et al. (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 18, 997–1006. doi: 10.1038/cr.2008.282

Colston, K., Colston, M. J., Fieldsteel, A. H., and Feldman, D. (1982). 1,25-dihydroxyvitamin D3 receptors in human epithelial cancer cell lines. *Cancer Res.* 42, 856–859.

Colston, K. W., Berger, U., and Coombes, R. C. (1989). Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet* 1, 188–191.

Consortium, E. P., Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigo, R., Gingeras, T. R., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799–816. doi: 10.1038/nature05874

Consortium, G. T. (2013). The Genotype-Tissue Expression (GTEx) project. *Nat. Genet.* 45, 580–585. doi: 10.1038/ng.2653

Costa, J. L., Eijk, P. P., van de Wiel, M. A., ten Berge, D., Schmitt, F., Narvaez, C. J., et al. (2009). Anti-proliferative action of vitamin D in MCF7 is still active after siRNA-VDR knock-down. *BMC Genomics* 10:499. doi: 10.1186/1471-2164-10-499

Cui, M., Klopot, A., Jiang, Y., and Fleet, J. C. (2009). The effect of differentiation on 1,25 dihydroxyvitamin D-mediated gene expression in the enterocyte-like cell line, Caco-2. *J. Cell. Physiol.* 218, 113–121. doi: 10.1002/jcp.21574

Despouy, G., Bastie, J. N., Deshaies, S., Balitrand, N., Mazharian, A., Rochette-Egly, C., et al. (2003). Cyclin D3 is a cofactor of retinoic acid receptors, modulating their activity in the presence of cellular retinoic acid-binding protein II. *J. Biol. Chem.* 278, 6355–6362. doi: 10.1074/jbc.M210697200

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Do, J. H., and Choi, D. K. (2006). Normalization of microarray data: single-labeled and dual-labeled arrays. *Mol. Cells* 22, 254–261.

Dobrzynski, M., and Bruggeman, F. J. (2009). Elongation dynamics shape bursty transcription and translation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2583–2588. doi: 10.1073/pnas.0803507106

Doig, C. L., Singh, P. K., Dhiman, V. K., Thorne, J. L., Battaglia, S., Sobolewski, M., et al. (2013). Recruitment of NCOR1 to VDR target genes is enhanced in prostate cancer cells and associates with altered DNA methylation patterns. *Carcinogenesis* 34, 248–256. doi: 10.1093/carcin/bgs331

Doolittle, W. F. (2013). Is junk DNA bunk? A critique of ENCODE. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5294–5300. doi: 10.1073/pnas.1221376110

Drake, M. T., Maurer, M. J., Link, B. K., Habermann, T. M., Ansell, S. M., Micallef, I. N., et al. (2010). Vitamin D insufficiency and prognosis in non-Hodgkin's lymphoma. *J. Clin. Oncol.* 28, 4191–4198. doi: 10.1200/JCO.2010.28.6674

Dunlop, T. W., Vaisanen, S., Frank, C., and Carlberg, C. (2004). The genes of the coactivator TIF2 and the corepressor SMRT are primary 1alpha,25(OH)2D3 targets. *J. Steroid Biochem. Mol. Biol.* 89–90, 257–260. doi: 10.1016/j.jsbmb.2004.03.079

Dwivedi, P. P., Omdahl, J. L., Kola, I., Hume, D. A., and May, B. K. (2000). Regulation of rat cytochrome P450C24 (CYP24) gene expression. Evidence for functional cooperation of Ras-activated Ets transcription factors with the vitamin D receptor in 1,25-dihydroxyvitamin D(3)-mediated induction. *J. Biol. Chem.* 275, 47–55. doi: 10.1074/jbc.275.1.47

Eelen, G., Verlinden, L., Van Camp, M., Mathieu, C., Carmeliet, G., Bouillon, R., et al. (2004). Microarray analysis of 1alpha,25-dihydroxyvitamin D3-treated MC3T3-E1 cells. *J. Steroid Biochem. Mol. Biol.* 89–90, 405–407. doi: 10.1016/j.jsbmb.2004.03.008

El-Hefnawy, T., Raja, S., Kelly, L., Bigbee, W. L., Kirkwood, J. M., Luketich, J. D., et al. (2004). Characterization of amplifiable, circulating RNA in plasma and its potential as a tool for cancer diagnostics. *Clin. Chem.* 50, 564–573. doi: 10.1373/clinchem.2003.028506

Ellison, T. I., Smith, M. K., Gilliam, A. C., and MacDonald, P. N. (2008). Inactivation of the vitamin D receptor enhances susceptibility of murine skin to UV-induced tumorigenesis. *J. Invest. Dermatol.* 128, 2508–2517. doi: 10.1038/jid.2008.131

Elstner, E., Campbell, M. J., Munker, R., Shintaku, P., Binderup, L., Heber, D., et al. (1999). Novel 20-epi-vitamin D3 analog combined with 9-cis-retinoic acid markedly inhibits colony growth of prostate cancer cells. *Prostate* 40, 141–149. doi: 10.1002/(SICI)1097-0045(19990801)40:3%3C141::AID-PROS1%3E3.0.CO;2-C

Engreitz, J. M., Chen, R., Morgan, A. A., Dudley, J. T., Mallelwar, R., and Butte, A. J. (2011). ProfileChaser: searching microarray repositories based on genome-wide patterns of differential expression. *Bioinformatics* 27, 3317–3318. doi: 10.1093/bioinformatics/btr548

Gatfield, D., Le Martelot, G., Vejnar, C. E., Gerlach, D., Schaad, O., Fleury-Olela, F., et al. (2009). Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes Dev.* 23, 1313–1326. doi: 10.1101/gad.1781009

Gkika, D., Mahieu, F., Nilius, B., Hoenderop, J. G., and Bindels, R. J. (2004). 80K-H as a new Ca2+ sensor regulating the activity of the epithelial Ca2+ channel transient receptor potential cation channel V5 (TRPV5). *J. Biol. Chem.* 279, 26351–26357. doi: 10.1074/jbc.M403801200

Goldberg, A. D., Allis, C. D., and Bernstein, E. (2007). Epigenetics: a landscape takes shape. *Cell* 128, 635–638. doi: 10.1016/j.cell.2007.02.006

Goyal, A., Delves, G. H., Chopra, M., Lwaleed, B. A., and Cooper, A. J. (2006). Prostate cells exposed to lycopene *in vitro* liberate lycopene-enriched exosomes. *BJU Int.* 98, 907–911. doi: 10.1111/j.1464-410X.2006.06434.x

Guan, H., Liu, C., Chen, Z., Wang, L., Li, C., Zhao, J., et al. (2013). 1,25-Dihydroxyvitamin D3 up-regulates expression of hsa-let-7a-2 through the interaction of VDR/VDRE in human lung cancer A549 cells. *Gene* 522, 142–146. doi: 10.1016/j.gene.2013.03.065

Gynther, P., Toropainen, S., Matilainen, J. M., Seuter, S., Carlberg, C., and Vaisanen, S. (2011). Mechanism of 1alpha,25-dihydroxyvitamin D(3)-dependent repression of interleukin-12B. *Biochim. Biophys. Acta* 1813, 810–818. doi: 10.1016/j.bbamcr.2011.01.037

Hayes, G. M., Carrigan, P. E., Beck, A. M., and Miller, L. J. (2006). Targeting the RNA splicing machinery as a novel treatment strategy for pancreatic carcinoma. *Cancer Res.* 66, 3819–3827. doi: 10.1158/0008-5472.CAN-05-4065

Heikkinen, S., Vaisanen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1alpha,25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Hengst, L., and Reed, S. I. (1996). Translational control of p27Kip1 accumulation during the cell cycle. *Science* 271, 1861–1864. doi: 10.1126/science.271.5257.1861

Hsieh, J. C., Sisk, J. M., Jurutka, P. W., Haussler, C. A., Slater, S. A., Haussler, M. R., et al. (2003). Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. *J. Biol. Chem.* 278, 38665–38674. doi: 10.1074/jbc.M304886200

Huang, Y. C., Chen, J. Y., and Hung, W. C. (2004). Vitamin D(3) receptor/Sp1 complex is required for the induction of p27(Kip1) expression by vitamin D(3). *Oncogene*. doi: 10.1038/sj.onc.1207621

Iosue, I., Quaranta, R., Masciarelli, S., Fontemaggi, G., Batassa, E. M., Bertolami, C., et al. (2013). Argonaute 2 sustains the gene expression program driving human monocytic differentiation of acute myeloid leukemia cells. *Cell Death Dis.* 4, e926. doi: 10.1038/cddis.2013.452

Ito, I., Waku, T., Aoki, M., Abe, R., Nagai, Y., Watanabe, T., et al. (2013). A nonclassical vitamin D receptor pathway suppresses renal fibrosis. *J. Clin. Invest.* 123, 4579–4594. doi: 10.1172/JCI67804

Ivanovska, I., Ball, A. S., Diaz, R. L., Magnus, J. F., Kibukawa, M., Schelter, J. M., et al. (2008). MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. *Mol. Cell. Biol.* 28, 2167–2174. doi: 10.1128/MCB.01977-07

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. *Science* 293, 1074–1080. doi: 10.1126/science.1063127

Jiang, F., Li, P., Fornace, A. J. Jr., Nicosia, S. V., and Bai, W. (2003). G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. *J. Biol. Chem.* 278, 48030–48040. doi: 10.1074/jbc.M308430200

Jiang, Y. J., and Bikle, D. D. (2014). LncRNA: a new player in 1alpha, 25(OH)2 vitamin D3 /VDR protection against skin cancer formation. *Exp. Dermatol.* 23, 147–150. doi: 10.1111/exd.12341

Kangaspeska, S., Stride, B., Metivier, R., Polycarpou-Schwarz, M., Ibberson, D., Carmouche, R. P., et al. (2008). Transient cyclical methylation of promoter DNA. *Nature* 452, 112–115. doi: 10.1038/nature06640

Kapranov, P., and St Laurent, G. (2012). Dark matter RNA: existence, function, and controversy. *Front. Genet.* 3:60. doi: 10.3389/fgene.2012.00060

Katoh, M., and Katoh, M. (2007). WNT signaling pathway and stem cell signaling network. *Clin. Cancer Res.* 13, 4042–4045. doi: 10.1158/1078-0432.CCR-06-2316

Khanim, F. L., Gommersall, L. M., Wood, V. H., Smith, K. L., Montalvo, L., O'Neill, L. P., et al. (2004). Altered SMRT levels disrupt vitamin D3 receptor signalling in prostate cancer cells. *Oncogene* 23, 6712–6725. doi: 10.1038/sj.onc.1207772

Kim, J. Y., Son, Y. L., and Lee, Y. C. (2009). Involvement of SMRT Corepressor in Transcriptional Repression by the Vitamin D Receptor. *Mol. Endocrinol.* 23, 251–264. doi: 10.1210/me.2008-0426

Kim, S., Shevde, N. K., and Pike, J. W. (2005). 1,25-Dihydroxyvitamin D3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J. Bone Miner. Res.* 20, 305–317. doi: 10.1359/JBMR.041112

Koike, M., Elstner, E., Campbell, M. J., Asou, H., Uskokovic, M., Tsuruoka, N., et al. (1997). 19-nor-hexafluoride analogue of vitamin D3: a novel class of potent inhibitors of proliferation of human breast cell lines. *Cancer Res.* 57, 4545–4550.

Kommagani, R., Caserta, T. M., and Kadakia, M. P. (2006). Identification of vitamin D receptor as a target of p63. *Oncogene* 25, 3745–3751. doi: 10.1038/sj.onc.1209412

Kovalenko, P. L., Zhang, Z., Yu, J. G., Li, Y., Clinton, S. K., and Fleet, J. C. (2011). Dietary vitamin D and vitamin D receptor level modulate epithelial cell proliferation and apoptosis in the prostate. *Cancer Prev. Res. (Phila.)* 4, 1617–1625. doi: 10.1158/1940-6207.CAPR-11-0035

Lai, Y., Zhang, F., Nayak, T. K., Modarres, R., Lee, N. H., and McCaffrey, T. A. (2014). Concordant integrative gene set enrichment analysis of multiple large-scale two-sample expression data sets. *BMC Genomics* 15(Suppl. 1), S6. doi: 10.1186/1471-2164-15-S1-S6

Lambert, J. R., Kelly, J. A., Shim, M., Huffer, W. E., Nordeen, S. K., Baek, S. J., et al. (2006). Prostate derived factor in human prostate cancer cells: gene induction by vitamin D via a p53-dependent mechanism and inhibition of prostate cancer cell growth. *J. Cell. Physiol.* 208, 566–574. doi: 10.1002/jcp.20692

Lazaro, J. B., Bailey, P. J., and Lassar, A. B. (2002). Cyclin D-cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation. *Genes Dev.* 16, 1792–1805. doi: 10.1101/gad.U-9988R

Le May, N., Mota-Fernandes, D., Velez-Cruz, R., Iltis, I., Biard, D., and Egly, J. M. (2010). NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. *Mol. Cell* 38, 54–66. doi: 10.1016/j.molcel.2010.03.004

Leong, G. M., Subramaniam, N., Figueroa, J., Flanagan, J. L., Hayman, M. J., Eisman, J. A., et al. (2001). Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. *J. Biol. Chem.* 276, 18243–18248. doi: 10.1074/jbc.M010815200

Li, P., Li, C., Zhao, X., Zhang, X., Nicosia, S. V., and Bai, W. (2004). p27(Kip1) stabilization and G(1) arrest by 1,25-dihydroxyvitamin D(3) in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase. *J. Biol. Chem.* 279, 25260–25267. doi: 10.1074/jbc.M311052200

Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., et al. (2002). Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. *Mol. Endocrinol.* 16, 1243–1256. doi: 10.1210/mend.16.6.0874

Ling, H., Fabbri, M., and Calin, G. A. (2013). MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat. Rev. Drug Discov.* 12, 847–865. doi: 10.1038/nrd4140

Lisse, T. S., Liu, T., Irmler, M., Beckers, J., Chen, H., Adams, J. S., et al. (2011). Gene targeting by the vitamin D response element binding protein reveals a role for vitamin D in osteoblast mTOR signaling. *FASEB J.* 25, 937–947. doi: 10.1096/fj.10-172577

Liu, C. G., Calin, G. A., Volinia, S., and Croce, C. M. (2008). MicroRNA expression profiling using microarrays. *Nat. Protoc.* 3, 563–578. doi: 10.1038/nprot.2008.14

Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. (1996). Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* 10, 142–153. doi: 10.1101/gad.10.2.142

Lupien, M., Eeckhoute, J., Meyer, C. A., Wang, Q., Zhang, Y., Li, W., et al. (2008). FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* 132, 958–970. doi: 10.1016/j.cell.2008.01.018

Lussier, Y. A., Stadler, W. M., and Chen, J. L. (2012). Advantages of genomic complexity: bioinformatics opportunities in microRNA cancer signatures. *J. Am. Med. Inform. Assoc.* 19, 156–160. doi: 10.1136/amiajnl-2011-000419

Mahajan, M. A., and Samuels, H. H. (2000). A new family of nuclear receptor coregulators that integrate nuclear receptor signaling through CREB-binding protein. *Mol. Cell. Biol.* 20, 5048–5063. doi: 10.1128/MCB.20.14.5048-5063.2000

Maher, B. (2012). ENCODE: The human encyclopaedia. *Nature* 489, 46–48. doi: 10.1038/489046a

Malinen, M., Saramaki, A., Ropponen, A., Degenhardt, T., Vaisanen, S., and Carlberg, C. (2008). Distinct HDACs regulate the transcriptional response of human cyclin-dependent kinase inhibitor genes to Trichostatin A and 1alpha,25-dihydroxyvitamin D3. *Nucleic Acids Res.* 36, 121–132. doi: 10.1093/nar/gkm913

Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. U.S.A.* 100, 11980–11985. doi: 10.1073/pnas.2133841100

Mangan, S., Itzkovitz, S., Zaslaver, A., and Alon, U. (2006). The incoherent feed-forward loop accelerates the response-time of the gal system of Escherichia coli. *J. Mol. Biol.* 356, 1073–1081. doi: 10.1016/j.jmb.2005.12.003

Marinelli, R. J., Montgomery, K., Liu, C. L., Shah, N. H., Prapong, W., Nitzberg, M., et al. (2008). The stanford tissue microarray database. *Nucleic Acids Res.* 36, D871–D877. doi: 10.1093/nar/gkm861

Martinez-Climent, J. A., Fontan, L., Fresquet, V., Robles, E., Ortiz, M., and Rubio, A. (2010). Integrative oncogenomic analysis of microarray data in hematologic malignancies. *Methods Mol. Biol.* 576, 231–277. doi: 10.1007/978-1-59745-545-9_13

Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., et al. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res.* 66, 4574–4583. doi: 10.1158/0008-5472.CAN-05-2562

Metivier, R., Gallais, R., Tiffoche, C., Le Peron, C., Jurkowska, R. Z., Carmouche, R. P., et al. (2008). Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 452, 45–50. doi: 10.1038/nature06544

Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M., et al. (2003). Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115, 751–763. doi: 10.1016/S0092-8674(03)00934-6

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2012). VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol. Endocrinol.* 26, 37–51. doi: 10.1210/me.2011-1109

Meyer, M. B., Watanuki, M., Kim, S., Shevde, N. K., and Pike, J. W. (2006). The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. *Mol. Endocrinol.* 20, 1447–1461. doi: 10.1210/me.2006-0031

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10513–10518. doi: 10.1073/pnas.0804549105

Mohn, F., and Schubeler, D. (2009). Genetics and epigenetics: stability and plasticity during cellular differentiation. *Trends Genet.* 25, 129–136. doi: 10.1016/j.tig.2008.12.005

Mohri, T., Nakajima, M., Takagi, S., Komagata, S., and Yokoi, T. (2009). MicroRNA regulates human vitamin D receptor. *Int. J. Cancer* 125, 1328–1333. doi: 10.1002/ijc.24459

Novershtern, N., Subramanian, A., Lawton, L. N., Mak, R. H., Haining, W. N., McConkey, M. E., et al. (2011). Densely interconnected transcriptional

circuits control cell states in human hematopoiesis. *Cell* 144, 296–309. doi: 10.1016/j.cell.2011.01.004

Orlov, I., Rochel, N., Moras, D., and Klaholz, B. P. (2012). Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. *EMBO J.* 31, 291–300. doi: 10.1038/emboj.2011.445

Osoegawa, K., Tateno, M., Woon, P. Y., Frengen, E., Mammoser, A. G., Catanese, J. J., et al. (2000). Bacterial artificial chromosome libraries for mouse sequencing and functional analysis. *Genome Res.* 10, 116–128.

Padi, S. K., Zhang, Q., Rustum, Y. M., Morrison, C., and Guo, B. (2013). MicroRNA-627 mediates the epigenetic mechanisms of vitamin D to suppress proliferation of human colorectal cancer cells and growth of xenograft tumors in mice. *Gastroenterology* 145, 437–446. doi: 10.1053/j.gastro.2013.04.012

Pálmer, H. G., González-Sancho, J. M., Espada, J., Berciano, M. T., Puig, I., Baulida, J., et al. (2001). Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J. Cell. Biol.* 154, 369–387. doi: 10.1083/jcb.200102028

Pálmer, H. G., Sánchez-Carbayo, M., Ordóñez-Morán, P., Larriba, M. J., Cordón-Cardó, C, and Muñoz, A. (2003). Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells. *Cancer Res.* 63, 7799–7806.

Parkinson, H., Kapushesky, M., Shojatalab, M., Abeygunawardena, N., Coulson, R., Farne, A., et al. (2007). ArrayExpress–a public database of microarray experiments and gene expression profiles. *Nucleic Acids Res.* 35, D747–D750. doi: 10.1093/nar/gkl995

Peehl, D. M., Skowronski, R. J., Leung, G. K., Wong, S. T., Stamey, T. A., and Feldman, D. (1994). Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. *Cancer Res.* 54, 805–810.

Peleg, S., and Nguyen, C. V. (2010). The importance of nuclear import in protection of the vitamin D receptor from polyubiquitination and proteasome-mediated degradation. *J. Cell. Biochem.* 110, 926–934. doi: 10.1002/jcb.22606

Pereira, F., Barbachano, A., Silva, J., Bonilla, F., Campbell, M. J., Munoz, A., et al. (2011). KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. *Hum. Mol. Genet.* 20, 4655–4665. doi: 10.1093/hmg/ddr399

Pihlajamaa, P., Sahu, B., Lyly, L., Aittomaki, V., Hautaniemi, S., and Janne, O. A. (2014). Tissue-specific pioneer factors associate with androgen receptor cistromes and transcription programs. *EMBO J.* 33, 312–326. doi: 10.1002/embj.201385895

Pike, J. W., Gooze, L. L., and Haussler, M. R. (1980). Biochemical evidence for 1,25-dihydroxyvitamin D receptor macromolecules in parathyroid, pancreatic, pituitary, and placental tissues. *Life Sci.* 26, 407–414. doi: 10.1016/0024-3205(80)90158-7

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000). VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *FASEB J.* 14, 1455–1463. doi: 10.1096/fj.14.10.1455

Quack, M., and Carlberg, C. (2000). The impact of functional vitamin D(3) receptor conformations on DNA-dependent vitamin D(3) signaling. *Mol. Pharmacol.* 57, 375–384.

Rabinowits, G., Gercel-Taylor, C., Day, J. M., Taylor, D. D., and Kloecker, G. H. (2009). Exosomal microRNA: a diagnostic marker for lung cancer. *Clin. Lung Cancer.* 10, 42–46. doi: 10.3816/CLC.2009.n.006

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Rantala, J. K., Edgren, H., Lehtinen, L., Wolf, M., Kleivi, K., Vollan, H. K., et al. (2010). Integrative functional genomics analysis of sustained polyploidy phenotypes in breast cancer cells identifies an oncogenic profile for GINS2. *Neoplasia* 12, 877–888.

Rashid, S. F., Moore, J. S., Walker, E., Driver, P. M., Engel, J., Edwards, C. E., et al. (2001). Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. *Oncogene* 20, 1860–1872. doi: 10.1038/sj.onc.1204269

Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., et al. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* 11, 695–707. doi: 10.1016/S1097-2765(03)00090-X

Ribas, J., Ni, X., Haffner, M., Wentzel, E. A., Salmasi, A. H., Chowdhury, W. H., et al. (2009). miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Res.* 69, 7165–7169. doi: 10.1158/0008-5472.CAN-09-1448

Rid, R., Wagner, M., Maier, C. J., Hundsberger, H., Hintner, H., Bauer, J. W., et al. (2013). Deciphering the calcitriol-induced transcriptomic response in keratinocytes: presentation of novel target genes. *J. Mol. Endocrinol.* 50, 131–149. doi: 10.1530/JME-11-0191

Rosenbloom, K. R., Sloan, C. A., Malladi, V. S., Dreszer, T. R., Learned, K., Kirkup, V. M., et al. (2013). ENCODE data in the UCSC genome browser: year 5 update. *Nucleic Acids Res.* 41, D56–D63. doi: 10.1093/nar/gks1172

Sahu, B., Laakso, M., Pihlajamaa, P., Ovaska, K., Sinielnikov, I., Hautaniemi, S., et al. (2013). FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells. *Cancer Res.* 73, 1570–1580. doi: 10.1158/0008-5472.CAN-12-2350

Saramaki, A., Banwell, C. M., Campbell, M. J., and Carlberg, C. (2006). Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res.* 34, 543–554. doi: 10.1093/nar/gkj460

Saramaki, A., Diermeier, S., Kellner, R., Laitinen, H., Vaisanen, S., and Carlberg, C. (2009). Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha,25-dihydroxyvitamin D3. *J. Biol. Chem.* 284, 8073–8082. doi: 10.1074/jbc.M808090200

Sarruf, D. A., Iankova, I., Abella, A., Assou, S., Miard, S., and Fajas, L. (2005). Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. *Mol. Cell Biol.* 25, 9985–9995. doi: 10.1128/MCB.25.22.9985-9995.2005

Savli, H., Aalto, Y., Nagy, B., Knuutila, S., and Pakkala, S. (2002). Gene expression analysis of 1,25(OH)2D3-dependent differentiation of HL-60 cells: a cDNA array study. *Br. J. Haematol.* 118, 1065–1070. doi: 10.1046/j.1365-2141.2002.03734.x

Schwaller, J., Koeffler, H. P., Niklaus, G., Loetscher, P., Nagel, S., Fey, M. F., et al. (1995). Posttranscriptional stabilization underlies p53-independent induction of p21WAF1/CIP1/SDI1 in differentiating human leukemic cells. *J. Clin. Invest.* 95, 973–979. doi: 10.1172/JCI117806

Scsucova, S., Palacios, D., Savignac, M., Mellstrom, B., Naranjo, J. R., and Aranda, A. (2005). The repressor DREAM acts as a transcriptional activator on Vitamin D and retinoic acid response elements. *Nucleic Acids Res.* 33, 2269–2279. doi: 10.1093/nar/gki503

Seo, Y. K., Chung, Y. T., Kim, S., Echchgadda, I., Song, C. S., and Chatterjee, B. (2007). Xenobiotic- and vitamin D-responsive induction of the steroid/bile acid-sulfotransferase Sult2A1 in young and old mice: the role of a gene enhancer in the liver chromatin. *Gene* 386, 218–223. doi: 10.1016/j.gene.2006.10.006

Serandour, A. A., Avner, S., Percevault, F., Demay, F., Bizot, M., Lucchetti-Miganeh, C., et al. (2011). Epigenetic switch involved in activation of pioneer factor FOXA1-dependent enhancers. *Genome Res.* 21, 555–565. doi: 10.1101/gr.111534.110

Shah, N. H., Jonquet, C., Chiang, A. P., Butte, A. J., Chen, R., and Musen, M. A. (2009). Ontology-driven indexing of public datasets for translational bioinformatics. *BMC Bioinform.* 10 (Suppl. 2), S1. doi: 10.1186/1471-2105-10-S2-S1

Shanafelt, T. D., Drake, M. T., Maurer, M. J., Allmer, C., Rabe, K. G., and Slager, S. L., et al. (2011). Vitamin D insufficiency and prognosis in chronic lymphocytic leukemia. *Blood* 117, 1492–1498. doi: 10.1182/blood-2010-07-295683

Shi, X. B., Xue, L., Ma, A. H., Tepper, C. G., Kung, H. J., and White, R. W. (2011). miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. *Prostate* 71, 538–549. doi: 10.1002/pros.21270

Singh, P. K., Preus, L., Hu, Q., Yan, L., Long, M. D., Morrison, C. D., et al. (2014). Serum microRNA expression patterns that predict early treatment failure in prostate cancer patients. *Oncotarget* 5, 824–840.

Song, G., and Wang, L. (2008). Transcriptional mechanism for the paired miR-433 and miR-127 genes by nuclear receptors SHP and ERRgamma. *Nucleic Acids Res.* 36, 5727–5735. doi: 10.1093/nar/gkn567

Stamatoyannopoulos, J. A. (2012). What does our genome encode? *Genome Res.* 22, 1602–1611. doi: 10.1101/gr.146506.112

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. *Cancer Cell* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Strausberg, R. L., Buetow, K. H., Greenhut, S. F., Grouse, L. H., and Schaefer, C. F. (2002). The cancer genome anatomy project: online resources to reveal the molecular signatures of cancer. *Cancer Invest.* 20, 1038–1050. doi: 10.1081/CNV-120005922

Sun, T., Wang, Q., Balk, S., Brown, M., Lee, G. S., and Kantoff, P. (2009). The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines. *Cancer Res.* 69, 3356–3363. doi: 10.1158/0008-5472.CAN-08-4112

Szeles, L., Poliska, S., Nagy, G., Szatmari, I., Szanto, A., Pap, A., et al. (2010). Research resource: transcriptome profiling of genes regulated by RXR and its permissive and nonpermissive partners in differentiating monocyte-derived dendritic cells. *Mol. Endocrinol.* 24, 2218–2231. doi: 10.1210/me.2010-0215

Tagami, T., Lutz, W. H., Kumar, R., and Jameson, J. L. (1998). The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. *Biochem. Biophys. Res. Commun.* 253, 358–363. doi: 10.1006/bbrc.1998.9799

Tagliafico, E., Tenedini, E., Manfredini, R., Grande, A., Ferrari, F., Roncaglia, E., et al. (2006). Identification of a molecular signature predictive of sensitivity to differentiation induction in acute myeloid leukemia. *Leukemia* 20, 1751–1758. doi: 10.1038/sj.leu.2404358

Taylor, D. D., and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* 110, 13–21. doi: 10.1016/j.ygyno.2008.04.033

Thorne, J. L., Maguire, O., Doig, C. L., Battaglia, S., Fehr, L., Sucheston, L. E., et al. (2011). Epigenetic control of a VDR-governed feed-forward loop that regulates p21(waf1/cip1) expression and function in non-malignant prostate cells. *Nucleic Acids Res.* 39, 2045–2056. doi: 10.1093/nar/gkq875

Ting, H. J., Messing, J., Yasmin-Karim, S., and Lee, Y. F. (2013). Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D. *J. Biol. Chem.* 288, 1–9. doi: 10.1074/jbc.M112.395947

Towsend, K., Trevino, V., Falciani, F., Stewart, P. M., Hewison, M., and Campbell, M. J. (2006). Identification of VDR-responsive gene signatures in breast cancer cells. *Oncology* 71, 111–123. doi: 10.1159/000100989

Tress, M. L., Martelli, P. L., Frankish, A., Reeves, G. A., Wesselink, J. J., Yeats, C., et al. (2007). The implications of alternative splicing in the ENCODE protein complement. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5495–5500. doi: 10.1073/pnas.0700800104

Turner, B. M. (1993). Decoding the nucleosome. *Cell* 75, 5–8. doi: 10.1016/0092-8674(93)90673-E

Turner, B. M. (1998). Histone acetylation as an epigenetic determinant of long-term transcriptional competence. *Cell Mol. Life Sci.* 54, 21–31. doi: 10.1007/s000180050122

Turner, B. M. (2002). Cellular memory and the histone code. *Cell* 111, 285–291. doi: 10.1016/S0092-8674(02)01080-2

Vaisanen, S., Dunlop, T. W., Sinkkonen, L., Frank, C., and Carlberg, C. (2005). Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1alpha,25-Dihydroxyvitamin D3. *J. Mol. Biol.* 350, 65–77. doi: 10.1016/j.jmb.2005.04.057

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Varjosalo, M., Keskitalo, S., Van Drogen, A., Nurkkala, H., Vichalkovski, A., Aebersold, R., et al. (2013). The protein interaction landscape of the human CMGC kinase group. *Cell Rep.* 3, 1306–1320. doi: 10.1016/j.celrep.2013.03.027

Wang, F., Marshall, C. B., and Ikura, M. (2013a). Transcriptional/epigenetic regulator CBP/p300 in tumorigenesis: structural and functional versatility in target recognition. *Cell. Mol. Life Sci.* 70, 3989–4008. doi: 10.1007/s00018-012-1254-4

Wang, J., Huo, K., Ma, L., Tang, L., Li, D., Huang, X., et al. (2011a). Toward an understanding of the protein interaction network of the human liver. *Mol. Syst. Biol.* 7, 536. doi: 10.1038/msb.2011.67

Wang, Q. M., Jones, J. B., and Studzinski, G. P. (1996). Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. *Cancer Res.* 56, 264–267.

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., et al. (2005). Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol. Endocrinol.* 19, 2685–2695. doi: 10.1210/me.2005-0106

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011b). Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10:58. doi: 10.1186/1476-4598-10-58

Wang, W. L., Welsh, J., and Tenniswood, M. (2013b). 1,25-Dihydroxyvitamin D3 modulates lipid metabolism in prostate cancer cells through miRNA mediated regulation of PPARA. *J. Steroid Biochem. Mol. Biol.* 136, 247–251. doi: 10.1016/j.jsbmb.2012.09.033

Wang, X., Gocek, E., Liu, C. G., and Studzinski, G. P. (2009). MicroRNAs181 regulate the expression of p27(Kip1) in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D(3). *Cell Cycle* 8, 736–741. doi: 10.4161/cc.8.5.7870

Welsh, J., Wietzke, J. A., Zinser, G. M., Smyczek, S., Romu, S., Tribble, E., et al. (2002). Impact of the Vitamin D3 receptor on growth-regulatory pathways in mammary gland and breast cancer. *J. Steroid Biochem. Mol. Biol.* 83, 85–92. doi: 10.1016/S0960-0760(02)00277-7

Yang, X., Bemis, L., Su, L. J., Gao, D., and Flaig, T. W. (2012). miR-125b Regulation of Androgen receptor signaling via modulation of the receptor complex co-repressor NCOR2. *Biores. Open Access,* 1, 55–62. doi: 10.1089/biores.2012.9903

Yang, X., Downes, M., Yu, R. T., Bookout, A. L., He, W., Straume, M., et al. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell* 126, 801–810. doi: 10.1016/j.cell.2006.06.050

Yuan, C. X., Ito, M., Fondell, J. D., Fu, Z. Y., and Roeder, R. G. (1998). The TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7939–7944. doi: 10.1073/pnas.95.14.7939

Zella, L. A., Kim, S., Shevde, N. K., and Pike, J. W. (2006). Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3. *Mol. Endocrinol.* 20, 1231–1247. doi: 10.1210/me.2006-0015

Zerr, P., Vollath, S., Palumbo-Zerr, K., Tomcik, M., Huang, J., Distler, A., et al. (2014). Vitamin D receptor regulates TGF-β signalling in systemic sclerosis. *Ann. Rheum. Dis.* doi: 10.1136/annrheumdis-2013-204378. [Epub ahead of print].

Zhang, C., Dowd, D. R., Staal, A., Gu, C., Lian, J. B., van Wijnen, A. J., et al. (2003). Nuclear coactivator-62 kDa/Ski-interacting protein is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing. *J. Biol. Chem.* 278, 35325–35336. doi: 10.1074/jbc.M305191200

Zhang, L., Stokes, N., Polak, L., and Fuchs, E. (2011). Specific microRNAs are preferentially expressed by skin stem cells to balance self-renewal and early lineage commitment. *Cell Stem Cell* 8, 294–308. doi: 10.1016/j.stem.2011.01.014

Zhang, Y., Szustakowski, J., and Schinke, M. (2009). Bioinformatics analysis of microarray data. *Methods Mol. Biol.* 573, 259–284. doi: 10.1007/978-1-60761-247-6_15

Zhi, H. Y., Hou, S. W., Li, R. S., Basir, Z., Xiang, Q., Szabo, A., et al. (2011). PTPH1 cooperates with vitamin D receptor to stimulate breast cancer growth through their mutual stabilization. *Oncogene* 30, 1706–1715. doi: 10.1038/onc.2010.543

Zhou, Y., Gong, W., Xiao, J., Wu, J., Pan, L., Li, X., et al. (2014). Transcriptomic analysis reveals key regulators of mammogenesis and the pregnancy-lactation cycle. *Sci. China Life Sci.* 57, 340–355. doi: 10.1007/s11427-013-4579-9

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Interaction of vitamin D with membrane-based signaling pathways

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Many studies in different biological systems have revealed that 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) modulates signaling pathways triggered at the plasma membrane by agents such as Wnt, transforming growth factor (TGF)-β, epidermal growth factor (EGF), and others. In addition, 1α,25$(OH)_2D_3$ may affect gene expression by paracrine mechanisms that involve the regulation of cytokine or growth factor secretion by neighboring cells. Moreover, post-transcriptional and post-translational effects of 1α,25$(OH)_2D_3$ add to or overlap with its classical modulation of gene transcription rate. Together, these findings show that vitamin D receptor (VDR) cannot be considered only as a nuclear-acting, ligand-modulated transcription factor that binds to and controls the transcription of target genes. Instead, available data support the view that much of the complex biological activity of 1α,25$(OH)_2D_3$ resides in its capacity to interact with membrane-based signaling pathways and to modulate the expression and secretion of paracrine factors. Therefore, we propose that future research in the vitamin D field should focus on the interplay between 1α,25$(OH)_2D_3$ and agents that act at the plasma membrane, and on the analysis of intercellular communication. Global analyses such as RNA-Seq, transcriptomic arrays, and genome-wide ChIP are expected to dissect the interactions at the gene and molecular levels.

Keywords: 1α,25$(OH)_2D_3$, VDR, membrane-based signaling, Wnt, growth factors, cytokines, paracrine effects

INTRODUCTION

The active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) is a key regulator of gene expression in higher organisms. It modulates the activity of the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors that regulate gene transcription. Genome-wide chromatin immunoprecipitation studies have shown that VDR binds to hundreds of genome sites even in the absence of 1α,25$(OH)_2D_3$ and that ligand binding increases and partially changes these binding sites, which depend on the cell type and the duration of treatment (Carlberg and Campbell, 2013). While a subset of VDR binding sites may be responsible for the control of gene expression (VDREs or vitamin D response elements), others might be temporary anchorage places for a population of unliganded "dormant" VDR. According to the classical view, VDR binds DNA as heterodimers with a retinoid X receptor (RXRα, β, or γ) and, upon ligand binding, changes the transcription rate of neighboring genes.

However, many genes whose expression is altered by 1α,25$(OH)_2D_3$ do not contain VDREs. Putative mechanisms of this action include post-transcriptional regulation via changes in the levels of microRNAs that modulate the half-life and/or translation of their messenger RNAs (Thorne et al., 2011; Wang et al., 2011; Alvarez-Díaz et al., 2012; Kasiappan et al., 2012; Guan et al., 2013). Also, 1α,25$(OH)_2D_3$ may regulate genes post-translationally via changes in the phosphorylation or other modifications of proteins which affect their stability (Lin et al., 2003; Li et al., 2004), or through changes in the level or activity of proteases that target them (Alvarez-Díaz et al., 2010).

Increasing importance has recently been accorded to another mechanism of 1α,25$(OH)_2D_3$ action: the modulation of signaling pathways triggered by other agents at the plasma membrane. Indeed, a number of studies have shown that 1α,25$(OH)_2D_3$ modulates the effects of growth factors and cytokines by altering either their cytosolic signaling pathways or the activity of target transcription factors in the nucleus, or even in a paracrine fashion by inhibiting their synthesis and secretion by neighboring cells.

Here we review the available data on these non-classical, alternative mechanisms by which 1α,25$(OH)_2D_3$ modulates gene expression. Notably, for specific genes such as c-*MYC*, both direct transcriptional and indirect modes of regulation by 1α,25$(OH)_2D_3$ have been described (Pan and Simpson, 1999; Pálmer et al., 2001; Toropainen et al., 2010; Salehi-Tabar et al., 2012).

INTERACTION OF 1α,25$(OH)_2D_3$ WITH WNT, HEDGEHOG, AND NOTCH PATHWAYS

Wnt, Hedgehog, and Notch signaling pathways, which have long been known to play crucial roles during development, are now considered critical for many tumorigenic processes in which they function abnormally due to mutation and/or changes in expression of components.

Wnt factors activate several signaling pathways upon binding to different plasma membrane receptors: the canonical or Wnt/β-catenin and the non-canonical (planar polarity, Ca^{2+} ...) pathways (Clevers and Nusse, 2012). Activation of the Wnt/β-catenin pathway by mutation of *APC* or *AXIN* tumor suppressor genes or of *CTNNB1*/β-catenin oncogene together with changes in the expression of a number of regulatory genes (*SFRPs*, *DICKKOPF (DKK)s*...) is a hallmark of most colorectal cancers and of a variable proportion of several other malignancies (Clevers and Nusse, 2012). A series of studies report that $1\alpha,25(OH)_2D_3$ antagonizes Wnt/β-catenin signaling in colon cancer cells by several mechanisms: the reduction of transcriptionally active β-catenin/T-cell factor complexes, the induction of β-catenin relocation from the nucleus toward the *adherens junctions* structures at the plasma membrane, and the increase in the level of the Wnt inhibitor DKK-1 (Pálmer et al., 2001; Shah et al., 2006; Aguilera et al., 2007) (**Figure 1**). In this way, the pathway endpoint, i.e., the activation of β-catenin target genes, is attenuated by $1\alpha,25(OH)_2D_3$ (Pálmer et al., 2001). Emphasizing the importance of this action, an additional indirect mechanism of Wnt/β-catenin antagonism in colon cancer has been proposed involving IL-1β, which will be reviewed in section $1\alpha,25(OH)_2D_3$ and Cytokines. Although $1,25(OH)_2D_3$ inhibits β-catenin/TCF transcriptional activity in colon and other cancer cells, the upregulation of the Wnt/β-catenin pathway by either ligand-activated or unliganded VDR has been described in osteoblasts and keratinocytes, where it promotes bone formation and hair follicle differentiation, respectively (Larriba et al., 2013). However, the results reported in keratinocytes are controversial: while VDR enhances Wnt signaling through direct binding to Lymphocyte Enhancer-binding Factor (LEF)-1 independently of ligand and β-catenin (Luderer et al., 2011), ligand-activated VDR is believed to inhibit Wnt/β-catenin signaling (Bikle, 2011; Jiang et al., 2012).

Inhibition of Hedgehog (Hh) signaling by vitamin D compounds has also been suggested. In a study combining experiments in zebrafish, the yeast *Pichia pastori* and mouse fibroblasts, secreted vitamin D_3, or its precursor 7-dehydrocholesterol (7-DHC), was shown to mediate the paracrine inhibition of Smoothened (Smo) by Patched (Ptch)1 which leads to pathway inactivation (Bijlsma et al., 2006). In the model proposed, which includes the binding of vitamin D_3 to Smo at high (micromolar) concentrations, Hh ligands activate the pathway by blocking the induction of the secretion of vitamin D_3/7-DHC by Ptch1 (Bijlsma et al., 2006) (**Figure 1**). The Hh pathway is aberrantly activated in basal cell carcinoma, the most frequent human tumor type. Interestingly, $1\alpha,25(OH)_2D_3$ inhibits proliferation and induces differentiation of mouse basal cell carcinomas and embryonal rhabdomyosarcomas with an activated Hh pathway due to *Ptch1* deletion (Uhmann et al., 2011, 2012). As in the previous study, $1\alpha,25(OH)_2D_3$ acts at the level of Smo in a VDR-independent manner (**Figure 1**). Curiously, Tang et al. found that vitamin D_3 inhibits Hh and cell proliferation more effectively than 7-DHC, $25(OH)D_3$, or $1\alpha,25(OH)_2D_3$ in murine basal cell carcinoma cells (Tang et al., 2011). Vitamin D_3 also inhibits proliferation and Hh pathway through inactivation of Smo in cultured mouse pancreatic adenocarcinoma cells, but has no antitumor activity *in vivo* (Brüggemann et al., 2010). A common concern in all these studies is the high concentration of vitamin D_3 required to observe the reported effects. Research in *Vdr*-deficient mice and in mouse skin explants has shown that lack of VDR increases the expression of several components of the Hh pathway such as *Shh*, *Smo*, *Gli1*, *Gli2*, and *Ptch1*, while $1\alpha,25(OH)_2D_3$ suppresses their expression (Bikle et al., 2013) (**Figure 1**). However, the interaction between $1\alpha,25(OH)_2D_3$ and Hh signaling in human skin remains to be elucidated.

Few studies link vitamin D with Notch signaling. Differentiation of human osteoblasts with vitamin D_3 and dexamethasone distinctly affects the expression of Notch receptor family members (Schnabel et al., 2002). In rodent osteoblasts, the transcription factor Hes-1, which is an effector of the Notch pathway, enhances the induction of *SPP1*/osteopontin transcription by $1\alpha,25(OH)_2D_3$, indicating the collaboration of $1\alpha,25(OH)_2D_3$ and Notch pathways in bone remodeling (Shen and Christakos, 2005) (**Figure 1**). Transcriptomic analyses in human RWPE1 immortalized non-tumorigenic prostate cells showed the reduction of the RNA levels of the NOTCH ligands *JAGGED* (*JAG)1*, *JAG2*, and *Delta-like* (*DLL)1* by $1\alpha,25(OH)_2D_3$ (Kovalenko et al., 2010) (**Figure 1**). By contrast, no changes in the expression of *NOTCH-1* and *JAG1* were detected in cultured human keratinocytes upon $1\alpha,25(OH)_2D_3$ treatment (Reichrath and Reichrath, 2012). As *JAG1* transcription and, consequently, Notch signaling are upregulated by Wnt/β-catenin in colorectal cancer cells (Rodilla et al., 2009), the repressive effect of $1\alpha,25(OH)_2D_3$ on the Notch pathway in this system may be secondary to the antagonism of the Wnt/β-catenin pathway (**Figure 1**).

INTERPLAY OF $1\alpha,25(OH)_2D_3$ WITH AGENTS THAT TRIGGER SIGNALING PATHWAYS VIA PLASMA MEMBRANE KINASE RECEPTORS

There is mutual antagonism between $1\alpha,25(OH)_2D_3$ and epidermal growth factor (EGF), a potent mitogen, in primary colon epithelial cells and in established colon (Caco-2) and breast (T47D) tumor cell lines. This is based on the cross-inhibition of the expression of their respective receptors, VDR and EGFR (Tong et al., 1998, 1999). However, it is a cell type-dependent effect as EGF increases VDR in the rat small intestine and $1\alpha,25(OH)_2D_3$ increases EGFR in BT-20 breast cancer cells (Bruns et al., 1989; Desprez et al., 1991). In addition, $1\alpha,25(OH)_2D_3$ inhibits EGFR signaling by increasing the level of E-cadherin protein at the plasma membrane, which downregulates EGFR (Pálmer et al., 2001; Andl and Rustgi, 2005), and by decreasing that of SPROUTY-2, a cytosolic protein that reduces EGFR ubiquitination, internalization and degradation (Cabrita and Christofori, 2008; Barbáchano et al., 2010).

Transforming growth factor (TGF)-β has opposite roles in carcinogenesis: it inhibits proliferation of normal epithelial cells, but it later induces epithelial-mesenchymal transition, immunosuppression and metastasis (Pickup et al., 2013). $1\alpha,25(OH)_2D_3$ induces the expression of the type I TGF-β receptor and both agents, $1\alpha,25(OH)_2D_3$ and TGF-β, cooperate in Caco-2 cell growth inhibition (Chen et al., 2002; Pálmer et al., 2003). Moreover, Smad3, a mediator of TGF-β signaling, is a co-activator of VDR and contributes to gene regulation by $1\alpha,25(OH)_2D_3$

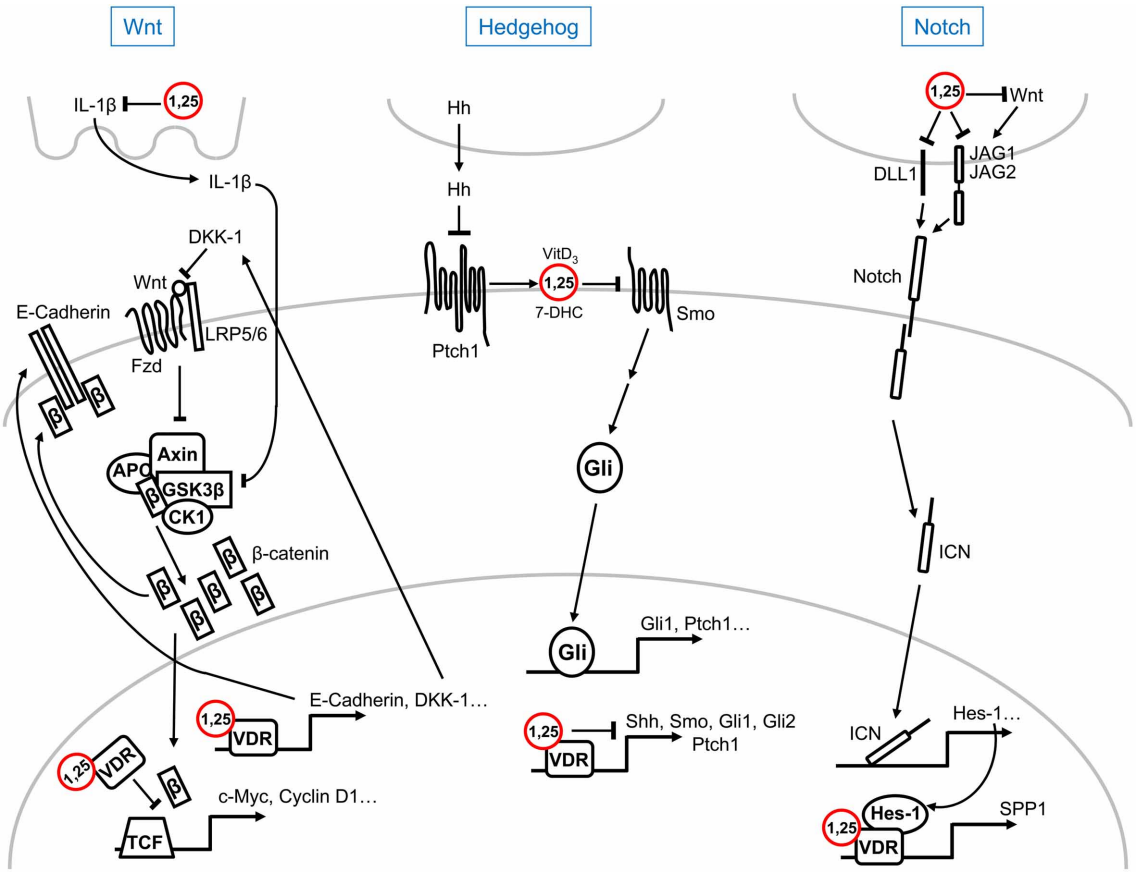

FIGURE 1 | Schematic representation of the multilevel crosstalk of 1α,25(OH)$_2$D$_3$ (1,25) with Wnt, Hedgehog, and Notch signaling pathways. For simplicity, only main components and regulators of the pathways are shown. Explanations, details, and references can be found in the text.

(Yanagisawa et al., 1999), an effect that is abrogated by Smad7 in transfected COS-7 cells (Yanagi et al., 1999). Reinforcing the interaction between both signaling pathways, 1α,25(OH)$_2$D$_3$ induces the expression of Smad anchor for receptor activation (SARA) (Pálmer et al., 2003), which maintains the epithelial phenotype by recruiting Smads 2/3 to the activated TGF-β receptors and regulates endocytic trafficking of EGFR and other proteins (Tang et al., 2011; Kostaras et al., 2013). Notably, a recent study of R. M. Evans' group has revealed a genome-wide overlap of VDR and Smad3 binding sites that is responsible for the abrogation by VDR ligands of the TGF-β1-mediated activation of hepatic stellate cells during liver fibrosis (Ding et al., 2013). These authors show that TGF-β1 signaling redistributes VDR-binding sites in the genome and facilitates VDR binding at Smad3 profibrotic target genes. Upon ligand activation, VDR binding at coregulated genes decreases Smad3 occupancy at these sites, causing inhibition of fibrosis (Ding et al., 2013). This is a regulatory feedback mechanism in which VDR ligands limit the fibrotic process and so ensure an appropriate non-pathological tissue response. Given the crucial roles of TGF-β in carcinogenesis, future studies should examine whether vitamin D compounds play similar roles in the maintenance of epithelial integrity opposing the onset of carcinomas.

1α,25(OH)$_2$D$_3$ and TGF-β interact also in bone. Curiously, in rat (UMR 106 and ROS 17/2.8) and human (MG-63) osteoblastic cells TGF-β increases VDR expression but inhibits the stimulation of osteocalcin and osteopontin transcription and RNA levels by 1α,25(OH)$_2$D$_3$ (Staal et al., 1994). TGF-β exerts this inhibitory effect by reducing the binding of VDR-RXR complexes to VDREs localized in the promoter of these genes without affecting the nuclear availability of VDR at least in ROS 17/2.8 cells (Staal et al., 1996). In contrast to the stimulation of osteocalcin synthesis in human and rat cells, 1α,25(OH)$_2$D$_3$ decreases osteocalcin production in mouse fetal long bone cultures and neonatal osteoblastic MC3T3 cells while stimulating bone resorption (Staal et al., 1998). This bone resorption action of 1α,25(OH)$_2$D$_3$ is dose-dependently inhibited by TGF-β (Staal et al., 1998).

A complex, cell type-, context- and sometimes age-dependent relation exists between 1α,25(OH)$_2$D$_3$ and insulin-like growth factors (IGF)-I and II. For instance, in C2C12 myoblasts 1α,25(OH)$_2$D$_3$ decreases IGF-I expression while it increases that of IGF-II (Garcia et al., 2011). In HT29 colon carcinoma cells several vitamin D compounds inhibit the secretion of IGF-II thus attenuating its cell proliferation activity (Oh et al., 2001). In addition, 1α,25(OH)$_2$D$_3$ blocks the mitogenic activity of insulin

and IGF-I in MCF7 breast cancer cells, at least in part due to the inhibition of c-FOS upregulation (Vink-van Wijngaarden et al., 1996). 1α,25$(OH)_2D_3$ and IGF-I have also opposite effects on mouse long bones: IGF-I increases osteocalcin production, which is completely blocked by 1α,25$(OH)_2D_3$, and inhibits the enhancement of bone resorption caused by 1α,25$(OH)_2D_3$ (Staal et al., 1998). Furthermore, 1α,25$(OH)_2D_3$ variably regulates the expression of several IGF binding proteins (IGFBPs), a group of molecules with pleiotropic actions that transport IGFs and also modulate cell survival/apoptosis: 1α,25$(OH)_2D_3$ induces IGFBP3 expression in SW480-ADH colon carcinoma, SaOS-2 osteosarcoma, PC3 prostate cancer, MCF7 breast carcinoma and MCF-10A normal mammary cells (Pálmer et al., 2003; Matilainen et al., 2005; Malinen et al., 2011; Brosseau et al., 2013), IGFBP6 in SaOS-2, SW480-ADH and colon carcinoma HT29 cells (Oh et al., 2001; Pálmer et al., 2003; Matilainen et al., 2005), IGFBP1 and IGFBP5 in SaOS-2 and PC3 cells, and IGFBP4 in SaOS-2 cells (Matilainen et al., 2005). Conversely, 1α,25$(OH)_2D_3$ represses IGFBP4 in HT29 and SW480-ADH cells, and IGFBP2 in HT29 cells (Oh et al., 2001; Pálmer et al., 2003). In ovarian cells, 1α,25$(OH)_2D_3$ alone induces IGFBP1 production but, conversely, it enhances the inhibitory effect of insulin (Parikh et al., 2010). Curiously, recent studies show that IGFBP3 interacts with VDR (Li et al., 2013) and that IGFBP6 binds VDR and blocks the induction of osteoblast differentiation by 1α,25$(OH)_2D_3$ (Cui et al., 2011).

Cell type-dependent effects of 1α,25$(OH)_2D_3$ have also been described for hepatocyte growth factor (HGF) signaling. 1α,25$(OH)_2D_3$ activates the *HGF* gene promoter and induces HGF expression and secretion in rat NRK-49F renal interstitial fibroblasts (Li et al., 2005) and in human keloid fibroblasts (Zhang et al., 2011). Consistently with these results, vitamin D deficiency reduces HGF and HGF receptor/c-Met expression during liver regeneration in rats (Goupil et al., 1997). Conversely, 1α,25$(OH)_2D_3$ decreases the level of *HGF* RNA in human HL-60 promyelocitic leukemia cells (Inaba et al., 1993), smooth muscle cells (Shalhoub et al., 2010) and MG-63 osteosarcoma cells (Chattopadhyay et al., 2003). Moreover, the expression of c-Met is inhibited by 1α,25$(OH)_2D_3$ in human MHCC97 hepatocellular cell line (Wu et al., 2007). Curiously, 1α,25$(OH)_2D_3$ and HGF cooperate to increase osteogenic differentiation of human bone marrow stem cells and maturation of chondrocyte progenitor cells (Grumbles et al., 1996; D'Ippolito et al., 2002; Chen et al., 2011, 2012). Also, 1α,25$(OH)_2D_3$ and HGF additively inhibit proliferation of androgen-unresponsive prostate cancer cells (Qadan et al., 2000).

In concordance with its regulatory role in the organism, 1α,25$(OH)_2D_3$ favors physiological and homeostatic angiogenesis but inhibits angiogenesis in pathological conditions. Thus, 1α,25$(OH)_2D_3$ promotes myogenic differentiation of C2C12 cells by increasing the expression of two key angiogenic factors: vascular endothelial growth factor (VEGF) and fibroblast growth factor-1 (Garcia et al., 2013). In addition, 1α,25$(OH)_2D_3$ stimulates pro-angiogenic properties of endothelial progenitor cells by increasing VEGF levels (Grundmann et al., 2012). 1α,25$(OH)_2D_3$ also upregulates VEGF expression in osteoblast-like cells but not in breast cancer cells (Schlaeppi et al., 1997). Likewise, ED-71, a vitamin D analog, enhances VEGF expression and promotes angiogenesis in a murine bone marrow ablation model (Okuda et al., 2007). Indeed, increased production of VEGF in vascular smooth muscle cells results from the activation of a VDRE present in the *VEGF* gene promoter (Cardus et al., 2009). By contrast, 1α,25$(OH)_2D_3$ downregulates hypoxia-inducible factor (HIF)-1 and VEGF protein expression in several human colon, prostate and breast cancer cell lines (Ben-Shoshan et al., 2007), decreases VEGF production by human lumbar annulus cells (Gruber et al., 2008), and protects against diabetic retinopathy in rats by inhibiting VEGF expression in the retina (Ren et al., 2012).

1α,25$(OH)_2D_3$ also modulates the activity of signaling pathways mediated by other types of plasma membrane receptors such us G protein-coupled receptors. Shen et al. found that 1α,25$(OH)_2D_3$ suppresses the expression of parathyroid hormone-related protein (PTHrP) in prostate cancer cells via a negative VDRE localized within the non-coding region of the gene, thus antagonizing the induction of cell proliferation and of the expression of the pro-invasive integrin $\alpha_6\beta_4$ exerted by PTHrP signaling (Shen et al., 2007).

1α,25$(OH)_2D_3$ AND CYTOKINES

The anti-inflammatory and immunomodulatory actions, and thus some of the anticancer and antimicrobial effects of 1α,25$(OH)_2D_3$, are mediated by the regulation of cytokine production and/or through the control of their receptors or downstream signaling pathways. Globally, 1α,25$(OH)_2D_3$contributes to the autocrine and paracrine control of innate and adaptative immune responses (Adorini and Penna, 2008).

1α,25$(OH)_2D_3$ regulates the function of antigen-presenting cells and T-lymphocytes. It inhibits Th1 cells differentiation and, therefore, the secretion of Th1-type cytokines, enhances the development of Th2 cells, and induces tolerogenic monocytes and dendritic cells. IL-4 and IL-10 are among the commonly increased cytokines, while IL-1, IL-2, IL-6, IL-17, tumor necrosis factor (TNF)-α and interferon (IFN)-γ are decreased (Adorini and Penna, 2008).

Mechanistically, ligand-activated VDR directly downregulates the expression of IL-10, IL-2, and IL-12B in lipopolysaccharide-treated human monocytes (THP-1) through its binding to VDREs located in the genomic regions of these genes and the recruitment of the co-repressor NCOR/SMRT and histone deacetylases (Matilainen et al., 2010a,b; Gynther et al., 2011). Remarkably, IL-10 is downregulated by short 1α,25$(OH)_2D_3$ treatment (8 h) but upregulated at late time points (48 h) (Matilainen et al., 2010a). In addition, direct VDR binding to a single VDRE mediates the upregulation of *IL-8* gene by 1α,25$(OH)_2D_3$ in undifferentiated and differentiated THP-1 cells (Ryynänen and Carlberg, 2013).

1α,25$(OH)_2D_3$ also changes the expression of target genes in immune cells by repressing crucial transcription factors such as nuclear factor *kappa* B (NFkB) and signaling pathways such as Janus kinase-signal transducer and activator of transcription (JAK-STAT) (Yu et al., 1995; Muthian et al., 2006; Geldmeyer-Hilt et al., 2011). 1α,25$(OH)_2D_3$ also represses NFκB activity in fibroblasts and adipocytes (Harant et al., 1998; Mutt et al., 2012), and fibroblasts lacking VDR have increased NFκB activity (Sun et al., 2006). Direct (increase in IκBα expression and reduction

of nuclear translocation of p65) and indirect (upregulation of IGFBP3 and clusterin) mechanisms contribute to the inhibition of NFκB activation (Krishnan and Feldman, 2010). D. Feldman's group has reported that, in addition to inhibiting NFκB, the anti-inflammatory effects of 1α,25(OH)$_2$D$_3$ in prostate cancer cells include the reduction of pro-inflammatory prostaglandins (PG) production via suppression of ciclooxygenase-2, downregulation of PG receptors, and upregulation of 15-hydroxyprostaglandin dehydrogenase, which inactivates PGs (Krishnan and Feldman, 2010). Moreover, 1α,25(OH)$_2$D$_3$ decreases the synthesis of pro-inflammatory IL-6 through the inactivation of p38 kinase due to the upregulation of the mitogen kinase phosphatase (MKP)5 and the blockade of TNF-α (Krishnan and Feldman, 2010). In Jurkat cells, the repression of *IL-2* gene by 1α,25(OH)$_2$D$_3$ is at least partially due to the blockade of NFATp/AP-1 complex formation at a positive regulatory NFAT-1 site, which is bound by VDR-RXR heterodimers (Alroy et al., 1995).

1α,25(OH)$_2$D$_3$ reduces the secretion of interleukin (IL)1-β in THP macrophages by blocking the activation of STAT1 (Kaler et al., 2009). As IL1-β activates the Wnt/β-catenin pathway in colon carcinoma cells via inhibition of GSK3β activity and subsequent stabilization and nuclear translocation of β-catenin, this mechanism may contribute to the antagonism of Wnt signaling by 1α,25(OH)$_2$D$_3$ (Kaler et al., 2009) (**Figure 1**). Curiously, IL-1α is believed to be upregulated and to mediate the antiproliferative effects of 1α,25(OH)$_2$D$_3$ in prostate progenitor/stem cells (Maund et al., 2011).

In human osteoblasts, 1α,25(OH)$_2$D$_3$ completely overrules the inhibitory effect of IFN-β on mineralization. This dominant effect on osteoblast differentiation and bone formation is reflected in the downregulation of IFN-related and -regulated genes by 1α,25(OH)$_2$D$_3$ (Woeckel et al., 2012). Concomitantly, 1α,25(OH)$_2$D$_3$ also induces activin A, a strong inhibitor of mineralization, and represses follistatin, the natural antagonist of activin A, to ensure a fine-tuned regulation of the mineralization process (Woeckel et al., 2013b).

Recent findings have underscored the complexity of 1α,25(OH)$_2$D$_3$ action and its role in the antimicrobial response as part of innate and adaptative immunity. Thus, activation of macrophage Toll-like receptors (TLRs) by intracellular bacteria such as *Mycobacterium tuberculosis* upregulates *VDR* and *CYP27B1* genes that allow the induction of the antimicrobial peptide cathelicidin by 1α,25(OH)$_2$D$_3$ (Liu et al., 2006). In monocytes, TLR activation triggers induction of defensin β4 (*DEFB4*) gene requiring the cooperation between IL-1β and 1α,25(OH)$_2$D$_3$, which is explained by the presence of one VDRE and two IL-1β-activatable NFkB sites in the *DEFB4* promoter (Liu et al., 2009). In addition, 1α,25(OH)$_2$D$_3$ is required for the antimicrobial effect of IFN-γ in human macrophages (Fabri et al., 2011). Moreover, by inducing the expression of TLR2 and CD14 receptors and cathelicidin, 1α,25(OH)$_2$D$_3$ mediates the effect of TGF-β favoring the response to microbial infection and wound injury by keratinocytes (Schauber et al., 2007). These findings show also unexpected cooperation of 1α,25(OH)$_2$D$_3$ with agents (IL-1β, TGF-β, IFN-γ) that are antagonistic in other cell types.

INTERPLAY OF 1α,25(OH)$_2$D$_3$/VDR WITH TRANSCRIPTION FACTORS

Liganded or unliganded VDR interacts with or regulates the expression of a number of transcription factors that are downstream effectors of different signaling pathways (**Table 1**). An interesting example is the upregulation by 1α,25(OH)$_2$D$_3$ of *CDKN1B*/p27^{Kip1}, a cell cycle regulator gene which lacks VDREs. 1α,25(OH)$_2$D$_3$ was first shown to induce *CDKN1B* transcription by stimulating the binding of Sp1 and NF-Y transcription factors to the *CDKN1B* promoter in the myelomonocytic U937 cell line (Inoue et al., 1999). Later, direct VDR-Sp1 interaction at the promoter Sp1 sites was described as responsible for this effect (Huang et al., 2004). In addition to the enhancement of transcription, 1α,25(OH)$_2$D$_3$ increases the stability of p27^{Kip1} protein by repressing p45^{Skp2}, an F-box protein, through the induction of VDR-Sp1 complexes that together with histone deacetylase 1 are recruited to Sp1 sites at the p45^{Skp2} gene promoter (Lin et al., 2003; Li et al., 2004; Huang and Hung, 2006).

The granulocyte-macrophage colony-stimulating factor (*GM-CSF*) gene is another example of unusual regulation by 1α,25(OH)$_2$D$_3$. Ligand-activated VDR represses *GM-CSF* through a composite DNA element recognized by Jun-Fos heterodimers (AP-1) and nuclear factor of activated T-cells (NFAT)1 (Towers et al., 1999). In the absence of RXR, VDR binds to c-Jun and stabilizes AP-1 bound to DNA, which outcompetes NFAT1 and decreases *GM-CSF* transcription. In Caco-2 cells, 1α,25(OH)$_2$D$_3$ stimulates AP-1 via activation of protein kinase C-α, ERK and JNK leading to cell differentiation (Chen et al., 1999).

Table 1 | Interplay between VDR and other transcription factors.

Transcription factor	Biological effect	References
Sp1/NF-Y	Potentiation	Inoue et al., 1999; Huang et al., 2004
AP-1/NFAT1	Repression	Towers et al., 1999
AP-1	Activation	Chen et al., 1999
CREB	Repression	Yuan et al., 2007
FOXO3a, FOXO4	Activation	An et al., 2010
p53	Mutual repression	Stambolsky et al., 2010; Chen et al., 2013
PPAR-α/δ	Activation	Sertznig et al., 2009a,b
PPAR-γ	Variable	Alimirah et al., 2012; Woeckel et al., 2013a
RAR	Variable	Jiménez-Lara and Aranda, 1999; Tavera-Mendoza et al., 2006; Anand et al., 2008; Ng et al., 2010
ER	Downregulation	Krishnan et al., 2010; Swami et al., 2013
AR	Crossregulation	Zhao et al., 1999; Ting et al., 2005
PIT-1	Downregulation	Seoane and Pérez-Fernández, 2006

In renal cells, $1\alpha,25(OH)_2D_3$ suppresses renin gene expression by blocking the cyclic AMP response element (CRE) through direct binding of VDR to CRE-binding protein (CREB) and so, inhibiting the binding of CREB to the CRE (Yuan et al., 2007). By a complex mechanism, $1\alpha,25(OH)_2D_3$ also regulates several Forkhead box (FOX) transcription factors. Ligand-activated VDR binds FOXO3a and FOXO4 together with their regulators, sirtuin 1 deacetylase and protein phosphatase 1, inducing deacetylation and dephosphorylation of FOXO proteins, thereby activating these (An et al., 2010). In the case of the p53 tumor suppressor protein a mutual regulation takes place: while mutated p53 interacts physically with VDR and changes VDR-target genes, converting $1\alpha,25(OH)_2D_3$ from a pro-apoptotic into an anti-apoptotic agent (Stambolsky et al., 2010), $1\alpha,25(OH)_2D_3$ activates the promoter of Mdm2 in a p53-dependent fashion promoting the expression of this negative regulator of p53 protein stability and function (Chen et al., 2013).

Multiple interplays between $1\alpha,25(OH)_2D_3$/VDR and other nuclear receptor ligands have been described. Among them, crosstalk between liganded VDR and peroxisome proliferator-activated receptor (PPAR)-α/δ in melanoma cells (Sertznig et al., 2009a,b) that may involve the stimulation of PPAR-δ expression by $1\alpha,25(OH)_2D_3$ (Dunlop et al., 2005). A synergistic action of $1\alpha,25(OH)_2D_3$ and rosiglitazone, a PPAR-γ ligand, has been shown during osteoblast-mediated mineralization (Woeckel et al., 2013a), while in human T47D breast cancer cells PPAR-γ binds VDR and represses its transcriptional activity, possibly also by competing for RXR heterodimerization (Alimirah et al., 2012). Titration out of common co-activators, but not of RXR, may be the mechanism by which ligand-bound VDR represses retinoic acid receptor (RAR) transactivation in GH4C1 pituitary cells (Jiménez-Lara and Aranda, 1999). The relation between $1\alpha,25(OH)_2D_3$ and retinoic acid is however complex, as cooperative effects on target genes and cellular outcome (proliferation inhibition and differentiation) have been described in other systems (Tavera-Mendoza et al., 2006; Anand et al., 2008; Ng et al., 2010). As for estrogen receptor (ER), D. Feldman's group has shown that $1\alpha,25(OH)_2D_3$ exerts a multilevel protective effect against breast cancer that includes the inhibition of estrogen synthesis through the direct and indirect repression of aromatase (CYP19) and the downregulation of ER-α expression through two VDREs in its promoter region (Krishnan et al., 2010; Swami et al., 2013). Likewise, there is a complex and unresolved relationship between $1\alpha,25(OH)_2D_3$ and androgen receptor (AR) synthesis and signaling. $1\alpha,25(OH)_2D_3$ induces AR in LNCaP cells (Zhao et al., 1999) while AR reduces VDR transcriptional activity (Ting et al., 2005), perhaps in some cells by a mechanism mediated by prohibitin (Mooso et al., 2010). In addition, $1\alpha,25(OH)_2D_3$ inhibits glucuronidation and so, inactivation of androgen in prostate cancer cells through the repression of UDP-glucuronosyltransferases (UGT) 2B15 and 2B17, which is counterintuitive given the growth promoting action of androgen and the antiproliferative effect of $1\alpha,25(OH)_2D_3$ in prostate cancer cells (Kaeding et al., 2008). In human bladder, $1\alpha,25(OH)_2D_3$ and analogs inhibit cell proliferation promoted by androgen and keratinocyte growth factor and induce apoptosis at least in part by repressing Bcl-2 expression (Crescioli et al., 2005).

Pituitary transcription factor (Pit)-1 activates growth hormone and prolactin genes in the anterior pituitary and also in breast cancer cells (Seoane and Pérez-Fernández, 2006). In MCF7 cells, VDR homodimers bind the *PIT-1* promoter and inhibit its expression in the presence of $1\alpha,25(OH)_2D_3$ without involvement of RXR (Seoane and Pérez-Fernández, 2006).

CONCLUSIONS

The available evidence shows that the classical view of VDR only as a nuclear-acting ligand-modulated transcription factor that regulates the rate of transcription of those genes to which it binds is outdated. Instead, VDR and its ligand constitute a multilevel main regulator of gene expression in higher cells acting directly or indirectly, and via a variety of different mechanisms, on many signaling pathways. Some of them are triggered from the plasma membrane by paracrine or endocrine agents, and $1\alpha,25(OH)_2D_3$ interacts at different levels: membrane receptors, cytosolic signaling molecules or effector nuclear transcription factors. In most cases $1\alpha,25(OH)_2D_3$ action is mediated by nuclear VDR but in a few others this is unclear and non-canonical VDR-independent or extranuclear effects have been proposed. Available studies show that $1\alpha,25(OH)_2D_3$ and these signaling pathways interact variably and with distinct outcomes in a cell/tissue-specific fashion and sometimes also differentially between normal and malignant cells.

PERSPECTIVES

The increasingly recognized importance of its non-cell autonomous actions has widened the scope of the study of $1\alpha,25(OH)_2D_3$. On the one hand, an in-depth study of the interplay between $1\alpha,25(OH)_2D_3$ and other agents, which seems to be cell-specific in terms of biological outcome, is necessary to elucidate the possibilities of combined therapies using vitamin D compounds and inhibitors or activators of a variety of signaling pathways. On the other hand, several of these interactions take place at the intercellular level. By using high-throughput techniques and genome-wide analyses, we expect to be able to identify secreted paracrine and intracellular mediators of the interaction between $1\alpha,25(OH)_2D_3$ and other signaling pathways responsible for the regulatory actions of $1\alpha,25(OH)_2D_3$ in the organism. Future research should aim to discern how vitamin D compounds modulate tissue and organ physiology and how they may be used to treat pathological processes such as infections, autoimmune disorders, or cancer.

AUTHOR CONTRIBUTIONS

María Jesús Larriba, José Manuel González-Sancho, Félix Bonilla, and Alberto Muñoz wrote the manuscript.

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REFERENCES

Adorini, L., and Penna, G. (2008). Control of autoimmune diseases by the vitamin D endocrine system. *Nat. Clin. Pract. Rheumatol.* 4, 404–412. doi: 10.1038/ncprheum0855

Aguilera, O., Peña, C., García, J. M., Larriba, M. J., Ordóñez-Morán, P., Navarro, D., et al. (2007). The Wnt antagonist DICKKOPF-1 gene is induced by 1α,25-dihydroxyvitamin D_3 associated to the differentiation of human colon cancer cells. *Carcinogenesis* 28, 1877–1884. doi: 10.1093/carcin/bgm094

Alimirah, F., Peng, X., Yuan, L., Mehta, R. R., von Knethen, A., Choubey, D., et al. (2012). Crosstalk between the peroxisome proliferator-activated receptor γ (PPARγ) and the vitamin D receptor (VDR) in human breast cancer cells: PPARγ binds to VDR and inhibits 1α,25-dihydroxyvitamin D_3 mediated transactivation. *Exp. Cell Res.* 318, 2490–2497. doi: 10.1016/j.yexcr.2012.07.020

Alroy, I., Towers, T. L., and Freedman, L. P. (1995). Transcriptional repression of the interleukin-2 gene by vitamin D_3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. *Mol. Cell. Biol.* 15, 5789–5799.

Alvarez-Díaz, S., Larriba, M. J., López-Otín, C., and Muñoz, A. (2010). Vitamin D: proteases, protease inhibitors and cancer. *Cell Cycle* 9, 32–37. doi: 10.4161/cc.9.1.10266

Alvarez-Díaz, S., Valle, N., Ferrer-Mayorga, G., Lombardía, L., Herrera, M., Domínguez, O., et al. (2012). MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells. *Hum. Mol. Genet.* 21, 2157–2165. doi: 10.1093/hmg/dds031

An, B. S., Tavera-Mendoza, L. E., Dimitrov, V., Wang, X., Calderon, M. R., Wang, H. J., et al. (2010). Stimulation of Sirt1-regulated FoxO protein function by the ligand-bound vitamin D receptor. *Mol. Cell. Biol.* 30, 4890–4900. doi: 10.1128/MCB.00180-10

Anand, P. K., Kaul, D., and Sharma, M. (2008). Synergistic action of vitamin D and retinoic acid restricts invasion of macrophages by pathogenic mycobacteria. *J. Microbiol. Immunol. Infect.* 41, 17–25.

Andl, C. D., and Rustgi, A. K. (2005). No one-way street: cross-talk between E-cadherin and receptor tyrosine kinase (RTK) signaling: a mechanism to regulate RTK activity. *Cancer Biol. Ther.* 4, 28–31. doi: 10.4161/cbt.4.1.1431

Barbáchano, A., Ordóñez-Morán, P., García, J. M., Sánchez, A., Pereira, F., Larriba, M. J., et al. (2010). SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumourigenicity. *Oncogene* 29, 4800–4813. doi: 10.1038/onc.2010.225

Ben-Shoshan, M., Amir, S., Dang, D. T., Dang, L. H., Weisman, Y., and Mabjeesh, N. J. (2007). 1α,25-dihydroxyvitamin D_3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells. *Mol. Cancer Ther.* 6, 1433–1439. doi: 10.1158/1535-7163.MCT-06-0677

Bijlsma, M. F., Spek, C. A., Zivkovic, D., van de Water, S., Rezaee, F., and Peppelenbosch, M. P. (2006). Repression of smoothened by patched-dependent (pro-)vitamin D_3 secretion. *PLoS Biol.* 4:e232. doi: 10.1371/journal.pbio.0040232

Bikle, D. D. (2011). The vitamin D receptor: a tumor suppressor in skin. *Discov. Med.* 11, 7–17.

Bikle, D. D., Elalieh, H., Welsh, J., Oh, D., Cleaver, J., and Teichert, A. (2013). Protective role of vitamin D signaling in skin cancer formation. *J. Steroid Biochem. Mol. Biol.* 136, 271–279. doi: 10.1016/j.jsbmb.2012.09.021

Brosseau, C., Pirianov, G., and Colston, K. W. (2013). Role of insulin-like growth factor binding protein-3 in 1,25-dihydroxyvitamin-D_3-induced breast cancer cell apoptosis. *Int. J. Cell Biol.* 2013, 960378. doi: 10.1155/2013/960378

Brüggemann, L. W., Queiroz, K. C., Zamani, K., van Straaten, A., Spek, C. A., and Bijlsma, M. F. (2010). Assessing the efficacy of the hedgehog pathway inhibitor vitamin D_3 in a murine xenograft model for pancreatic cancer. *Cancer Biol. Ther.* 10, 79–88. doi: 10.4161/cbt.10.1.12165

Bruns, D. E., Krishnan, A. V., Feldman, D., Gray, R. W., Christakos, S., Hirsch, G. N., et al. (1989). Epidermal growth factor increases intestinal calbindin-D9k and 1,25-dihydroxyvitamin D receptors in neonatal rats. *Endocrinology* 125, 478–485. doi: 10.1210/endo-125-1-478

Cabrita, M. A., and Christofori, G. (2008). Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* 11, 53–62. doi: 10.1007/s10456-008-9089-1

Cardus, A., Panizo, S., Encinas, M., Dolcet, X., Gallego, C., Aldea, M., et al. (2009). 1,25-dihydroxyvitamin D_3 regulates VEGF production through a vitamin D response element in the VEGF promoter. *Atherosclerosis* 204, 85–89. doi: 10.1016/j.atherosclerosis.2008.08.020

Carlberg, C., and Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78, 127–136. doi: 10.1016/j.steroids.2012.10.019

Chattopadhyay, N., MacLeod, R. J., Tfelt-Hansen, J., and Brown, E. M. (2003). 1α,25$(OH)_2$-vitamin D_3 inhibits HGF synthesis and secretion from MG-63 human osteosarcoma cells. *Am. J. Physiol. Endocrinol. Metab.* 284, E219–E227. doi: 10.1152/ajpendo.00247.2002

Chen, A., Davis, B. H., Bissonnette, M., Scaglione-Sewell, B., and Brasitus, T. A. (1999). 1,25-Dihydroxyvitamin D_3 stimulates activator protein-1-dependent Caco-2 cell differentiation. *J. Biol. Chem.* 274, 35505–35513. doi: 10.1074/jbc.274.50.35505

Chen, A., Davis, B. H., Sitrin, M. D., Brasitus, T. A., and Bissonnette, M. (2002). Transforming growth factor-β 1 signaling contributes to Caco-2 cell growth inhibition induced by 1,25$(OH)_2D_3$. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283, G864–G874. doi: 10.1152/ajpgi.00524.2001

Chen, H., Reed, G., Guardia, J., Lakhan, S., Couture, O., Hays, E., et al. (2013). Vitamin D directly regulates Mdm2 gene expression in osteoblasts. *Biochem. Biophys. Res. Commun.* 430, 370–374. doi: 10.1016/j.bbrc.2012.11.003

Chen, K., Aenlle, K. K., Curtis, K. M., Roos, B. A., and Howard, G. A. (2012). Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. *Bone* 51, 69–77. doi: 10.1016/j.bone.2012.04.002

Chen, K., Perez-Stable, C., D'Ippolito, G., Schiller, P. C., Roos, B. A., and Howard, G. A. (2011). Human bone marrow-derived stem cell proliferation is inhibited by hepatocyte growth factor via increasing the cell cycle inhibitors p53, p21 and p27. *Bone* 49, 1194–1204. doi: 10.1016/j.bone.2011.08.023

Clevers, H., and Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell* 149, 1192–1205. doi: 10.1016/j.cell.2012.05.012

Crescioli, C., Morelli, A., Adorini, L., Ferruzzi, P., Luconi, M., Vannelli, G. B., et al. (2005). Human bladder as a novel target for vitamin D receptor ligands. *J. Clin. Endocrinol. Metab.* 90, 962–972. doi: 10.1210/jc.2004-1496

Cui, J., Ma, C., Qiu, J., Ma, X., Wang, X., Chen, H., et al. (2011). A novel interaction between insulin-like growth factor binding protein-6 and the vitamin D receptor inhibits the role of vitamin D_3 in osteoblast differentiation. *Mol. Cell. Endocrinol.* 338, 84–92. doi: 10.1016/j.mce.2011.03.011

Desprez, P. Y., Poujol, D., Falette, N., Lefebvre, M. F., and Saez, S. (1991). 1,25-Dihydroxyvitamin D_3 increases epidermal growth factor receptor gene expression in BT-20 breast carcinoma cells. *Biochem. Biophys. Res. Commun.* 176, 1–6. doi: 10.1016/0006-291X(91)90880-G

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

D'Ippolito, G., Schiller, P. C., Perez-stable, C., Balkan, W., Roos, B. A., and Howard, G. A. (2002). Cooperative actions of hepatocyte growth factor and 1,25-dihydroxyvitamin D_3 in osteoblastic differentiation of human vertebral bone marrow stromal cells. *Bone* 31, 269–275. doi: 10.1016/S8756-3282(02)00820-7

Dunlop, T. W., Vaisanen, S., Frank, C., Molnar, F., Sinkkonen, L., and Carlberg, C. (2005). The human peroxisome proliferator-activated receptor δ gene is a primary target of 1α,25-dihydroxyvitamin D_3 and its nuclear receptor. *J. Mol. Biol.* 349, 248–260. doi: 10.1016/j.jmb.2005.03.060

Fabri, M., Stenger, S., Shin, D. M., Yuk, J. M., Liu, P. T., Realegeno, S., et al. (2011). Vitamin D is required for IFN-γ-mediated antimicrobial activity of human macrophages. *Sci. Transl. Med.* 3, 104ra102. doi: 10.1126/scitranslmed.3003045

Garcia, L. A., Ferrini, M. G., Norris, K. C., and Artaza, J. N. (2013). 1,25$(OH)_2$vitamin D_3 enhances myogenic differentiation by modulating the expression of key angiogenic growth factors and angiogenic inhibitors in C_2C_{12} skeletal muscle cells. *J. Steroid Biochem. Mol. Biol.* 133, 1–11. doi: 10.1016/j.jsbmb.2012.09.004

Garcia, L. A., King, K. K., Ferrini, M. G., Norris, K. C., and Artaza, J. N. (2011). 1,25$(OH)_2$vitamin D_3 stimulates myogenic differentiation by inhibiting cell proliferation and modulating the expression of promyogenic growth factors and myostatin in C2C12 skeletal muscle cells. *Endocrinology* 152, 2976–2986. doi: 10.1210/en.2011-0159

Geldmeyer-Hilt, K., Heine, G., Hartmann, B., Baumgrass, R., Radbruch, A., and Worm, M. (2011). 1,25-dihydroxyvitamin D_3 impairs NF-κB activation in human naïve B cells. *Biochem. Biophys. Res. Commun.* 407, 699–702. doi: 10.1016/j.bbrc.2011.03.078

Goupil, D., Ethier, C., Zarnegar, R., and Gascon-Barre, M. (1997). Hepatic expression of regeneration marker genes following partial hepatectomy in the rat. Influence of 1,25-dihydroxyvitamin D_3 in hypocalcemia. *J. Hepatol.* 26, 659–668.

Gruber, H. E., Hoelscher, G., Ingram, J. A., Chow, Y., Loeffler, B., and Hanley, E. N. Jr. (2008). 1,25$(OH)_2$-vitamin D_3 inhibits proliferation and decreases production of monocyte chemoattractant protein-1, thrombopoietin, VEGF, and angiogenin by human annulus cells *in vitro*. *Spine* 33, 755–765. doi: 10.1097/BRS.0b013e3181695d59

Grumbles, R. M., Howell, D. S., Wenger, L., Altman, R. D., Howard, G. A., and Roos, B. A. (1996). Hepatocyte growth factor and its actions in growth plate chondrocytes. *Bone* 19, 255–261. doi: 10.1016/8756-3282(96)00180-9

Grundmann, M., Haidar, M., Placzko, S., Niendorf, R., Darashchonak, N., Hubel, C. A., et al. (2012). Vitamin D improves the angiogenic properties of endothelial progenitor cells. *Am. J. Physiol. Cell Physiol.* 303, C954–C962. doi: 10.1152/ajpcell.00030.2012

Guan, H., Liu, C., Chen, Z., Wang, L., Li, C., Zhao, J., et al. (2013). 1,25-Dihydroxyvitamin D_3 up-regulates expression of hsa-let-7a-2 through the interaction of VDR/VDRE in human lung cancer A549 cells. *Gene* 522, 142–146. doi: 10.1016/j.gene.2013.03.065

Gynther, P., Toropainen, S., Matilainen, J. M., Seuter, S., Carlberg, C., and Väisänen, S. (2011). Mechanism of 1α,25-dihydroxyvitamin D_3-dependent repression of interleukin-12B. *Biochim. Biophys. Acta* 1813, 810–818. doi: 10.1016/j.bbamcr.2011.01.037

Harant, H., Wolff, B., and Lindley, I. J. (1998). 1α,25-Dihydroxyvitamin D_3 decreases DNA binding of nuclear factor-κB in human fibroblasts. *FEBS Lett.* 436, 329–334. doi: 10.1016/S0014-5793(98)01153-3

Huang, Y.-C., Chen, J.-Y., and Hung, W.-C. (2004). Vitamin D_3 receptor/Sp1 complex is required for the induction of $p27^{KIP1}$ expression by vitamin D_3. *Oncogene* 23, 4856–4861. doi: 10.1038/sj.onc.1207621

Huang, Y.-C., and Hung, W.-C. (2006). 1,25-Dihydroxyvitamin D_3 transcriptionally represses $p45^{Skp2}$ expression via the Sp1 sites in human prostate cancer cells. *J. Cell. Physiol.* 209, 363–369. doi: 10.1002/jcp.20741

Inaba, M., Koyama, H., Hino, M., Okuno, S., Terada, M., Nishizawa, Y., et al. (1993). Regulation of release of hepatocyte growth factor from human promyelocytic leukemia cells, HL-60, by 1,25-dihydroxyvitamin D_3, 12-O-tetradecanoylphorbol 13-acetate, and dibutyryl cyclic adenosine monophosphate. *Blood* 82, 53–59.

Inoue, T., Kamiyama, J., and Sakai, T. (1999). Sp1 and NF-Y synergistically mediate the effect of vitamin D_3 in the $p27^{KIP1}$ gene promoter that lacks vitamin D response elements. *J. Biol. Chem.* 274, 32309–32317. doi: 10.1074/jbc.274.45.32309

Jiang, Y. J., Teichert, A. E., Fong, F., Oda, Y., and Bikle, D. D. (2012). 1α,25$(OH)_2$-Dihydroxyvitamin D_3/VDR protects the skin from UVB-induced tumor formation by interacting with the β-catenin pathway. *J. Steroid Biochem. Mol. Biol.* 136, 229–232. doi: 10.1016/j.jsbmb.2012.09.024

Jiménez-Lara, A. M., and Aranda, A. (1999). Vitamin D represses retinoic acid-dependent transactivation of the retinoic acid receptor-β2 promoter: the AF-2 domain of the vitamin D receptor is required for transrepression. *Endocrinology* 140, 2898–2907. doi: 10.1210/endo.140.6.6770

Kaeding, J., Belanger, J., Caron, P., Verreault, M., Belanger, A., and Barbier, O. (2008). Calcitrol (1α,25-dihydroxyvitamin D_3) inhibits androgen glucuronidation in prostate cancer cells. *Mol. Cancer Ther.* 7, 380–390. doi: 10.1158/1535-7163.MCT-07-0455

Kaler, P., Augenlicht, L., and Klampfer, L. (2009). Macrophage-derived IL-1β stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D_3. *Oncogene* 28, 3892–3902. doi: 10.1038/onc.2009.247

Kasiappan, R., Shen, Z., Tse, A. K., Jinwal, U., Tang, J., Lungchukiet, P., et al. (2012). 1,25-Dihydroxyvitamin D_3 suppresses telomerase expression and human cancer growth through microRNA-498. *J. Biol. Chem.* 287, 41297–41309. doi: 10.1074/jbc.M112.407189

Kostaras, E., Sflomos, G., Pedersen, N. M., Stenmark, H., Fotsis, T., and Murphy, C. (2013). SARA and RNF11 interact with each other and ESCRT-0 core proteins and regulate degradative EGFR trafficking. *Oncogene* 32, 5220–5232. doi: 10.1038/onc.2012.554

Kovalenko, P. L., Zhang, Z., Cui, M., Clinton, S. K., and Fleet, J. C. (2010). 1,25 dihydroxyvitamin D-mediated orchestration of anticancer, transcript-level effects in the immortalized, non-transformed prostate epithelial cell line, RWPE1. *BMC Genomics* 11:26. doi: 10.1186/1471-2164-11-26

Krishnan, A. V., and Feldman, D. (2010). Molecular pathways mediating the anti-inflammatory effects of calcitriol: implications for prostate cancer chemoprevention and treatment. *Endocr. Relat. Cancer* 17, R19–R38. doi: 10.1677/ERC-09-0139

Krishnan, A. V., Swami, S., Peng, L., Wang, J., Moreno, J., and Feldman, D. (2010). Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy. *Endocrinology* 151, 32–42. doi: 10.1210/en.2009-0855

Larriba, M. J., González-Sancho, J. M., Barbáchano, A., Niell, N., Ferrer-Mayorga, G., and Muñoz, A. (2013). Vitamin D is a multilevel repressor of Wnt/β-catenin signaling in cancer cells. *Cancers* 5, 1242–1260. doi: 10.3390/cancers5041242

Li, J., Jin, D., Fu, S., Mei, G., Zhou, J., Lei, L., et al. (2013). Insulin-like growth factor binding protein-3 modulates osteoblast differentiation via interaction with vitamin D receptor. *Biochem. Biophys. Res. Commun.* 436, 632–637. doi: 10.1016/j.bbrc.2013.04.111

Li, P., Li, C., Zhao, X., Zhang, X., Nicosia, S. V., and Bai, W. (2004). $p27^{Kip1}$ stabilization and G_1 arrest by 1,25-dihydroxyvitamin D_3 in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase. *J. Biol. Chem.* 279, 25260–25267. doi: 10.1074/jbc.M311052200

Li, Y., Spataro, B. C., Yang, J., Dai, C., and Liu, Y. (2005). 1,25-dihydroxyvitamin D inhibits renal interstitial myofibroblast activation by inducing hepatocyte growth factor expression. *Kidney Int.* 68, 1500–1510. doi: 10.1111/j.1523-1755.2005.00562.x

Lin, R., Wang, T. T., Miller, W. H., and White, J. H. (2003). Inhibition of F-Box protein $p45^{SKP2}$ expression and stabilization of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in vitamin D analog-treated cancer cells. *Endocrinology* 144, 749–753. doi: 10.1210/en.2002-0026

Liu, P. T., Schenk, M., Walker, V. P., Dempsey, P. W., Kanchanapoomi, M., Wheelwright, M., et al. (2009). Convergence of IL-1β and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS ONE* 4:e5810. doi: 10.1371/journal.pone.0005810

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., et al. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311, 1770–1773. doi: 10.1126/science.1123933

Luderer, H. F., Gori, F., and Demay, M. B. (2011). Lymphoid enhancer-binding factor-1 (LEF1) interacts with the DNA-binding domain of the vitamin D receptor. *J. Biol. Chem.* 286, 18444–18451. doi: 10.1074/jbc.M110.188219

Malinen, M., Ryynänen, J., Heinäniemi, M., Väisänen, S., and Carlberg, C. (2011). Cyclical regulation of the insulin-like growth factor binding protein 3 gene in response to 1α,25-dihydroxyvitamin D_3. *Nucleic Acids Res.* 39, 502–512. doi: 10.1093/nar/gkq820

Matilainen, J. M., Husso, T., Toropainen, S., Seuter, S., Turunen, M. P., Gynther, P., et al. (2010a). Primary effect of 1α,25$(OH)_2D_3$ on IL-10 expression in monocytes is short-term down-regulation. *Biochim. Biophys. Acta* 1803, 1276–1286. doi: 10.1016/j.bbamcr.2010.07.009

Matilainen, J. M., Räsänen, A., Gynther, P., and Väisänen, S. (2010b). The genes encoding cytokines IL-2, IL-10 and IL-12B are primary 1α,25$(OH)_2D_3$ target genes. *J. Steroid Biochem. Mol. Biol.* 121, 142–145. doi: 10.1016/j.jsbmb.2010.03.020

Matilainen, M., Malinen, M., Saavalainen, K., and Carlberg, C. (2005). Regulation of multiple insulin-like growth factor binding protein genes by 1α,25-dihydroxyvitamin D_3. *Nucleic Acids Res.* 33, 5521–5532. doi: 10.1093/nar/gki872

Maund, S. L., Barclay, W. W., Hover, L. D., Axanova, L. S., Sui, G., Hipp, J. D., et al. (2011). Interleukin-1α mediates the antiproliferative effects of 1,25-dihydroxyvitamin D_3 in prostate progenitor/stem cells. *Cancer Res.* 71, 5276–5286. doi: 10.1158/0008-5472.CAN-10-2160

Mooso, B., Madhav, A., Johnson, S., Roy, M., Moore, M. E., Moy, C., et al. (2010). Androgen Receptor regulation of Vitamin D Receptor in response of castration-resistant prostate cancer cells to 1α-Hydroxyvitamin D_5 - a calcitriol analog. *Genes Cancer* 1, 927–940. doi: 10.1177/1947601910385450

Muthian, G., Raikwar, H. P., Rajasingh, J., and Bright, J. J. (2006). 1,25 Dihydroxyvitamin-D_3 modulates JAK-STAT pathway in IL-12/IFNγ axis leading to Th1 response in experimental allergic encephalomyelitis. *J. Neurosci. Res.* 83, 1299–1309. doi: 10.1002/jnr.20826

Mutt, S. J., Karhu, T., Lehtonen, S., Lehenkari, P., Carlberg, C., Saarnio, J., et al. (2012). Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D_3 via the NF-κB pathway. *FASEB J.* 26, 4400–4407. doi: 10.1096/fj.12-210880

Ng, K. Y., Ma, M. T., Leung, K. K., and Leung, P. S. (2010). Vitamin D and vitamin A receptor expression and the proliferative effects of ligand activation of these receptors on the development of pancreatic progenitor cells derived from human fetal pancreas. *Stem Cell Rev.* 7, 53–63. doi: 10.1007/s12015-010-9146-1

Oh, Y. S., Kim, E. J., Schaffer, B. S., Kang, Y. H., Binderup, L., MacDonald, R. G., et al. (2001). Synthetic low-calcaemic vitamin D_3 analogues inhibit secretion of insulin-like growth factor II and stimulate production of insulin-like growth factor-binding protein-6 in conjunction with growth suppression of HT-29 colon cancer cells. *Mol. Cell. Endocrinol.* 183, 141–149. doi: 10.1016/S0303-7207(01)00598-6

Okuda, N., Takeda, S., Shinomiya, K., Muneta, T., Itoh, S., Noda, M., et al. (2007). ED-71, a novel vitamin D analog, promotes bone formation and angiogenesis and inhibits bone resorption after bone marrow ablation. *Bone* 40, 281–292. doi: 10.1016/j.bone.2006.08.017

Pálmer, H. G., González-Sancho, J. M., Espada, J., Berciano, M. T., Puig, I., Baulida, J., et al. (2001). Vitamin D_3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. *J. Cell Biol.* 154, 369–387. doi: 10.1083/jcb.200102028

Pálmer, H. G., Sánchez-Carbayo, M., Ordóñez-Morán, P., Larriba, M. J., Cordón-Cardó, C., and Muñoz, A. (2003). Genetic signatures of differentiation induced by 1α,25-dihydroxyvitamin D_3 in human colon cancer cells. *Cancer Res.* 63, 7799–7806.

Pan, Q., and Simpson, R. U. (1999). c-Myc intron element-binding proteins are required for 1,25-dihydroxyvitamin D_3 regulation of c-myc during HL-60 cell differentiation and the involvement of HOXB4. *J. Biol. Chem.* 274, 8437–8444. doi: 10.1074/jbc.274.13.8437

Parikh, G., Varadinova, M., Suwandhi, P., Araki, T., Rosenwaks, Z., Poretsky, L., et al. (2010). Vitamin D regulates steroidogenesis and insulin-like growth factor binding protein-1 (IGFBP-1) production in human ovarian cells. *Horm. Metab. Res.* 42, 754–757. doi: 10.1055/s-0030-1262837

Pickup, M., Novitskiy, S., and Moses, H. L. (2013). The roles of TGFβ in the tumour microenvironment. *Nat. Rev. Cancer* 13, 788–799. doi: 10.1038/nrc3500

Qadan, L. R., Perez-Stable, C. M., Schwall, R. H., Burnstein, K. L., Ostenson, R. C., Howard, G. A., et al. (2000). Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines. *Endocrinology* 141, 2567–2573. doi: 10.1210/endo.141.7.7546

Reichrath, S., and Reichrath, J. (2012). No evidence for induction of key components of the Notch signaling pathway (Notch-1, Jagged-1) by treatment with UV-B, $1,25(OH)_2D_3$, and/or epigenetic drugs (TSA, 5-Aza) in human keratinocytes *in vitro*. *Dermatoendocrinology* 4, 44–52. doi: 10.4161/derm.19027

Ren, Z., Li, W., Zhao, Q., Ma, L., and Zhu, J. (2012). The impact of 1,25-dihydroxyvitamin D_3 on the expressions of vascular endothelial growth factor and transforming growth factor-β_1 in the retinas of rats with diabetes. *Diabetes Res. Clin. Pract.* 98, 474–480. doi: 10.1016/j.diabres.2012.09.028

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernandez-Majada, V., Grilli, A., et al. (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6315–6320. doi: 10.1073/pnas.0813221106

Ryynänen, J., and Carlberg, C. (2013). Primary 1,25-dihydroxyvitamin D_3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells. *PLoS ONE* 8:e78170. doi: 10.1371/journal.pone.0078170

Salehi-Tabar, R., Nguyen-Yamamoto, L., Tavera-Mendoza, L. E., Quail, T., Dimitrov, V., An, B. S., et al. (2012). Vitamin D receptor as a master regulator of the c-MYC/MXD1 network. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18827–18832. doi: 10.1073/pnas.1210037109

Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., et al. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* 117, 803–811. doi: 10.1172/JCI30142

Schlaeppi, J. M., Gutzwiller, S., Finkenzeller, G., and Fournier, B. (1997). 1,25-Dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. *Endocr. Res.* 23, 213–229.

Schnabel, M., Fichtel, I., Gotzen, L., and Schlegel, J. (2002). Differential expression of Notch genes in human osteoblastic cells. *Int. J. Mol. Med.* 9, 229–232.

Seoane, S., and Pérez-Fernández, R. (2006). The vitamin D receptor represses transcription of the pituitary transcription factor Pit-1 gene without involvement of the retinoid X receptor. *Mol. Endocrinol.* 20, 735–748. doi: 10.1210/me.2005-0253

Sertznig, P., Dunlop, T., Seifert, M., Tilgen, W., and Reichrath, J. (2009a). Cross-talk between vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling in melanoma cells. *Anticancer Res.* 29, 3647–3658.

Sertznig, P., Seifert, M., Tilgen, W., and Reichrath, J. (2009b). Activation of vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling pathways through $1,25(OH)_2D_3$ in melanoma cell lines and other skin-derived cell lines. *Dermatoendocrinology* 1, 232–238. doi: 10.4161/derm.1.4.9629

Shah, S., Islam, M. N., Dakshanamurthy, S., Rizvi, I., Rao, M., Herrell, R., et al. (2006). The molecular basis of vitamin D receptor and β-catenin crossregulation. *Mol. Cell* 21, 799–809. doi: 10.1016/j.molcel.2006.01.037

Shalhoub, V., Shatzen, E. M., Ward, S. C., Young, J. I., Boedigheimer, M., Twehues, L., et al. (2010). Chondro/osteoblastic and cardiovascular gene modulation in human artery smooth muscle cells that calcify in the presence of phosphate and calcitriol or paricalcitol. *J. Cell. Biochem.* 111, 911–921. doi: 10.1002/jcb.22779

Shen, Q., and Christakos, S. (2005). The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin. *J. Biol. Chem.* 280, 40589–40598. doi: 10.1074/jbc.M504166200

Shen, X., Mula, R. V. R., Li, J., Weigel, N. L., and Falzon, M. (2007). PTHrP contributes to the anti-proliferative and integrin α6β4-regulating effects of 1,25-dihydroxyvitamin D_3. *Steroids* 72, 930–938. doi: 10.1016/j.steroids.2007.08.003

Staal, A., Birkenhäger, J. C., Pols, H. A. P., Buurman, C. J., Vink-van Wijngaarden, T., Kleinekoort, W. M., et al. (1994). Transforming growth factor β-induced dissociation between vitamin D receptor level and 1,25-dihydroxyvitamin D_3 action in osteoblast-like cells. *Bone Miner.* 26, 27–42. doi: 10.1016/S0169-6009(08)80160-2

Staal, A., Geertsma-Kleinekoort, W. M. C., Van Den Bemd, G. J. C. M., Buurman, C. J., Birkenhäger, J. C., Pols, H. A. P., et al. (1998). Regulation of osteocalcin production and bone resorption by 1,25-dihydroxyvitamin D_3 in mouse long bones: interaction with the bone-derived growth factors TGF-β and IGF-I. *J. Bone Miner. Res.* 13, 36–43. doi: 10.1359/jbmr.1998.13.1.36

Staal, A., Van Wijnen, A. J., Desai, R. K., Pols, H. A. P., Birkenhäger, J. C., Deluca, H. F., et al. (1996). Antagonistic effects of transforming growth factor-β on vitamin D_3 enhancement of osteocalcin and osteopontin transcription: reduced interactions of vitamin D receptor/retinoid X receptor complexes with vitamin E response elements. *Endocrinology* 137, 2001–2011. doi: 10.1210/endo.137.5.8612541

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D_3 response by cancer-associated mutant p53. *Cancer Cell* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Sun, J., Kong, J., Duan, Y., Szeto, F. L., Liao, A., Madara, J. L., et al. (2006). Increased NF-κB activity in fibroblasts lacking the vitamin D receptor. *Am. J. Physiol. Endocrinol. Metab.* 291, E315–E322. doi: 10.1152/ajpendo.00590.2005

Swami, S., Krishnan, A. V., Peng, L., Lundqvist, J., and Feldman, D. (2013). Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. *Endocr. Relat. Cancer* 20, 565–577. doi: 10.1530/ERC-12-0281

Tang, J. Y., Xiao, T. Z., Oda, Y., Chang, K. S., Shpall, E., Wu, A., et al. (2011). Vitamin D_3 inhibits hedgehog signaling and proliferation in murine basal cell carcinomas. *Cancer Prev. Res.* 4, 744–751. doi: 10.1158/1940-6207.CAPR-10-0285

Tang, W. B., Ling, G. H., Sun, L., and Liu, F. Y. (2010). Smad anchor for receptor activation (SARA) in TGF-β signaling. *Front. Biosci.* 2, 857–860. doi: 10.2741/E147

Tavera-Mendoza, L., Wang, T. T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., et al. (2006). Convergence of vitamin D and retinoic acid signalling at a common hormone response element. *EMBO Rep.* 7, 180–185. doi: 10.1038/sj.embor.7400594

Thorne, J. L., Maguire, O., Doig, C. L., Battaglia, S., Fehr, L., Sucheston, L. E., et al. (2011). Epigenetic control of a VDR-governed feed-forward loop that regulates $p21^{waf1/cip1}$ expression and function in non-malignant prostate cells. *Nucleic Acids Res.* 39, 2045–2056. doi: 10.1093/nar/gkq875

Ting, H. J., Bao, B. Y., Hsu, C. L., and Lee, Y. F. (2005). Androgen-receptor coregulators mediate the suppressive effect of androgen signals on vitamin D receptor activity. *Endocrine* 26, 1–9. doi: 10.1385/ENDO:26:1:001

Tong, W.-M., Hofer, H., Ellinger, A., Peterlik, M., and Cross, H. S. (1999). Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells: relevance for suppression of epidermal growth factor-stimulated cell growth. *Oncol. Res.* 11, 77–84.

Tong, W.-M., Kállay, E., Hofer, H., Hulla, W., Manhardt, T., Peterlik, M., et al. (1998). Growth regulation of human colon cancer cells by epidermal growth factor and 1,25-dihydroxyvitamin D_3 is mediated by mutual modulation of receptor expression. *Eur. J. Cancer* 34, 2119–2125. doi: 10.1016/S0959-8049(98)00267-6

Toropainen, S., Väisänen, S., Heikkinen, S., and Carlberg, C. (2010). The down-regulation of the human MYC gene by the nuclear hormone 1α,25-dihydroxyvitamin D_3 is associated with cycling of corepressors and histone deacetylases. *J. Mol. Biol.* 400, 284–294. doi: 10.1016/j.jmb.2010.05.031

Towers, T. L., Staeva, T. P., and Freedman, L. P. (1999). A two-hit mechanism for vitamin D3-mediated transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-Jun. *Mol. Cell. Biol.* 19, 4191–4199.

Uhmann, A., Niemann, H., Lammering, B., Henkel, C., Hess, I., Nitzki, F., et al. (2011). Antitumoral effects of calcitriol in basal cell carcinomas involve inhibition of hedgehog signaling and induction of vitamin D receptor signaling and differentiation. *Mol. Cancer Ther.* 10, 2179–2188. doi: 10.1158/1535-7163.MCT-11-0422

Uhmann, A., Niemann, H., Lammering, B., Henkel, C., Hess, I., Rosenberger, A., et al. (2012). Calcitriol inhibits Hedgehog signaling and induces vitamin D receptor signaling and differentiation in the patched mouse model of embryonal rhabdomyosarcoma. *Sarcoma* 2012, 357040. doi: 10.1155/2012/357040

Vink-van Wijngaarden, T., Pols, H. A. P., Buurman, C. J., Birkenhäger, J. C., and van Leeuwen, J. P. T. M. (1996). Inhibition of insulin- and insulin-like growth factor-I-stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D_3 and the vitamin D_3 analogue EB1089. *Eur. J. Cancer* 32A, 842–848. doi: 10.1016/0959-8049(95)00647-8

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011). Effects of 1α,25 dihydroxyvitamin D_3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10, 58. doi: 10.1186/1476-4598-10-58

Woeckel, V. J., Bruedigam, C., Koedam, M., Chiba, H., van der Eerden, B. C. J., and van Leeuwen, J. P. T. M. (2013a). 1α,25-dihydroxyvitamin D_3 and rosiglitazone synergistically enhance osteoblast-mediated mineralization. *Gene* 512, 438–443. doi: 10.1016/j.gene.2012.07.051

Woeckel, V. J., Koedam, M., van de Peppel, J., Chiba, H., van der Eerden, B. C. J., and van Leeuwen, J. P. T. M. (2012). Evidence of vitamin D and interferon-β cross-talk in human osteoblasts with 1α,25-dihydroxyvitamin D_3 being dominant over interferon-β in stimulating mineralization. *J. Cell. Physiol.* 227, 3258–3266. doi: 10.1002/jcp.24020

Woeckel, V. J., van der Eerden, B. C. J., Schreuders-Koedam, M., Eijken, M., and Van Leeuwen, J. P. T. M. (2013b). 1α,25-dihydroxyvitamin D_3 stimulates activin A production to fine-tune osteoblast-induced mineralization. *J. Cell. Physiol.* 228, 2167–2174. doi: 10.1002/jcp.24388

Wu, F. S., Zheng, S. S., Wu, L. J., Teng, L. S., Ma, Z. M., Zhao, W. H., et al. (2007). Calcitriol inhibits the growth of MHCC97 heptocellular cell lines by down-modulating c-met and ERK expressions. *Liver Int.* 27, 700–707. doi: 10.1111/j.1478-3231.2007.01487.x

Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J., and Kato, S. (1999). Positive and negative modulation of vitamin D receptor function by transforming growth factor-β signaling through Smad proteins. *J. Biol. Chem.* 274, 12971–12974.

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., et al. (1999). Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321.

Yu, X. P., Bellido, T., and Manolagas, S. C. (1995). Down-regulation of NF-κB protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10990–10994.

Yuan, W., Pan, W., Kong, J., Zheng, W., Szeto, F. L., Wong, K. E., et al. (2007). 1,25-dihydroxyvitamin D_3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J. Biol. Chem.* 282, 29821–29830. doi: 10.1074/jbc.M705495200

Zhang, G. Y., Cheng, T., Luan, Q., Liao, T., Nie, C. L., Zheng, X., et al. (2011). Vitamin D: a novel therapeutic approach for keloid, an *in vitro* analysis. *Br. J. Dermatol.* 164, 729–737. doi: 10.1111/j.1365-2133.2010.10130.x

Zhao, X. Y., Ly, L. H., Peehl, D. M., and Feldman, D. (1999). Induction of androgen receptor by 1α,25-dihydroxyvitamin D_3 and 9-cis retinoic acid in LNCaP human prostate cancer cells. *Endocrinology* 140, 1205–1212. doi: 10.1210/endo.140.3.6561

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Vitamin D and the epigenome

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Epigenetic mechanisms play a crucial role in regulating gene expression. The main mechanisms involve methylation of DNA and covalent modifications of histones by methylation, acetylation, phosphorylation, or ubiquitination. The complex interplay of different epigenetic mechanisms is mediated by enzymes acting in the nucleus. Modifications in DNA methylation are performed mainly by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) proteins, while a plethora of enzymes, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) regulate covalent histone modifications. In many diseases, such as cancer, the epigenetic regulatory system is often disturbed. Vitamin D interacts with the epigenome on multiple levels. Firstly, critical genes in the vitamin D signaling system, such as those coding for vitamin D receptor (*VDR*) and the enzymes 25-hydroxylase (*CYP2R1*), 1α-hydroxylase (*CYP27B1*), and 24-hydroxylase (*CYP24A1*) have large CpG islands in their promoter regions and therefore can be silenced by DNA methylation. Secondly, VDR protein physically interacts with coactivator and corepressor proteins, which in turn are in contact with chromatin modifiers, such as HATs, HDACs, HMTs, and with chromatin remodelers. Thirdly, a number of genes encoding for chromatin modifiers and remodelers, such as HDMs of the Jumonji C (JmjC)-domain containing proteins and lysine-specific demethylase (LSD) families are primary targets of VDR and its ligands. Finally, there is evidence that certain VDR ligands have DNA demethylating effects. In this review we will discuss regulation of the vitamin D system by epigenetic modifications and how vitamin D contributes to the maintenance of the epigenome, and evaluate its impact in health and disease.

Keywords: VDR, VDRE, 1,25-dihydroxyvitamin D3, CYP27B1, CYP24A1, DNA methylation, histone modifications, CpG island

INTRODUCTION

The role of vitamin D in regulating gene expression has become increasingly evident since the discovery of the transcription factor vitamin D receptor (VDR), a member of the steroid nuclear receptor superfamily. The effect of liganded VDR depends on the epigenetic landscape of the target gene. Genome wide analysis in the human leukemia cell line THP-1 showed that VDR binds mainly at loci of open chromatin. Upon treatment with the VDR ligand 1,25-dihydroxyvitamin D_3 (1,25-D_3), chromatin accessibility further increases in more than 30% of these loci (Seuter et al., 2013). The mechanism of action of the liganded VDR is dependent on binding and action of histone acetyltransferases (HATs) and histone methyltransferases (HMTs). It has been shown that co-treatment of cells with 1,25-D_3, and histone deacetylase or DNA methyltransferase inhibitors often have synergistic effects (Pan et al., 2010).

Many common diseases have both genetic and epigenetic components, which communicate in an intricate and multilayered manner. Currently, it is not clear to what extent epigenetic alterations contribute to onset and progress of common diseases, such as cancer. Epigenetics refers to processes that alter gene activity without changing the DNA sequence. They play an important role in regulating key processes during development, including embryonic developmental events, gene imprinting, and inactivation of chromosome X in females (Bird, 2002; Meissner et al., 2008; Tsai and Baylin, 2011). Maintenance of normal functioning of these biological processes is dependent on the intricate interaction between several epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNAs (Jones and Baylin, 2007). Therefore, at a given promoter the marks arising from DNA methylation and histone modifications determine whether the chromatin is in an open (active) or a closed (repressed) state. Deregulation of the epigenetic mechanisms can lead to aberrant DNA methylation patterns and chromatin architecture, which is a common feature in cancer (Baylin and Jones, 2011; Tsai and Baylin, 2011; Helin and Dhanak, 2013).

EPIGENETIC CHANGES MEDIATED BY THE VITAMIN D RECEPTOR AND ITS LIGANDS

The effect of nutrition on the methylation equilibrium of the genome is already accepted as one of the mechanisms preventing either promoter hyper- or global hypomethylation. Several nutrients are renowned for their impact on DNA methylation, such as folic acid, vitamin B, green tea, and alcohol (Arasaradnam et al., 2008). The effect of vitamin D is currently under debate.

Primary epigenetic effects of vitamin D are linked to histone modifications, mainly acetylation. The VDR/RXR dimer interacts with HATs to induce transcriptional activation (Karlic and Varga,

2011). Several studies have suggested that vitamin D may affect also DNA methylation. A recent study associated severe vitamin D deficiency with methylation changes in leukocyte DNA, although the observed differences were relatively small (Zhu et al., 2013). This study suggested that subjects with vitamin D deficiency were more likely to show reduced synthesis and increased catabolism of active vitamin D. Whether this was the cause of the vitamin D deficiency or the consequence thereof is not clear and needs further studies.

EFFECT OF VITAMIN D ON DNA METHYLATION

DNA methylation is the most extensively studied epigenetic mark (Esteller, 2008). In humans, DNA methylation occurs on cytosines followed by guanine (CpG) (Bird, 1980; Gruenbaum et al., 1981). Regions of DNA enriched in CpG clusters form CpG islands (CGI) (Wang and Leung, 2004). DNA methylation is necessary for regulating and orchestrating key biological processes, including cell cycle, differentiation, as well as genomic imprinting (Feinberg et al., 2002; Reik and Lewis, 2005; Jones and Baylin, 2007). DNA hypermethylation is mainly found in intergenic regions and repetitive genomic sequences to maintain these in a transcriptionally inactive chromatin state (Herman and Baylin, 2003).

DNA methyltransferases (DNMTs) mediate DNA methylation (Robertson, 2005). DNMT1 encodes for a maintenance methyltransferase, whereas DNTM3A/3B encode for de novo methyltransferases, which are pivotal to maintain and establish genomic methylation (Okano et al., 1998, 1999; Jin and Robertson, 2013). However, *in vivo* studies suggest that all three DNMTs might exert both de novo and maintenance functions (Rhee et al., 2000, 2002; Kim et al., 2002; Esteller, 2007a). Recently, a new group of enzymes that induce active demethylation of the DNA was discovered, the ten-eleven translocation (TET) enzyme family, which plays an important role both in development and tumorigenesis (Kriaucionis and Heintz, 2009; Ficz et al., 2011; Williams et al., 2011; Yamaguchi et al., 2012; Hackett et al., 2013).

Alterations in the cancer epigenome are generally associated with loss of global DNA methylation and gain of methylation in specific gene promoters (Ting et al., 2006). Loss of global methylation may lead to chromosomal instability (Eden et al., 2003), loss of imprinting (Cui et al., 2003; Bjornsson et al., 2007), and activation of transposable elements, thereby leading to disturbances in the genome (Bestor, 2005; Esteller, 2008). Conversely, hypermethylation of promoter regions of tumor suppressor genes (Greger et al., 1989; Sakai et al., 1991; Esteller, 2008) leads to loss of expression of key genes affecting pathways involved in maintenance of cellular functions, including cell cycle, apoptosis, and DNA repair (Esteller, 2007b). Several bona fide tumor suppressor genes are silenced by promoter hypermethylation in tumors. For instance, hypermethylation of the promoter of the DNA repair gene *hMLH1* is associated with early stages of endometrial and colon cancer, and microsatellite instability phenotype (Esteller et al., 1999). Epigenetically mediated silencing of cyclin-dependent kinase inhibitor 2A, which is crucial for control of cell cycle has been reported in several cancers (Brock et al., 2008; Liau et al., 2014). Additionally, pathways regulated by microRNAs have been associated with DNA hypermethylation-dependent silencing (Saito et al., 2006).

Besides methylating cytosines, DNMTs may coordinate other chromatin-mediated aspects of gene expression at sites of gene promoters (Herman and Baylin, 2003). For example, hypermethylation of promoters of tumor suppressor genes is associated with recruitment of proteins belonging to the methyl CpG-binding domain (MBD) family, MeCP2, MBD1, MBD2, MBD3, and MBD4 (Ballestar and Esteller, 2005). It has been shown that MeCP2 represses transcription of methylated DNA by recruiting histone deacetylases (HDACs), providing the first evidence for interactions between DNA methylation and histone modifications (Jones et al., 1998; Nan et al., 1998).

There is evidence that 1,25-D_3 is able to induce DNA demethylation, however, the mechanisms behind the effect of 1,25-D_3 on DNA methylation are not clear. In most cases it is probably passive demethylation that happens over several cycles of DNA replication. However, in some cases demethylation occurs within 1–4 h, indicative of an active process (Doig et al., 2013). The fact that vitamin D can alter methylation of DNA in the promoter of certain genes is novel. Tapp and colleagues suggested that in healthy subjects global, age-related CGI methylation of human rectal mucosa was influenced not only by gender, folate availability, and selenium, but also by vitamin D status (Tapp et al., 2013). The authors show negative association between serum 25-D_3 level and CGI methylation of the adenomatous polyposis coli (*APC*) promoter region, a tumor suppressor often inactive in colorectal cancer. Interestingly, they observed a weak positive correlation of vitamin D level with methylation of *LINE-1* (genomic long interspersed nuclear element-1), a mammalian autonomous retrotransposon, increasing stability of this region (Tapp et al., 2013). A recent study in colorectal cancer patients investigating two Canadian populations (from Newfoundland and Ontario) found that high dietary vitamin D intake was associated with lower methylation of the two WNT antagonists *dickkopf 1* (*DKK1*) and *WNT5A* (Rawson et al., 2012). This relationship became even more significant in females in the Newfoundland population, while in the Ontario population the association between vitamin D intake and lower methylation was observed only in early stage tumors, but not in late stage tumors (Rawson et al., 2012). These data confer further insights in the mechanisms regulating the transcriptional activating effect of vitamin D on *DKK1* expression described *in vitro* (Aguilera et al., 2007; Pendas-Franco et al., 2008).

Moreover, treatment of the triple negative breast cancer cell line MDA-MB-231 with 1,25-D_3 reduced DNA methylation of the *e-cadherin* promoter (Lopes et al., 2012), while another study showed that 1,25-D_3 induced demethylation of the *PDZ-LIM* domain-containing protein 2 promoter, leading to increased expression (Vanoirbeek et al., 2014). In non-malignant and malignant prostate epithelial cells, treatment with 1,25-D_3 caused clear changes in site-specific methylation of the *p21* promoter, in a cell line-specific manner (Doig et al., 2013).

An interesting interaction between vitamin D and DNA methylation is induction of the expression of GADD45 (growth arrest and DNA damage) protein by 1,25-D_3 in several tumor

cells (Jiang et al., 2003; Zhang et al., 2006; Bremmer et al., 2012). GADD45A is one of the enzymes that promote epigenetic gene activation by repair mediated DNA demethylation in Xenopus laevis (Barreto et al., 2007).

In summary, alterations in DNA methylation lead to aberrant gene expression and disruptions of genomic integrity, which contribute to development and progression of diseases. Vitamin D can regulate these processes; the mechanisms behind need further investigations.

INTERACTIONS OF VITAMIN D WITH CHROMATIN MODULATORS AND REMODELERS

Nuclear receptors, such as the VDR contain DNA-binding domains that mediate binding to the DNA, presuming the DNA is available and is not wound tightly around nucleosomes. The chromatin context determines nuclear receptor binding and determines which epigenetic modifications will occur thereafter. Upon binding to their genomic response elements, nuclear hormone receptors will then recruit different regulatory cofactor complexes (Lee et al., 2001). The unliganded VDR is able to bind also genomic DNA, where it usually forms complexes with corepressor proteins that either exert HDAC activity, e.g., ALIEN (Polly et al., 2000), or are associated with HDACs, such as NCOR1 and SMRT. The corepressors dissociate upon binding of 1,25-D_3, and are replaced by coactivator complexes.

The chromatin environment dictates gene activity throughout the genome. Post-translational modifications of the N-terminal tails of histone proteins allow nucleosomes to shift, the chromatin to relax, and genes to become activated. Histone modifications change in response to environmental stimuli (Meyer et al., 2013). Histones are major protein components of chromatin that undergo post-translational modifications, including acetylation of lysines, methylation of lysines and arginines, and phosphorylation of serine and threonine residues (Esteller, 2008). In epigenetically silenced genes, hypermethylation of CGIs is often associated with loss of acetylation on histone 3 and 4 (H3 and H4), loss of methylation of lysine (K) 4 on H3 (H3K4), and gain of methylation of K9 and K27 on H3 (H3K9 and H3K27) (Esteller, 2008).

Histone acetylation generally correlates with transcriptional activation (Hebbes et al., 1988; Kouzarides, 2007) and is dependent on a dynamic interaction between histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Marks et al., 2001). The balance between the actions of these enzymes is crucial in controlling gene expression, and governs several developmental processes and disease states (Haberland et al., 2009). Generally, HATs are defined as activators of transcription, whereas HDACs as transcription repressors (Parbin et al., 2014). In various cancer types, including prostate, gastric, and breast cancers, overexpression of HDAC1 is often associated with poor clinical outcome (Choi et al., 2001; Halkidou et al., 2004; Zhang et al., 2005). In colorectal cancer patients HDAC1, 2, and 3 are overexpressed, and high HDAC1 and 2 expression is linked with reduced patient survival (Zhu et al., 2004; Wilson et al., 2006). Overexpression of HDAC1 plays a crucial role in regulating proliferation by repressing the expression of the cyclin-dependent kinase inhibitor p21 (Lagger et al., 2003). Additionally, silencing of HDAC4 leads to re-expression of p21, which in turn induces cell growth arrest and tumor growth inhibition, both *in vitro* and *in vivo* in a human glioblastoma model (Mottet et al., 2009). In addition to classical HDACs, another group of enzymes, the sirtuins (silent information regulator 2 proteins) are involved in histone deacetylation (Schwer and Verdin, 2008). Sirtuins have been linked to metabolic disorders, cancer, aging, and also regulation of the circadian rhythm (Guarente, 2006; Longo and Kennedy, 2006; Jung-Hynes et al., 2010).

Many of the coactivators recruited by the VDR, including p160 steroid receptor coactivator proteins (SRC1, 2, and 3), p300, or CBP have lysine acetyltransferase activity (**Figure 1**). Indeed, treatment of THP-1 cells with 1,25-D_3 increased H3K27ac at the promoter of several early VDR target genes (Seuter et al., 2013). In genetic hypercalciuric stone forming rats inhibition of bone morphogenetic protein 2 (BMP2) by 1,25-D_3, seems to involve H3 deacetylation and H3K9 di-methylation (Fu et al., 2013).

In MDA-MB453 breast cancer cells 1,25-D_3 treatment regulates expression of p21 through a mechanism involving both histone acetylation and methylation, probably by dynamic

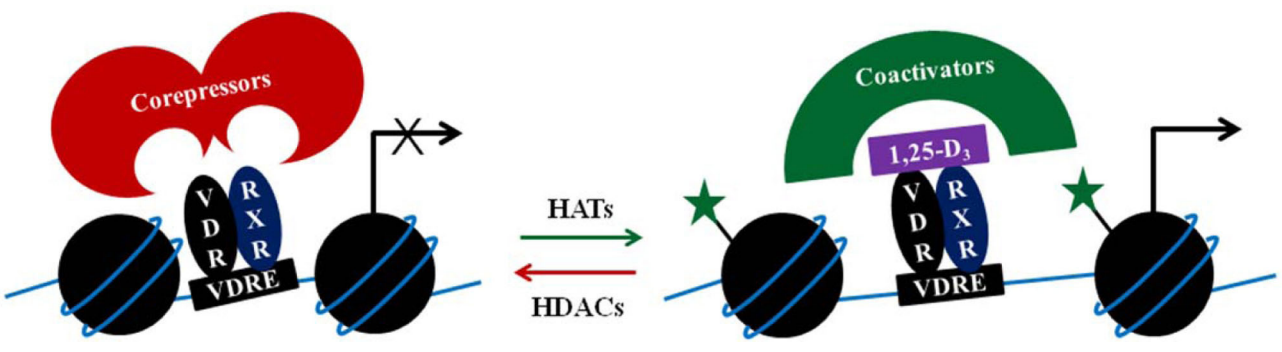

FIGURE 1 | Simplified illustration of a two-step coregulator model. The VDR/RXR complex in absence of the 1,25-D_3 may bind corepressors that would lead/maintain gene repression, partly by attracting histone deacetylases (HDACs). Upon ligand binding to the VDR/RXR complex, corepressors are replaced by coactivators, which include histone acetyltransferases (HATs). Acetylation of histones (indicated by the green stars) enables chromatin relaxation and gene transcription.

chromatin looping from distal 1,25-D_3 responsive elements to the TSS of *p21* (Saramäki et al., 2009).

Histone methylation can lead either to gene activation or repression, depending on the histone site that is methylated, the degree of methylation (e.g., mono-methylation, di-methylation, or tri-methylation), amino acid residues affected, and their position in the histone tail (Esteller, 2008). Methylation of histones depends on a dynamic process arising from the actions of methyltransferases (HMTs) and demethylases (HDMs) (Shi and Whetstine, 2007; Mosammaparast and Shi, 2010; Greer and Shi, 2012). So far, two protein families capable of demethylating lysines are known, the amine oxidases (Shi et al., 2004) and jumonji C (JmjC)-domain-containing proteins (Cloos et al., 2006; Tsukada et al., 2006). The first histone demethylase discovered was the lysine-specific demethylase 1 (LSD1/KDM1A), an amine oxidase, which demethylates H3K4me2/me1 (**Table 1**) (Shi et al., 2004). High expression of KDM1A in various cancers, including colorectal cancer, prostate cancer, and neuroblastomas is associated with increased cancer recurrence and poor clinical outcome (Kahl et al., 2006; Schulte et al., 2009; Ding et al., 2013). Additionally, it has been shown that LSD1 is essential for androgen and estrogen receptor-dependent gene activation via H3K9me2/me1 demethylation (Metzger et al., 2005; Garcia-Bassets et al., 2007; Perillo et al., 2008). There is a reciprocal regulatory effect between the activity of VDR and histone demethylases. In the colon cancer cell line SW480-ADH 1,25-D_3 increased the expression of the lysine-specific demethylase 1 and 2 (Pereira et al., 2012).

1,25-D_3 treatment affected also the expression of a series of different JmjC histone demethylases. The first identified member of the JmjC family was KDM2A/JHDM1A (Tsukada et al., 2006). Expression profiling data showed altered expression of KDM2A and KDM2B in several tumors, however, it seems that their pro- or antioncogenic functions are tissue-dependent (Frescas et al., 2007, 2008; Pfau et al., 2008). 1,25-D_3 inhibited the expression of several histone demethylases (e.g., KDM4A/4C/4D/5A/2B, JMJD5/6, PLA2G4B), and induced the expression of others, JARID2 and KDM5B (Pereira et al., 2012). Members of the KDM4 family catalyze tri-demethylation of H3K9 and/or H3K36 (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006; Whetstine et al., 2006; Lin et al., 2008). H3K9me3 is a mark for heterochromatin and demethylation of H3K9 is suggested to be linked with chromosomal instability (Cloos et al., 2006). Inhibition of expression of KDM4 family members by 1,25-D_3 could thus contribute to genome stability. Members of KDM5 cluster catalyze demethylation of H3K4me3/me2, which is a mark for open chromatin (Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007; Tahiliani et al., 2007) and their upregulaton upon 1,25-D_3 treatment might lead to gene repression (Pereira et al., 2011). Overexpression of KDM5B has been reported in breast and prostate cancers (Lu et al., 1999; Xiang et al., 2007). Deletion of *kdm5b* inhibits tumor growth in a syngeneic mouse mammary

Table 1 | A simplified list of the members of the two classes of histone demethylases (mentioned in the manuscript).

Class of histone demethylases	Histone demethylase family	Histone demethylase	Histone substrate	Gene expression
Amine oxidases	KDM1	KDM1A	H3K4me2/me1 H3K9me2/me1	Repression Activation
		KDM1B	H3K4me2/me1	Repression
Jumonji C-domain-containing proteins	KDM2	KDM2A	H3K36me2/me1	Repression
		KDM2B	H3K4me3 H3K36me2/me1	
	KDM3	KDM3A	H3K9me2/me1	Activation
		KDM3B	H3K9me3/me2/me1	
	KDM4	KDM4A	H3K9me3/me2	Activation
		KDM4B	H3K36me3/me2	Repression
		KDM4C		
		KDM4D	H3K9me3/me2	Activation
	KDM5	KDM5A	H3K4me3/me2	Repression
		KDM5B		
		KDM5C		
		KDM5D		
	KDM6	KDM6A	H3K27me3/me2	Activation
		KDM6B		
	PHF	JHDM1D	H3K9me2/me1 H3K27me2/me1	Activation
		PHF8	H3K9me2/me1	

Reviewed in Pedersen and Helin (2010), Greer and Shi (2012). The main histone demethylase families and submembers are indicated. Degree of methylation and site of lysine residue are given. References for individual enzymes can be found throughout the text. H, Histone; K, lysine; me1, mono-methylation; me2, di-methylation; me3, tri-methylation; KDM1A, lysine-specific demethylase 1A; JHDM1D, JmjC-domain-containing histone demethylation protein 1D; PHF, plant homeodomain finger protein.

tumor (Yamane et al., 2007), suggestive of its potential role in tumor development. 1,25-D_3 induced the expression of the histone demethylase KDM6B as well, which is the only other known enzyme, besides KDM6A that is able to demethylate H3K27me3, a histone mark that correlates with gene repression. Furthermore, the authors showed positive correlation between KDM6B and VDR in 96 colon tumor patients, and inverse correlation of KDM6B with SNAIL1, which is involved in epithelial to mesenchymal transition, indicating that probably the antiproliferative role of 1,25-D_3 via KDM6B upregulation might take place *in vivo* (Pereira et al., 2011). Interestingly, treatment of SW480-ADH cells with 1,25-D_3 had no effect on global H3K27me3 levels, in spite of KDM6B upregulation (Pereira et al., 2011, 2012). The effect of 1,25-D_3 on the expression of histone demethylases may well be indirect and could be mediated by microRNAs (Padi et al., 2013). KDM2A is one of the direct targets of microRNA-627. 1,25-D_3-dependent upregulation of the microRNA-627 expression both *in vitro*, in the HT-29 colorectal cancer cells and *in vivo*, in tumor xenografts, led to lower KDM2A levels (Padi et al., 2013).

In different pathologies, the expression pattern of the nuclear receptor cofactors is altered, compromising the effect of 1,25-D_3 (Doig et al., 2013; Singh et al., 2013). The initial interactions between VDR and coactivators are the seed for the assembly of intricate multiprotein complexes that remodel the chromatin structure, recruit the core transcriptional machinery, and induce expression of 1,25-D_3 target genes (**Figure 1**). Often, differences in responsiveness to 1,25-D_3 depend on the expression pattern of the coregulators of VDR. In prostate cancer cells, the temporal distribution of the nuclear corepressor NCOR1 at VDR target genes is different in 1,25-D_3 responsive cells compared with unresponsive cells (Doig et al., 2013; Singh et al., 2013).

The liganded VDR is able both to transactivate and transrepress target genes. The mechanisms of action are probably different between transactivation and transrepression, and also highly dependent on the motifs of the vitamin D response elements. A highly complex mechanism regulates the ligand-dependent repression of CYP27B1 (Kim et al., 2007a). CYP27B1 repression requires two epigenetic modifications: deacetylation of histones and methylation of the *CYP27B1* gene promoter and exon regions. This is dependent on the presence of the VDR interacting repressor (VDIR) and the chromatin remodeler Williams Syndrome transcription factor. In the absence of 1,25-D_3, VDIR is bound directly to the E-box motifs in the negative VDRE and recruits histone acetyltransferases to induce *CYP27B1* gene transcription. In the presence of 1,25-D_3, VDIR acts as a scaffold for the 1,25-D_3-VDR complex to repress transcription of *CYP27B1* through recruitment of HDAC2, DNMT1, and DNMT3B (Kim et al., 2007a). It seems that VDIR and HDAC2 are involved also in the 1,25-D_3-dependent transrepression of the human parathyroid hormone gene (Kim et al., 2007b). It is not clear, whether this mechanism of transrepression by liganded VDR also applies to other genes. In mesenchymal stem cells 1,25-D_3 represses gene expression by binding to promoters with enhanced H3K9Ac and H3K9me2 (Tan et al., 2009). Whether H3K9 acetylation/methylation enabled VDR binding or VDR binding caused H3K9 acetylation, is not clear.

Proper orchestration of histone modifications in crosstalk with other chromatin regulators is crucial in maintaining the epigenetic landscape and governing gene expression. Any disturbances in these constellations may lead to aberrant gene expression. Whether 1,25-D_3 affects regulation of other chromatin modulators as well, is not yet known.

REGULATION OF THE VITAMIN D SYSTEM

The vitamin D system has pleiotropic functions and regulates approximately 3% of the human genome (Bouillon et al., 2008). To maintain balance, a strict regulation of the vitamin D system genes is of utmost importance. The main role of liganded VDR in tissues not involved in calcium homeostasis is to control expression of genes that regulate cell proliferation, differentiation, and apoptosis. One major limitation in the therapeutic exploitation of these effects is the resistance of cancer cells to 1,25-D_3. Epigenetic corruption of VDR signaling is suggested to be one of the mechanisms that leads to reduced responsiveness to 1,25-D_3 actions. This can be caused by promoter methylation of key vitamin D system genes or by skewed accumulation of VDR-associated corepressors, preferentially at promoters of anti-proliferative target genes (Abedin et al., 2006).

Expression of the vitamin D degrading and metabolizing enzymes is regulated through binding of 1,25-D_3-liganded VDR to vitamin D responsive elements (VDREs). However, the major regulators of 1,25-D_3 levels and signaling CYP2R1, CYP24A1, CYP27B1, and VDR, "the vitamin D tool" genes, are prone to epigenetic regulation. CpG islands span the promoters of *CYP2R1*, *CYP24A1*, and *VDR*, while a CpG island is located within the *CYP27B1* gene locus (**Figure 2**). Therefore, DNA methylation and histone modifications in these regions can change the chromatin state from an open to closed conformation and lead to transcriptional repression of these genes. Expression of vitamin D tool genes becomes deregulated in various types of cancer, and these changes may be partially attributed to epigenetic alterations (reviewed in Hobaus et al., 2013). As early as 1984, Yoneda et al. (1984) have shown that the histone acetyltransferase inhibitor butyrate augments 1,25-D_3 actions. Several studies confirmed these findings (e.g., Rashid et al., 2001) and have suggested that the action of butyrate could be through upregulation of VDR or CYP27B1 expression (Gaschott and Stein, 2003). Whether this effect is mediated by direct acetylation of the *VDR* or *CYP27B1* promoters, has not been determined.

In this chapter we discuss evidence for epigenetic regulation through DNA methylation of these genes in health and disease.

EPIGENETIC REGULATION OF THE VITAMIN D RECEPTOR

The VDR is a nuclear receptor mediating 1,25-D_3 signaling. It is expressed by at least 38 cell types in the human body (Norman and Bouillon, 2010). In the absence of its ligand 1,25-D_3, VDR is mainly found in the cytoplasm (Nagpal et al., 2005). Upon ligand binding, VDR heterodimerizes with the retinoid X receptor (RXR) and translocates to the nucleus, where it binds to vitamin D responsive elements (VDREs) to regulate transcription of 1,25-D_3 target genes. This is achieved through recruitment of coactivators or corepressors to the VDR-RXR complex bound to DNA (Nagpal et al., 2005; Pike et al., 2012; Haussler et al., 2013;

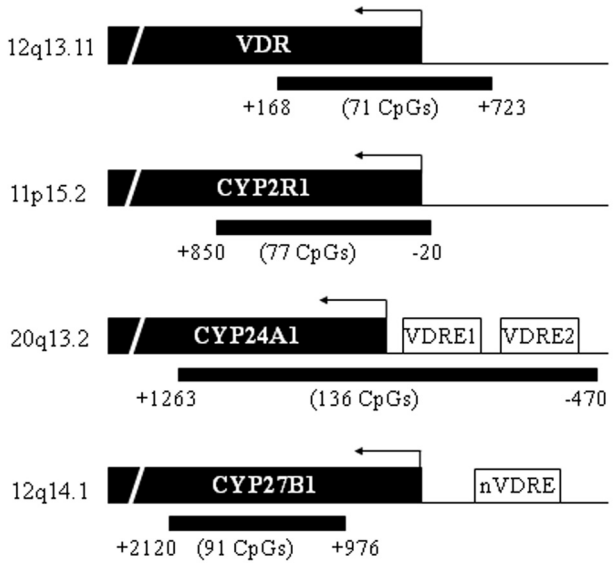

FIGURE 2 | Location of CpG islands in the promoter region of vitamin D tools genes. VDR is located on 12q13.11 (chr12:48235320-48298814), CYP2R1 on chromosome 11p15.2 (chr11:14899556-14913751), CYP24A1 on chromosome 20q13.2 (chr20:52769988-52790516), and CYP27B1 on 12q14.1 (chr12:58156117-58160976). The locations of the CpG islands are indicated (black bars) relative to start of the gene locus (not to TSS) according to UCSC Genome Browser Homepage (GRCh37/hg19) (Karolchik et al., 2014). Number of CpGs located within each island is stated. Two vitamin D responsive elements (VDRE) are located in the proximal CYP24A1 promoter region and one nVDRE is located in the CYP27B1 promoter region.

Pike and Meyer, 2013). As VDR is rarely mutated during carcinogenesis (Miller et al., 1997), the disturbance of the vitamin D signaling and apparent 1,25-D_3 insensitivity in cancer (Marik et al., 2010) must be attributed to other alterations, which may include epigenetic changes, such as promoter methylation.

The *VDR* gene is located on the long arm of chromosome 12 (12q13.11) and contains 4 potential promoter regions. Exons 1a, 1c, and 1d of the *VDR* are well conserved, while 1b, 1e, and 1f show low homology (Halsall et al., 2007). Exon 1a appears to contain a strong promoter, including several transcription factor binding sites (AP-2 and SP1). Transcription was reported to originate also in exons 1d, 1e, and 1f, while translation starts in exon 2 (Halsall et al., 2007). Marik and colleagues performed an *in silico* analysis of the *VDR* gene sequence and reported three CpG islands located in exon 1a spanning from −790 bp to 380 bp relative to the TSS in exon 1a (Marik et al., 2010). According to the UCSC Genome browser, however, only one large CpG island spanning 892 bp in length is found in the *VDR* promoter region (Gardiner-Garden and Frommer, 1987; Karolchik et al., 2014). This discrepancy is likely due to the different search parameters used for CpG island identification.

Epigenetic silencing of VDR was suggested to cause the slow normalization of VDR levels in the parathyroid glands of uremic rats after kidney transplantation (Lewin et al., 2002; Hofman-Bang et al., 2012). However, sequencing of the *VDR* promoter [−250 to 300 bp relative to exon 1 (43 CpGs)] in normal and uremic rats showed no difference between methylation patterns (Hofman-Bang et al., 2012). Further, the authors reported that methylation levels coincided with the negative control, thus showing that promoter methylation does not play a role in regulating VDR expression in the parathyroid glands.

In contrast, promoter methylation was reported to cause repression of *VDR* gene expression in HIV infected T cells. In normal T cells, activation or priming causes an upregulation of VDR expression (Von Essen et al., 2010). In comparison, infection of previously activated T cells with human immunodeficiency virus (HIV) led to upregulation of DNMT3B, increased promoter methylation of *VDR* (45–70%), and decreased *VDR* gene expression (Chandel et al., 2013). This downregulation of VDR could be reversed upon treatment with 5-azacytidine (AZA) suggesting that the decreased expression of VDR by HIV is, at least partially, caused by DNA methylation (Chandel et al., 2013). There is evidence for an inverse correlation between the vitamin D status and infections, however, many trials failed to show a protective effect of vitamin D (reviewed in Yamshchikov et al., 2009). Thus, reduced sensitivity to vitamin D metabolites due to, e.g., downregulation of VDR may account for inconclusive trials. This is supported by a study investigating methylation of the 3′end of *VDR* in two South African groups revealing differences with respect to ethnicity and tuberculosis status of the patients (Andraos et al., 2011).

In breast tumors, methylation of exon 1a of the *VDR* gene was significantly higher (65% of CpGs methylated) compared with normal breast tissue (15% of CpGs methylated) (Marik et al., 2010). *In vitro*, in breast cancer cell lines, three hypermethylated regions in exon 1a became demethylated after treatment with the DNMT1 inhibitor 5-aza-2′-deoxycytidine (DAC) and VDR mRNA expression increased. These regions were in proximity to the SP1 binding sites (approximately 790 bp from TSS), NFκB binding sites (approximately −480 from TSS), and the exon 1a TSS. Treatment with 1,25-D_3 had no effect on methylation of these regions (Marik et al., 2010). In other types of cancer, e.g., the choriocarcinoma-derived trophoblast cell lines JEG-3 and JAR, the *VDR* promoter was densely methylated (Novakovic et al., 2009). In contrast, no methylation of the VDR promoter region was observed in colon cancer cell lines, and treatment with DAC did not increase gene expression (Habano et al., 2011; Höbaus et al., 2013a). In parathyroid tumors the expression of VDR is decreased (Gogusev et al., 1997; Carling et al., 2000), however, no differences in DNA methylation of *VDR* were observed between parathyroid tumors and healthy controls (Sulaiman et al., 2013). Similar results were seen in parathyroid adenoma samples, which showed decreased expression of VDR, but showed no promoter methylation (Varshney et al., 2013).

Additionally, it has been suggested that expression of 5′ truncated variants of VDR is linked to methylation of the *VDR* promoter. These variants are predominantly found in breast cancer compared with the full length variants expressed in normal breast tissue. Treatment with DAC restored expression of the active transcript variant of VDR in breast cancer cell lines, indicating promoter methylation as cause of truncated protein expression (Marik et al., 2010). The significance of these potentially untranslated truncated variants remains to be investigated, however, as

they are not found in normal breast tissue aberrant expression of truncated isoforms may further disrupt vitamin D signaling in tumor tissue.

A recent study suggested that in colorectal cancer metastases, VDR becomes the target of the polycomb group protein enhancer of zeste homolog 2 (EZH2) that mediates VDR downregulation by H3K27 trimethylation in the VDR promoter (Lin et al., 2013). The histone deacetylase HDAC3, one of the most frequently upregulated genes in cancer, seems to inhibit VDR expression. In two colorectal cancer cell lines, HCT116 and SW480 knock down of HDAC3 increased VDR expression and restored sensitivity of these cells to 1,25-D_3 (Godman et al., 2008).

Taken together, there is evidence that in various diseases the decreased tissue sensitivity to 1,25-D_3 could have been caused by the epigenetic silencing of the VDR.

EPIGENETIC REGULATION OF THE CYP2R1

CYP2R1 is a microsomal P450 enzyme, which hydroxylates both vitamin D_2 and D_3 at position C-25 to form the circulating storage form of vitamin D 25-D_3. The promoter region of *CYP2R1* is located within a CpG island, which can be subjected to epigenetic regulation. So far, only two studies investigated the promoter methylation status of this gene. Genome wide association studies found increased *CYP2R1* promoter methylation in leukocyte DNA from individuals with severe vitamin D deficiency compared with control group (Zhu et al., 2013). Further, methylation levels of *CYP2R1* promoter decreased within 12 months of vitamin D supplementation in DNA extracted from serum of non-Hispanic white American post-menopausal women aged $\geq$55 years (Zhu et al., 2013), indicating an effect of vitamin D supplementation on *CYP2R1* promoter methylation. These data indicate that under low vitamin D serum levels, the promoter of the major 25-hydroxylase *CYP2R1* may become methylated, and that event appears to be reversible upon exposure to increased vitamin D.

EPIGENETIC REGULATION OF THE CYP27B1

CYP27B1 is an inner mitochondrial membrane P450 enzyme that converts 25-D3 to its active form 1,25-D3. It is mainly expressed in the proximal tubule of the kidneys, but it is also expressed in many vitamin D target tissues, albeit at lower levels (Hendrix et al., 2004). The *CYP27B1* gene harbors a CpG island. However, recent sequence updates (Ensembl 74, November 2013) shift the CpG island from the *CYP27B1* promoter region into the gene coding sequence (Flicek et al., 2014). This explains the differences between the location of the CpG island depicted in **Figure 2** and the location of the CpG island described in literature. For simplicity, statements on nVDRES and CpG island location below refer to reports in the published articles and not to **Figure 2**.

The promoter region of *CYP27B1* contains a negative VDRE (nVDRE) located at around 500 bp, consisting of two E-box like motifs (Murayama et al., 2004). This region is responsible for 1,25-D_3-dependent transrepression, which seems to be achieved through recruitment of both HDACs and DNMTs by VDR/RXR to the promoter region of *CYP27B1* (Takeyama and Kato, 2011). For further details see subsection Interactions of Vitamin D with Chromatin Modulators and Remodelers.

In cancer, expression of CYP27B1 is often downregulated. This may be explained by increased methylation of the CpG island located within *CYP27B1*. In the breast cancer cells MDA-MB-231, *CYP27B1* hypermethylation led to gene silencing, which could be reversed by treatment with deoxyC (Shi et al., 2002). In prostate cancer cell lines, combination of the DNMT1 inhibitor DAC and the HDAC inhibitor TSA resulted in increased activity of CYP27B1 (Khorchide et al., 2005). In the choriocarcinoma cell lines BeWo and JAR the promoter of *CYP27B1* was densely methylated (Novakovic et al., 2009). The *CYP27B1* promoter was hypermethylated (61%) in Non-Hodgkin's lymphoma, but not in benign follicular hyperplasia. Two out of four non-Hodgkin's lymphoma cell lines showed strong methylation of the *CYP27B1* promoter. Interestingly, all four responded to DAC-TSA treatments with upregulation of gene expression independent of the methylation status of their *CYP27B1* promoter, which may be explained by other regions prone to methylation not investigated in this study or by differences in silencing mechanisms (Shi et al., 2007). Further, the methylation level of *CYP27B1* was increased in primary lymphoma and leukemia cells also compared with normal peripheral blood lymphocytes (Lagger et al., 2003; Wjst et al., 2010).

Methylation of *CYP27B1* in diseases might cause reduced local activation of 25-D_3 to 1,25-D_3, thus reducing local 1,25-D_3 levels and restricting its functions.

EPIGENETIC REGULATION OF CYP24A1

The 1,25-dihydroxyvitamin D_3 24-hydroxylase is an inner mitochondrial membrane P450 enzyme, which catalyzes both 25-D_3 and 1,25-D_3 (Kawashima et al., 1981; Pedersen et al., 1983; Sakaki et al., 2000). Its primary site of expression are the kidneys, where it plays a crucial role in regulating systemic vitamin D metabolite levels, however, expression is found in many other vitamin D target tissues.

The promoter of *CYP24A1* is spanned by a CpG island making it prone to regulation by DNA methylation. Several responsive elements are located within this area, including two VDREs, a vitamin stimulating element (VSE), and SP1 binding sites (see **Figure 2**).

In healthy kidney, skeletal muscle, whole blood, brain, skin fibroblasts, and sperm the *CYP24A1* promoter is not methylated (Novakovic et al., 2009), although the expression levels are highly variable. In peripheral blood lymphocytes methylation of *CYP24A1* was low (5%) (Wjst et al., 2010). Interestingly, in full term human placenta 56.5% of the *CYP24A1* promoter is methylated. *CYP24A1* methylation was also observed in the placenta of the marmoset and mouse, however, at a lower level.

In the choriocarcinoma cell lines JEG-3, BeWo, and JAR the promoter of *CYP24A1* was densely methylated and the methylation level correlated inversely with the low gene expression (Novakovic et al., 2009). Treatment of osteoblastic ROS cells with 1,25-D_3 did not induce CYP24A1 expression. Considering the strong methylation of the *CYP24A1* promoter region, epigenetic silencing of CYP24A1 may account for the unresponsiveness of this gene to 1,25-D_3 (Ohyama et al., 2002). In the human prostate cancer cell line PC3, methylation of the *CYP24A1*

promoter reduced reporter gene expression in a methylation-dependent manner (Luo et al., 2010). In prostate cancer cells, the methylation status of *CYP24A1* promoter inversely correlated with gene expression. Demethylating agents restored CYP24A1 expression only in cell lines where the *CYP24A1* promoter was methylated prior to treatment (Luo et al., 2010). Only DNA demethylation by DAC treatment permitted recruitment of VDR to the *CYP24A1* promoter (Luo et al., 2010). In patients, development from benign toward malignant prostate lesions was paralleled by increasing methylation levels of the *CYP24A1* promoter (Luo et al., 2010). Prostate tumor derived endothelial cells (TDEC) expressed less CYP24A1 compared with endothelial cells derived from normal cells or matrigel plugs, which may be attributed to increased *CYP24A1* promoter methylation in TDECs (Johnson et al., 2010). We have shown recently that in colon cancer cell lines DAC induced CYP24A1 expression in a cell line-specific manner, independent of the methylation level of the promoter. In these cells induction of CYP24A1 expression by DAC seems to be independent of *CYP24A1* promoter methylation (Höbaus et al., 2013a). Moreover, the methylation level of the *CYP24A1* promoter was comparably low both in colon adenocarcinomas and the adjacent mucosa, although the expression of CYP24A1 was significantly higher in the tumors (Höbaus et al., 2013b).

Taken together, the regulation of CYP24A1 by DNA methylation appears to be tissue-dependent, both in health and disease.

CONCLUSIONS

There is a strong reciprocity between the vitamin D system and epigenetic mechanisms. The vitamin D system is, on the one hand regulated by epigenetic mechanisms and, on the other hand, is involved in regulating epigenetic events. Critical vitamin D tool genes can be silenced by DNA methylation. The VDR protein interacts, directly or indirectly, with chromatin modifiers and remodelers. Liganded VDR regulates expression of several of these chromatin modifiers and remodelers, and it might even regulate DNA methylation.

Epigenetic regulation of gene expression is a fine-tuned mechanism and its deregulation can lead to pathological conditions. The impact of vitamin D in the maintenance of the normal epigenetic landscape underlines the central role of this hormone in physiology.

PERSPECTIVES

One of the most fundamental questions in the control of gene expression is the way how epigenetic marks are established, erased, and recognized. Regulating epigenetic events could be a further mechanism by which 1,25-D_3 may prevent or delay tumorigenesis and onset of chronic diseases. Therefore, we need to understand better the impact of vitamin D on the epigenome, and plan thorough and comprehensive studies to examine this interplay.

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REFERENCES

Abedin, S. A., Banwell, C. M., Colston, K. W., Carlberg, C., and Campbell, M. J. (2006). Epigenetic corruption of VDR signalling in malignancy. *Anticancer Res.* 26, 2557–2566.

Aguilera, O., Pena, C., Garcia, J. M., Larriba, M. J., Ordonez-Moran, P., Navarro, D., et al. (2007). The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. *Carcinogenesis* 28, 1877–1884. doi: 10.1093/carcin/bgm094

Andraos, C., Koorsen, G., Knight, J. C., and Bornman, L. (2011). Vitamin D receptor gene methylation is associated with ethnicity, tuberculosis, and TaqI polymorphism. *Hum. Immunol.* 72, 262–268. doi: 10.1016/j.humimm.2010.12.010

Arasaradnam, R. P., Commane, D. M., Bradburn, D., and Mathers, J. C. (2008). A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics* 3, 193–198. doi: 10.4161/epi.3.4.6508

Ballestar, E., and Esteller, M. (2005). Methyl-CpG-binding proteins in cancer: blaming the DNA methylation messenger. *Biochem. Cell Biol.* 83, 374–384. doi: 10.1139/o05-035

Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S. K., Handa, V., et al. (2007). Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* 445, 671–675. doi: 10.1038/nature05515

Baylin, S. B., and Jones, P. A. (2011). A decade of exploring the cancer epigenome - biological and translational implications. *Nat. Rev. Cancer* 11, 726–734. doi: 10.1038/nrc3130

Bestor, T. H. (2005). Transposons reanimated in mice. *Cell* 122, 322–325. doi: 10.1016/j.cell.2005.07.024

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* 16, 6–21. doi: 10.1101/gad.947102

Bird, A. P. (1980). DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res.* 8, 1499–1504. doi: 10.1093/nar/8.7.1499

Bjornsson, H. T., Brown, L. J., Fallin, M. D., Rongione, M. A., Bibikova, M., Wickham, E., et al. (2007). Epigenetic specificity of loss of imprinting of the IGF2 gene in Wilms tumors. *J. Natl. Cancer Inst.* 99, 1270–1273. doi: 10.1093/jnci/djm069

Bouillon, R., Carmeliet, G., Verlinden, L., Van Etten, E., Verstuyf, A., Luderer, H. F., et al. (2008). Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr. Rev.* 29, 726–776. doi: 10.1210/er.2008-0004

Bremmer, F., Thelen, P., Pottek, T., Behnes, C. L., Radzun, H. J., and Schweyer, S. (2012). Expression and function of the vitamin D receptor in malignant germ cell tumour of the testis. *Anticancer Res.* 32, 341–349.

Brock, M. V., Hooker, C. M., Ota-Machida, E., Han, Y., Guo, M., Ames, S., et al. (2008). DNA methylation markers and early recurrence in stage I lung cancer. *N. Engl. J. Med.* 358, 1118–1128. doi: 10.1056/NEJMoa0706550

Carling, T., Rastad, J., Szabó, E., Westin, G., and Akerström, G. (2000). Reduced parathyroid vitamin D receptor messenger ribonucleic acid levels in primary and secondary hyperparathyroidism. *J. Clin. Endocrinol. Metab.* 85, 2000–2003. doi: 10.1210/jc.85.5.2000

Chandel, N., Husain, M., Goel, H., Salhan, D., Lan, X., Malhotra, A., et al. (2013). VDR hypermethylation and HIV-induced T cell loss. *J. Leukoc. Biol.* 93, 623–631. doi: 10.1189/jlb.0812383

Choi, J. H., Kwon, H. J., Yoon, B. I., Kim, J. H., Han, S. U., Joo, H. J., et al. (2001). Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn. J. Cancer Res.* 92, 1300–1304. doi: 10.1111/j.1349-7006.2001.tb02153.x

Christensen, J., Agger, K., Cloos, P. A., Pasini, D., Rose, S., Sennels, L., et al. (2007). RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. *Cell* 128, 1063–1076. doi: 10.1016/j.cell.2007.02.003

Cloos, P. A., Christensen, J., Agger, K., Maiolica, A., Rappsilber, J., Antal, T., et al. (2006). The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* 442, 307–311. doi: 10.1038/nature04837

Cui, H., Cruz-Correa, M., Giardiello, F. M., Hutcheon, D. F., Kafonek, D. R., Brandenburg, S., et al. (2003). Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 299, 1753–1755. doi: 10.1126/science.1080902

Ding, J., Zhang, Z. M., Xia, Y., Liao, G. Q., Pan, Y., Liu, S., et al. (2013). LSD1-mediated epigenetic modification contributes to proliferation and metastasis of colon cancer. *Br. J. Cancer* 109, 994–1003. doi: 10.1038/bjc.2013.364

Doig, C. L., Singh, P. K., Dhiman, V. K., Thorne, J. L., Battaglia, S., Sobolewski, M., et al. (2013). Recruitment of NCOR1 to VDR target genes is enhanced in prostate cancer cells and associates with altered DNA methylation patterns. *Carcinogenesis* 34, 248–256. doi: 10.1093/carcin/bgs331

Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 300, 455. doi: 10.1126/science.1083557

Esteller, M. (2007a). Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* 16, Spec No 1, R50–R59. doi: 10.1093/hmg/ddm018

Esteller, M. (2007b). Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br. J. Cancer* 96(Suppl.), R26–R30. doi: 10.1038/sj.bjc.6602918

Esteller, M. (2008). Epigenetics in cancer. *N. Engl. J. Med.* 358, 1148–1159. doi: 10.1056/NEJMra072067

Esteller, M., Catasus, L., Matias-Guiu, X., Mutter, G. L., Prat, J., Baylin, S. B., et al. (1999). hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am. J. Pathol.* 155, 1767–1772. doi: 10.1016/S0002-9440(10)65492-2

Feinberg, A. P., Cui, H., and Ohlsson, R. (2002). DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin. Cancer Biol.* 12, 389–398. doi: 10.1016/S1044-579X(02)00059-7

Ficz, G., Branco, M. R., Seisenberger, S., Santos, F., Krueger, F., Hore, T. A., et al. (2011). Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473, 398–402. doi: 10.1038/nature10008

Flicek, P., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., et al. (2014). Ensembl 2014. *Nucleic Acids Res.* 42, D749–D755. doi: 10.1093/nar/gkt1196

Fodor, B. D., Kubicek, S., Yonezawa, M., O'Sullivan, R. J., Sengupta, R., Perez-Burgos, L., et al. (2006). Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. *Genes Dev.* 20, 1557–1562. doi: 10.1101/gad.388206

Frescas, D., Guardavaccaro, D., Bassermann, F., Koyama-Nasu, R., and Pagano, M. (2007). JHDM1B/FBXL10 is a nucleolar protein that represses transcription of ribosomal RNA genes. *Nature* 450, 309–313. doi: 10.1038/nature06255

Frescas, D., Guardavaccaro, D., Kuchay, S. M., Kato, H., Poleshko, A., Basrur, V., et al. (2008). KDM2A represses transcription of centromeric satellite repeats and maintains the heterochromatic state. *Cell Cycle* 7, 3539–3547. doi: 10.4161/cc.7.22.7062

Fu, B., Wang, H., Wang, J., Barouhas, I., Liu, W., Shuboy, A., et al. (2013). Epigenetic regulation of BMP2 by 1,25-dihydroxyvitamin D3 through DNA methylation and histone modification. *PLoS ONE* 8:e61423. doi: 10.1371/journal.pone.0061423

Garcia-Bassets, I., Kwon, Y. S., Telese, F., Prefontaine, G. G., Hutt, K. R., Cheng, C. S., et al. (2007). Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* 128, 505–518. doi: 10.1016/j.cell.2006.12.038

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* 196, 261–282. doi: 10.1016/0022-2836(87)90689-9

Gaschott, T., and Stein, J. (2003). Short-chain fatty acids and colon cancer cells: the vitamin D receptor–butyrate connection. *Recent Results Cancer Res.* 164, 247–257. doi: 10.1007/978-3-642-55580-0_18

Godman, C. A., Joshi, R., Tierney, B. R., Greenspan, E., Rasmussen, T. P., Wang, H. W., et al. (2008). HDAC3 impacts multiple oncogenic pathways in colon cancer cells with effects on Wnt and vitamin D signaling. *Cancer Biol. Ther.* 7, 1570–1580. doi: 10.4161/cbt.7.10.6561

Gogusev, J., Duchambon, P., Hory, B., Giovannini, M., Goureau, Y., Sarfati, E., et al. (1997). Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. *Kidney Int.* 51, 328–336. doi: 10.1038/ki.1997.41

Greer, E. L., and Shi, Y. (2012). Histone methylation: a dynamic mark in health, disease and inheritance. *Nat. Rev. Genet.* 13, 343–357. doi: 10.1038/nrg3173

Greger, V., Passarge, E., Höpping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* 83, 155–158. doi: 10.1007/BF00286709

Gruenbaum, Y., Stein, R., Cedar, H., and Razin, A. (1981). Methylation of CpG sequences in eukaryotic DNA. *FEBS Lett.* 124, 67–71. doi: 10.1016/0014-5793(81)80055-5

Guarente, L. (2006). Sirtuins as potential targets for metabolic syndrome. *Nature* 444, 868–874. doi: 10.1038/nature05486

Habano, W., Gamo, T., Terashima, J., Sugai, T., Otsuka, K., Wakabayashi, G., et al. (2011). Involvement of promoter methylation in the regulation of Pregnane X receptor in colon cancer cells. *BMC Cancer* 11:81. doi: 10.1186/1471-2407-11-81

Haberland, M., Montgomery, R. L., and Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* 10, 32–42. doi: 10.1038/nrg2485

Hackett, J. A., Sengupta, R., Zylicz, J. J., Murakami, K., Lee, C., Down, T. A., et al. (2013). Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. *Science* 339, 448–452. doi: 10.1126/science.1229277

Halkidou, K., Gaughan, L., Cook, S., Leung, H. Y., Neal, D. E., and Robson, C. N. (2004). Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* 59, 177–189. doi: 10.1002/pros.20022

Halsall, J. A., Osborne, J. E., Hutchinson, P. E., and Pringle, J. H. (2007). *In silico* analysis of the 5' region of the Vitamin D receptor gene: functional implications of evolutionary conservation. *J. Steroid. Biochem. Mol. Biol.* 103, 352–356. doi: 10.1016/j.jsbmb.2006.12.046

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J. C., et al. (2013). Molecular mechanisms of vitamin D action. *Calcif. Tissue Int.* 92, 77–98. doi: 10.1007/s00223-012-9619-0

Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* 7, 1395–1402.

Helin, K., and Dhanak, D. (2013). Chromatin proteins and modifications as drug targets. *Nature* 502, 480–488. doi: 10.1038/nature12751

Hendrix, I., Anderson, P., May, B., and Morris, H. (2004). Regulation of gene expression by the CYP27B1 promoter-study of a transgenic mouse model. *J. Steroid Biochem. Mol. Biol.* 89–90, 139–142. doi: 10.1016/j.jsbmb.2004.03.093

Herman, J. G., and Baylin, S. B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* 349, 2042–2054. doi: 10.1056/NEJMra023075

Höbaus, J., Fetahu, I. S. H., Khorchide, M., Manhardt, T., and Kallay, E. (2013a). Epigenetic regulation of the 1,25-dihydroxyvitamin D3 24-hydroxylase (CYP24A1) in colon cancer cells. *J. Steroid Biochem. Mol. Biol.* 136, 296–299. doi: 10.1016/j.jsbmb.2012.08.003

Höbaus, J., Hummel, D. M., Thiem, U., Fetahu, I. S., Aggarwal, A., Müllauer, L., et al. (2013b). Increased copy-number and not DNA hypomethylation causes overexpression of the candidate proto-oncogene CYP24A1 in colorectal cancer. *Int. J. Cancer* 133, 1380–1388. doi: 10.1002/ijc.28143

Hobaus, J., Thiem, U., Hummel, D. M., and Kallay, E. (2013). Role of calcium, vitamin d, and the extrarenal vitamin d hydroxylases in carcinogenesis. *Anticancer Agents Med. Chem.* 13, 20–35. doi: 10.2174/187152013804487434

Hofman-Bang, J., Gravesen, E., Olgaard, K., and Lewin, E. (2012). Epigenetic methylation of parathyroid CaR and VDR promoters in experimental secondary hyperparathyroidism. *Int. J. Nephrol.* 2012:123576. doi: 10.1155/2012/123576

Iwase, S., Lan, F., Bayliss, P., De La Torre-Ubieta, L., Huarte, M., Qi, H. H., et al. (2007). The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. *Cell* 128, 1077–1088. doi: 10.1016/j.cell.2007.02.017

Jiang, F., Li, P., Fornace, A. J., Nicosia, S. V., and Bai, W. (2003). G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. *J. Biol. Chem.* 278, 48030–48040. doi: 10.1074/jbc.M308430200

Jin, B., and Robertson, K. D. (2013). DNA methyltransferases, DNA damage repair, and cancer. *Adv. Exp. Med. Biol.* 754, 3–29. doi: 10.1007/978-1-4419-9967-2_1

Johnson, C. S., Chung, I., and Trump, D. L. (2010). Epigenetic silencing of CYP24 in the tumor microenvironment. *J. Steroid Biochem. Mol. Biol.* 121, 338–342. doi: 10.1016/j.jsbmb.2010.03.046

Jones, P. A., and Baylin, S. B. (2007). The epigenomics of cancer. *Cell* 128, 683–692. doi: 10.1016/j.cell.2007.01.029

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19, 187–191. doi: 10.1038/561

Jung-Hynes, B., Reiter, R. J., and Ahmad, N. (2010). Sirtuins, melatonin and circadian rhythms: building a bridge between aging and cancer. *J. Pineal Res.* 48, 9–19. doi: 10.1111/j.1600-079X.2009.00729.x

Kahl, P., Gullotti, L., Heukamp, L. C., Wolf, S., Friedrichs, N., Vorreuther, R., et al. (2006). Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res.* 66, 11341–11347. doi: 10.1158/0008-5472.CAN-06-1570

Karlic, H., and Varga, F. (2011). Impact of vitamin D metabolism on clinical epigenetics. *Clin. Epigenetics* 2, 55–61. doi: 10.1007/s13148-011-0021-y

Karolchik, D., Barber, G. P., Casper, J., Clawson, H., Cline, M. S., Diekhans, M., et al. (2014). The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res.* 42, D764–770. doi: 10.1093/nar/gkt1168

Kawashima, H., Torikai, S., and Kurokawa, K. (1981). Localization of 25-hydroxyvitamin D3 1 alpha-hydroxylase and 24-hydroxylase along the rat nephron. *Proc. Natl. Acad. Sci. U.S.A.* 78, 1199–1203. doi: 10.1073/pnas.78.2.1199

Khorchide, M., Lechner, D., and Cross, H. S. (2005). Epigenetic regulation of vitamin D hydroxylase expression and activity in normal and malignant human prostate cells. *J. Steroid Biochem. Mol. Biol.* 93, 167–172. doi: 10.1016/j.jsbmb.2004.12.022

Kim, G. D., Ni, J., Kelesoglu, N., Roberts, R. J., and Pradhan, S. (2002). Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. *EMBO J.* 21, 4183–4195. doi: 10.1093/emboj/cdf401

Kim, M. S., Fujiki, R., Kitagawa, H., and Kato, S. (2007a). 1alpha,25(OH)2D3-induced DNA methylation suppresses the human CYP27B1 gene. *Mol. Cell Endocrinol.* 265–266, 168–173. doi: 10.1016/j.mce.2006.12.014

Kim, M. S., Fujiki, R., Murayama, A., Kitagawa, H., Yamaoka, K., Yamamoto, Y., et al. (2007b). 1Alpha,25(OH)2D3-induced transrepression by vitamin D receptor through E-box-type elements in the human parathyroid hormone gene promoter. *Mol. Endocrinol.* 21, 334–342. doi: 10.1210/me.2006-0231

Kim, S., Li, M., Paik, H., Nephew, K., Shi, H., Kramer, R., et al. (2008). Predicting DNA methylation susceptibility using CpG flanking sequences. *Pac. Symp. Biocomput.* 13, 315–326.

Klose, R. J., Yamane, K., Bae, Y., Zhang, D., Erdjument-Bromage, H., Tempst, P., et al. (2006). The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. *Nature* 442, 312–316. doi: 10.1038/nature04853

Klose, R. J., Yan, Q., Tothova, Z., Yamane, K., Erdjument-Bromage, H., Tempst, P., et al. (2007). The retinoblastoma binding protein RBP2 is an H3K4 demethylase. *Cell* 128, 889–900. doi: 10.1016/j.cell.2007.02.013

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* 128, 693–705. doi: 10.1016/j.cell.2007.02.005

Kriaucionis, S., and Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324, 929–930. doi: 10.1126/science.1169786

Lagger, G., Doetzlhofer, A., Schuettengruber, B., Haidweger, E., Simboeck, E., Tischler, J., et al. (2003). The tumor suppressor p53 and histone deacetylase 1 are antagonistic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene. *Mol. Cell Biol.* 23, 2669–2679. doi: 10.1128/MCB.23.8.2669-2679.2003

Lee, J. W., Lee, Y. C., Na, S. Y., Jung, D. J., and Lee, S. K. (2001). Transcriptional coregulators of the nuclear receptor superfamily: coactivators and corepressors. *Cell Mol. Life Sci.* 58, 289–297. doi: 10.1007/PL00000856

Lewin, E., Garfia, B., Recio, F. L., Rodriguez, M., and Olgaard, K. (2002). Persistent downregulation of calcium-sensing receptor mRNA in rat parathyroids when severe secondary hyperparathyroidism is reversed by an isogenic kidney transplantation. *J. Am. Soc. Nephrol.* 13, 2110–2116. doi: 10.1097/01.ASN.0000024439.38838.03

Liau, J. Y., Liao, S. L., Hsiao, C. H., Lin, M. C., Chang, H. C., and Kuo, K. T. (2014). Hypermethylation of the CDKN2A gene promoter is a frequent epigenetic change in periocular sebaceous carcinoma and is associated with younger patient age. *Hum Pathol.* 45, 533–539. doi: 10.1016/j.humpath.2013.10.019

Lin, C. H., Li, B., Swanson, S., Zhang, Y., Florens, L., Washburn, M. P., et al. (2008). Heterochromatin protein 1a stimulates histone H3 lysine 36 demethylation by the Drosophila KDM4A demethylase. *Mol. Cell* 32, 696–706. doi: 10.1016/j.molcel.2008.11.008

Lin, Y. W., Ren, L. L., Xiong, H., Du, W., Yu, Y. N., Sun, T. T., et al. (2013). Role of STAT3 and vitamin D receptor in EZH2-mediated invasion of human colorectal cancer. *J. Pathol.* 230, 277–290. doi: 10.1002/path.4179

Longo, V. D., and Kennedy, B. K. (2006). Sirtuins in aging and age-related disease. *Cell* 126, 257–268. doi: 10.1016/j.cell.2006.07.002

Lopes, N., Carvalho, J., Duraes, C., Sousa, B., Gomes, M., Costa, J. L., et al. (2012). 1Alpha,25-dihydroxyvitamin D3 induces de novo E-cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation. *Anticancer Res.* 32, 249–257.

Lu, P. J., Sundquist, K., Baeckstrom, D., Poulsom, R., Hanby, A., Meier-Ewert, S., et al. (1999). A novel gene (PLU-1) containing highly conserved putative DNA/chromatin binding motifs is specifically up-regulated in breast cancer. *J. Biol. Chem.* 274, 15633–15645. doi: 10.1074/jbc.274.22.15633

Luo, W., Karpf, A. R., Deeb, K. K., Muindi, J. R., Morrison, C. D., Johnson, C. S., et al. (2010). Epigenetic regulation of vitamin D 24-hydroxylase/CYP24A1 in human prostate cancer. *Cancer Res.* 70, 5953–5962. doi: 10.1158/0008-5472.CAN-10-0617

Marik, R., Fackler, M., Gabrielson, E., Zeiger, M. A., Sukumar, S., Stearns, V., et al. (2010). DNA methylation-related vitamin D receptor insensitivity in breast cancer. *Cancer Biol. Ther.* 10, 44–53. doi: 10.4161/cbt.10.1.11994

Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001). Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* 1, 194–202. doi: 10.1038/35106079

Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454, 766–770. doi: 10.1038/nature07107

Metzger, E., Wissmann, M., Yin, N., Müller, J. M., Schneider, R., Peters, A. H., et al. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 437, 436–439. doi: 10.1038/nature04020

Meyer, M. B., Benkusky, N. A., and Pike, J. W. (2013). 1,25-Dihydroxyvitamin D3 induced histone profiles guide discovery of VDR action sites. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.09.005. [Epub ahead of print].

Miller, C. W., Morosetti, R., Campbell, M. J., Mendoza, S., and Koeffler, H. P. (1997). Integrity of the 1,25-dihydroxyvitamin D3 receptor in bone, lung, and other cancers. *Mol. Carcinog.* 19, 254–257. doi: 10.1002/(SICI)1098-2744(199708)19:4<254::AID-MC6>3.0.CO;2-C

Mosammaparast, N., and Shi, Y. (2010). Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. *Annu. Rev. Biochem.* 79, 155–179. doi: 10.1146/annurev.biochem.78.070907.103946

Mottet, D., Pirotte, S., Lamour, V., Hagedorn, M., Javerzat, S., Bikfalvi, A., et al. (2009). HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. *Oncogene* 28, 243–256. doi: 10.1038/onc.2008.371

Murayama, A., Kim, M. S., Yanagisawa, J., Takeyama, K., and Kato, S. (2004). Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. *EMBO J.* 23, 1598–1608. doi: 10.1038/sj.emboj.7600157

Nagpal, S., Na, S., and Rathnachalam, R. (2005). Noncalcemic actions of vitamin D receptor ligands. *Endocr. Rev.* 26, 662–687. doi: 10.1210/er.2004-0002

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., et al. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, 386–389. doi: 10.1038/30764

Norman, A. W., and Bouillon, R. (2010). Vitamin D nutritional policy needs a vision for the future. *Exp. Biol. Med. (Maywood)* 235, 1034–1045. doi: 10.1258/ebm.2010.010014

Novakovic, B., Sibson, M., Ng, H. K., Manuelpillai, U., Rakyan, V., Down, T., et al. (2009). Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J. Biol. Chem.* 284, 14838–14848. doi: 10.1074/jbc.M809542200

Ohyama, Y., Kusada, T., Yamasaki, T., and Ide, H. (2002). Extensive methylation of CpG island of CYP24 gene in osteoblastic ROS17/2.8 cells. *Nucleic Acids Res. Suppl.* 249–250. doi: 10.1093/nass/2.1.249

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247–257. doi: 10.1016/S0092-8674(00)81656-6

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.* 19, 219–220. doi: 10.1038/890

Padi, S. K., Zhang, Q., Rustum, Y. M., Morrison, C., and Guo, B. (2013). MicroRNA-627 mediates the epigenetic mechanisms of vitamin D to suppress proliferation of human colorectal cancer cells and growth of xenograft tumors in mice. *Gastroenterology* 145, 437–446. doi: 10.1053/j.gastro.2013.04.012

Pan, L., Matloob, A. F., Du, J., Pan, H., Dong, Z., Zhao, J., et al. (2010). Vitamin D stimulates apoptosis in gastric cancer cells in synergy with trichostatin A /sodium butyrate-induced and 5-aza-2′-deoxycytidine-induced PTEN upregulation. *FEBS J.* 277, 989–999. doi: 10.1111/j.1742-4658.2009.07542.x

Parbin, S., Kar, S., Shilpi, A., Sengupta, D., Deb, M., Rath, S. K., et al. (2014). Histone deacetylases: a saga of perturbed acetylation homeostasis in cancer. *J. Histochem. Cytochem.* 62, 11–33. doi: 10.1369/0022155413506582

Pedersen, J. I., Shobaki, H. H., Holmberg, I., Bergseth, S., and Björkhem, I. (1983). 25-Hydroxyvitamin D3-24-hydroxylase in rat kidney mitochondria. *J. Biol. Chem.* 258, 742–746.

Pedersen, M. T., and Helin, K. (2010). Histone demethylases in development and disease. *Trends Cell Biol.* 20, 662–671. doi: 10.1016/j.tcb.2010.08.011

Pendas-Franco, N., Aguilera, O., Pereira, F., Gonzalez-Sancho, J. M., and Munoz, A. (2008). Vitamin D and Wnt/beta-catenin pathway in colon cancer: role and regulation of DICKKOPF genes. *Anticancer Res.* 28, 2613–2623.

Pereira, F., Barbachano, A., Silva, J., Bonilla, F., Campbell, M. J., Munoz, A., et al. (2011). KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. *Hum. Mol. Genet.* 20, 4655–4665. doi: 10.1093/hmg/ddr399

Pereira, F., Barbachano, A., Singh, P. K., Campbell, M. J., Munoz, A., and Larriba, M. J. (2012). Vitamin D has wide regulatory effects on histone demethylase genes. *Cell Cycle* 11, 1081–1089. doi: 10.4161/cc.11.6.19508

Perillo, B., Ombra, M. N., Bertoni, A., Cuozzo, C., Sacchetti, S., Sasso, A., et al. (2008). DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. *Science* 319, 202–206. doi: 10.1126/science.1147674

Pfau, R., Tzatsos, A., Kampranis, S. C., Serebrennikova, O. B., Bear, S. E., and Tsichlis, P. N. (2008). Members of a family of JmjC domain-containing oncoproteins immortalize embryonic fibroblasts via a JmjC domain-dependent process. *Proc. Natl. Acad. Sci. U.SA.* 105, 1907–1912. doi: 10.1073/pnas.0711865105

Pike, J. W., and Meyer, M. B. (2013). Fundamentals of vitamin D hormone-regulated gene expression. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.11.004. [Epub ahead of print].

Pike, J. W., Meyer, M. B., and Bishop, K. A. (2012). Regulation of target gene expression by the vitamin D receptor - an update on mechanisms. *Rev. Endocr. Metab. Disord.* 13, 45–55. doi: 10.1007/s11154-011-9198-9

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000). VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *FASEB J.* 14, 1455–1463. doi: 10.1096/fj.14.10.1455

Rashid, S. F., Moore, J. S., Walker, E., Driver, P. M., Engel, J., Edwards, C. E., et al. (2001). Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. *Oncogene* 20, 1860–1872. doi: 10.1038/sj.onc.1204269

Rawson, J. B., Sun, Z., Dicks, E., Daftary, D., Parfrey, P. S., Green, R. C., et al. (2012). Vitamin D intake is negatively associated with promoter methylation of the Wnt antagonist gene DKK1 in a large group of colorectal cancer patients. *Nutr. Cancer* 64, 919–928. doi: 10.1080/01635581.2012.711418

Reik, W., and Lewis, A. (2005). Co-evolution of X-chromosome inactivation and imprinting in mammals. *Nat. Rev. Genet.* 6, 403–410. doi: 10.1038/nrg1602

Rhee, I., Bachman, K. E., Park, B. H., Jair, K. W., Yen, R. W., Schuebel, K. E., et al. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416, 552–556. doi: 10.1038/416552a

Rhee, I., Jair, K. W., Yen, R. W., Lengauer, C., Herman, J. G., Kinzler, K. W., et al. (2000). CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* 404, 1003–1007. doi: 10.1038/35010000

Robertson, K. D. (2005). DNA methylation and human disease. *Nat. Rev. Genet.* 6, 597–610. doi: 10.1038/nrg1655

Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A., et al. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 9, 435–443. doi: 10.1016/j.ccr.2006.04.020

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., and Dryja, T. P. (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet.* 48, 880–888.

Sakaki, T., Sawada, N., Komai, K., Shiozawa, S., Yamada, S., Yamamoto, K., et al. (2000). Dual metabolic pathway of 25-hydroxyvitamin D3 catalyzed by human CYP24. *Eur. J. Biochem.* 267, 6158–6165. doi: 10.1046/j.1432-1327.2000.01680.x

Saramäki, A., Diermeier, S., Kellner, R., Laitinen, H., Vaïsänen, S., and Carlberg, C. (2009). Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha,25-dihydroxyvitamin D3. *J. Biol. Chem.* 284, 8073–8082. doi: 10.1074/jbc.M808090200

Schulte, J. H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R., et al. (2009). Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. *Cancer Res.* 69, 2065–2071. doi: 10.1158/0008-5472.CAN-08-1735

Schwer, B., and Verdin, E. (2008). Conserved metabolic regulatory functions of sirtuins. *Cell Metab.* 7, 104–112. doi: 10.1016/j.cmet.2007.11.006

Seuter, S., Pehkonen, P., Heikkinen, S., and Carlberg, C. (2013). Dynamics of 1α,25-dihydroxyvitamin D3-dependent chromatin accessibility of early vitamin D receptor target genes. *Biochim. Biophys. Acta* 1829, 1266–1275. doi: 10.1016/j.bbagrm.2013.10.003

Shi, H., Guo, J., Duff, D. J., Rahmatpanah, F., Chitima-Matsiga, R., Al-Kuhlani, M., et al. (2007). Discovery of novel epigenetic markers in non-Hodgkin's lymphoma. *Carcinogenesis* 28, 60–70. doi: 10.1093/carcin/bgl092

Shi, H., Yan, P. S., Chen, C. M., Rahmatpanah, F., Lofton-Day, C., Caldwell, C. W., et al. (2002). Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells. *Cancer Res.* 62, 3214–3220.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., et al. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953. doi: 10.1016/j.cell.2004.12.012

Shi, Y., and Whetstine, J. R. (2007). Dynamic regulation of histone lysine methylation by demethylases. *Mol. Cell* 25, 1–14. doi: 10.1016/j.molcel.2006.12.010

Singh, P. K., Doig, C. L., Dhiman, V. K., Turner, B. M., Smiraglia, D. J., and Campbell, M. J. (2013). Epigenetic distortion to VDR transcriptional regulation in prostate cancer cells. *J. Steroid Biochem. Mol. Biol.* 136, 258–263. doi: 10.1016/j.jsbmb.2012.10.002

Sulaiman, L., Juhlin, C. C., Nilsson, I. L., Fotouhi, O., Larsson, C., and Hashemi, J. (2013). Global and gene-specific promoter methylation analysis in primary hyperparathyroidism. *Epigenetics* 8, 646–655. doi: 10.4161/epi.24823

Tahiliani, M., Mei, P., Fang, R., Leonor, T., Rutenberg, M., Shimizu, F., et al. (2007). The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature* 447, 601–605. doi: 10.1038/nature05823

Takeyama, K., and Kato, S. (2011). The vitamin D3 1alpha-hydroxylase gene and its regulation by active vitamin D3. *Biosci. Biotechnol. Biochem.* 75, 208–213. doi: 10.1271/bbb.100684

Tan, J., Lu, J., Huang, W., Dong, Z., Kong, C., Li, L., et al. (2009). Genome-wide analysis of histone H3 lysine9 modifications in human mesenchymal stem cell osteogenic differentiation. *PLoS ONE* 4:e6792. doi: 10.1371/journal.pone.0006792

Tapp, H. S., Commane, D. M., Bradburn, D. M., Arasaradnam, R., Mathers, J. C., Johnson, I. T., et al. (2013). Nutritional factors and gender influence age-related DNA methylation in the human rectal mucosa. *Aging Cell* 12, 148–155. doi: 10.1111/acel.12030

Ting, A. H., McGarvey, K. M., and Baylin, S. B. (2006). The cancer epigenome–components and functional correlates. *Genes Dev.* 20, 3215–3231. doi: 10.1101/gad.1464906

Tsai, H. C., and Baylin, S. B. (2011). Cancer epigenetics: linking basic biology to clinical medicine. *Cell Res.* 21, 502–517. doi: 10.1038/cr.2011.24

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempst, P., et al. (2006). Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 439, 811–816. doi: 10.1038/nature04433

Vanoirbeek, E., Eelen, G., Verlinden, L., Carmeliet, G., Mathieu, C., Bouillon, R., et al. (2014). PDLIM2 expression is driven by vitamin D and is involved in the pro-adhesion, and anti-migration and -invasion activity of vitamin D. *Oncogene* 33, 1904–1911. doi: 10.1038/onc.2013.123

Varshney, S., Bhadada, S. K., Sachdeva, N., Arya, A. K., Saikia, U. N., Behera, A., et al. (2013). Methylation status of the CpG islands in vitamin D and calcium-sensing receptor gene promoters does not explain the reduced gene expressions in parathyroid adenomas. *J. Clin. Endocrinol. Metab.* 98, E1631–E1635. doi: 10.1210/jc.2013-1699

Von Essen, M. R., Kongsbak, M., Schjerling, P., Olgaard, K., Odum, N., and Geisler, C. (2010). Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat. Immunol.* 11, 344–349. doi: 10.1038/ni.1851

Wang, Y., and Leung, F. C. (2004). An evaluation of new criteria for CpG islands in the human genome as gene markers. *Bioinformatics* 20, 1170–1177. doi: 10.1093/bioinformatics/bth059

Whetstine, J. R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., et al. (2006). Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* 125, 467–481. doi: 10.1016/j.cell.2006.03.028

Williams, K., Christensen, J., Pedersen, M. T., Johansen, J. V., Cloos, P. A., Rappsilber, J., et al. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473, 343–348. doi: 10.1038/nature10066

Wilson, A. J., Byun, D. S., Popova, N., Murray, L. B., L'Italien, K., Sowa, Y., et al. (2006). Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J. Biol. Chem.* 281, 13548–13558. doi: 10.1074/jbc.M510023200

Wjst, M., Heimbeck, I., Kutschke, D., and Pukelsheim, K. (2010). Epigenetic regulation of vitamin D converting enzymes. *J. Steroid Biochem. Mol. Biol.* 121, 80–83. doi: 10.1016/j.jsbmb.2010.03.056

Xiang, Y., Zhu, Z., Han, G., Ye, X., Xu, B., Peng, Z., et al. (2007). JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19226–19231. doi: 10.1073/pnas.0700735104

Yamaguchi, S., Hong, K., Liu, R., Shen, L., Inoue, A., Diep, D., et al. (2012). Tet1 controls meiosis by regulating meiotic gene expression. *Nature* 492, 443–447. doi: 10.1038/nature11709

Yamane, K., Tateishi, K., Klose, R. J., Fang, J., Fabrizio, L. A., Erdjument-Bromage, H., et al. (2007). PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. *Mol. Cell* 25, 801–812. doi: 10.1016/j.molcel.2007.03.001

Yamshchikov, A. V., Desai, N. S., Blumberg, H. M., Ziegler, T. R., and Tangpricha, V. (2009). Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocr. Pract.* 15, 438–449. doi: 10.4158/EP09101.ORR

Yoneda, T., Aya, S., and Sakuda, M. (1984). Sodium butyrate (SB) augments the effects of 1,25 dihydroxyvitamin D3 (1,25(OH)2D3) on neoplastic and osteoblastic phenotype in clonal rat osteosarcoma cells. *Biochem. Biophys. Res. Commun.* 121, 796–801. doi: 10.1016/0006-291X(84)90748-4

Zhang, X., Nicosia, S. V., and Bai, W. (2006). Vitamin D receptor is a novel drug target for ovarian cancer treatment. *Curr. Cancer Drug. Targets* 6, 229–244. doi: 10.2174/156800906776842939

Zhang, Z., Yamashita, H., Toyama, T., Sugiura, H., Ando, Y., Mita, K., et al. (2005). Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast*. *Breast Cancer Res. Treat.* 94, 11–16. doi: 10.1007/s10549-005-6001-1

Zhu, H., Wang, X., Shi, H., Su, S., Harshfield, G. A., Gutin, B., et al. (2013). A genome-wide methylation study of severe vitamin D deficiency in African American adolescents. *J. Pediatr.* 162, 1004–1009.e1001. doi: 10.1016/j.jpeds.2012.10.059

Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K. P., and Göttlicher, M. (2004). Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer Cell* 5, 455–463. doi: 10.1016/S1535-6108(04)00114-X

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Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling

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P53 and its family members have been implicated in the direct regulation of the vitamin D receptor (VDR). Vitamin D- and p53-signaling pathways have a significant impact on spontaneous or carcinogen-induced malignant transformation of cells, with VDR and p53 representing important tumor suppressors. VDR and the p53/p63/p73 proteins all function typically as receptors or sensors that turn into transcriptional regulators upon stimulus, with the main difference being that the nuclear VDR is activated as a transcription factor after binding its naturally occurring ligand 1,25-dihydroxyvitamin D with high affinity while the p53 family of transcription factors, mostly in the nucleoplasm, responds to a large number of alterations in cell homeostasis commonly referred to as stress. An increasing body of evidence now convincingly demonstrates a cross-talk between vitamin D- and p53-signaling that occurs at different levels, has genome-wide implications and that should be of high importance for many malignancies, including non-melanoma skin cancer. One interaction involves the ability of p53 to increase skin pigmentation via POMC derivatives including alpha-MSH and ACTH. Pigmentation protects the skin against UV-induced DNA damage and skin carcinogenesis, yet on the other hand reduces cutaneous synthesis of vitamin D. A second level of interaction may be through the ability of 1,25-dihydroxyvitamin D to increase the survival of skin cells after UV irradiation. UV irradiation-surviving cells show significant reductions in thymine dimers in the presence of 1,25-dihydroxyvitamin D that are associated with increased nuclear p53 protein expression, and significantly reduced NO products. A third level of interaction is documented by the ability of vitamin D compounds to regulate the expression of the *murine double minute 2* (*MDM2*) gene in dependence of the presence of wild-type p53. MDM2 has a well-established role as a key negative regulator of p53 activity. Finally, p53 and family members have been implicated in the direct regulation of VDR. This overview summarizes some of the implications of the cross-talk between vitamin D- and p53-signaling for carcinogenesis in the skin and other tissues.

Keywords: vitamin D, vitamin D receptor, p53, MDM2, cancer

SKIN, VDR AND THE VITAMIN D ENDOCRINE SYSTEM/REGULATORY NETWORK: AN INTRODUCTION

The skin is the largest organ of the human body, consisting of several compartments that are named epidermis, dermis and subcutis. The epidermis contains a basal layer (stratum basale), that is composed of self-renewing cells (keratinocytes) with limited proliferative capacity (transient amplifying cells), of stem cells with high proliferative capacity that need to be preserved, and of outwardly migrating layers (stratum spinosum, stratum granulosum and stratum corneum) of mostly resting keratinocytes at different stages of differentiation. The skin is one of the key tissues of the human body's vitamin D regulatory network (VDRN) (Lehmann et al., 2004; Holick, 2007; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). First, vitamin D is synthesized in the skin (**Figure 1**) by the action of solar or artificial ultraviolet B (UVB) radiation (under most living conditions, only a small amount of vitamin D is taken up by the diet) (Lehmann et al., 2004; Holick, 2007). Second, the skin represents an important target tissue for 1,25-dihydroxyvitamin D, the biologically active natural vitamin D metabolite, that is formed from vitamin D by consecutive hydroxylations at position 25 in the liver (mediated by CYP2R1 and by CYP27A1, resulting in 25-hydroxyvitamin D) and at position 1 in the kidneys and in many other tissues (mediated by CYP27B1) (Lehmann et al., 2004; Holick, 2007; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). 1,25-Dihydroxyvitamin D represents a potent seco-steroid hormone that regulates, via various independent mechanisms growth, many non-malignant and malignant cell types, including human keratinocytes (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). It exerts its effects through the binding with high affinity to a corresponding receptor (VDR) that is located intranuclear in target tissues (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath,

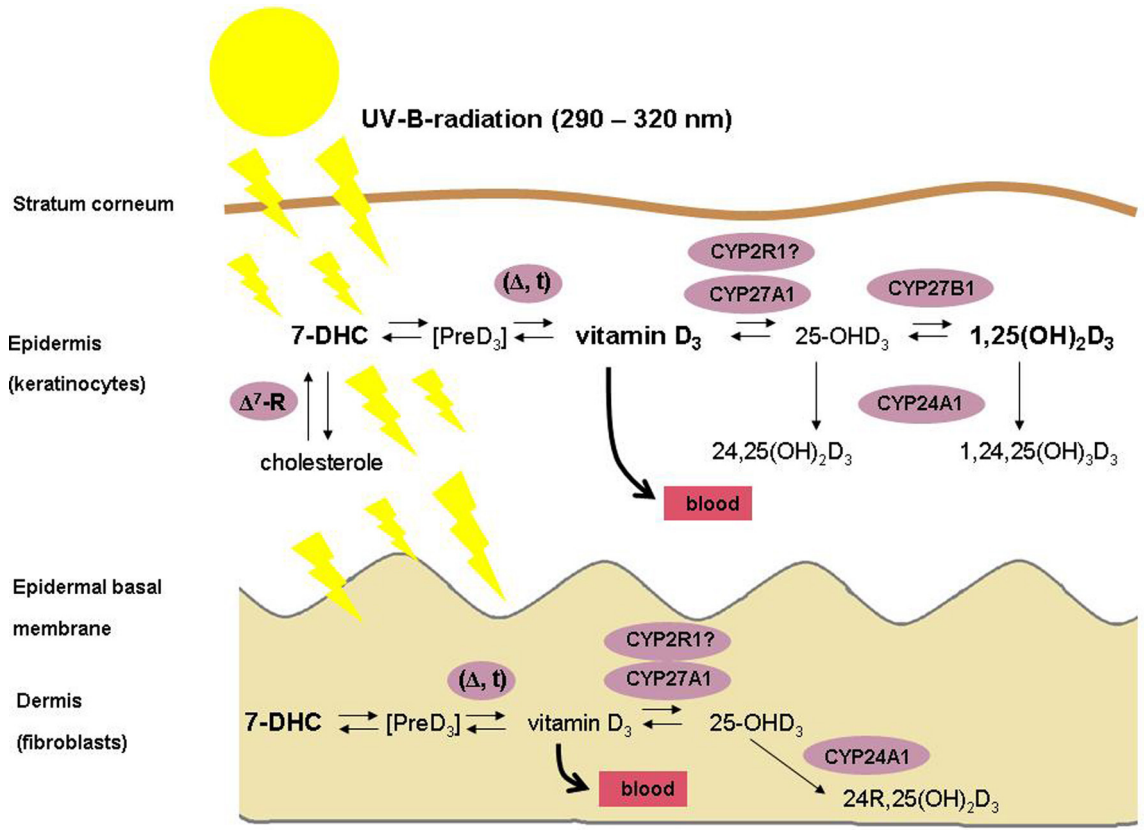

FIGURE 1 | Schematic illustration of the vitamin D metabolism in human skin. Please note that, in contrast to fibroblasts, keratinocytes possess the enzymatic machinery for the complete synthesis of 1,25-dihydroxyvitamin D from 7-dehydrocholesterole (7-DHC).

2012; Mason and Reichrath, 2013). VDR is a member of a superfamily named trans-acting transcriptional regulatory factors, that also contains the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs), as well as the thyroid and steroid hormone receptors (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). The farnesoid-X receptor (FXR) that controls bile acid metabolism and the pregnane-X receptor (PXR) which regulates xenobiotic detoxification are evolutionarily most closely related to the VDR (Haussler et al., 2012). Binding of its ligand 1,25-dihydroxyvitamin D induces conformational changes of the VDR that lead to heterodimerization with RXR and to zinc finger-mediated binding to vitamin D response elements (VDREs) that are located in regulatory regions of target genes (Haussler et al., 2012). As a result, vitamin D activity in a particular cell largely depends upon sufficient expression of VDR and RXR proteins, the autocrine/paracrine production or the endocrine delivery of adequate amounts of the 1,25-dihydroxyvitamin D ligand, and of cell-specific programming of gene transcription to regulate expression of distinctive genes that encode proteins that finally exert the vitamin D effect (Haussler et al., 2012). cDNA microarray analyses of mRNAs and other investigations suggest that as many as 500–1000 coding genes may be regulated by the VDR, which may contact up to ∼8000 loci in the human genome (Haussler et al., 2012). 1,25-Dihydroxyvitamin D-mediated transcriptional regulation of many genes involved in cellular growth and differentiation has been demonstrated, including the genes for ß$_3$-integrin, fibronectin, and cell cycle regulatory proteins such as p21/WAF-1 (CDKN1A) (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012). Like most other skin cells, keratinocytes express VDR (Lehmann et al., 2004; Holick, 2007); in these cells, 1,25-dihydroxyvitamin D, blocks proliferation and promotes differentiation *in vitro* (Lehmann et al., 2004; Holick, 2007; Haussler et al., 2012). Interestingly, it has been reported that the combination of 1,25-dihydroxyvitamin D and the retinoic acid metabolite isotretinoin is efficient in the therapy of precancerous skin lesions and of non-melanoma skin cancer (cutaneous squamous and basal cell carcinomas) (Tang et al., 2012a,b; Mason and Reichrath, 2013). Moreover, it has been demonstrated that VDR ablation promotes chemically induced skin carcinogenesis (Tang et al., 2012a,b; Mason and Reichrath, 2013).

VDR-signaling comprises much more than just ligand/receptor triggering of gene expression. Distinct and fine-tuned responses indicate a complex regulation of this signaling pathway. Moreover, chemical and other modifications of the VDR signaling pathway govern such important parameters as intracellular trafficking, duration of interaction between the receptor and

cofactors, the receptor and ligand, as well as turnover and stability of other relevant proteins (Haussler et al., 2012). Not least, regulation of VDR target genes is controlled by stability and turnover of relevant microRNAs and RNAs (Haussler et al., 2012).

Depending on cell type and context, both VDR- and p53-signaling regulate many cellular functions that are of relevance for cancer development, including proliferation, differentiation, apoptosis and cell survival (Murray-Zmijewski et al., 2006; Holick, 2007; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009; Haussler et al., 2012; Mason and Reichrath, 2013). Consequently, vitamin D- and p53-signaling pathways have a significant impact on spontaneous or carcinogen-induced malignant transformation of cells, with vitamin D receptor (VDR) and p53 representing important tumor suppressors (Murray-Zmijewski et al., 2006; Holick, 2007; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009; Haussler et al., 2012; Mason and Reichrath, 2013). Mutations in genes encoding for proteins of the p53 pathway represent a hallmark of many if not all types of cancer (Vousden and Lane, 2007; Vousden and Prives, 2009). Low serum 25(OH)D concentrations and distinct polymorphisms (SNPs) in the *VDR* gene and other vitamin D-related genes, on the other hand, are associated with an increased incidence and an unfavorable outcome of various malignancies (Mason and Reichrath, 2013). The VDR and the p53 family all function typically as activatable transcriptional regulators, with the main difference being that VDR is activated after binding its naturally occurring ligand 1,25-dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol) with high affinity (Haussler et al., 2012) while p53, mostly in the nucleoplasm, responds to a large and still growing number of alterations in cell homeostasis (Murray-Zmijewski et al., 2006; McKeon and Melino, 2007; Vousden and Lane, 2007; Vousden and Prives, 2009). In any event is the result of such activation—manifested by conformational changes and heterodimerization with retinoid X receptor (RXR) of VDR and by chemical modifications and oligomerization of the p53 family—the direct contact with regulatory DNA. In both pathways the cell type- and context-dependent recruitment of nuclear co-regulators entails the stimulation or repression of a very large number, typically hundreds, of genes (Lin et al., 2005; Holick, 2007; Perez and Pietenpol, 2007; Sbisa et al., 2007; Riley et al., 2008; Haussler et al., 2012). Several of these code themselves for transcriptional regulators, adding a further level of complexity to the networks. It is obvious that transcription factor pathways may cross-talk, for instance, through the sharing of target genes or co-regulators, and through the engagement in interdependent regulatory loops. Indeed, all of these mechanisms, plus several others, seem to have been realized in the cross-talk of VDR and the p53 family (**Table 1**).

Intriguingly, both pathways are critically involved in cellular processes that are important for carcinogenesis such as cell differentiation/proliferation, in the regulation of stem cell maintenance, and in cell homeostasis. While VDR controls proliferation/differentiation of many cell types (Holick, 2007; Haussler et al., 2012), some members and isoforms of the p53 family, and in particular p53 itself, reduce the stem cell potential and stimulate differentiation (Lin et al., 2005). Interestingly, on the side of the p53 family, all three members (p53/p63/p73) can be expressed as truncated isoforms capable of counteracting their siblings' transactivating effects (Murray-Zmijewski et al., 2006). Not too surprising, VDR and p53 have been linked to many malignancies, including non-melanoma skin cancer (Mason and Reichrath, 2013). The present review aims at providing an overview on this interesting signaling network, with a focus on non-melanoma skin cancer. Future genome-wide analyses of the target genes will shed further light on the interaction of these pleiotropic regulators. Before the cross-talk is discussed, the p53 pathway shall be briefly outlined.

THE p53 FAMILY OF TRANSCRIPTIONAL REGULATORS

p53, p63, and p73 (the p53 family hereafter) are homotetrameric transcriptional regulators that bind to very closely related DNA motifs, consisting of two consecutive 10-mers (half-sites),

Table 1 | Overview of the cross talk between vitamin D- and p53 signaling.

Crooss talk/interaction	Mechanism	References
p53 modulates cutaneous vitamin D synthesis	p53 upregulates skin pigmentation via POMC derivatives including alpha-MSH and ACTH.	Rev. in Yamaguchi and Hearing, 2009 (77)
p53 regulates VDR expression	p53 and its family members have been implicated in the direct regulation of the VDR.	Maruyama et al., 2006 (97)
	p53 protein binds to highly conserved intron-sequences of the VDR gene.	Kommagani et al., 2007 (96)
1,25-D increases survival of UV-irradiated skin cells	Significant reductions in thymine dimers in the presence of 1,25-D in UV-irradiated, surviving cells that are associated with increased nuclear p53 protein expression.	Gupta et al., 2007 (78)
1,25-D regulates MDM2 expression	Dependent on presence of wild type p53, 1,25-D regulates expression of the MDM2 gene.	Chen et al., 2013 (79)
	Interaction between VDRE and p53Res in the P2 promoter region of the MDM2 gene.	

1,25-D, 1,25-dihydroxyvitamin D; MDM2, murine double minute 2.

preferentially spaced by no more than zero to 2 base pairs, with the consensus r,r,r,C,A/T,T,G,y,c,y (p53); r,r,r,C,G,T,G,y,y,y; t/a,a/t,a,C,A/T,T,G,t,t/a,t or r,r,r,C,A/G,T/A,G,y,y,y (p63), and a/c/g,g/a,g,C,A,T,G,c/t,c,c/t (p73; r = purines; y = pyrimidines) (Osada et al., 2005; Riley et al., 2008; Brandt et al., 2009; Roemer, 2012). They share a large number of target sequences, as expected given the high degree of homology within the DNA binding domains, among the consensus sequence motifs, and the degeneracy of the individual binding sites (Roemer, 2012). It is therefore perhaps no surprise that the regulation of a defined sequence by any of these transcription factors is controlled at several levels including posttranslational modifications and protein/protein interactions. Many of these are specific for each family paralog. Moreover, the binding of p53, p63, and p73 to DNA is affected by additional parameters such as the number of the half-sites, their orientation, their position relative to the target gene, and their overlap with binding sites for other transcription factors. Finally, differential recruitment of co-activator/co-repressor complexes to promoters has been documented. These may be coded, for example, by specific spacings between the 10-mers of the DNA binding motifs (Riley et al., 2008). Epigenetic CpG methylation does not seem to affect the binding to DNA significantly (Brandt et al., 2009; Roemer, 2012).

The p53 family proteins display a modular organization that is quite different from that of the VDR (see above). Typically, an N-terminal transactivation domain (TD), a central DNA binding domain (DBD) and C-terminal regulatory and protein/protein interaction domain is present. The DBDs are the most highly conserved regions among the paralogs, sharing ∼60% homology (Murray-Zmijewski et al., 2006; Roemer, 2012). In addition to the full-length variants, a large number of isoforms exists, owing to transcription initiation from internal promoters, alternative splicing and the use of alternative translation initiation sites; however, in most cases the DBD is maintained. More than 10 different isoforms of p53, more than six of p63 and at least 29 of p73 are currently known (Murray-Zmijewski et al., 2006; Hollstein and Hainaut, 2010; Roemer, 2012). In most cases, their biological functions are not fully understood. Furthermore, an arsenal of posttranslational modifications that are in part interdependent has evolved. These include phosphorylations, acetylations, ubiquitinations, sumoylations, neddylations, methylations, glycosylations, and oxidation/reduction, and they control the proteins' abundance, DNA binding, level of activity as transcription factor, cross-talk with other proteins and subcellular localization (Murray-Zmijewski et al., 2006; Toledo and Wahl, 2006; Kruse and Gu, 2009). All these levels of regulation are best studied in p53 and have revealed an enormous degree of complexity (Vousden and Prives, 2009; Roemer, 2012) which may be exemplified by the chemical modification "code" that seems to regulate p53 function in a tissue-specific manner through the sequential build-up of poly-phosphorylation patterns at different sites and that may even be accompanied by other chemical changes such as acetylations (Gu and Roeder, 1997; Ashcroft et al., 2000; Wang et al., 2004; Roemer, 2012). At the level of the cell, p53 is involved in the regulation of the cell cycle (Wang and El-Deiry, 2006), cell survival and autophagy, DNA repair, respiration, oxidative stress protection, glucose metabolism, cell adhesion/motility, the cytoskeleton and endo/exosome compartments, and of angiogenesis. At the organismal level, p53 is involved in tumor suppression and maintenance of genome stability, and the control of stem cell compartments, female fertility and ageing (Riley et al., 2008; Roemer, 2012).

The complexity of the regulation of the p53 family is further highlighted by the antagonistic partnership between p53 and its central negative regulators, the E3 ubiquitin ligases murine double minute 2 (MDM2) and MDM4 (Roemer, 2012). Activation of p53 is almost always involving inhibition of MDM2/4. For example, acetylation of p53 and MDM2 overcomes the inhibitory ubiquitination of p53 by MDM2 through the blocking of MDM2 enzymatic function, the dissociation of the p53/MDM2 complex and thereby, the stimulation of p53's interaction with DNA as well as the recruitment of co-activators (Gu and Roeder, 1997; Ashcroft et al., 2000; Wang et al., 2004; Roemer, 2012). MDM4 is not functioning as a ubiquitin ligase for p53 but can inhibit p53's transcriptional activity and modulate the p53/MDM2 interaction (Toledo and Wahl, 2006; Roemer, 2012). Since p53 can transactivate the *MDM2* gene, a negative feedback loop is formed (Toledo and Wahl, 2006; Kruse and Gu, 2009; Roemer, 2012). Such a loop is also established with p63 and p73; however, MDM2 inhibits these transcription factors at promoters yet in contrast to p53 cannot ubiquitin-mark them for degradation (Murray-Zmijewski et al., 2006; Roemer, 2012).

Cell context determines the respective function of individual p53 family members. In the absence of extra stress, i.e., under physiological background stress induced, for instance, by reactive oxygen species (ROS) as a by-product of respiration, p53, p63, and p73 primarily control cell fate, differentiation and development. Intriguingly, these functions seem to be predominantly mediated by the DNA binding competent yet transactivation impaired delta-N isoforms of the proteins (ΔNp63, ΔNp73). In cells or tissue that have been challenged by further stresses, as for example by overt ROS production, radiation, hypoxia, hypo/hyperthermia, metabolite shortages and imbalances, oncogene dysregulation, and virus/bacterial/parasite infections, the p53 family members, and in particular p53 itself, seem to mainly control repair, proliferative capacity and survival. Central to these functions are the transactivation-proficient isoforms (p53, TAp63, TAp73). Since many of the damaging stresses can support cell transformation, the p53 family, and here again, mostly p53 itself, thus act as tumor suppressors by inducing cell cycle arrest, temporary or permanent senescence, apoptosis, and differentiation (Vousden and Lane, 2007; Levine and Oren, 2009; Vousden and Prives, 2009; Roemer, 2012). Conversely, lack of proper function of p53 or p73, or overproduction of dominant-negative ΔNp63, support tumor formation in animals and humans. Along the same line, tumor-inducing viruses encode proteins that target p53, and perhaps there is no tumor in which the p53 pathway itself plus all ascending/descending pathways are fully intact (Gatza et al., 2007; Vousden and Lane, 2007; Feng et al., 2008; Hu et al., 2008; Roemer, 2012).

Like p53, p63, and p73 can act as tumor suppressors, although this does not seem to be their primary functions (Murray-Zmijewski et al., 2006; Roemer, 2012). For example, p63 and p73 are not as frequently mutated in human cancers as is p53.

Rather, p63 is often overproduced in tumors (Park et al., 2000), which seems to contradict its function as a tumor suppressor, yet as mentioned above, this is often due to p63 isoforms that lack the transactivation domain but not their ability to bind to DNA and that thereby may act dominant-negatively (Candi et al., 2007; Roemer, 2012). In contrast and as expected from a tumor suppressor, transactivation competent p63 (TAp63) can sensitize cells to apoptosis in response to DNA damaging stress (Gressner et al., 2005; Roemer, 2012). Moreover, some p63± mice are tumor-prone, and the resulting tumors often display loss of the remaining wild-type allele (Flores, 2007; Roemer, 2012). Mice with a specific deficiency for TAp73 show genomic instability and a higher tumor incidence (Tomasini et al., 2008; Roemer, 2012). Furthermore, p63 and p73 seem to have p53-independent roles in DNA repair (Talos et al., 2007; Lin et al., 2009; Roemer, 2012).

p63 and p73, but not p53, are crucial for embryonic development in all organisms examined so far (Danilova et al., 2008; Roemer, 2012). Although p53-deficiency interferes with mesoderm/endoderm fate determination in the frog *Xenopus* (Wallingford et al., 1997; Roemer, 2012), this condition fails to generate significant early phenotypes in mice or humans (Choi and Donehower, 1999; Varley, 2003; Roemer, 2012). However, at a more subtle level, and since p53 can induce stem cell differentiation, lack of p53 function may cause unrestrained stem cell proliferation (Gil-Perotin et al., 2006; Dumble et al., 2007; Roemer, 2012). Other more subtle functions of p53 are in mitochondrial respiration and glucose metabolism (Matoba et al., 2006; Roemer, 2012). Overactivity of p53, by contrast, does indeed entail immediate and dramatic consequences in the development of the early mouse embryo—its apoptotic loss—and one of the most striking functions of the p53 inhibitors MDM2 and MDM4 during embryonic development is the prevention of this consequence (Marine et al., 2006; Roemer, 2012). Later in embryonal development, for example during neurogenesis, the DNA binding-proficient yet transactivation-incompetent dominant-negative isoform of p73, ΔNp73, may serve as a p53 and p63 restraining factor to inhibit apoptosis (Jacobs et al., 2004; Roemer, 2012). p53 and possibly p63, but most importantly p73, help shape the nervous system during life, perhaps primarily by controlling apoptosis (Jacobs et al., 2005; Miller and Kaplan, 2007; Roemer, 2012).

p63 function during development is critical for epithelial stem cell maintenance (Yi et al., 2008; Roemer, 2012), squamous epithelial differentiation and skin renewal (Truong et al., 2006; Koster et al., 2007; Mikkola, 2007; Roemer, 2012). The ΔNp63 isoform acts mainly through controlling the expansion of epithelial layers while TAp63 seems to support differentiation, and it functions as the guardian of the female germ line by inducing apoptosis in damaged resting oocytes (Suh et al., 2006; Roemer, 2012). p73 deficiency in mice results in neuronal and olfactory dysfunctions as well as in chronic infection and inflammation (Murray-Zmijewski et al., 2006; Roemer, 2012). Collectively, the stem cell/differentiated cell decision seem to be regulated in part by the balance between the ΔNp63/TAp63 antagonists in the skin and - in an analogous manner - by the balance between the ΔNp73/TAp73 antagonists in the developing nervous and immune systems. Thus, p73 may be to neuronal development and homeostasis what p63 is to the development and homeostasis of the skin (De Laurenzi et al., 2000; Jacobs et al., 2004; Roemer, 2012). Since the pleiotropic VDR is important for tumor suppression as well as skin development and differentiation, it is perhaps no surprise that both transcriptional regulator pathways talk to each other.

CROSS-TALK BETWEEN THE VDR AND THE p53 FAMILY IN CANCER

An increasing body of evidence points to a cross-talk between vitamin D- and p53-signaling occuring at different levels that might be of great importance for many malignancies, including non-melanoma skin cancer (**Table 1**). Both p53 and VDR act as tumor suppressors in several tissues, including skin. Much of the tumor suppressor function in the skin may be mediated through the interaction of the VDR and p53 pathways—either by mutual activation or inhibition. What is known about this interaction, in particular in non-melanoma skin cancer? DNA damage induced by solar or artificial ultraviolet (UV) radiation represents the most important environmental risk factor for carcinogenesis of cutaneous squamous cell carcinoma (SCC) (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). The predominant types of DNA damage which are directly induced by UV are promutagenic pyrimidine dimers (Wikonkal and Brash, 1999; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Thymine-thymine dimers, which represent cys-syn cyclobutane pyrimidine dimers (CPDs), are the major form of pyrimidine dimers that are identified in human skin following UV-B exposure. In contrast, other types of DNA damage, including cytosine-cytosine, thymine-cytosine bipyrimidines, and 6–4 photoproducts are less frequently detected (Douki et al., 2000; Cooke et al., 2003; Courdavault et al., 2004a; Mouret et al., 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). CPDs are caused via disruption of the 5–6 double bonds in two adjacent pyrimidine bases, thereby inducing atypical covalent binding which connects the 2 bases by a stable ring configuration, resulting in a bipyrimidine (Ravanat et al., 2001; Pattison and Davies, 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). In general, CPDs are induced by UV-B (290–320 nm) (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013), although the production of thymine dimer by UV-A (320–400 nm) wavelengths below 330 nm has also been reported (Applegate et al., 1999; Jiang et al., 1999; Rochette et al., 2003; Courdavault et al., 2004b; Mouret et al., 2006; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). UV-radiation induces gene mutations which may result in photocarcinogenesis (Hart et al., 1977; Sutherland et al., 1985; Brash et al., 1991; Agar et al., 2004; Besaratinia et al., 2008; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Moreover, it has been shown that DNA damage initiates and promotes cellular mechanisms which block the detection and elimination of transformed cells by immune surveillance (Applegate et al., 1989; Kripke et al., 1992; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). UV radiation induces different forms of DNA lesions which are generated either photochemically and directly or indirectly by UV activation of several photoreceptors which have the capacity to modulate the cellular redox equilibrium, thereby generating reactive oxygen species (ROS) (Reichrath and Reichrath, 2012;

Mason and Reichrath, 2013). ROS induced cellular damage then leads both to oxidative DNA damage, and to lipid peroxidation (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). Additionally, UV-induced increased levels of nitric oxide synthase (Deliconstantinos et al., 1995; Bruch-Gerharz et al., 1998; Cals-Grierson and Ormerod, 2004; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013) cause excess levels of nitric oxide (NO) (Paunel et al., 2005; Mowbray et al., 2009; Reichrath and Reichrath, 2012; Mason and Reichrath, 2013). It has been shown that these pathophysiologically increased concentrations of NO and ROS combine to generate genotoxic NO products, including peroxynitrite, which modify the bases and the sugar-phosphate scaffold of DNA via nitrosative and oxidative damage (Reichrath and Reichrath, 2012; Mason and Reichrath, 2013) Both UV- and ROS-induced damages activate p53.

UV-irradiation induces p53 to stimulate skin pigmentation via POMC derivatives including alpha-MSH and ACTH (Yamaguchi and Hearing, 2009), thereby protecting the skin against further UV-induced DNA damage and skin carcinogenesis. However, this reduces cutaneous synthesis of vitamin D. This may be important because, on a second level, 1,25-dihydroxyvitamin D can increase the survival of UV-irradiated keratinocytes and prevent further accumulation of DNA damage in these surviving skin cells (Gupta et al., 2007). Following UVR, the survival of 1,25-dihydroxyvitamin D-treated skin cells was significantly higher as compared to vehicle-treated cells ($P < 0.01$) (Gupta et al., 2007). In that study, UVR-surviving and 1,25-dihydroxyvitamin D-treated keratinocytes showed significantly reduced levels of thymine dimers (TDs) as compared to vehicle-treated cells ($P < 0.001$) (Gupta et al., 2007). Following UVR, nuclear p53 protein levels were elevated and, notably, became elevated to significantly higher levels in the presence of 1,25-dihydroxyvitamin D ($P < 0.01$). In contrast, NO derivatives were significantly decreased in 1,25-dihydroxyvitamin D-treated keratinocytes ($P < 0.05$) (Gupta et al., 2007). Both the elevated levels of nuclear p53 protein and the decreased production of nitric oxide products were suggested to be responsible at least in part for the decrease in TDs seen with 1,25-dihydroxyvitamin D-treatment after UVR (Gupta et al., 2007). In addition, a reduction in the number of TDs ($P < 0.05$) and in sunburn cells ($P < 0.01$) were demonstrated in skin sections from Skh:hr1 mice that had been treated with 1,25-dihydroxyvitamin D, at 24 h after UVR (Gupta et al., 2007). It was concluded that the vitamin D system in skin, in combination with p53, may represent an intrinsic mechanism that protects against UV damage (Gupta et al., 2007).

As a further molecular level of interaction it has been demonstrated that vitamin D compounds can regulate the expression of the MDM2 gene in dependence of p53 (Chen et al., 2013). As outlined above, *MDM2* represents a p53-inducible gene that encodes an E3 ubiquitin ligase mainly responsible for the degradation of p53 by the 26S proteasome (Roemer, 2012). A major function of MDM2 is its role as a key negative feed-back regulator of p53 activity (Bond et al., 2004), p53 activates *MDM2* expression via binding to corresponding p53 response elements (p53REs) in the P2 promoter of the *MDM2* gene. The increase of MDM2 protein then leads to its binding to p53 (primarily at the N-terminal 1–52 residues), which causes degradation of p53 or inhibition of p53 activity as a transcription factor (Chen et al., 1993). Vitamin D may thus prevent a lasting and overt p53 response in the face of damage and may thereby protect reparable cells from p53-induced apoptosis. However, MDM2 also exerts many p53-independent functions, and interacts with a broad variety of other proteins (including insulin like growth factor receptor, androgen receptor, estrogen receptor, Numb, RB, p300, etc.) that are of importance for various cellular functions including proliferation/differentiation, cell fate determination, and signaling (Ries et al., 2000; Ganguli and Wasylyk, 2003; Steinman et al., 2004; Zhang and Zhang, 2005; Lengner et al., 2006; Araki et al., 2010). VDR may activate the *MDM2* gene directly, through a VDR-response element in the promoter P2 of the *MDM2* gene (Barak et al., 1994; Zauberman et al., 1995; Roemer, 2012; Chen et al., 2013) However, even with this direct binding to *MDM2* sequences, p53 seems to be required for the induction of *MDM2* expression by VDR (Chen et al., 2013). Perhaps this is reflecting a cross-talk between the VDR and p53 bound to DNA since the p53 response element in the *MDM2* gene is also located in promoter P2.

VDR and p53 family members act first and foremost as transcription factors, and accordingly, much of the highly complex cross-regulation between them seems to happen at this level. For example, members of the p53 family including ΔNp63 can modulate VDR signaling through competitive binding to various VDR target genes including *p21Waf1/Cip (CDKN1A)*. Multiple VDREs have recently been identified in the promoter region of the *CDKN1A* gene, which is a transcriptional target of p53 and encodes a powerful blocker of the cell cycle in G1 and G2 phases (Saramaki et al., 2006). Notably, like with the *MDM2* gene, the VDR and p53 binding sites are in close proximity in the *CDKN1A* promoter (Saramaki et al., 2006). A much more detailed and unbiased (pathway-independent) genome-wide analysis of the VDR:p53 family interactions bound to DNA is in need. To this end, it shall be useful to employ chromatin immunoprecipitations (ChIPs) with either ChIPping with p53-antibodies first and re-ChIPping with VDR antibodies, or *vice versa*. Also, knock-in studies in which VDR response elements or p53 response elements in close proximity are deleted, should provide a deeper insight into the cooperativity or antagonism between these important tumor-suppressing transcription factors.

In the skin, p53/p63 play an important regulatory role in the maintenance of the stem cells as well as in the establishment of the differentiation gradient. In the undifferentiated proliferating basal layer of the skin, the dominant negatively acting, because DNA binding but transactivation impaired, ΔNp63 rules. Most effects exerted by the transactivation-competent p53 family members are inhibited by it (Yang et al., 1998; Lee and Kimelman, 2002; Roemer, 2012) In addition, ΔNp63 may inhibit differentiation by the blunting of VDR signaling through binding to various VDR target genes including *CDKN1A* (Pellegrini et al., 2001; Westfall et al., 2003; Roemer, 2012) TAp63 that is minor to ΔNp63 in this proliferating compartment of the skin, may become more dominant as ΔNp63 levels decrease in the course of differentiation (Nylander et al., 2002; Roemer, 2012).

Finally, p53 family members may regulate VDR directly (Maruyama et al., 2006; Kommagani et al., 2007). In a

comparative genomics investigation in the human and mouse genome designed to locate conserved p53 binding sites, the VDR and 31 other genes were newly described as putative p53 targets. Reverse transcription-PCR and real-time PCR confirmed the responsiveness of these genes to p53 in human cancer cell lines (Maruyama et al., 2006). It was shown that VDR is upregulated by p53 and some other members of the p53 family. For example, an isoform of p63 (p63 gamma) can specifically upregulate VDR by directly associating with the VDR promoter *in vivo* (Kommagani et al., 2007). Moreover, ChIP analysis demonstrated that wild-type p53 protein binds to a conserved intronic site of the VDR gene (Maruyama et al., 2006). Conversely, transfection of VDR into cells resulted in upregulation of several p53 target genes and in growth suppression of colorectal cancer cells. In addition and as discussed above, p53 stimulated several VDR target genes in a 1,25-dihydroxyvitamin D-dependent manner, that is, in cooperation with VDR (Maruyama et al., 2006). Future, whole transcriptome-including studies will identify new transcripts that are initiated by VDR and p53 in concert.

An increasing body of evidence highlights the relevance of the cross-talk between VDR- and p53-signaling under various physiological and pathophysiological conditions. One study identified the VDRE as overrepresented in promoter sequences bound by mutated p53 (mutp53), and showed that mutp53 can interact functionally and physically with VDR (Stambolsky et al., 2010). In that investigation, mutp53 was recruited to VDR target genes and modulated their expression (increasing transactivation or relieving repression) (Stambolsky et al., 2010). Moreover, mutp53 promoted the nuclear accumulation of VDR and converted 1,25-dihydroxyvitamin D into an anti-apoptotic agent (Stambolsky et al., 2010).

Several investigations analyzed the cross-talk between VDR- and p53-signaling in bone. It was demonstrated that hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D act together to induce osteogenic differentiation of human bone marrow stem cells (hMSC) potentially through elevating p53 (Chen et al., 2012). The authors of this study hypothesized that the combination of HGF and 1,25-dihydroxyvitamin D can promote hMSC differentiation by up-regulation of 1,25-dihydroxyvitamin D and/or VDR expression to booster cell response(s) to 1,25-dihydroxyvitamin D. In line with this hypothesis, it was shown that HGF up-regulated gene expression of VDR and p63 and that p63 gene knockdown by siRNA eliminated the effects of HGF on VDR gene expression (Chen et al., 2012). Moreover, recent findings suggest that the cross-talk of VDR and p53 may directly target the human osteocalcin gene and positively affect osteocalcin gene expression. It was reported that osteocalcin promoter activity can be up-regulated both by exogenous and endogenous p53 and downregulated by p53-specific siRNA (Chen et al., 2011). It was shown that p53 binds to the human osteocalcin promoter *in vitro* and a p53 response element within the osteocalcin promoter region was identified (Chen et al., 2011). In this investigation, an additive effect of p53 and VDR on the regulation of osteocalcin promoter activity was observed. Another study demonstrated that p73 acts as an upstream regulator of 1,25-dihydroxyvitamin D-induced osteoblastic differentiation (Kommagani et al., 2010). In that investigation, silencing p73 significantly decreased 1,25-dihydroxyvitamin D-mediated osteoblastic differentiation; although p73 induced by DNA-damage increased 1,25-dihydroxyvitamin D-mediated differentiation of osteosarcoma cells (Kommagani et al., 2010).

CONCLUSIONS AND PERSPECTIVES

VDR and the members of the p53 family are activatable transcriptional regulators that are at the hub of a common molecular network to control cell homeostasis, proliferation, differentiation and survival, and that way, act as classical tumor suppressors. Malfunction of either entails elevated susceptibility to transformation. A tissue archetypical of this interaction is the skin. Here, VDR as well as p53 and p63 control differentiation and the maintenance of the stem cell compartment. Accordingly, damage to skin cells such as induced by UV irradiation, or transformation of skin cells, typically come with characteristic responses of these proteins in the form of specific gene expression profiles to control differentiation, proliferation and survival. Since both classes of nuclear transcription factors act primarily through the regulation of genes, it is thus no great surprise to find functional interaction at several levels. Future, ChIP- and transcriptome analysis-based genome-wide studies of the DNA sequences that are targeted individually or by both factors together should provide us with new insights into this fascinating network.

REFERENCES

Agar, N. S., Halliday, G. M., Barnetson, R. S., Ananthaswamy, H. N., Wheeler, M., and Jones, A. M. (2004). The basal layer in human squamous tumours harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4954–4959. doi: 10.1073/pnas.0401141101

Applegate, L. A., Ley, R. D., Alcalay, J., and Kripke, M. L. (1989). Identification of the molecular target for the suppression of contact hypersensitivity by ultraviolet radiation. *J. Exp. Med.* 170, 1117–1131. doi: 10.1084/jem.170.4.1117

Applegate, L. A., Scaletta, C., Panizzon, R., Niggli, H., and Frenk, E. (1999). *In vivo* induction of pyrimidine dimers in human skin by UVA radiation: initiation of cell damage and/or intercellular communication? *Int. J. Mol. Med.* 3, 467–472. doi: 10.3892/ijmm.3.5.467

Araki, S., Eitel, J. A., Batuello, C. N., Bijangi-Vishehsaraei, K., Xie, X. J., Danielpour, D., et al. (2010). TGF-beta1-induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. *J. Clin. Invest.* 120, 290–302. doi: 10.1172/JCI39194

Ashcroft, M., Taya, Y., and Vousden, K. H. (2000). Stress signals utilize multiple pathways to stabilize p53. *Mol. Cell. Biol.* 20, 3224–3233. doi: 10.1128/MCB.20.9.3224-3233.2000

Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994). Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.* 8, 1739–1749. doi: 10.1101/gad.8.15.1739

Besaratinia, A., Kim, S. I., and Pfeifer, G. P. (2008). Rapid repair of UVA-induced oxidized purines and persistence of UVB-induced dipyrimidine lesions determine the mutagenicity of sunlight in mouse cells. *FASEB J.* 22, 2379–2392. doi: 10.1096/fj.07-105437

Bond, G. L., Hu, W., Bond, E. E., Robins, H., Lutzker, S. G., Arva, N. C., et al. (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumour suppressor pathway and accelerates tumour formation in humans. *Cell* 119, 591–602. doi: 10.1016/j.cell.2004.11.022

Brandt, T., Petrovich, M., Joerger, A. C., and Veprintsev, D. B. (2009). Conservation of DNA-binding specificity and oligomerisation properties within the p53 family. *BMC Genomics* 10:628. doi: 10.1186/1471-2164-10-628

Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., et al. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 88, 10124–10128. doi: 10.1073/pnas.88.22.10124

Bruch-Gerharz, D., Ruzicka, T., and Kolb-Bachofen, V. (1998). Nitric oxide in human skin: current status and future prospects. *J. Invest. Dermatol.* 110, 1–7. doi: 10.1046/j.1523-1747.1998.00084.x

Cals-Grierson, M. M., and Ormerod, A. D. (2004). Nitric oxide function in the skin. *Nitric Oxide* 10, 179–193. doi: 10.1016/j.niox.2004.04.005

Candi, E., Dinsdale, D., Rufini, A., Salomoni, P., Knight, R. A., Mueller, M., et al. (2007). TAp63 and DeltaNp63 in cancer and epidermal development. *Cell Cycle* 6, 274–285. doi: 10.4161/cc.6.3.3797

Chen, H., Hays, E., Liboon, J., Neely, C., Kolman, K., and Chandar, N. (2011). Osteocalcin gene expression is regulated by wild-type p53. *Calcif. Tissue Int.* 89, 411–418. doi: 10.1007/s00223-011-9533-x

Chen, H., Reed, G., Guardia, J., Lakhan, S., Couture, O., Hays, E., et al. (2013). Vitamin D directly regulates Mdm2 gene expression in osteoblasts. *Biochem. Biophys. Res. Commun.* 430, 370–374. doi: 10.1016/j.bbrc.2012.11.003

Chen, J., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* 13, 4107–4114.

Chen, K., Aenlle, K. K., Curtis, K. M., Roos, B. A., and Howard, G. A. (2012). Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. *Bone* 51, 69–77. doi: 10.1016/j.bone.2012.04.002

Choi, J., and Donehower, L. A. (1999). p53 in embryonic development: maintaining a fine balance. *Cell. Mol. Life Sci.* 55, 38–47. doi: 10.1007/s000180050268

Cooke, M. S., Podmore, I. D., Mistry, N., Evans, M. D., Herbert, K. E., Griffiths, H. R. et al. (2003). Immunochemical detection of UV-induced DNA damage and repair. *J Immunol Methods.* 280, 125–133. doi: 10.1016/S0022-1759(03)00269-2

Courdavault, S., Baudouin, C., Charveron, M., Favier, A., Cadet, J., and Douki, T. (2004a). Larger yield of cyclobutane dimers than 8-oxo-7,8-dihydroguanine in the DNA of UVA-irradiated human skin cells. *Mutat. Res.* 556, 135–142. doi: 10.1016/j.mrfmmm.2004.07.011

Courdavault, S., Baudouin, C., Sauvaigo, S., Mouret, S., Candéias, S., Charveron, M., et al. (2004b). Unrepaired cyclobutane pyrimidine dimers do not prevent proliferation of UV-B-irradiated cultured human fibroblasts. *Photochem. Photobiol.* 79, 145–151. doi: 10.1562/0031-8655(2004)079<0145:UCPDDN>2.0.CO;2

Danilova, N., Sakamoto, K. M., and Lin, S. (2008). p53 family in development. *Mech. Dev.* 125, 919–931. doi: 10.1016/j.mod.2008.09.003

De Laurenzi, V., Raschella, G., Barcaroli, D., Annicchiarico-Petruzzelli, M., Ranalli M, Catani, M. V., et al. (2000). Induction of neuronal differentiation by p73 in a neuroblastoma cell line. *J. Biol. Chem.* 275, 15226–15231. doi: 10.1074/jbc.275.20.15226

Deliconstantinos, G., Villiotou, V., and Stravrides, J. C. (1995). Release by ultraviolet B (u.v.B) radiation of nitric oxide (NO) from human keratinocytes: a potential role for nitric oxide in erythema production. *Br. J. Pharmacol.* 114, 1257–1265. doi: 10.1111/j.1476-5381.1995.tb13341.x

Douki, T., Court, M., Sauvaigo, S., Odin, F., and Cadet, J. (2000). Formation of the main UV-induced thymine dimeric lesions within isolated and cellular DNA as measured by high performance liquid chromatography-tandem mass spectrometry. *J. Biol. Chem.* 275, 11678–11685. doi: 10.1074/jbc.275.16.11678

Dumble, M., Moore, L., Chambers, S. M., Geiger, H., Van Zant, G., Goodell, M. A., et al. (2007). The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging. *Blood* 109, 1736–1742. doi: 10.1182/blood-2006-03-010413

Feng, Z., Hu, W., Rajagopal, G., and Levine, A. J. (2008). The tumour suppressor p53: cancer and aging. *Cell Cycle* 7, 842–847. doi: 10.4161/cc.7.7.5657

Flores, E. R. (2007). The roles of p63 in cancer. *Cell Cycle* 6, 300–304. doi: 10.4161/cc.6.3.3793

Ganguli, G., and Wasylyk, B. (2003). p53-independent functions of MDM2. *Mol. Cancer Res.* 1, 1027–1035.

Gatza, C., Moore, L., Dumble, M., and Donehower, L. A. (2007). Tumour suppressor dosage regulates stem cell dynamics during aging. *Cell Cycle* 6, 52–55. doi: 10.4161/cc.6.1.3667

Gil-Perotin, S., Marin-Husstege, M., Li, J., Soriano-Navarro, M., Zindy, F., Roussel, M. F., et al. (2006). Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumours. *J. Neurosci.* 26, 1107–1116. doi: 10.1523/JNEUROSCI.3970-05.2006

Gressner, O., Schilling, T., Lorenz, K., Schulze Schleithoff, E., Koch, A., Schulze-Bergkamen, H., et al. (2005). TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J.* 24, 2458–2471. doi: 10.1038/sj.emboj.7600708

Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90, 595–606. doi: 10.1016/S0092-8674(00)80521-8

Gupta, R., Dixon, K. M., Deo, S. S., Holliday, C. J., Slater, M., Halliday, G. M., et al. (2007). Photoprotection by 1,25 dihydroxyvitamin D3 is associated with an increase in p53 and a decrease in nitric oxide products. *J. Invest. Dermatol.* 27, 707–715. doi: 10.1038/sj.jid.5700597

Hart, R. W., Setlow, R. B., and Woodhead, A. D. (1977). Evidence that pyrimidine dimers in DNA can give rise to tumours. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5574–5578. doi: 10.1073/pnas.74.12.5574

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J. C., et al. (2012). Molecular mechanisms of vitamin D action. *Calcif. Tissue Int.* 92, 77–98. doi: 10.1007/s00223-012-9619-0

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Hollstein, M., and Hainaut, P. (2010). Massively regulated genes: the example of TP53. *J. Pathol.* 220, 164–173. doi: 10.1002/path.2637

Hu, W., Feng, Z., Atwal, G. S., and Levine, A. J. (2008). p53: a new player in reproduction. *Cell Cycle* 7, 848–852. doi: 10.4161/cc.7.7.5658

Jacobs, W. B., Govoni, G., Ho, D., Atwal, J. K., Barnabe-Heider, F., Keyes, W. M., et al. (2005). p63 is an essential proapoptotic protein during neural development. *Neuron*. 48, 743–756. doi: 10.1016/j.neuron.2005.10.027

Jacobs, W. B., Walsh, G. S., and Miller, F. D. (2004). Neuronal survival and p73/p63/p53: a family affair. *Neuroscientist* 10, 443–455. doi: 10.1177/1073858404263456

Jiang, W., Ananthaswamy, H. N., Muller, H. K., and Kripke, M. L. (1999). p53 protects against skin cancer induction by UV-B radiation. *Oncogene* 18, 4247–4253. doi: 10.1038/sj.onc.1202789

Kommagani, R., Payal, V., and Kadakia, M. P. (2007). Differential regulation of vitamin D receptor (VDR) by the p53 Family: p73-dependent induction of VDR upon DNA damage. *J. Biol. Chem.* 282, 29847–29854. doi: 10.1074/jbc.M703641200

Kommagani, R., Whitlatch, A., Leonard, M. K., and Kadakia, M. P. (2010). p73 is essential for vitamin D-mediated osteoblastic differentiation. *Cell Death Differ.* 17, 398–407. doi: 10.1038/cdd.2009.135

Koster, M. I., Dai, D., and Roop, D. R. (2007). Conflicting roles for p63 in skin development and carcinogenesis. *Cell Cycle* 6, 269–273. doi: 10.4161/cc.6.3.3792

Kripke, M. L., Cox, P. A., Alas, L. G., and Yarosh, D. B. (1992). Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7516–7520. doi: 10.1073/pnas.89.16.7516

Kruse, J. P., and Gu, W. (2009). Modes of p53 regulation. *Cell* 137, 609–622. doi: 10.1016/j.cell.2009.04.050

Lee, H., and Kimelman, D. (2002). A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev. Cell.* 2, 607–616. doi: 10.1016/S1534-5807(02)00166-1

Lehmann, B., Querings, K., and Reichrath, J. (2004). Vitamin D and skin: new aspects for dermatology. *Exp. Dermatol.* 13, 11–15. doi: 10.1111/j.1600-0625.2004.00257.x

Lengner, C. J., Steinman, H. A., Gagnon, J., Smith, T. W., Henderson, J. E., Kream, B. E., et al. (2006). Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. *J. Cell Biol.* 172, 909–921. doi: 10.1083/jcb.200508130

Levine, A. J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. *Nat. Rev. Cancer*. 9, 749–758. doi: 10.1038/nrc2723

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., et al. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat. Cell Biol.* 7, 165–171. doi: 10.1038/ncb1211

Lin, Y. L., Sengupta, S., Gurdziel, K., Bell, G. W., Jacks, T., and Flores, E. R. (2009). p63 and p73 transcriptionally regulate genes involved in DNA repair. *PLoS Genet*. 5:e1000680. doi: 10.1371/journal.pgen.1000680

Marine, J. C., Francoz, S., Maetens, M., Wahl, G., Toledo, F., and Lozano, G. (2006). Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. *Cell Death Differ.* 13, 927–934. doi: 10.1038/sj.cdd.4401912

Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., et al. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res.* 66, 4574–4583. doi: 10.1158/0008-5472.CAN-05-2562

Mason, R. S., and Reichrath, J. (2013). Sunlight vitamin D and skin cancer. *Anticancer Agents Med. Chem.* 13, 83–97. doi: 10.2174/187152013804487272

Matoba, S., Kang, J. G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., et al. (2006). p53 regulates mitochondrial respiration. *Science* 312, 1650–1653. doi: 10.1126/science.1126863

McKeon, F., and Melino, G. (2007). Fog of war: the emerging p53 family. *Cell Cycle* 6, 229–232. doi: 10.4161/cc.6.3.3876

Mikkola, M. L. (2007). p63 in skin appendage development. *Cell Cycle* 6, 285–290. doi: 10.4161/cc.6.3.3798

Miller, F. D., and Kaplan, D. R. (2007). To die or not to die: neurons and p63. *Cell Cycle* 6, 312–317. doi: 10.4161/cc.6.3.3795

Mouret, S., Baudouin, C., Charveron, M., Favier, A., Cadet, J., and Douki, T. (2006). Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13765–13770. doi: 10.1073/pnas.0604213103

Mowbray, M., McLintock, S., Weerakoon, R., Lomatschinsky, N., Jones, S., Rossi, A. G., et al. (2009). Enzyme-independent NO stores in human skin: quantification and influence of UV radiation. *J. Invest. Dermatol.* 129, 834–842. doi: 10.1038/jid.2008.296

Murray-Zmijewski, F., Lane, D. P., and Bourdon, J. C. (2006). p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ.* 13, 962–972. doi: 10.1038/sj.cdd.4401914

Nylander, K., Vojtesek, B., Nenutil, R., Lindgren, B., Roos, G., Zhanxiang, W., et al. (2002). Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J. Pathol.* 198, 417–427. doi: 10.1002/path.1231

Osada, M., Park, H. L., Nagakawa, Y., Yamashita, K., Fomenkov, A., Kim, M. S., et al. (2005). Differential recognition of response elements determines target gene specificity for p53 and p63. *Mol. Cell. Biol.* 25, 6077–6089. doi: 10.1128/MCB.25.14.6077-6089.2005

Park, B. J., Lee, S. J., Kim, J. I., Lee, S. J., Lee, C. H., Chang, S. G., et al. (2000). Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res.* 60, 3370–3374.

Pattison, D. I., and Davies, M. J. (2006). Actions of ultraviolet light on cellular structures. *EXS* 96, 131–157. doi: 10.1007/3-7643-7378-4_6

Paunel, A., Dejam, A., Thelen, S., Kirsch, M., Horstjann, M., Gharini, P., et al. (2005). UVA induces immediate and enzyme-independent nitric oxide formation in healthy human skin leading to NO-specific signalling. *J. Invest. Dermatol.* 125:A3.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., et al. (2001). p63 identifies keratinocyte stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3156–3161. doi: 10.1073/pnas.061032098

Perez, C. A., and Pietenpol, J. A. (2007). Transcriptional programs regulated by p63 in normal epithelium and tumours. *Cell Cycle* 6, 246–254. doi: 10.4161/cc.6.3.3801

Ravanat, J. L., Douki, T., and Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *J. Photochem. Photobiol. B.* 63, 88–102. doi: 10.1016/S1011-1344(01)00206-8

Reichrath, J., and Reichrath, S. (2012). Hope and challenge: the importance of ultraviolet (UV) radiation for cutaneous vitamin D synthesis and skin cancer. *Scand. J. Clin. Lab. Invest. Suppl.* 243, 112–119. doi: 10.3109/00365513.2012.682876

Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., et al. (2000). Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell* 103, 321–330. doi: 10.1016/S0092-8674(00)00123-9

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. *Nat. Rev. Mol. Cell Biol.* 9, 402–412. doi: 10.1038/nrm2395

Rochette, P. J., Therrien, J., Drouin, R., Perdiz, D., Bastien, N., and Drobetsky, E. A., et al. (2003). UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine-thymine dipyrimidines and correlate with the mutation spectrum in rodent cells. *Nucleic Acids Res.* 31, 2786–2794. doi: 10.1093/nar/gkg402

Roemer, K. (2012). Notch and the p53 clan of transcription factors. *Adv. Exp. Med. Biol.* 727, 223–240. doi: 10.1007/978-1-4614-0899-4_17

Saramaki, A., Banwell, C. M., Campbell, M. J., and Carlberg, C. (2006). Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res.* 34, 543–554. doi: 10.1093/nar/gkj460

Sbisa, E., Catalano, D., Grillo, G., Licciulli, F., Turi, A., Liuni, S., et al. (2007). p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining *in silico* prediction and microarray data. *BMC Bioinformatics* 8(Suppl. 1):S20. doi: 10.1186/1471-2105-8-S1-S20

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., et al. (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. *Cancer Cell.* 17, 273–285. doi: 10.1016/j.ccr.2009.11.025

Steinman, H. A., Burstein, E., Lengner, C., Gosselin, J., Pihan, G., Duckett, C. S., et al. (2004). An alternative splice form of Mdm2 induces p53-independent cell growth and tumourigenesis. *J. Biol. Chem.* 279, 4877–4886. doi: 10.1074/jbc.M305966200

Suh, E. K., Yang, A., Kettenbach, A., Bamberger, C., Michaelis, A. H., Zhu, Z., et al. (2006). p63 protects the female germ line during meiotic arrest. *Nature* 444, 624–628. doi: 10.1038/nature05337

Sutherland, B. M., Blackett, A. D., Feng, N. I., Freeman, S. E., Ogut, E. S., Gange, R. W., et al. (1985). Photoreactivation and other ultraviolet/visible light effects on DNA in human skin. *Ann. N.Y. Acad. Sci.* 453, 73–79. doi: 10.1111/j.1749-6632.1985.tb11799.x

Talos, F., Nemajerova, A., Flores, E. R., Petrenko, O., and Moll, U. M. (2007). p73 suppresses polyploidy and aneuploidy in the absence of functional p53. *Mol. Cell.* 27, 647–659. doi: 10.1016/j.molcel.2007.06.036

Tang, J. Y., Fu, T., Lau, C., Oh, D. H., Bikle, D. D., and Asgari, M. M. (2012a). Vitamin D in cutaneous carcinogenesis: Part I. *J. Am. Acad. Dermatol.* 67, 803.e1–803.e12. doi: 10.1016/j.jaad.2012.05.044

Tang, J. Y., Fu, T., Lau, C., Oh, D. H., Bikle, D. D., and Asgari, M. M. (2012b). Vitamin D in cutaneous carcinogenesis: Part II. *J. Am. Acad. Dermatol.* 67, 817.e1–817.e11. doi: 10.1016/j.jaad.2012.07.022

Toledo, F., and Wahl, G. M. (2006). Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. *Nat. Rev. Cancer*. 6, 909–923. doi: 10.1038/nrc2012

Tomasini, R., Tsuchihara, K., Wilhelm, M., Fujitani, M., Rufini, A., Cheung, C. C., et al. (2008). TAp73 knockout shows genomic instability with infertility and tumour suppressor functions. *Genes Dev.* 22, 2677–2691. doi: 10.1101/gad.1695308

Truong, A. B., Kretz, M., Ridky, T. W., Kimmel, R., and Khavari, P. A. (2006). p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev.* 20, 3185–3197. doi: 10.1101/gad.1463206

Varley, J. M. (2003). Germline TP53 mutations and Li-Fraumeni syndrome. *Hum. Mutat.* 21, 313–320. doi: 10.1002/humu.10185

Vousden, K. H., and Lane, D. P. (2007). p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* 8, 275–283. doi: 10.1038/nrm2147

Vousden, K. H., and Prives, C. (2009). Blinded by the light: the growing complexity of p53. *Cell.* 137, 413–431. doi: 10.1016/j.cell.2009.04.037

Wallingford, J. B., Seufert, D. W., Virta, V. C., and Vize, P. D. (1997). p53 activity is essential for normal development in Xenopus. *Curr. Biol.* 7, 747–757. doi: 10.1016/S0960-9822(06)00333-2

Wang, S., and El-Deiry, W. S. (2006). p73 or p53 directly regulates human p53 transcription to maintain cell cycle checkpoints. *Cancer Res.* 66, 6982–6989. doi: 10.1158/0008-5472.CAN-06-0511

Wang, X., Taplick, J., Geva, N., and Oren, M. (2004). Inhibition of p53 degradation by Mdm2 acetylation. *FEBS Lett.* 561, 195–201. doi: 10.1016/S0014-5793(04)00168-1

Westfall, M. D., Mays, D. J., Sniezek, J. C., and Pietenpol, J. A. (2003). The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters *in vivo* and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol. Cell. Biol.* 23, 2264–2276. doi: 10.1128/MCB.23.7.2264-2276.2003

Wikonkal, N. M., and Brash, D. E. (1999). Ultraviolet radiation induced signature mutations in photocarcinogenesis. *J. Invest. Dermatol. Symp. Proc.* 4, 6–10. doi: 10.1038/sj.jidsp.5640173

Yamaguchi, Y., and Hearing, V. J. (2009). Physiological factors that regulate skin pigmentation. *Biofactors* 35, 193–199. doi: 10.1002/biof.29

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dötsch, V., et al. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.* 2, 305–316. doi: 10.1016/S1097-2765(00)80275-0

Yi, R., Poy, M. N., Stoffel, M., and Fuchs, E. (2008). A skin microRNA promotes differentiation by repressing "stemness." *Nature* 452, 225–229. doi: 10.1038/nature06642

Zauberman, A., Flusberg, D., Haupt, Y., Barak, Y., and Oren, M. (1995). A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res.* 23, 2584–2592. doi: 10.1093/nar/23.14.2584

Zhang, Z., and Zhang, R. (2005). p53-independent activities of MDM2 and their relevance to cancer therapy. *Curr. Cancer Drug Targets*. 5, 9–20. doi: 10.2174/1568009053332618

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Vitamin D: a critical and essential micronutrient for human health

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Vitamin D is a micronutrient that is needed for optimal health throughout the whole life. Vitamin D_3 (cholecalciferol) can be either synthesized in the human skin upon exposure to the UV light of the sun, or it is obtained from the diet. If the photoconversion in the skin due to reduced sun exposure (e.g., in wintertime) is insufficient, intake of adequate vitamin D from the diet is essential to health. Severe vitamin D deficiency can lead to a multitude of avoidable illnesses; among them are well-known bone diseases like osteoporosis, a number of autoimmune diseases, many different cancers, and some cardiovascular diseases like hypertension are being discussed. Vitamin D is found naturally in only very few foods. Foods containing vitamin D include some fatty fish, fish liver oils, and eggs from hens that have been fed vitamin D and some fortified foods in countries with respective regulations. Based on geographic location or food availability adequate vitamin D intake might not be sufficient on a global scale. The International Osteoporosis Foundation (IOF) has collected the 25-hydroxy-vitamin D plasma levels in populations of different countries using published data and developed a global vitamin D map. This map illustrates the parts of the world, where vitamin D did not reach adequate 25-hydroxyvitamin D plasma levels: 6.7% of the papers report 25-hydroxyvitamin D plasma levels below 25 nmol/L, which indicates vitamin D deficiency, 37.3% are below 50 nmol/Land only 11.9% found 25-hydroxyvitamin D plasma levels above 75 nmol/L target as suggested by vitamin D experts. The vitamin D map is adding further evidence to the vitamin D insufficiency pandemic debate, which is also an issue in the developed world. Besides malnutrition, a condition where the diet does not match to provide the adequate levels of nutrients including micronutrients for growth and maintenance, we obviously have a situation where enough nutrients were consumed, but lacked to reach sufficient vitamin D micronutrient levels. The latter situation is known as hidden hunger. The inadequate vitamin D status impacts on health care costs, which in turn could result in significant savings, if corrected. Since little is known about the effects on the molecular level that accompany the pandemic like epigenetic imprinting, the insufficiency-triggered gene regulations or the genetic background influence on the body to maintain metabolic resilience, future research will be needed. The nutrition community is highly interested in the molecular mechanism that underlies the vitamin D insufficiency caused effect. In recent years, novel large scale technologies have become available that allow the simultaneous acquisition of transcriptome, epigenome, proteome, or metabolome data in cells of organs. These important methods are now used for nutritional approaches summarized in emerging scientific fields of nutrigenomics, nutrigenetics, or nutriepigenetics. It is believed that with the help of these novel concepts further understanding can be generated to develop future sustainable nutrition solutions to safeguard nutrition security.

Keywords: vitamin D, 25-hydroxyvitamin D, nutrition, micronutrients, hidden hunger, nutrition security, nutritional pathways, nutrigenomics

INTRODUCTION

Vitamin D is needed to maintain calcium concentrations within a narrow physiological range. This function is vital as the calcium ion is essential for a large variety of cellular and metabolic processes in the body (Berridge, 2012). To secure the calcium supplies besides intestinal absorption, calcium is stored in the skeleton and acts as a large calcium reservoir that is mainly controlled by PTH and vitamin D (Bouillon et al., 2014). Humans produce vitamin D by exposure to sunlight that includes ultraviolet B radiation; if ultraviolet B radiation is not available in sufficient amounts, vitamin D needs to be obtained from the diet or dietary supplements (Holick, 2007). The start of the vitamin D endocrine system is believed to have been initiated before the start of vertebrates and evolved over millions of years

(Bouillon and Suda, 2014). Therefore, the vitamin D micronutrient either synthesized through the sun by the skin or through dietary uptake is well-adapted to the human body. The endogenously conjugated vitamin D metabolites have taken over many important roles in the maintenance of human health, of which many still await to be discovered.

In this paper, we summarize the knowledge on vitamin D as an essential micronutrient important for human health and discuss the new nutritional research on its way to gain further knowledge on the function of vitamin D for nutrition.

VITAMIN D PART OF NUTRITION AND CONTENT IN FOODS

The history of vitamin D is linked to first scientific description of the classic bone disease rickets by Whistler in 1645 (Norman, 2012). Two centuries later it was Schütte who observed the usefulness of cod liver oil in the treatment of rickets and osteomalacia in 1824. The hunt for the anti-rachitic factor ended in early twentieth century, when Mellanby could demonstrate in a series of hallmark studies (1919–1924) that a nutritional component in the diet was the anti-rachitic factor to prevent rickets (Mellanby, 1919, 1976; Platt, 1956). Shortly after, vitamin D was inaugurated without the characterization of the chemical structure. In 1919, Hudschinsky showed in parallel that UV light was able to ameliorate rickets by increasing calcification in rachitic children (Huldschinsky, 1919, 1926). Both findings of the cod liver oil and the UV light preventing rickets remained independent observations until Hess and Weinstock elegantly could demonstrate that the anti-rachitic vitamin D was produce by UV irradiation in skin (Hess and Weinstock, 1925a,b). In 1936, Windaus and colleagues determined the chemical structure of the fat-soluble seco-steroid vitamin D (Windaus et al., 1936).

The vitamin D definition comprises a group of molecules called the calciferols. The main forms present in foods are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), whereas the metabolite 25-hydroxycholecalciferol (25-hydroxyvitamin D_3) is a natural part of the food chain by its occurrence in animal products. Vitamin D_3 is unique by the fact that the same nutrient can be synthesized in the skin through the action of sunlight or being taken up by diet. This dual source of intake secures the body to maintain sufficient vitamin D levels in the body. The production in skin is usually the major vitamin D_3 source for the body. However, in countries that receive insufficient sun exposure, people rely on dietary vitamin D as a major source. Exposure of the precursor 7-dehydrocholesterol in the basal and suprabasal layers of the epidermis to ultraviolet B (UVB) light with a wavelength of 290–315 nm is needed for the formation of the previtamin D_3. The subsequent conversion is a non-enzymatic process that includes a thermal isomerization of the previtamin D_3 to produce vitamin D_3 (Collins and Norman, 2001; Holick, 2011). This vitamin D_3 is rapidly converted to 25-hydroxyvitamin D_3 in the liver. The vitamin D status is evaluated by measuring the circulating levels of serum 25-hydroxyvitamin D, which is the sum of cutaneous synthesis (vitamin D_3) or dietary contribution (vitamin D_3 and vitamin D_2). The 25-hydroxyvitamin D_3 needs to be further hydroxylated in the kidney (or locally in other organs Lehmann et al., 2001) to form 1,25-dihydroxyvitamin D_3, the active endogenous hormone, which is responsible for most of the physiological actions of vitamin D through the binding to the vitamin D receptor (VDR). The plant-derived vitamin D_2 is processed in the same way. For both vitamers, vitamin D_2, and vitamin D_3, the consecutive molecular action is believed to be identical, whereas only 1,25-dihydroxy vitamin D_3 is the endogenous hormone, the activated vitamer 1,25-dihydroxyvitamin D_2 is hormone mimetic. Therefore, it was not surprising that vitamin D_3 has been reported to be superior to vitamin D_2 in terms of bioavailability and maintaining the vitamin D status by the majority of studies (Trang et al., 1998; Armas et al., 2004; Romagnoli et al., 2008; Glendenning et al., 2009; Heaney et al., 2011; Lehmann et al., 2013). Only one study reported that the two vitamers were essentially equipotent (Holick et al., 2008).

The level of cutaneous vitamin D_3 synthesis is mainly affected by the amount of solar UVB radiation reaching the human skin, which is a function that needs to take into account the wavelength, thickness of the ozone layer in the atmosphere and solar zenith angle. Furthermore, the geographic latitude, season of the year and time of day influence and restrict the skin-borne synthesis of vitamin D_3 (Webb et al., 1988; Holick, 2011). It was described that vitamin D_3 synthesis in the skin declines with age, which is due in part to a fall of 7-dehydrocholesterol and the morphological changes due to biological aging (MacLaughlin and Holick, 1985; Holick et al., 1989). Matsuoka et al. (1991) have shown that in Caucasians and Asian subjects having a lighter skin pigmentation UVB radiation produce significantly higher vitamin D_3 serum levels than in African American and East Indian groups. It is not of a surprise that skin pigmentation reduces vitamin D_3 formation. This skin tone dependent down regulation is easily overcome by increased sun exposures (Armas et al., 2007). Apart to darker pigmented skin, cutaneous vitamin D_3 production can be reduced for many other reasons like severe air pollution in large cities, less outdoor activity as a consequence of an unhealthy lifestyle change, immobility of institutionalized elderly populations, topical application of sunscreens with a high sun protection factors or cultural dress codes (e.g., veiling). Therefore, dietary intake of vitamin D through foods or supplements plays a vital part to maintain healthy vitamin D levels.

Through nutrition, vitamin D intake is limited. There are few naturally-occurring food sources containing relevant levels of vitamin D. **Table 1** summarizes the vitamin D content in selected foods. Vegetarian diets are limited to the plant vitamin D_2 that is only present in some mushrooms. Commercially dark cultivated white button mushrooms contain low amounts of vitamin D_2, only wild mushrooms or sun-dried mushrooms contain elevated amounts of ergocalciferol (Mattila et al., 1994, 1999b, 2001; Teichmann et al., 2007). Some commercial producers include an UVB radiation step to increase the vitamin D_2 content in their products (Mau et al., 1998; Roberts et al., 2008). Vitamin D_2 is formed out from ergosterol in the mushrooms. Some plants that are used as foods however can contain ergosterol, but this provitamin form is not converted to vitamin D_2. Vitamin D_3 is not found in food-borne plants. In plants, the occurrence of vitamin D_3-related compounds is scarce. Interestingly, species belonging to the botanical *Solanaceae* family, like *Solanum malacoxylon* (*Solanum glaucophyllum* and *Solanum glaucum*), contain a glycoside of the active 1,25-dihydroxyvitamin D_3 hormone

Table 1 | Vitamin D content in raw products, processed foods, and fortified foods.

Category	Foodstuff	Range		References
		(μg vitamin D per 100 g)	(IU vitamin D per 100 g)	
RAW PRODUCTS				
Fish	Herring	2.2–38.0	88–1,520	Kobayashi et al., 1995; Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Salmon	4.2–34.5	168–1,380	Kobayashi et al., 1995; Ostermeyer and Schmidt, 2006; Lu et al., 2007; Byrdwell et al., 2013
	Halibut	4.7–27.4	188–1,094	Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Perch	0.3–25.2	12–1,012	Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Trout	3.8–19.0	152–760	Mattila et al., 1995a; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
	Tuna	1.7–18.7	68–748	Takeuchi et al., 1984, 1986; Kobayashi et al., 1995; Byrdwell et al., 2013
	Mackerel	0.5–15.5	20–620	Egaas and Lambertsen, 1979; Aminullah Bhuiyan et al., 1993; Kobayashi et al., 1995; Ostermeyer and Schmidt, 2006; Lu et al., 2007
	Cod	0.5–6.9	20–276	Kobayashi et al., 1995; Mattila et al., 1995a; Ostermeyer and Schmidt, 2006; Byrdwell et al., 2013
Mushrooms	Morel	4.2–6.3	168–252	Phillips et al., 2011
	Dark cultivated white bottom mushrooms	0–0.2	0–8	Mattila et al., 2001; Teichmann et al., 2007; Phillips et al., 2011
	Wild grown mushrooms	0.3–29.8	10–1,192	Mattila et al., 1994, 1999b, 2001; Kobayashi et al., 1995; Teichmann et al., 2007
Animal products	Pork meat	0.1–0.7	4–28	Kobayashi et al., 1995; Bilodeau et al., 2011; Strobel et al., 2013
	Beef meat	0–0.95	0–38	Kobayashi et al., 1995; Montgomery et al., 2000, 2002; Bilodeau et al., 2011; Strobel et al., 2013
	Chicken meat	0–0.3	0–12	Kobayashi et al., 1995; Mattila et al., 1995b; Bilodeau et al., 2011; Strobel et al., 2013
	Beef liver	0–14.1	0–560	Kobayashi et al., 1995; Mattila et al., 1995b; Montgomery et al., 2000, 2002
	Eggs	0.4–12.1	28–480	Mattila et al., 1992, 1999a; Kobayashi et al., 1995; Bilodeau et al., 2011; Exler et al., 2013
PROCESSED FOODS				
Fish	Tuna (skipjack) liver oil	144,400	5,776,000	Takeuchi et al., 1984
	Halibut liver oil	13,400	536,000	Egaas and Lambertsen, 1979
	Cod liver oil	137.5–575.0	5,500–23,000	Egaas and Lambertsen, 1979; Takeuchi et al., 1984
	Canned pink salmon	12.7–43.5	508–1,740	Bilodeau et al., 2011
	Canned sardines	3.2–10	128–400	Mattila et al., 1995a
	Smoked salmon	4.9–27.2	196–1,088	Ostermeyer and Schmidt, 2006
Mushrooms	Irradiated mushrooms	6.6–77.4	264–3,094	Mau et al., 1998; Roberts et al., 2008
Dairy	Butter	0.2–2.0	8–80	Kobayashi et al., 1995; Mattila et al., 1995b; Jakobsen and Saxholt, 2009
	Cheese	0–0.1	0–4	Mattila et al., 1995b; Wagner et al., 2008
FORTIFIED FOODS				
Cereals	Corn flakes	2–4.7	87–189	Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
Beverages	Orange juice	1.1	44	Wacker and Holick, 2013
	Malted drink mix, powder	3	123	Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
Dairy	Milk	1.1–2.0	42–79	Calvo et al., 2004; Haytowitz et al., 2009; U.S. Department of Agriculture, 2013
	Cheese	2.6–25.0	102–1,000	Haytowitz et al., 2009; Tippetts et al., 2012; U.S. Department of Agriculture, 2013

(Boland, 1986; Boland et al., 2003; Japelt et al., 2013). This deciduous shrub (1.5–3.0 m stem length) is widely distributed in the provinces of Buenos Aires in Argentina and in Brazil and is responsible for the calcinotic disease in cattle and other grazing animal.

Animal food products are the main dietary source for naturally occurring vitamin D_3 (Schmid and Walther, 2013). Since the discovery of vitamin D, vitamin D was associated with oily fish products. It was driven by the early observation that the amount of vitamin D in a teaspoon of cod liver oil was sufficient to prevent rickets in infants. It is still the fish liver oil that contains the highest amounts of vitamin D_3. The highest reported concentration was found in skipjack liver oil 144,400 μg/100 g (Takeuchi et al., 1984). The fish liver oils besides other nutritional ingredients might contain high levels of vitamin A. The vitamin A to vitamin D ratio in the fish liver oils is species and fishing area dependent. The ratio range starts with a factor of 0.5 for skipjack liver oil and can even reach an extreme ratio of 119 (pollack liver oil) (Takeuchi et al., 1984). This wide vitamin A to vitamin D ratio range is the reason why fish liver oils often need further processing. In fresh fish products we observe a huge variation in the vitamin D_3 content per 100 g wet weight (Egaas and Lambertsen, 1979; Takeuchi et al., 1984, 1986; Kobayashi et al., 1995; Mattila et al., 1995a, 1997; Ostermeyer and Schmidt, 2006; Lu et al., 2007; Byrdwell et al., 2013) (**Table 1**). Large variations in vitamin D_3 content were found within the same species, but also between the different fish species. Fish obtain their vitamin D_3 requirements through their diet (Holick, 2003). Therefore, the vitamin D_3 levels in the zooplankton, the primary food source of fish, or seasonal changes in the zooplankton reservoirs in the different habitats, might be the reasons for the observed fluctuation in the fish product. Interestingly, the weight, the sex, or the age of the fish could not be correlated to the vitamin D_3 content. Furthermore, no significant correlation between the tissue fat content and vitamin D levels was detected (Mattila et al., 1995a, 1997). Significant differences in vitamin D_3 content were found between muscle and skin tissues and even more pronounced between muscle and liver tissues (Takeuchi et al., 1986). The 25-hydroxyvitamin D_3 compound was also detected, though at low concentrations (Takeuchi et al., 1986; Mattila et al., 1995a; Ovesen et al., 2003; Bilodeau et al., 2011).

Wild and sun-dried mushrooms can be a good dietary source of vitamin D_2 (Mattila et al., 1994, 1999b, 2001; Kobayashi et al., 1995; Teichmann et al., 2007; Phillips et al., 2011). However, the commercially produced mushrooms, e.g., the white button mushroom, do not contain or contain only very low amounts of vitamin D_2 (Mattila et al., 2001; Teichmann et al., 2007; Phillips et al., 2011). The vitamin D_2 content in commercially produced mushrooms can be increased by UVB exposure during the culturing or the postharvest process (Mau et al., 1998; Roberts et al., 2008). The concentration of vitamin D in eggs can vary from 0.4 to 12.1 μg (Parrish, 1979; Mattila et al., 1992, 1999a; Bilodeau et al., 2011; Exler et al., 2013), it is in a similar range like offal (Mattila et al., 1995b; Montgomery et al., 2000, 2002). Other animal products like pork, beef, and chicken muscle meat are low in vitamin D content (Mattila et al., 1995b; Montgomery et al., 2000, 2002; Bilodeau et al., 2011; Strobel et al., 2013). By adding vitamin D_3 into the feed, the vitamin D_3 content can be increased in muscle and liver of cattle, to 4.6 μg per 100 g of tissue and 99.6 μg per 100 g of tissue, respectively (Montgomery et al., 2004). Milk, unless fortified, has been shown to contain no or very little amounts of vitamin D, whereas in dairy products like butter and cheese the vitamin D content is higher, but in serving size amounts still very low (Kobayashi et al., 1995; Mattila et al., 1995b; Jakobsen and Saxholt, 2009; Trenerry et al., 2011). In general, household cooking seems to have some effect on vitamin D stability depending on the actual foodstuffs and the heating process used (Mattila et al., 1999b; Jakobsen and Knuthsen, 2014).

To meet the vitamin D needs in the countries some states fortify foods. Dairy products are ideal for vitamin D fortification. In Canada vitamin D fortification is mandatory for milk (1 μg/100 ml) and margarine (13.3 μg/100 g) (Health Canada, 2014). In other countries, like the United States, vitamin D fortification is optional for products like milk, breakfast cereals, and fruit juices (Calvo et al., 2004). In the U.S. Department of Agriculture (2013) of the US Department of Agriculture (USDA)'s Nutrient Databank System (Haytowitz et al., 2009), 5036 foods have been determined for their vitamin D content, of which only 259 food items had detectable vitamin D levels. The data showed that per serving only seven fish products had >15 μg vitamin D. All 29 foods that contained between 2.5 μg 15 μg vitamin D per serving were either fortified foods (21) or fish produce (8). Two-thirds of all vitamin D containing foods were far below the 1.0 μg level, whereas 20 percent had even negligible vitamin D content per serving (below 0.1 μg).

Despite the fact that moderate sun exposure of arms and legs in summer for 5–30 min between the hours of 10 a.m. and 3 p.m. twice a week is enough to produce sufficient vitamin D_3 in the body (Holick, 2007), it is astonishing that many populations that live at these privileged latitudes fail to achieve this goal (Holick and Chen, 2008; Lips, 2010; Wahl et al., 2012; Hilger et al., 2014). During winter time, when vitamin D_3 production by the sun ceased, adequate vitamin D levels can only be achieved by UVB exposure from indoor tanning units, or by a daily diet of fortified foods or a few selected food items. This restricted list of options to achieve sufficient levels is one of the reasons, why the use of dietary vitamin D supplements has become so popular. It is currently the most applied and secure option to reach adequate vitamin D intake levels (Holick, 2007).

VITAMIN D MAP, MALNUTRITION, HIDDEN HUNGER, AND NUTRITION SECURITY

An accepted biomarker for the vitamin D status in the general population is to measure the serum concentration of 25-hydroxyvitamin D levels, which is the major circulating form of vitamin D and reflects both dietary vitamin D intake and the endogenous vitamin D production (Lips, 2001, 2007). The serum concentration of 25-hydroxyvitamin D is linked to the serum level of the active hormone 1,25-dihydroxyvitamin D and also to the clinical relevant parathyroid hormone level. Lips has classified the 25-hydroxyvitamin D levels into four stages (Lips, 2001; Lips et al., 2013): severe deficiency (<12.5 nmol/L), deficiency (12.5–25 nmol/L), insufficiency (25–50 nmol/L), repletion

(>50 nmol/L). The thresholds for severe deficiency and deficiency are undisputed; however, a controversy has arisen for defining the border between insufficiency and repletion. In 2011, the Institute of Medicine (IOM) suggested a serum level of 50 nmol/L as the value at which 97.5% of the vitamin D needs of the population would be covered (Institute of Medicine, 2011; Ross et al., 2011), whereas, the Endocrine Society (ES) defined it to be higher: 75 nmol/L (Holick et al., 2011). All deficiency levels including insufficiency, as so-called mild deficiency, must be prevented through focused supplementation.

In 2010, the Institute of Medicine (IOM) introduced new dietary reference intake (DRI) values for vitamin D after comprehensive reviewing of more than 1000 high quality research articles to renew thereby their first settings from 1997 (Institute of Medicine, 2011). The DRIs address an adequate nutritional intake of all sources. The IOM has set the dietary allowance (RDA) to 600 IU per day for the general population and at 800 IU per day for persons 70 years and older, whereas 1 IU is the biological equivalent of 0.025 μg vitamin D_3. The tolerable upper intake level or UL (Upper Level of Intake), which represents the safe upper limit, was set to 4000 IU per day for vitamin D intake (Ross et al., 2011). The new RDAs reflect the scientific outcome from large dietary studies that revealed vitamin D insufficiency (Looker et al., 2002; Zadshir et al., 2005). In 2012, Troesch et al. analyzed the vitamin intake from different dietary surveys that included the German Nutritional Intake Study (Nationale Verzehrstudie II) 2008 (Max Rubner-Institut, 2008), the US National Health and Nutrition Examination Survey (NHANES) from 2003 to 2008 (Centers for Disease Control and Prevention & National Center for Health Statistics, 2009), the UK (The British National Diet and Nutrition Survey, 2003) (Henderson et al., 2003) and the Netherlands (van Rossum et al., 2011), and could confirm that vitamin D is one of the critical vitamins, which intake is below the recommendation (Troesch et al., 2012).

A gap exists between the intake and the recommendation of vitamin D. The chronic insufficient intake of micronutrients like vitamin D without seeing immediate clinical signs is called Hidden Hunger. Hidden Hunger, in particular for vitamin D, is more prevalent in the populations of the developed countries as anticipated (Biesalski, 2013). Hidden Hunger is a threat for the nutrition security for a given country. Nutrition security mandates sufficient micronutrients in an adequate food supply and is required to safeguard an optimal nutritional status of a population.

Many groups have identified vitamin D deficiency or insufficiency to become a public health problem worldwide (Holick, 2007; Holick and Chen, 2008; Mithal et al., 2009; Lips and Van Schoor, 2011; Wahl et al., 2012; Hilger et al., 2014). Mithal et al. (2009) described in their global report that most populations do not achieve a desirable vitamin D status and particular people at risk and elderly people suffer from vitamin D deficiency. In two reports, the International Osteoporosis Foundation (IOF) and its partners published the global vitamin D status map (Wahl et al., 2012; Hilger et al., 2014). The vitamin D map was based on a systematic review of the worldwide vitamin D levels, using all available publications published between 1990 and February 2011 (Hilger et al., 2014). Eligible studies include 168,389 participants from the general populations throughout the world where the mean or median serum 25-hydroxyvitamin D levels were measured. Studies included had a cross-sectional design or were based on a population based cohorts. The analysis identified nearly 200 studies from 44 countries, whereas only half of the studies were included in the global vitamin D status map as 50.2% of the studies were not representative for the target populations. **Figure 1** shows the global vitamin D status map listed by countries and by continents. The largest numbers of studies were performed in Europe, followed by North America and Asia-Pacific. Available data from Latin America and even more from Africa are limited. Results of this review showed that 6.7% of the population were vitamin D deficient (mean 25-hydroxyvitamin D values <25 nmol/L), 37.3% were vitamin D insufficient according to IOM (mean values below 50 nmol/L) and 88.1% of the population showed an insufficient vitamin D status according to the ES (mean values below 75 nmol/L). No significant differences were found for gender or age, when looking at the worldwide data, but some regional differences could be identified (Hilger et al., 2014). The 25-hydroxyvitamin D serum levels were higher in Europe and the US, when compared to Middle East and Africa. This might be due to the vitamin D food fortification programs in North America (Prentice, 2008). Furthermore, the systematic analysis revealed that institutionalized elderly were more at risk to have low 25-hydroxyvitamin D levels in Europe and Asia/Pacific. The compared non-institutionalized elderly group showed higher levels, possibly due to spending more of time outdoors. The group of institutionalized elderly is therefore at high risk to become vitamin D deficient. Further research is needed to inform public health policy makers to reduce the risk for potential health consequences of low vitamin D status.

In the past few years the national recommendations for dietary vitamin D were adjusted in several countries; they are not harmonized across the European Union yet and vary from 200 to 800 IU. The higher recommendations for dietary vitamin D intake are increasingly being suggested in government documents, position statements and clinical practice guidelines for bone health. In 2008, the US Food and Drug Administration updated the health claim for the prevention of osteoporosis by including vitamin D to the consumption of calcium (Food and Drug Administration, 2008). In 2008, the American Academy of Pediatrics also reacted and issued an update of their guidelines for vitamin D intake and rickets prevention (Perrine et al., 2010). They doubled the recommended dose of vitamin D for children to 400 IU per day, beginning in the first few days of life and continuing throughout adolescence. In 2010, the Institute of Medicine (IOM) released the revised Dietary Reference Intakes (DRI's) for calcium and vitamin D and tripled the recommendations for vitamin D intakes to 600 IU per day for children and all adults up to age 69 years (Institute of Medicine, 2011). The IOM stated that there was insufficient evidence to make recommendations for non-skeletal benefits.

In 2012, the German, Austrian, and Swiss Nutrition Societies raised the recommended vitamin D intake to 800 IU per day, in case of absent UVB exposure, for all age groups starting from 1 year of age (German Nutrition Society, 2012). Furthermore, key opinion leaders are increasingly recommending higher daily intakes for vitamin D, between 800 and 1000 IU or even higher for people at risk or older adults. The recent statement by the IOF and the guidelines by the US ES suggest that higher vitamin D doses

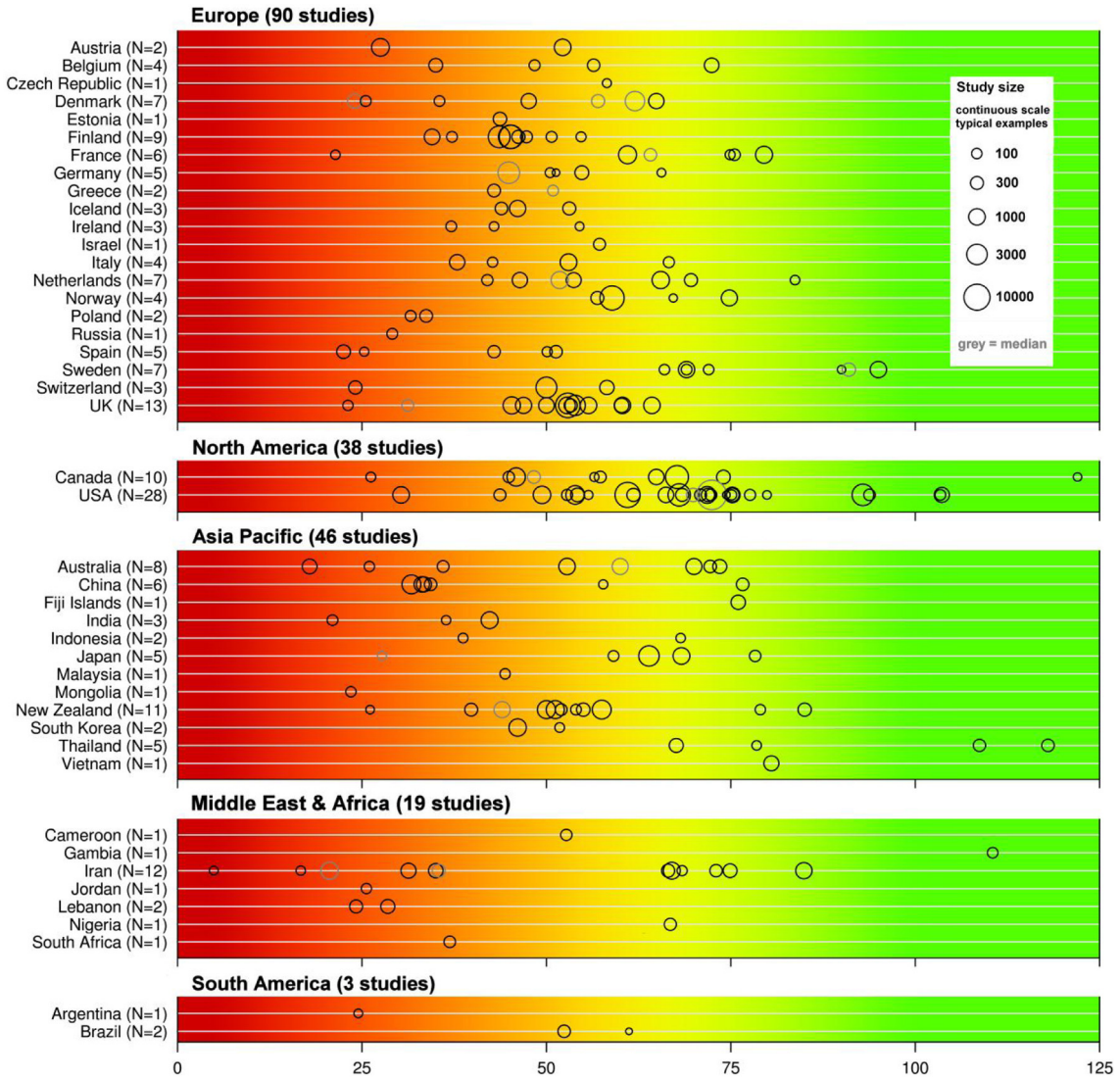

FIGURE 1 | Overview of published 25-hydroxyvitamin D mean/median values by countries (modified from Hilger et al., 2014). The color trend from red, yellow to green shown above the graphical diagram represents the current uncertainty around the definition of 25-hydroxyvitamin D_3 serum thresholds starting from severe deficiency (red), deficiency, insufficiency to total repletion (green). The reported means are shown as black circles, studies that reported medians are given in gray circle. The study size is indicated by the circle size. Mean/median values falling within the intensely red zone are most consistent with severe vitamin D deficiency.

would be needed to achieve the desirable 25-hydroxyvitamin D serum level of 75 nmol/L for people at risk or older individuals.

Increasing the vitamin D levels in the population would also ameliorate health economics. Grant and colleagues calculated the benefit of increasing vitamin D levels to reduce the economic burden of diseases (Grant et al., 2009). A rise in the vitamin D serum level of all Europeans to 40 nmol/L would reduce the economic burden of different diseases and could save health care costs of up to 16.7%. Besides reducing the economic costs, vitamin D intake could in addition also reduce mortality rates and maintain a longer healthy life style.

NUTRITIONAL RESEARCH TO ADDRESS AND UNDERSTAND VITAMIN D INSUFFICIENCY

Vitamin D deficiency is undoubtedly linked to severe consequences in the growing child by causing incomplete mineralization of the bone and in the adult accounting to wasteful osteomalacia. In the vitamin D insufficiency stage, this severity gets gradually less, but the outcome remains unchanged. Besides the established and accepted functional skeletal health relationship, more and more evidence is accumulating for falls (Pfeifer et al., 2000, 2009; Bischoff et al., 2003; Flicker et al., 2005; Broe et al., 2007; Prince et al., 2008; Bischoff-Ferrari et al., 2009) and physical performance (Bischoff-Ferrari et al., 2004; Houston et al., 2011; Ceglia et al., 2013; Redzic et al., 2013; Sohl et al., 2013;

Tieland et al., 2013), which has been recognized by a health claim of the European Food and Safety Authority in 2011: "Vitamin D may reduce the risk of falling. Falling is a risk factor for bone fractures." This health claim is targeting men and women 60 years of age and older and the dose required is a daily consumption of 800 IU vitamin D, which can come from all sources. Further emerging vitamin D health relationships include physiological parameters like improved immune response (Baeke et al., 2010; Schwalfenberg, 2011; Hewison, 2012; White, 2012), improved respiratory health(Berry et al., 2011; Charan et al., 2012; Choi et al., 2013; Hirani, 2013) possibly also relate to reduced tuberculosis incidence (Nnoaham and Clarke, 2008; Martineau et al., 2011; Mitchell et al., 2011; Coussens et al., 2012; Salahuddin et al., 2013; Huaman et al., 2014); and reduced risk to develop autoimmune diseases like multiple sclerosis (Solomon and Whitham, 2010; Cantorna, 2012; Dobson et al., 2013) or type 1 diabetes (Hypponen et al., 2001; Holick, 2003; Ramos-Lopez et al., 2006; Baeke et al., 2010; De Boer et al., 2012; Dong et al., 2013; Van Belle et al., 2013). In chronic, non-communicable diseases, vitamin D deficiency is being discussed to possibly ameliorate the incidence of some neoplastic diseases like colorectal, lung, prostate, and breast cancers (Ng et al., 2008; Rosen et al., 2012; Welsh, 2012; Cheng et al., 2013); cardiovascular diseases (CVDs) including hypertension, myocardial infarction, stroke (Forman et al., 2007; Giovannucci et al., 2008; Gardner et al., 2011; Bischoff-Ferrari et al., 2012; Tamez and Thadhani, 2012; Karakas et al., 2013; Pilz et al., 2013a; Schroten et al., 2013); life-style diseases like obesity and type 2 diabetes (Pittas et al., 2007; González-Molero et al., 2012; Khan et al., 2013; Pilz et al., 2013b; Schottker et al., 2013; Tsur et al., 2013; Van Belle et al., 2013; Bouillon et al., 2014); diseases related to the decline in sight function including age-related macular degeneration (Parekh et al., 2007; Millen et al., 2011; Lee et al., 2012); and neurological disorders including Alzheimer and Parkinson disease (Buell and Dawson-Hughes, 2008; Annweiler et al., 2012; Eyles et al., 2013; Zhao et al., 2013). One may wonder about the width of possible implications being looked at, but considering the more than 1000 genes which vitamin D is regulating through the VDR (Carlberg and Campbell, 2013), this may actually not be a surprise. To determine the potential role of vitamin D supplementation in the prevention or treatment of chronic non-skeletal diseases notwithstanding, large-scale clinical trials are demanded. In this respect for the nutrition field, four new large-scale ongoing long-term supplementation studies are expected to deliver results in near future (**Table 2**). The two very large studies, VITAL trial ($n = 20,000$) and FIND study ($n = 18,000$), are meant to deliver clinical evidence for the effect of vitamin D_3 on cancer, CVD and diabetes outcomes. The two smaller trials, CAPS and DO-HEALTH, each having more than 2,000 participants are including cancer, infections, fractures, hypertension, cognitive function, and physical performance outcomes. In all four studies the placebo group will produce vitamin D_3 in the skin and will possibly consume vitamin D through food, and therefore this will narrow the vitamin D serum level gap between the placebo and treatment groups. It remains to be seen whether the applied supplementation doses (2000 IU and 1600 IU, 3200 IU) will be sufficient to see a clear difference between the treatment and the control groups. An open likelihood will remain for the placebo group potentially obtaining sufficient vitamin D_3 (600–800 IU) levels that are considered to be sufficient for skeletal effects. In such a case only an incremental increase of an additional ∼1000 IU can be considered as the effective dose, for which no power calculation was available at the time before study begun. In light of such a situation, it will be of interest whether the micronutrient triage theory of Bruce Ames can be validated with vitamin D_3 (Ames, 2006; McCann and Ames, 2009). The triage theory postulates, as a result of recurrent shortages of micronutrients during evolution, that the body has selected and developed a metabolic rebalancing response to shortage. These rebalancing favored micronutrient-needs for short term survival, while those only required for long-term health were starved. In the case of the micronutrient vitamin D_3, calcium and bone metabolism can be considered to be secured with highest priority, therefore, it might be speculated that the 600–800 IU intake would satisfy this vitamin D_3 serum level threshold. For the chronic non-skeletal diseases however, which have only secondary priority in an evolutionary perspective, higher serum vitamin D_3 levels would be required. The ongoing four vitamin D_3 studies that have chronic diseases as their main outcomes and use nutritionally relevant ∼2000 IU are therefore well-suited to address whether the triage theory holds also true for the micronutrient vitamin D_3.

Vitamin D_3 once in the blood immediately binds to the vitamin D-binding protein (DBP) and gets transported into the liver (Holick, 2007). The first hydroxylation at position 25 generates the major circulating metabolites 25-hydroxyvitamin D_3. This metabolite circulates throughout all organs and undergoes hydroxylation at position 1, which occurs mainly in the kidney, but also in other organs, to form 1,25-dihydroxyvitamin D_3, the active hormone. Besides the major circulating metabolite 25-hydroxyvitamin D_3 and the hormonally active metabolite 1,25-dihydroxyvitamin D_3, more than 35 additional vitamin D_3 metabolites are formed by the body (Bouillon et al., 1995; Norman et al., 2001). It is speculated that they might be intermediates in the catabolism of 1,25-dihydroxyvitamin D_3. The human body has evolved many CYP enzymes and invests energy to form these additional 35 vitamin D_3 metabolites, whether this is for the purpose to catabolize 1,25-dihydroxyvitamin D_3, remains still to be answered. More appealing is the theory that these metabolites are formed to fulfill yet unknown functions of vitamin D_3. This perspective could potentially also account to the pleiotropic non-skeletal health benefits reported by the many vitamin D intake studies. For some of the vitamin D_3 metabolites like the 24*R*,25-dihydroxyvitamin D_3 potential function was explored *in vitro* (Norman et al., 2002).

The 24*R*,25-dihydroxyvitamin D_3 has been shown to be an essential hormone in the process of bone fracture healing. The 24*R*,25-dihydroxyvitamin D_3 most likely initiates its biological responses via binding to the ligand binding domain of a postulated cell membrane receptor $VDR_{mem24,25}$, similar to the better studied, but still not cloned cell membrane receptor for 1,25-dihydroxyvitamin D_3, $VDR_{mem1,25}$ (Norman et al., 2002). From the nutritional point of view, it will be of interest to investigate the function of the all vitamin D_3 metabolites and relate the function to the level of vitamin D_3 intake to secure the health benefit according to the triage theory.

Table 2 | List of ongoing large nutritional vitamin D_3 supplementation trials (>2,000 subjects) using nutrition-related daily vitamin D_3 doses (1,600–3,200 IU).

Acronym	Name, clinical trial identifier	Principal investigator	Place	Participants	Dose	Duration	Main outcomes	Results expected	Web link
CAPS	Clinical Trial of Vitamin D_3 to Reduce Cancer Risk in Postmenopausal Women NCT01052051	Joan Lappe, Creighton University	USA	2,332, healthy postmenopausal women: 55+	2,000 IU D_3 (and 1,500 mg calcium) daily	5 years	All cancers	2015	http://clinicaltrials.gov/ct2/show/NCT01052051?term=NCT01052051&rank=1
VITAL	Vitamin D and Omega-3 Trial NCT01169259	JoAnn E. Manson, Brigham and Women's Hospital	USA	20,000, men: 50+ women: 55+	2,000 IU D_3, daily omega-3 fatty acids	5 years	Cancer, Cardiovascular disease	2017	http://clinicaltrials.gov/show/NCT01169259
DO-HEALTH	Vitamin D3—Omega3—Home Exercise—Healthy Ageing and Longevity Trial NCT01745263	Heike Bischoff-Ferrari, University Zürich	8 European Cities	2,152, 70+	2,000 IU D_3 daily omega-3 fatty acids	3 years	Infections, Fractures, Blood pressure, Cognitive function, Lower extremity function	2017	http://clinicaltrials.gov/ct2/show/NCT01745263?term=bischoff-ferrari&rank=1;
FIND	Finnish Vitamin D Trial NCT01463813	Tomi-Pekka Tuomainen, University of Eastern Finland	Finland	18,000 men: 60+, women: 65+	1,600 IU D_3 daily or 3,200 IU D_3 daily	5 years	Cancer, Cardiovascular disease Diabetes	2020	http://clinicaltrials.gov/show/NCT01463813

According to the current knowledge, the vitamin D endocrine system is funneled through the biologically most active metabolite 1,25-dihydroxyvitamin D_3 that is mainly produced in the kidney, but also in other organs (Bouillon et al., 2013). Mechanistically 1,25-dihydroxyvitamin D_3 binds the VDR directly on a DNA sequence, the 1,25-dihydroxyvitamin D_3 response element (VDRE), in the regulatory region of primary 1,25-dihydroxyvitamin D_3 target genes (Carlberg and Campbell, 2013). The VDR forms together with the retinoid X receptor or putative other transcription factors a heterodimer on the VDRE, recruiting tissue-specific transcriptional co-activators and regulates through a conformational change upon 1,25-dihydroxyvitamin D_3 binding the downstream gene. The VDR is widespread in more than 30 tissues (Bouillon et al., 1995) and may trigger expression of more than 1000 genes through 1,25-dihydroxyvitamin (Carlberg et al., 2013; Hossein-Nezhad et al., 2013). The regulation of tissue-specific gene expression by 1,25-dihydroxyvitamin D_3 is of high interest, as it guides us toward the better understanding of the mechanistic action of vitamin D_3 in the different tissues. The gained knowledge from the mechanistic studies can help to design smaller and more focused nutritional intervention RCTs to answer whether vitamin D contributes to a specific health benefit of interest. In this respect the GeneChip-based transcriptomics methodology using high-density microarrays demonstrated the expression of genes in a variety of important functions of more than 100 different pathways that could be linked to vitamin D deficiency (Bossé et al., 2007; Tarroni et al., 2012; Hossein-Nezhad et al., 2013). The development of chromatin immunoprecipitation (ChiP) methodology linked to site-specific PCR amplification of the VDR bound genomic DNA fragment, and later the methods using tiled microarrays (ChiP-chip) applying the first unbiased genome-wide approach, which then was followed by the massive parallel NGS sequencing approach of the immunoprecipitated DNA segments, opened up new avenues to investigate 1,25-dihydroxyvitamin D_3 target genes in selected tissues (Ramagopalan et al., 2010; Heikkinen et al., 2011; Carlberg et al., 2012, 2013; Pike et al., 2014). In an elegant study, Carlberg et al. identified in samples of 71 pre-diabetic individuals of the VitDmet study changes in serum 25-hydroxyvitamin D_3 concentrations that were associated to primary vitamin D target genes (Carlberg et al., 2013). Based on their finding the authors proposed the genes CD14 and THBD as transcriptomics biomarkers, from which the effects of a successful vitamin D_3 supplementation can be evaluated. These biomarkers are potentially suitable for displaying the transcriptomics response of human tissues to vitamin D_3 supplementation.

Epigenetic alterations of the genome refer to heritable and modifiable changes in gene expression that are not affecting the DNA sequence. They may be inherited as Mendelian, non-Mendelian, or environmentally caused traits. One of the 1,25-dihydroxyvitamin D_3 induced epigenetic modification was shown for the hypo-methylating effect on the osteocalcin promoter (Haslberger et al., 2006). 1,25-Dihydroxyvitamin D_3 was associated with the demethylation of the osteocalcin promoter and induced the osteocalcin gene expression. The activity of VDR can be modulated by epigenetic histone acetylation. The VDR alone or in concert with other transcription factors can recruit histone-modifying enzymes like histone acetyl transferases (HATs) or histone deacetylases (HDACs) and epigenically direct transcriptional expression of downstream genes (Burrell et al., 2011; Karlic and Varga, 2011; Sundar and Rahman, 2011; Hossein-Nezhad et al., 2013). The trans-generational epigenetic inheritance of vitamin D_3 triggered epigenome modification is not fully explored, however maternal vitamin D deficiency has been discussed with adverse pregnancy outcomes or potential susceptibility for diseases (Burrell et al., 2011; Hossein-Nezhad and Holick, 2012). For future nutritional research it would be of great value to identify and validate epigenetic biomarkers that could serve as risk assessment tool for vitamin D insufficiency related susceptibility to develop a disease later in life.

Variations in vitamin D status have been shown to be related to inheritance. The disparity of vitamin D levels according to ethnicity given skin pigmentation is well-established (Cashman, 2014; Ng et al., 2014). Dark skinned population individuals have compared to Caucasian descendants almost one-half the serum concentrations of 25-hydroxyvitamin D (Nesby-O'dell et al., 2002). From twin studies it has been estimated that the heritability of genetic regulation of vitamin D levels to be between 23 and 80% (Dastani et al., 2013). In addition, large-scale genetic association studies using linkage disequilibrium analysis have identified genetic loci correlating with serum vitamin D level within five candidate genes (Dastani et al., 2013). The identified SNPs are within the 1alpha-hydroxylase of 25-hydroxyvitamin D (CYP27B1) gene, the 25-hydroxylase of vitamin D (CYP2R1) gene, the vitamin D carrier protein (GC) gene, the VDR gene, and the cytochrome P-450 (CYP24A1) gene coding for an enzyme that inactivates 1,25-dihydroxyvitamin D. It is important to note that replication studies in separate populations have to follow to verify the validity of the identified SNPs. The SNP information will provide the additional guidance toward a personalized nutritional advice to reach a sufficient vitamin D status.

CONCLUSION AND FUTURE PERSPECTIVES

In the recent years the knowledge about vitamin D and its implications have extended far beyond its classical role in bone health in either fields of basic research as well as in human trials. In particular, the evidence for the role of vitamin D in reducing the risk of fractures as well as decreasing the risk for falling is convincing and authorities have responded to it. Besides a health claim issued by the EFSA on the risk reduction for falling the dietary intake recommendations have been significantly increased in several countries such as the US and in Europe (Austria, Germany, Switzerland). A number of other countries around the globe are in the process of establishing new dietary intake recommendations as well. It turns out that on average a daily intake of 600–800 IU vitamin D appears to be required to meet fundamental needs of the human body, for specific applications higher daily intakes may be necessary, which will become clearer as the results of a number of ongoing clinical studies will become available.

The obvious question to answer is: do people obtain the recommended amounts of vitamin D? The diet is typically only a minor vitamin D source as only few food items contain relevant amounts of vitamin D, such as fatty sea fish. The primary vitamin

D source for humans is the vitamin D synthesis in the skin from vitamin D precursors by the sunlight—provided the skin is sufficiently exposed to strong enough sun radiation. Several groups have reviewed the published results on 25-hydroxyvitamin D serum levels the established marker of the vitamin D status, showing that low 25-hydroxyvitamin D levels are found in many cohorts around the world. A recent systematic review of the global vitamin D status (Hilger et al., 2014) showed that 6.7% of the overall populations reported deficient 25-hydroxyvitamin D levels below 25 nmol/L, 37% had 25-hydroxyvitamin D levels below 50 nmol/L, and only 11% were above 75 nmol/L, which is considered an adequate status by the IOF and the ES. So a very important task ahead of us is to find efficient ways to improve the vitamin D status on the population level, be it by dietary means, food fortification, or dietary supplements.

In addition, it will be very important to gather sound and convincing evidence for the many additional implicated health benefits of vitamin D besides the ones that already reached a health claim status and to see which of them will actually hold up. This will require appropriate human studies on the one hand, and also involve the appropriate use of the novel experimental approaches like nutrigenomics, nutrigenetics, and nutriepigenetics on the other hand. In conclusion, the evidence we have for vitamin D in human health is exciting, however we have to make sure that appropriate measures are taken to improve the vitamin D status to the levels required to be beneficial for human health. In future, we will also need to further apply, exploit and invest in novel, innovative and break-through technologies in the vitamin D research to understand the underlying mechanisms by which vitamin D is exerting so many effects in the human body, which is knowledge needed to the purpose to obtain and secure optimal public health through nutrition.

REFERENCES

Ames, B. N. (2006). Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17589–17594. doi: 10.1073/pnas.0608757103

Aminullah Bhuiyan, A. K. M., Ratnayake, W. M. N., and Ackman, R. G. (1993). Nutritional composition of raw and smoked atlantic mackerel (scomber scombrus): oil- and water-soluble vitamins. *J. Food Compos. Anal.* 6, 172–184. doi: 10.1006/jfca.1993.1019

Annweiler, C., Rolland, Y., Schott, A. M., Blain, H., Vellas, B., Herrmann, F. R., et al. (2012). Higher vitamin D dietary intake is associated with lower risk of Alzheimer's disease: a 7-year follow-up. *J. Gerontol. A Biol. Sci. Med. Sci.* 67, 1205–1211. doi: 10.1093/gerona/gls107

Armas, L. A., Dowell, S., Akhter, M., Duthuluru, S., Huerter, C., Hollis, B. W., et al. (2007). Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: the effect of UVB dose and skin color. *J. Am. Acad. Dermatol.* 57, 588–593. doi: 10.1016/j.jaad.2007.03.004

Armas, L. A., Hollis, B. W., and Heaney, R. P. (2004). Vitamin D_2 is much less effective than vitamin D_3 in humans. *J. Clin. Endocrinol. Metab.* 89, 5387–5391. doi: 10.1210/jc.2004-0360

Baeke, F., Takiishi, T., Korf, H., Gysemans, C., and Mathieu, C. (2010). Vitamin D: modulator of the immune system. *Curr. Opin. Pharmacol.* 10, 482–496. doi: 10.1016/j.coph.2010.04.001

Berridge, M. J. (2012). Calcium signalling remodelling and disease. *Biochem. Soc. Trans.* 40, 297–309. doi: 10.1042/BST20110766

Berry, D. J., Hesketh, K., Power, C., and Hyppönen, E. (2011). Vitamin D status has a linear association with seasonal infections and lung function in British adults. *Br. J. Nutr.* 106, 1433–1440. doi: 10.1017/S0007114511001991

Biesalski, H. K. (2013). "Hidden hunger in the developed world," in *The Road to Good Nutrition, A Global Perspective, 1st Edn.*, eds M. Eggersdorfer, K. Kraemer, M. Ruel, M. Van Ameringen, H. K. Biesalski, M. Bloem, et al. (Basel: Karger), 39–50.

Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., et al. (2011). Determination of vitamin D_3 and 25-hydroxyvitamin D_3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. *J. Food Compos. Anal.* 24, 441–448. doi: 10.1016/j.jfca.2010.08.002

Bischoff, H. A., Stahelin, H. B., Dick, W., Akos, R., Knecht, M., Salis, C., et al. (2003). Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. *J. Bone Miner. Res.* 18, 343–351. doi: 10.1359/jbmr.2003.18.2.343

Bischoff-Ferrari, H. A., Dawson-Hughes, B., Staehelin, H. B., Orav, J. E., Stuck, A. E., Theiler, R., et al. (2009). Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. *BMJ* 339:b3692. doi: 10.1136/bmj.b3692

Bischoff-Ferrari, H. A., Dawson-Hughes, B., Stocklin, E., Sidelnikov, E., Willett, W. C., Edel, J. O., et al. (2012). Oral supplementation with 25(OH)D_3 versus vitamin D_3: effects on 25(OH)D levels, lower extremity function, blood pressure, and markers of innate immunity. *J. Bone Miner. Res.* 27, 160–169. doi: 10.1002/jbmr.551

Bischoff-Ferrari, H. A., Dietrich, T., Orav, E. J., Hu, F. B., Zhang, Y., Karlson, E. W., et al. (2004). Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged $\geq$60 y. *Am. J. Clin. Nutr.* 80, 752–758.

Boland, R., Skliar, M., Curino, A., and Milanesi, L. (2003). Vitamin D compounds in plants. *Plant Sci.* 164, 357–369. doi: 10.1016/S0168-9452(02)00420-X

Boland, R. L. (1986). Plants as a source of vitamin D_3 metabolites. *Nutr. Rev.* 44, 1–8. doi: 10.1111/j.1753-4887.1986.tb07543.x

Bossé, Y., Maghni, K., and Hudson, T. J. (2007). 1α,25-dihydroxy-vitamin D_3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. *Physiol. Genomics* 29, 161–168. doi: 10.1152/physiolgenomics.00134.2006

Bouillon, R., Carmeliet, G., Lieben, L., Watanabe, M., Perino, A., Auwerx, J., et al. (2014). Vitamin D and energy homeostasis—Of mice and men. *Nat. Rev. Endocrinol.* 10, 79–87. doi: 10.1038/nrendo.2013.226

Bouillon, R., Lieben, L., Mathieu, C., Verstuyf, A., and Carmeliet, G. (2013). Vitamin D action: lessons from VDR and Cyp27b1 null mice. *Pediatr. Endocrinol. Rev.* 10(Suppl. 2), 354–366.

Bouillon, R., Okamura, W. H., and Norman, A. W. (1995). Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16, 200–257.

Bouillon, R., and Suda, T. (2014). Vitamin D: calcium and bone homeostasis during evolution. *Bonekey Rep.* 3:480. doi: 10.1038/bonekey.2013.214

Broe, K. E., Chen, T. C., Weinberg, J., Bischoff-Ferrari, H. A., Holick, M. F., and Kiel, D. P. (2007). A higher dose of vitamin D reduces the risk of falls in nursing home residents: a randomized, multiple-dose study. *J. Am. Geriatr. Soc.* 55, 234–239. doi: 10.1111/j.1532-5415.2007.01048.x

Buell, J. S., and Dawson-Hughes, B. (2008). Vitamin D and neurocognitive dysfunction: preventing "D"ecline? *Mol. Aspects Med.* 29, 415–422. doi: 10.1016/j.mam.2008.05.001

Burrell, A. M., Handel, A. E., Ramagopalan, S. V., Ebers, G. C., and Morahan, J. M. (2011). Epigenetic mechanisms in multiple sclerosis and the major histocompatibility complex (MHC). *Discov. Med.* 11, 187–196.

Byrdwell, W. C., Horst, R. L., Phillips, K. M., Holden, J. M., Patterson, K. Y., Harnly, J. M., et al. (2013). Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection and mass spectrometry. *J. Food Compos. Anal.* 30, 109–119. doi: 10.1016/j.jfca.2013.01.005

Calvo, M. S., Whiting, S. J., and Barton, C. N. (2004). Vitamin D fortification in the United States and Canada: current status and data needs. *Am. J. Clin. Nutr.* 80, 1710S–1716S.

Cantorna, M. T. (2012). Vitamin D, multiple sclerosis and inflammatory bowel disease. *Arch. Biochem. Biophys.* 523, 103–106. doi: 10.1016/j.abb.2011.11.001

Carlberg, C., and Campbell, M. J. (2013). Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78, 127–136. doi: 10.1016/j.steroids.2012.10.019

Carlberg, C., Seuter, S., De Mello, V. D., Schwab, U., Voutilainen, S., Pulkki, K., et al. (2013). Primary vitamin d target genes allow a categorization of possible benefits of vitamin D_3 supplementation. *PLoS ONE* 8:e71042. doi: 10.1371/journal.pone.0071042

Carlberg, C., Seuter, S., and Heikkinen, S. (2012). The first genome-wide view of vitamin D receptor locations and their mechanistic implications. *Anticancer Res.* 32, 271–282.

Cashman, K. D. (2014). The vitamin D RDA for African American adults: higher than that for white persons? *Am. J. Clin. Nutr.* 99, 427–428. doi: 10.3945/ajcn.113.082271

Ceglia, L., Niramitmahapanya, S., Da Silva Morais, M., Rivas, D. A., Harris, S. S., Bischoff-Ferrari, H., et al. (2013). A randomized study on the effect of vitamin D_3 supplementation on skeletal muscle morphology and vitamin d receptor concentration in older women. *J. Clin. Endocrinol. Metab.* 98, E1927–E1935. doi: 10.1210/jc.2013-2820

Centers for Disease Control and Prevention & National Center for Health Statistics. (2009). *NHANES 2003-2004, NHANES 2005-2006, NHANES 2007-2008. Data, Documentation, Codebooks, SAS Code. Dietary Interview. Individual Foods, Total Nutrient Intakes First and Second Day*. Hyattsville, MD: U.S. Department of Health and Human Services & Centers for Disease Control and Prevention. Available online at: http://www.cdc.gov/nchs/nhanes/nhanes_questionnaires.htm

Charan, J., Goyal, J. P., Saxena, D., and Yadav, P. (2012). Vitamin D for prevention of respiratory tract infections: a systematic review and meta-analysis. *J. Pharmacol. Pharmacother.* 3, 300–303. doi: 10.4103/0976-500X.103685

Cheng, T. Y., Lacroix, A. Z., Beresford, S. A., Goodman, G. E., Thornquist, M. D., Zheng, Y., et al. (2013). Vitamin D intake and lung cancer risk in the Women's Health Initiative. *Am. J. Clin. Nutr.* 98, 1002–1011. doi: 10.3945/ajcn.112.055905

Choi, C. J., Seo, M., Choi, W. S., Kim, K. S., Youn, S. A., Lindsey, T., et al. (2013). Relationship between serum 25-hydroxyvitamin D and lung function among Korean adults in Korea National Health and Nutrition Examination Survey, 2008–2010. *J. Clin. Endocrinol. Metab.* 98, 1703–1710. doi: 10.1210/jc.2012-3901

Collins, E. D., and Norman, A. W. (2001). "Vitamin D," in *Handbook of Vitamins*, eds R. B. Rucker, J. W. Suttie, D. B. Mccormick, and L. J. Machlin (New York, NY: Marcel Dekker Inc.), 51–113.

Coussens, A. K., Wilkinson, R. J., Hanifa, Y., Nikolayevskyy, V., Elkington, P. T., Islam, K., et al. (2012). Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15449–15454. doi: 10.1073/pnas.1200072109

Dastani, Z., Li, R., and Richards, B. (2013). Genetic regulation of vitamin D levels. *Calcif. Tissue Int.* 92, 106–117. doi: 10.1007/s00223-012-9660-z

De Boer, I. H., Sachs, M. C., Cleary, P. A., Hoofnagle, A. N., Lachin, J. M., Molitch, M. E., et al. (2012). Circulating Vitamin D metabolites and kidney disease in type 1 diabetes. *J. Clin. Endocrinol. Metab.* 97, 4780–4788. doi: 10.1210/jc.2012-2852

Dobson, R., Giovannoni, G., and Ramagopalan, S. (2013). The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude. *J. Neurol. Neurosurg. Psychiatry* 84, 427–432. doi: 10.1136/jnnp-2012-303934

Dong, J. Y., Zhang, W. G., Chen, J. J., Zhang, Z. L., Han, S. F., and Qin, L. Q. (2013). Vitamin D intake and risk of type 1 diabetes: a meta-analysis of observational studies. *Nutrients* 5, 3551–3562. doi: 10.3390/nu5093551

Egaas, E., and Lambertsen, G. (1979). Naturally occurring vitamin D_3 in fish products analysed by HPLC, using vitamin D2 as an international standard. *Int. J. Vitam. Nutr. Res.* 49, 35–42.

Exler, J., Phillips, K. M., Patterson, K. Y., and Holden, J. M. (2013). Cholesterol and vitamin D content of eggs in the U.S. retail market. *J. Food Compos. Anal.* 29, 110–116. doi: 10.1016/j.jfca.2012.11.001

Eyles, D. W., Burne, T. H. J., and McGrath, J. J. (2013). Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front. Neuroendocrinol.* 34, 47–64. doi: 10.1016/j.yfrne.2012.07.001

Flicker, L., Macinnis, R. J., Stein, M. S., Scherer, S. C., Mead, K. E., Nowson, C. A., et al. (2005). Should older people in residential care receive vitamin D to prevent falls? Results of a randomized trial. *J. Am. Geriatr. Soc.* 53, 1881–1888. doi: 10.1111/j.1532-5415.2005.00468.x

Food and Drug Administration. (2008). Food labeling: health claims; calcium and osteoporosis, and calcium, vitamin D, and osteoporosis. *Fed. Regist*. 73, 56477–56487.

Forman, J. P., Giovannucci, E., Holmes, M. D., Bischoff-Ferrari, H. A., Tworoger, S. S., Willett, W. C., et al. (2007). Plasma 25-hydroxyvitamin D levels and risk of incident hypertension. *Hypertension* 49, 1063–1069. doi: 10.1161/HYPERTENSIONAHA.107.087288

Gardner, D. G., Chen, S., Glenn, D. J., and Ni, W. (2011). *Vitamin D and The Cardiovascular System*. Amsterdam: Elsevier Inc.

German Nutrition Society. (2012). New reference values for vitamin D. *Ann. Nutr. Metab.* 60, 241–246. doi: 10.1159/000337547

Giovannucci, E., Liu, Y., Hollis, B. W., and Rimm, E. B. (2008). 25-hydroxyvitamin d and risk of myocardial infarction in men: a prospective study. *Arch. Intern. Med.* 168, 1174–1180. doi: 10.1001/archinte.168.11.1174

Glendenning, P., Chew, G. T., Seymour, H. M., Gillett, M. J., Goldswain, P. R., Inderjeeth, C. A., et al. (2009). Serum 25-hydroxyvitamin D levels in vitamin D-insufficient hip fracture patients after supplementation with ergocalciferol and cholecalciferol. *Bone* 45, 870–875. doi: 10.1016/j.bone.2009.07.015

González-Molero, I., Rojo-Martínez, G., Morcillo, S., Gutiérrez-Repiso, C., Rubio-Martín, E., Almaraz, M. C., et al. (2012). Vitamin D and incidence of diabetes: a prospective cohort study. *Clin. Nutr.* 31, 571–573. doi: 10.1016/j.clnu.2011.12.001

Grant, W. B., Cross, H. S., Garland, C. F., Gorham, E. D., Moan, J., Peterlik, M., et al. (2009). Estimated benefit of increased vitamin D status in reducing the economic burden of disease in western Europe. *Prog. Biophys. Mol. Biol.* 99, 104–113. doi: 10.1016/j.pbiomolbio.2009.02.003

Haslberger, A., Varga, F., and Karlic, H. (2006). Recursive causality in evolution: a model for epigenetic mechanisms in cancer development. *Med. Hypotheses* 67, 1448–1454. doi: 10.1016/j.mehy.2006.05.047

Haytowitz, D. B., Lemar, L. E., and Pehrsson, P. R. (2009). USDA's Nutrient Databank System -a tool for handling data from diverse sources. *J. Food Compos. Anal.* 22, 433–441. doi: 10.1016/j.jfca.2009.01.003

Health Canada. (2014). *Consolidation Food and Drugs Regulation.* Ottawa, ON: The Minister of Justice Canada.

Heaney, R. P., Recker, R. R., Grote, J., Horst, R. L., and Armas, L. A. (2011). Vitamin D_3 is more potent than vitamin D_2 in humans. *J. Clin. Endocrinol. Metab.* 96, E447–E452. doi: 10.1210/jc.2010-2230

Heikkinen, S., Vaisanen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1alpha,25-dihydroxyvitamin D_3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Henderson, L., Irving, K., Gregory, J. F., Bates, C. J., Prentice, A., Perks, J., et al. (2003). *The National Diet and Nutrition Survey: Adults Aged 19–64 Years. Vol. 3: Vitamin and Mineral Intake and Urinary Analytes*. London: The Stationery Office.

Hess, A. F., and Weinstock, M. (1925a). The antirachitic value of irradiated cholesterol an phytosterol. II. Further evidence of change in biological activity. *Methods Enzymol.* 64, 181–191.

Hess, A. F., and Weinstock, M. (1925b). The antirachitic value of irradiated cholesterol an phytosterol. III. Evidence of chemical change as shown by absorption spectra. *Methods Enzymol.* 64, 193–201.

Hewison, M. (2012). An update on vitamin D and human immunity. *Clin. Endocrinol.* 76, 315–325. doi: 10.1111/j.1365-2265.2011.04261.x

Hilger, J., Friedel, A., Herr, R., Rausch, T., Roos, F., Wahl, D. A., et al. (2014). A systematic review of vitamin D status in populations worldwide. *Br. J. Nutr.* 111, 23–45. doi: 10.1017/S0007114513001840

Hirani, V. (2013). Associations between vitamin D and self-reported respiratory disease in older people from a nationally representative population survey. *J. Am. Geriatr. Soc.* 61, 969–973. doi: 10.1111/jgs.12254

Holick, M. F. (2003). Vitamin D: a millenium perspective. *J. Cell. Biochem.* 88, 296–307. doi: 10.1002/jcb.10338

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl J. Med* 357, 266–281. doi: 10.1056/NEJMra070553

Holick, M. F. (2011). "Photobiology of Vitamin D," in *Vitamin D, 3rd Edn.,* eds D. Feldman, J. W. Pike, and J. S. Adams (London, GB: Elsevier), 13–22.

Holick, M. F., Biancuzzo, R. M., Chen, T. C., Klein, E. K., Young, A., Bibuld, D., et al. (2008). Vitamin D_2 is as effective as vitamin D_3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *J. Clin. Endocrinol. Metab.* 93, 677–681. doi: 10.1210/jc.2007-2308

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., et al. (2011). Evaluation, treatment, and prevention of vitamin d deficiency: an endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 96, 1911–1930. doi: 10.1210/jc.2011-0385

Holick, M. F., and Chen, T. C. (2008). Vitamin D deficiency: a worldwide problem with health consequences. *Am. J. Clin. Nutr.* 87, 1080S–1086S.

Holick, M. F., Matsuoka, L. Y., and Wortsman, J. (1989). Age, vitamin D, and solar ultraviolet. *Lancet* 2, 1104–1105. doi: 10.1016/S0140-6736(89)91124-0

Hossein-Nezhad, A., and Holick, M. F. (2012). Optimize dietary intake of vitamin D: an epigenetic perspective. *Curr. Opin. Clin. Nutr. Metab. Care* 15, 567–579. doi: 10.1097/MCO.0b013e3283594978

Hossein-Nezhad, A., Spira, A., and Holick, M. F. (2013). Influence of vitamin D status and vitamin D_3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. *PLoS ONE* 8:e58725. doi: 10.1371/journal.pone.0058725

Houston, D. K., Tooze, J. A., Hausman, D. B., Johnson, M. A., Nicklas, B. J., Miller, M. E., et al. (2011). Change in 25-hydroxyvitamin D and physical performance in older adults. *J. Gerontol. A Biol. Sci. Med. Sci.* 66, 430–436. doi: 10.1093/gerona/glq235

Huaman, M. A., Sterling, T. R., Shepherd, B. E., and Fiske, C. T. (2014). 25-Hydroxyvitamin D levels after recovery from tuberculosis: insights into pathogenesis. *Tuberculosis (Edinb)* 94, 51–54. doi: 10.1016/j.tube.2013.10.009

Huldschinsky, K. (1919). Heilung von Rachitis durch künstliche Höhensonne. *Dtsch. Med. Wochenschr.* 45, 712–713. doi: 10.1055/s-0028-1137830

Huldschinsky, K. (1926). The antirachitic: zone of the ultra-violet. *Klinische Wochenschrift* 5, 1972–1973. doi: 10.1007/BF01710213

Hypponen, E., Laara, E., Reunanen, A., Jarvelin, M. R., and Virtanen, S. M. (2001). Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet* 358, 1500–1503. doi: 10.1016/S0140-6736(01)06580-1

Institute of Medicine, F. A. N. B. (2011). *Dietary Reference Intakes for Calcium and Vitamin D.* Washington, DC: The National Academies Press.

Jakobsen, J., and Knuthsen, P. (2014). Stability of vitamin D in foodstuffs during cooking. *Food Chem.* 148, 170–175. doi: 10.1016/j.foodchem.2013.10.043

Jakobsen, J., and Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *J. Food Compos. Anal.* 22, 472–478. doi: 10.1016/j.jfca.2009.01.010

Japelt, R. B., Silvestro, D., Smedsgaard, J., Jensen, P. E., and Jakobsen, J. (2013). Quantification of vitamin D_3 and its hydroxylated metabolites in waxy leaf nightshade (*Solanum glaucophyllum* Desf.), tomato (*Solanum lycopersicum* L.) and bell pepper (*Capsicum annuum* L.). *Food Chem.* 138, 1206–1211. doi: 10.1016/j.foodchem.2012.11.064

Karakas, M., Thorand, B., Zierer, A., Huth, C., Meisinger, C., Roden, M., et al. (2013). Low levels of serum 25-hydroxyvitamin d are associated with increased risk of myocardial infarction, especially in women: results from the MONICA/KORA augsburg case-cohort study. *J. Clin. Endocrinol. Metab.* 98, 272–280. doi: 10.1210/jc.2012-2368

Karlic, H., and Varga, F. (2011). Impact of vitamin D metabolism on clinical epigenetics. *Clin. Epigenetics* 2, 55–61. doi: 10.1007/s13148-011-0021-y

Khan, H., Kunutsor, S., Franco, O. H., and Chowdhury, R. (2013). Vitamin D, type 2 diabetes and other metabolic outcomes: a systematic review and meta-analysis of prospective studies. *Proc. Nutr. Soc.* 72, 89–97. doi: 10.1017/S0029665112002765

Kobayashi, T., Takeuchi, A., and Okano, T. (1995). "Vitamin D contents in various kinds of Japanese foods," in *Challenges of Modern Medicine* (Rome: Ares-Serono Symposia), 345–350.

Lee, V., Rekhi, E., Hoh Kam, J., and Jeffery, G. (2012). Vitamin D rejuvenates aging eyes by reducing inflammation, clearing amyloid beta and improving visual function. *Neurobiol. Aging* 33, 2382–2389. doi: 10.1016/j.neurobiolaging.2011.12.002

Lehmann, B., Genehr, T., Knuschke, P., Pietzsch, J., and Meurer, M. (2001). UVB-induced conversion of 7-dehydrocholesterol to 1alpha,25-dihydroxyvitamin D_3 in an *in vitro* human skin equivalent model. *J. Invest. Dermatol.* 117, 1179–1185. doi: 10.1046/j.0022-202x.2001.01538.x

Lehmann, U., Hirche, F., Stangl, G. I., Hinz, K., Westphal, S., and Dierkes, J. (2013). Bioavailability of vitamin D_2 and D_3 in healthy volunteers, a randomized placebo-controlled trial. *J. Clin. Endocrinol. Metab.* 98, 4339–4345. doi: 10.1210/jc.2012-4287

Lips, P. (2001). Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr. Rev.* 22, 477–501. doi: 10.1210/edrv.22.4.0437

Lips, P. (2007). Relative value of 25(OH)D and 1,25(OH)2D measurements. *J. Bone Miner. Res.* 22, 1668–1671. doi: 10.1359/jbmr.070716

Lips, P. (2010). Worldwide status of vitamin D nutrition. *J. Steroid Biochem. Mol. Biol.* 121, 297–300. doi: 10.1016/j.jsbmb.2010.02.021

Lips, P., and Van Schoor, N. (2011). "Worldwide vitamin D status," in *Vitamin D, 3rd Edn.,* eds D. Feldman, J. W. Pike, and J. S. Adams (London, GB: Elsevier Inc.), 947–963.

Lips, P., Van Schoor, N. M., and Bravenboer, N. (2013). "Vitamin D-related disorders," in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism 8th Edn.,* eds R. B. Clifford J. Rosen, J. E. Compston, V. Rosen (Singapore: John Wiley & Sons, Inc.), 613–623.

Looker, A. C., Dawson-Hughes, B., Calvo, M. S., Gunter, E. W., and Sahyoun, N. R. (2002). Serum 25-hydroxyvitamin D status of adolescents and adults in two seasonal subpopulations from NHANES III. *Bone* 30, 771–777. doi: 10.1016/S8756-3282(02)00692-0

Lu, Z., Chen, T. C., Zhang, A., Persons, K. S., Kohn, N., Berkowitz, R., et al. (2007). An evaluation of the vitamin D_3 content in fish: is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *J. Steroid Biochem. Mol. Biol.* 103, 642–644. doi: 10.1016/j.jsbmb.2006.12.010

MacLaughlin, J., and Holick, M. F. (1985). Aging decreases the capacity of human skin to produce vitamin D_3. *J. Clin. Invest.* 76, 1536–1538. doi: 10.1172/JCI112134

Martineau, A. R., Timms, P. M., Bothamley, G. H., Hanifa, Y., Islam, K., Claxton, A. P., et al. (2011). High-dose vitamin D_3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377, 242–250. doi: 10.1016/S0140-6736(10)61889-2

Matsuoka, L. Y., Wortsman, J., Haddad, J. G., Kolm, P., and Hollis, B. W. (1991). Racial pigmentation and the cutaneous synthesis of vitamin D. *Arch. Dermatol.* 127, 536–538. doi: 10.1001/archderm.1991.04510010104011

Mattila, P., Konko, K., Eurola, M., Pihlava, J. M., Astola, J., Vahteristo, L., et al. (2001). Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J. Agric. Food Chem.* 49, 2343–2348. doi: 10.1021/jf001525d

Mattila, P., Lehikoinen, K., Kiiskinen, T., and Piironen, V. (1999a). Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. *J. Agric. Food Chem.* 47, 4089–4092. doi: 10.1021/jf990183c

Mattila, P., Piironen, V., Bäckman, C., Asunmaa, A., Uusi-Rauva, E., and Koivistoinen, P. (1992). Determination of vitamin D_3 in egg yolk by high-performance liquid chromatography with diode array detection. *J. Food Compost. Anal.* 5, 281–290. doi: 10.1016/0889-1575(92)90062-O

Mattila, P., Piironen, V., Haapala, R., Hirvi, T., and Uusi-Rauva, E. (1997). Possible factors responsible for the high variation in the cholecalciferol contents of fish. *J. Agric. Food Chem.* 45, 3891–3896. doi: 10.1021/jf970243j

Mattila, P., Piironen, V., Uusi-Rauva, E., and Koivistoinen, P. (1995a). Cholecalciferol and 25-hydroxycholecalciferol contents in fish and fish products. *J. Food Compost. Anal.* 8, 232–243. doi: 10.1006/jfca.1995.1017

Mattila, P., Ronkainen, R., Lehikoinen, K., and Piironen, V. (1999b). Effect of Household Cooking on the Vitamin D content in Fish, Eggs, and Wild Mushrooms. *J. Food Compost. Anal.* 12, 153–160. doi: 10.1006/jfca.1999.0828

Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E. (1994). Vitamin D contents in edible mushrooms. *J. Agric. Food Chem.* 42, 2449–2453. doi: 10.1021/jf00047a016

Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., and Koivistoinen, P. E. (1995b). Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and raw meat and liver as determined by HPLC. *J. Agric. Food Chem.* 43, 2394–2399. doi: 10.1021/jf00057a015

Mau, J. L., Chen, P. R., and Yang, J. H. (1998). Ultraviolet irradiation increased vitamin D_2 content in edible mushrooms. *J. Agric. Food Chem.* 46, 5269–5272. doi: 10.1021/jf980602q

Max Rubner-Institut. (2008). *Nationale Verzehrsstudie II. Ergebnisbericht Teil 2. Die bundesweite Befragung zur Ernaehrung von Jugendlichen und Erwachsenen (National Food Intake Study II. Results Part 2. Countrywide Assessment of Nutrition in Adolescents and Adults)*. Karlsruhe: Max Rubner-Institut Available online at: http://www.was-esse-ich.de/uploads/media/NVSII_Abschlussbericht_Teil_2.pdf

McCann, J. C., and Ames, B. N. (2009). Vitamin K, an example of triage theory: is micronutrient inadequacy linked to diseases of aging? *Am. J. Clin. Nutr.* 90, 889–907. doi: 10.3945/ajcn.2009.27930

Mellanby, E. (1919). An experimental investigation on rickets. *Lancet* 193, 407–412. doi: 10.1016/S0140-6736(01)25465-8

Mellanby, E. (1976). Nutrition classics. the lancet 1:407-12, 1919. An experimental investigation on rickets. *Nutr. Rev.* 34, 338–340. doi: 10.1111/j.1753-4887.1976.tb05815.x

Millen, A. E., Voland, R., Sondel, S. A., Parekh, N., Horst, R. L., Wallace, R. B., et al. (2011). Vitamin D status and early age-related macular degeneration in postmenopausal women. *Arch. Ophthalmol.* 129, 481–489. doi: 10.1001/archophthalmol.2011.48

Mitchell, K., Griffiths, C. J., and Martineau, A. R. (2011). Vitamin D and tuberculosis. *Curr. Respir. Med. Rev.* 7, 435–439. doi: 10.2174/157339811798072685

Mithal, A., Wahl, D. A., Bonjour, J. P., Burckhardt, P., Dawson-Hughes, B., Eisman, J. A., et al. (2009). Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos Int.* 20, 1807–1820. doi: 10.1007/s00198-009-0954-6

Montgomery, J. L., Carr, M. A., Kerth, C. R., Hilton, G. G., Price, B. P., Galyean, M. L., et al. (2002). Effect of vitamin D_3 supplementation level on the postmortem tenderization of beef from steers. *J. Anim. Sci.* 80, 971–981.

Montgomery, J. L., King, M. B., Gentry, J. G., Barham, A. R., Barham, B. L., Hilton, G. G., et al. (2004). Supplemental vitamin D_3 concentration and biological type of steers. II. Tenderness, quality, and residues of beef. *J. Anim. Sci.* 82, 2092–2104.

Montgomery, J. L., Parrish, F. C., Beitz, D. C., Horst, R. L., Huff-Lonergan, E. J., and Trenkle, A. H. (2000). The use of vitamin D_3 to improve beef tenderness. *J. Anim. Sci.* 78, 2615–2621.

Nesby-O'dell, S., Scanlon, K. S., Cogswell, M. E., Gillespie, C., Hollis, B. W., Looker, A. C., et al. (2002). Hypovitaminosis D prevalence and determinants among African American and white women of reproductive age: third National Health and Nutrition Examination Survey, 1988–1994. *Am. J. Clin. Nutr.* 76, 187–192.

Ng, K., Meyerhardt, J. A., Wu, K., Feskanich, D., Hollis, B. W., Giovannucci, E. L., et al. (2008). Circulating 25-hydroxyvitamin d levels and survival in patients with colorectal cancer. *J. Clin. Oncol.* 26, 2984–2991. doi: 10.1200/JCO.2007.15.1027

Ng, K., Scott, J. B., Drake, B. F., Chan, A. T., Hollis, B. W., Chandler, P. D., et al. (2014). Dose response to vitamin D supplementation in African Americans: results of a 4-arm, randomized, placebo-controlled trial. *Am. J. Clin. Nutr.* 99, 587–598. doi: 10.3945/ajcn.113.067777

Nnoaham, K. E., and Clarke, A. (2008). Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis. *Int. J. Epidemiol.* 37, 113–119. doi: 10.1093/ije/dym247

Norman, A. W. (2012). The history of the discovery of vitamin D and its daughter steroid hormone. *Ann. Nutr. Metab.* 61, 199–206. doi: 10.1159/000343104

Norman, A. W., Ishizuka, S., and Okamura, W. H. (2001). Ligands for the vitamin D endocrine system: different shapes function as agonists and antagonists for genomic and rapid response receptors or as a ligand for the plasma vitamin D binding protein. *J. Steroid. Biochem. Mol. Biol.* 76, 49–59. doi: 10.1016/S0960-0760(00)00145-X

Norman, A. W., Okamura, W. H., Bishop, J. E., and Henry, H. L. (2002). Update on biological actions of 1alpha,25$(OH)_2$-vitamin D_3 (rapid effects) and 24R,25$(OH)_2$-vitamin D_3. *Mol. Cell. Endocrinol.* 197, 1–13. doi: 10.1016/S0303-7207(02)00273-3

Ostermeyer, U., and Schmidt, T. (2006). Vitamin D and provitamin D in fish: determination by HPLC with electrochemical detection. *Eur. Food Res. Technol.* 222, 403–413. doi: 10.1007/s00217-005-0086-y

Ovesen, L., Brot, C., and Jakobsen, J. (2003). Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? *Ann. Nutr. Metab.* 47, 107–113. doi: 10.1159/000070031

Parekh, N., Chappell, R. J., Millen, A. E., Albert, D. M., and Mares, J. A. (2007). Association between Vitamin D and age-related macular degeneration in the third National Health and Nutrition Examination Survey, 1988 through 1994. *Arch. Ophthalmol.* 125, 661–669. doi: 10.1001/archopht.125.5.661

Parrish, D. B. (1979). Determination of vitamin D in foods: a review. *CRC Crit. Rev. Food Sci. Nutr.* 12, 29–57. doi: 10.1080/10408397909527272

Perrine, C. G., Sharma, A. J., Jefferds, M. E. D., Serdula, M. K., and Scanlon, K. S. (2010). Adherence to vitamin D recommendations among US infants. *Pediatrics* 125, 627–632. doi: 10.1542/peds.2009-2571

Pfeifer, M., Begerow, B., Minne, H. W., Abrams, C., Nachtigall, D., and Hansen, C. (2000). Effects of a short-term vitamin D and calcium supplementation on body sway and secondary hyperparathyroidism in elderly women. *J. Bone Miner. Res.* 15, 1113–1118. doi: 10.1359/jbmr.2000.15.6.1113

Pfeifer, M., Begerow, B., Minne, H. W., Suppan, K., Fahrleitner-Pammer, A., and Dobnig, H. (2009). Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals. *Osteoporos. Int.* 20, 315–322. doi: 10.1007/s00198-008-0662-7

Phillips, K. M., Ruggio, D. M., Horst, R. L., Minor, B., Simon, R. R., Feeney, M. J., et al. (2011). Vitamin D and sterol composition of 10 types of mushrooms from retail suppliers in the United States. *J. Agric. Food Chem.* 59, 7841–7853. doi: 10.1021/jf104246z

Pike, J. W., Lee, S. M., and Meyer, M. B. (2014). Regulation of gene expression by 1,25-dihydroxyvitamin D in bone cells: exploiting new approaches and defining new mechanisms. *Bonekey Rep.* 3, 482. doi: 10.1038/bonekey.2013.216

Pilz, S., Gaksch, M., O'Hartaigh, B., Tomaschitz, A., and März, W. (2013a). The role of vitamin D deficiency in cardiovascular disease: where do we stand in 2013? *Arch. Toxicol.* 87, 2083–2103. doi: 10.1007/s00204-013-1152-z

Pilz, S., Kienreich, K., Rutters, F., De Jongh, R., Van Ballegooijen, A. J., Grubler, M., et al. (2013b). Role of vitamin D in the development of insulin resistance and type 2 diabetes. *Curr. Diab. Rep.* 13, 261–270. doi: 10.1007/s11892-012-0358-4

Pittas, A. G., Lau, J., Hu, F. B., and Dawson-Hughes, B. (2007). The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* 92, 2017–2029. doi: 10.1210/jc.2007-0298

Platt, B. S. (1956). Sir Edward Mellanby, 8 April 1884-30 January 1955. *Br. J. Nutr.* 10, 177–181.

Prentice, A. (2008). Vitamin D deficiency: a global perspective. *Nutr. Rev.* 66, S153–S164. doi: 10.1111/j.1753-4887.2008.00100.x

Prince, R. L., Austin, N., Devine, A., Dick, I. M., Bruce, D., and Zhu, K. (2008). Effects of ergocalciferol added to calcium on the risk of falls in elderly high-risk women. *Arch. Int. Med.* 168, 103–108. doi: 10.1001/archinternmed.2007.31

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Ramos-Lopez, E., Jansen, T., Ivaskevicius, V., Kahles, H., Klepzig, C., Oldenburg, J., et al. (2006). Protection from type 1 diabetes by vitamin D receptor haplotypes. *Ann. N Y. Acad. Sci.* 1079, 327–334. doi: 10.1196/annals.1375.050

Redzic, M., Lewis, R. M., and Thomas, D. T. (2013). Relationship between 25-hydoxyvitamin D, muscle strength, and incidence of injury in healthy adults: a systematic review. *Nutr. Res.* 33, 251–258. doi: 10.1016/j.nutres.2013.02.007

Roberts, J. S., Teichert, A., and McHugh, T. H. (2008). Vitamin D2 formation from post-harvest UV-B treatment of mushrooms (Agaricus bisporus) and retention during storage. *J. Agricultur. Food Chem.* 56, 4541–4544. doi: 10.1021/jf0732511

Romagnoli, E., Mascia, M. L., Cipriani, C., Fassino, V., Mazzei, F., D'Erasmo, E., et al. (2008). Short and long-term variations in serum calciotropic hormones after a single very large dose of ergocalciferol (vitamin D2) or cholecalciferol (vitamin D_3) in the elderly. *J. Clin. Endocrinol. Metab.* 93, 3015–3020. doi: 10.1210/jc.2008-0350

Rosen, C. J., Adams, J. S., Bikle, D. D., Black, D. M., Demay, M. B., Manson, J. E., et al. (2012). The nonskeletal effects of vitamin D: an Endocrine Society scientific statement. *Endocr. Rev.* 33, 456–492. doi: 10.1210/er.2012-1000

Ross, A. C., Manson, J. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., et al. (2011). The 2011 report on dietary reference intakes for calcium and vitamin d from the institute of medicine: what clinicians need to know. *J. Clin. Endocrinol. Metab.* 96, 53–58. doi: 10.1210/jc.2010-2704

Salahuddin, N., Ali, F., Hasan, Z., Rao, N., Aqeel, M., and Mahmood, F. (2013). Vitamin D accelerates clinical recovery from tuberculosis: results of the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamin D supplementation in patients with pulmonary tuberculosis'. *BMC Infect. Dis.* 13:22. doi: 10.1186/1471-2334-13-22

Schmid, A., and Walther, B. (2013). Natural vitamin D content in animal products. *Adv. Nutr.* 4, 453–462. doi: 10.3945/an.113.003780

Schottker, B., Herder, C., Rothenbacher, D., Perna, L., Muller, H., and Brenner, H. (2013). Serum 25-hydroxyvitamin D levels and incident diabetes mellitus type 2: a competing risk analysis in a large population-based cohort of older adults. *Eur. J. Epidemiol.* 28, 267–275. doi: 10.1007/s10654-013-9769-z

Schroten, N. F., Ruifrok, W. P., Kleijn, L., Dokter, M. M., Sillje, H. H., Lambers Heerspink, H. J., et al. (2013). Short-term vitamin D_3 supplementation lowers plasma renin activity in patients with stable chronic heart failure: an open-label, blinded end point, randomized prospective trial (VitD-CHF trial). *Am. Heart J.* 166, 357–364.e352. doi: 10.1016/j.ahj.2013.05.009

Schwalfenberg, G. K. (2011). A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol. Nutr. Food Res.* 55, 96–108. doi: 10.1002/mnfr.201000174

Sohl, E., Van Schoor, N. M., De Jongh, R. T., Visser, M., Deeg, D. J., and Lips, P. (2013). Vitamin d status is associated with functional limitations and functional decline in older individuals. *J. Clin. Endocrinol. Metab.* 98, E1483–E1490. doi: 10.1210/jc.2013-1698

Solomon, A. J., and Whitham, R. H. (2010). Multiple sclerosis and vitamin D: a review and recommendations. *Curr. Neurol. Neurosci. Rep.* 10, 389–396. doi: 10.1007/s11910-010-0131-5

Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., and Greenfield, H. (2013). Vitamin D and 25-hydroxyvitamin D determination in meats by LC-IT-MS. *Food Chem.* 138, 1042–1047. doi: 10.1016/j.foodchem.2012.08.041

Sundar, I. K., and Rahman, I. (2011). Vitamin D and susceptibility of chronic lung diseases: role of epigenetics. *Front. Pharmacol.* 2:50. doi: 10.3389/fphar.2011.00050

Takeuchi, A., Okano, T., Ayame, M., Yoshikawa, H., Teraoka, S., Murakami, Y., et al. (1984). High-performance liquid chromatographic determination of vitamin D_3 in fish liver oils and eel body oils. *J. Nutr. Sci. Vitaminol.* 30, 421–430. doi: 10.3177/jnsv.30.421

Takeuchi, A., Okano, T., Sayamoto, M., Sawamura, S., Kobayashi, T., Motosugi, M., et al. (1986). Tissue distribution of 7-dehydrocholesterol, vitamin D_3 and 25-hydroxyvitamin D_3 in several species of fishes. *J. Nutr. Sci. Vitaminol.* 32, 13–22. doi: 10.3177/jnsv.32.13

Tamez, H., and Thadhani, R. I. (2012). Vitamin D and hypertension: an update and review. *Curr. Opin. Nephrol. Hypertens.* 21, 492–499. doi: 10.1097/MNH.0b013e3283557bf0

Tarroni, P., Villa, I., Mrak, E., Zolezzi, F., Mattioli, M., Gattuso, C., et al. (2012). Microarray analysis of $1,25(OH)_2D_3$ regulated gene expression in human primary osteoblasts. *J. Cell. Biochem.* 113, 640–649. doi: 10.1002/jcb.23392

Teichmann, A., Dutta, P. C., Staffas, A., and Jägerstad, M. (2007). Sterol and vitamin D_2 concentrations in cultivated and wild grown mushrooms: effects of UV irradiation. *LWT Food Sci. Technol.* 40, 815–822. doi: 10.1016/j.lwt.2006.04.003

Tieland, M., Brouwer-Brolsma, E. M., Nienaber-Rousseau, C., Van Loon, L. J., and De Groot, L. C. (2013). Low vitamin D status is associated with reduced muscle mass and impaired physical performance in frail elderly people. *Eur. J. Clin. Nutr.* 67, 1050–1055. doi: 10.1038/ejcn.2013.144

Tippetts, M., Martini, S., Brothersen, C., and McMahon, D. J. (2012). Fortification of cheese with vitamin D_3 using dairy protein emulsions as delivery systems. *J. Dairy Sci.* 95, 4768–4774. doi: 10.3168/jds.2011-5134

Trang, H. M., Cole, D. E., Rubin, L. A., Pierratos, A., Siu, S., and Vieth, R. (1998). Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. *Am. J. Clin. Nutr.* 68, 854–858.

Trenerry, V. C., Plozza, T., Caridi, D., and Murphy, S. (2011). The determination of vitamin D_3 in bovine milk by liquid chromatography mass spectrometry. *Food Chem.* 125, 1314–1319. doi: 10.1016/j.foodchem.2010.09.097

Troesch, B., Hoeft, B., McBurney, M., Eggersdorfer, M., and Weber, P. (2012). Dietary surveys indicate vitamin intakes below recommendations are common in representative Western countries. *Br. J. Nutr.* 108, 692–698. doi: 10.1017/S0007114512001808

Tsur, A., Feldman, B. S., Feldhammer, I., Hoshen, M. B., Leibowitz, G., and Balicer, R. D. (2013). Decreased serum concentrations of 25-hydroxycholecalciferol are associated with increased risk of progression to impaired fasting glucose and diabetes. *Diabetes Care* 36, 1361–1367. doi: 10.2337/dc12-1050

U.S. Department of Agriculture. (2013). *USDA National Nutrient Database for Standard Reference, Release 26. Nutrient Data Laboratory Home Page*. Available online at: http://www.ars.usda.gov/ba/ bhnrc/ndl (Accessed February 10, 2014).

Van Belle, T. L., Gysemans, C., and Mathieu, C. (2013). Vitamin D and diabetes: the odd couple. *Trends Endocrinol. Metab.* 24, 561–568. doi: 10.1016/j.tem.2013.07.002

van Rossum, C. T. M., Fransen, H. P., Verkaik-Kloosterman J., Buurma-Rethans, E. J. M., and Ocke, M. C. (2011). *Dutch National Food Consumption Survey 2007–2010: Diet of Children and Adults Aged 7 to 69 Years*. Bilthoven: RIVM.

Wacker, M., and Holick, M. (2013). Vitamin D—effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients* 5, 111–148. doi: 10.3390/nu5010111

Wagner, D., Sidhom, G., Whiting, S. J., Rousseau, D., and Vieth, R. (2008). The bioavailability of vitamin D from fortified cheeses and supplements is equivalent in adults. *J. Nutr.* 138, 1365–1371.

Wahl, D. A., Cooper, C., Ebeling, P. R., Eggersdorfer, M., Hilger, J., Hoffmann, K., et al. (2012). A global representation of vitamin D status in healthy populations. *Arch. Osteoporos.* 7, 155–172. doi: 10.1007/s11657-012-0093-0

Webb, A. R., Kline, L., and Holick, M. F. (1988). Influence of season and latitude on the cutaneous synthesis of vitamin D_3: exposure to winter sunlight in boston and edmonton will not promote vitamin D_3 synthesis in human skin. *J. Clin. Endocrinol. Metab.* 67, 373–378. doi: 10.1210/jcem-67-2-373

Welsh, J. (2012). Cellular and molecular effects of vitamin D on carcinogenesis. *Arch. Biochem. Biophys.* 523, 107–114. doi: 10.1016/j.abb.2011.10.019

White, J. H. (2012). Vitamin D metabolism and signaling in the immune system. *Rev. Endocr. Metab. Disord.* 13, 21–29. doi: 10.1007/s11154-011-9195-z

Windaus, A., Schenck, F., and Von Werder, F. (1936). Über das antirachitisch wirksame Bestrahlungsprodukt aus 7-Dehydrocholesterin. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 241, 100–103. doi: 10.1515/bchm2.1936.241.1-3.100

Zadshir, A., Tareen, N., Pan, D., Norris, K., and Martins, D. (2005). The prevalence of hypovitaminosis D among US adults: data from the NHANES III. *Ethn. Dis.* 15, S5-97–S95-101.

Zhao, Y., Sun, Y., Ji, H. F., and Shen, L. (2013). Vitamin D levels in Alzheimer's and Parkinson's diseases: a meta-analysis. *Nutrition* 29, 828–832. doi: 10.1016/j.nut.2012.11.018

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Impact of vitamin D on immune function: lessons learned from genome-wide analysis

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Immunomodulatory responses to the active form of vitamin D (1,25-dihydroxyvitamin D, 1,25D) have been recognized for many years, but it is only in the last 5 years that the potential role of this in normal human immune function has been recognized. Genome-wide analyses have played a pivotal role in redefining our perspective on vitamin D and immunity. The description of increased vitamin D receptor (VDR) and 1α-hydroxylase (CYP27B1) expression in macrophages following a pathogen challenge, has underlined the importance of intracrine vitamin D as key mediator of innate immune function. It is now clear that both macrophages and dendritic cells (DCs) are able to respond to 25-hydroxyvitamin D (25D), the major circulating vitamin D metabolite, thereby providing a link between the function of these cells and the variations in vitamin D status common to many humans. The identification of hundreds of primary 1,25D target genes in immune cells has also provided new insight into the role of vitamin D in the adaptive immune system, such as the modulation of antigen-presentation and T cells proliferation and phenotype, with the over-arching effects being to suppress inflammation and promote immune tolerance. In macrophages 1,25D promotes antimicrobial responses through the induction of antibacterial proteins, and stimulation of autophagy and autophagosome activity. In this way variations in 25D levels have the potential to influence both innate and adaptive immune responses. More recent genome-wide analyses have highlighted how cytokine signaling pathways can influence the intracrine vitamin D system and either enhance or abrogate responses to 25D. The current review will discuss the impact of intracrine vitamin D metabolism on both innate and adaptive immunity, whilst introducing the concept of disease-specific corruption of vitamin D metabolism and how this may alter the requirements for vitamin D in maintaining a healthy immune system in humans.

Keywords: macrophage, dendritic cell, intracrine, antigen-presentation, antibacterial, CYP27B1, VDR

INTRODUCTION

Amongst the many reported extra-skeletal effects of vitamin D, its ability to regulate immunity through effects on both the innate and adaptive systems has received considerable attention. This stems in part from homage to studies carried out more than a century ago by a then relatively unknown scientist, Dr. Nils Finsen. In 1903 Dr. Finsen won the Nobel Prize for Medicine or Physiology for showing that he could cure the epidermal form of tuberculosis (TB), lupus vulgaris, using concentrated light irradiation (Moller et al., 2005). The subsequent discovery that exposure to ultra-violet light promotes epidermal synthesis of vitamin D led to further studies describing the successful use of oral vitamin D supplementation to treat lupus vulgaris, and other mycobacterial infections such as leprosy (Airey, 1946; Herrera, 1949). The advent of antibiotic therapies for infectious diseases appeared to have consigned these studies to the history books. However, in 2006 the work of Finsen returned to center stage as a consequence of a series of genome-wide analyses that revealed pathogen-induction of an intracrine vitamin D system in monocytes (Liu et al., 2006), and an associated mechanism for anti-mycobacterial actions of vitamin D (Wang et al., 2004), whilst also shedding light on how these responses may vary according to the vitamin D "status" of any given individual. With increasing awareness of vitamin D-deficiency across the globe (Holick, 2007), and ongoing discussions concerning the physiological and clinical relevance of this (Holick et al., 2011; Ross et al., 2011), these genome-wide analyses have played a pivotal role in defining our new perspective on non-classical vitamin D physiology. The current review will detail these developments and how they have helped to define a role for vitamin D in normal immune function.

ANTIBACTERIAL RESPONSES TO VITAMIN D

Despite its early use in the treatment of mycobacterial diseases such as TB and leprosy (Airey, 1946; Herrera, 1949), the immunomodulatory actions of vitamin D did not become clear until much later. Elucidation of this important non-classical action of vitamin D stemmed from two key observations. Firstly, most proliferating cells within the immune system express the nuclear receptor for active 1,25-dihydroxyvitamin D (1,25D)—the vitamin D receptor (VDR). Initial studies focused on 1,25D

binding capacity in cells from the adaptive immune system such as T and B lymphocytes (T and B cells) (Bhalla et al., 1983; Provvedini et al., 1983), with subsequent reports describing specific intracellular binding of 1,25D in cells from the innate immune system such as monocytes/macrophages (Kreutz et al., 1993), dendritic cells (DC) (Brennan et al., 1987), neutrophils (Takahashi et al., 2002), and monocytic cell lines (Mangelsdorf et al., 1984). The functional significance of these data was not immediately clear but, nevertheless, it was assumed that VDR-expressing immune cells were able to respond the circulating active 1,25D in a similar fashion to classical vitamin D target tissues such as the intestine, kidney, and bone. However, this assumption was challenged by the second major observation linking vitamin D and the immunity, namely the discovery of active vitamin D metabolism by cells from the immune system.

Elevated serum levels of 1,25D reported for some patients with the granulomatous disease sarcoidosis were shown to be due to conversion of pro-hormone 25D to 1,25D by tissue and systemic macrophages in these patients (Barbour et al., 1981; Adams et al., 1983). Similar observations for other inflammatory and granulomatous diseases (Kallas et al., 2010) suggested that immune activity of the enzyme that catalyzes metabolism of 25D to 1,25D, 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) was a disease-related phenomenon. However, other studies, *in vitro*, highlighting the potential for macrophage 1α-hydroxylase activity in the absence of disease (Koeffler et al., 1985; Reichel et al., 1986) supported the exciting possibility that synthesis of 1,25D is part of normal immune function. Despite this, it was another 20 years before evidence to support this proposal was reported. Significantly, the major advances that provided this evidence involved genome-wide strategies that explored both the regulation and function of vitamin D by immune cells.

The first of the genome-wide studies to shed light on extra-skeletal actions of vitamin D was published by John White and colleagues at McGill University in Montreal and utilized a combination of DNA array and *in silico* strategies. In this report, DNA array analysis of 1,25D-regulated genes in squamous cell carcinoma cells *in vitro* (Akutsu et al., 2001; Lin et al., 2002) was combined with *in silico* analysis of genomic VDR binding sites to provide a comprehensive overview of potential 1,25D-VDR target genes (Wang et al., 2005). Genome-wide analysis of DNA sequences that are able to bind liganded VDR revealed consensus vitamin D response elements (VDRE) within the gene promoters for two antibacterial proteins, cathelicidin (*CAMP*) and β-defensin 2 (*DEFB4*) (Wang et al., 2005). Interestingly, although both of these genes exhibited classical proximal promoter direct-repeat 3 (DR3) consensus VDREs, only *CAMP* appeared to be transcriptionally induced by 1,25D in monocytes (Wang et al., 2005). The underlying mechanism for the differential regulation of monocyte *CAMP* and *DEFB4* by 1,25D was elucidated in subsequent studies, the first of which described increased expression of monocyte *DEFB4* following co-treatment with 1,25D and the inflammatory cytokine interleukin-1 (IL-1) (Liu et al., 2009). Based on these observations and promoter analysis for the *CAMP* and *DEFB4* genes, it was concluded that transcriptional induction of *DEFB4* requires cooperative occupancy of nuclear factor-κB (NF-κB) response elements as well as VDRE within the *DEFB4* gene promoter. By contrast, induction of *CAMP* appears to be primarily dependent on binding of VDR to promoter VDRE (Liu et al., 2009). The importance of NF-κB and VDR as co-inducers of *DEFB4* transcription was further emphasized by studies of the intracellular pathogen sensing protein NOD2 which is itself transcriptionally induced by 1,25D (Wang et al., 2010b). Cells co-treated with 1,25D and the ligand for NOD2, muramyl dipeptide (MDP), a cell wall product of Gram-positive and Gram-negative bacteria, showed potent NF-κB-dependent induction of *DEFB4* (Wang et al., 2010b). In these studies expression of *CAMP* was also enhanced by 1,25D-MDP co-treatment, suggesting that NF-κB may cooperate with VDR in a variety of immunomodulatory functions (**Figure 1**).

Subsequent functional studies confirmed that dose-dependent vitamin D induction of *CAMP* transcription involves direct interaction between liganded VDR and VDRE in the *CAMP* gene promoter (Wang et al., 2004; Gombart et al., 2005). Intriguingly, the VDRE initially identified within the *CAMP* promoter appear to be specific for subhuman and human primates, as there are no similar motifs within equivalent genes for lower mammals (Gombart et al., 2005). Acquisition of a VDRE by the *CAMP* gene appears to have occurred following the introduction of an

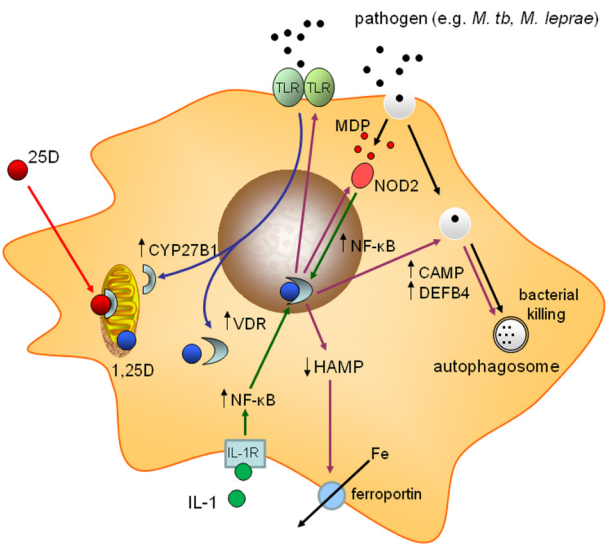

FIGURE 1 | Mechanisms for induction of vitamin D-mediated antibacterial responses in monocytes. Schematic representation of monocyte/macrophage responses to infection with a pathogen such as *Mycobacterium tuberculosis* (*M. tb*). Pattern recognition receptors (TLR2/1) sense *M. tb* and signal to induce expression of 1α-hydroxylase (CYP27B1) and the vitamin D receptor (VDR). The resulting intracrine system for vitamin D (blue arrows) converts 25-hydroxyvitamin D (25D) to 1,25-dihydroxyvitamin D (1,25D), which then binds to VDR and promotes transcriptional regulation. Prominent responses to intracrine activation of vitamin D (pink arrows) include: induction of antibacterial cathelicidin (CAMP) and β-defensin 2 (DEFB4); suppression of iron-regulatory hepcidin (HAMP); promotion of autophagy; induction of NOD2 expression; feedback regulation of toll-like receptor (TLR) expression; increased bacterial killing. For some responses (e.g., induction of DEFB4) accessory immune signals (MDP binding to NOD2, and IL-1 responsiveness) cooperate with intracrine vitamin D via nuclear factor-κB (NF-κB) (green arrows).

Alu short interspersed nuclear element (SINE) that placed *CAMP* under the control of 1,25D-VDR (Gombart et al., 2009b). This primate-specific adaptation has been conserved in humans and apes as well as Old World and New World primates, suggesting that a mechanism for transcriptional regulation of *CAMP* by vitamin D confers biological advantages. The assumption is that this mechanism will be potently activated by the relatively high circulating levels of 25D and 1,25D that are characteristic of non-human primates (Adams et al., 1985). A similar mechanism would also have been advantageous in early *Homo sapiens* whose existence is likely to have been defined by routine exposure to ultra-violet light and increased cutaneous vitamin D production, with associated high circulating levels of 25D. Conversely, in modern man where serum 25D status is more variable, this antibacterial mechanism may be less effective. Whilst this hypothetical mechanism broadly supports beneficial innate immune effects of vitamin D supplementation, the important question still remaining is how variations in serum levels of inactive 25D are able to influence immune responses driven by intracellular 1,25D and VDR. The answer to this question has been pivotal to our perspective on the non-classical actions of vitamin D and was, again, provided by genome-wide analyses.

For cells from both within and outside the classical immune system, recognition of and response to pathogens involves surveillance of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR). Prominent amongst these PRRs is the extended family of Toll-like receptor (TLR) non-catalytic transmembrane receptors which interact with specific PAMPs (Moresco et al., 2011). To clarify the role of the TLR2/1 heterodimer in mediating innate immune responses to the TB pathogen, *Mycobacterium tuberculosis* (*M. tb*), Liu and colleagues carried out DNA array analysis of gene expression in human macrophages and DCs following treatment with one of the putative PRRs for *M. tb* (Liu et al., 2006). Amongst the many macrophage genes shown to be differentially regulated by 19 kDa lipoprotein (a TLR2-interacting PAMP), expression of both *CYP27B1* and *VDR* was increased (Liu et al., 2006) (see **Figure 1**). This genome-wide approach provided the first unbiased evidence of a role for vitamin D metabolism and signaling in innate immune responses to a pathogen. Crucially, the TLR2/1-stimulus induced expression of both *CYP27B1* and *VDR*, suggesting that macrophage responses to *M. tb* involve an endogenous, intracrine, vitamin D system. Further studies showed that macrophages co-treated with the TLR2/1-ligand, 19 kDa lipoprotein were responsive to both active 1,25D and inactive 25D, confirming the functional efficacy of the intracrine model. Moreover, inhibition of either 1α-hydroxylase activity, or VDR function blocked the actions of 25D, underlining the importance of cell-specific metabolism as a determinant of vitamin D immunoregulation.

The specific functional read-outs used in the TLR2/1-*M.tb* study were induction of mRNA for the vitamin D catabolic enzyme 24-hydroxylase (*CYP24A1)* and the antibacterial protein *CAMP* (Liu et al., 2006). Parallel analysis of the other antibacterial target gene for 1,25D, *DEFB4,* did not reveal significant induction of this gene in the presence of 19 kDa lipoprotein and 25D. However, as outlined above, subsequent experiments demonstrated that co-treatment with either IL-1 (Liu et al., 2009), or the NOD2 ligand MDP (Wang et al., 2010b) cooperates with the TLR2/1 ligand and 25D to stimulate expression of *DEFB4*. Antibacterial proteins such as CAMP and DEFB4 play a crucial role in vitamin D-mediated killing of intracellular bacteria. Monocytes treated with increasing concentrations of CAMP peptide show a dose-dependent decrease in the viability of internalized *M. tb* (Liu et al., 2006); a similar inhibition of macrophage *M. tb* viability occurs in the presence of 25D, with this effect being abrogated by a VDR antagonist. In other studies knockdown of either *CAMP* or *DEFB4* decreased killing of *M. tb* in macrophages, suggesting that both antibacterial proteins are important in mediating vitamin D-induced responses to mycobacterial infection (Liu et al., 2009). Both CAMP and DEFB4 are detectable in the circulation where they are able to support innate immune responses to extra-cellular pathogens including anti-viral responses (Barlow et al., 2011; Tripathi et al., 2013). However, pathogens such as mycobacteria are internalized by phagocytosis, and bacterial killing can then take place following fusion of the resulting phagosome with a lysosome to form a phagolysosome. To evade this antibacterial process and maintain intracellular viability *M. tb* can subvert the transition of phagosomes to phagolysosomes (Vergne et al., 2004). In this situation, the host cell can restore pathogen encapsulation by evoking alternative mechanisms such as autophagy, in which encapsulation of organelles, cell proteins or intracellular pathogens in a double-membrane autophagosome occurs prior to fusion with lysosomes (Gutierrez et al., 2004). Although a well-recognized feature of eukaryotic cells cytosolic homeostasis (Klionsky and Emr, 2000), autophagy also appears to play a pivotal role in cellular response to infection (Gutierrez et al., 2004; Deretic and Levine, 2009). The ability of 1,25D and its synthetic analogs to promote autophagy is well established (Hoyer-Hansen et al., 2005; Wang et al., 2008), but recent data suggest that induction of autophagy may be particularly important for vitamin D-induced antibacterial responses to *M. tb* infection (Yuk et al., 2009; Shin et al., 2011) (**Figure 1**). The precise mechanism for this is not clear and may involve inhibition of the mammalian target of rapamycin (mTOR) intracellular signaling system (O'Kelly et al., 2006; Lisse et al., 2011) with mTOR acting to suppress the induction of autophagy (Sanjuan et al., 2009). It has also been suggested that vitamin D-induced autophagy occurs via an indirect mechanism, in that RNA-interference (RNAi) knockdown of antibacterial *CAMP* was sufficient to abrogate 1,25D-induced autophagy in monocytes (Yuk et al., 2009). In common with effects on expression of antibacterial proteins, it was noted that monocyte autophagy following activation of TLR2/1 involves enhanced expression of VDR and CYP27B1 (Shin et al., 2011), further highlighting the importance of intracrine 25D metabolism and action in normal human innate immunity.

Intracrine synthesis of 1,25D has also been shown to regulate expression of another antibacterial protein, hepcidin antibacterial protein (HAMP) (Bacchetta et al., 2013b). However, in contrast to CAMP and DEFB4, the direct microbiocidal properties of HAMP appear to be relatively weak. Instead, the major function of HAMP appears to be suppression of the cell membrane protein ferroportin, the only known exporter of intracellular iron

(Ganz, 2011). This link between HAMP and ferroportin in cells such as enterocytes, hepatocytes and monocytes plays a key role in the so-called anemia of infection or chronic disease (Ganz, 2009). Because pathogens such as bacteria utilize iron to maintain growth, restriction of circulating iron concentrations provides an important host response to systemic infection (Drakesmith and Prentice, 2012). However, for pathogens such as *M. tb* that attempt to evade immune surveillance at the intracellular level, accumulation of iron within this environment may promote the growth of internalized pathogens such as *Salmonella typhimurium* (Nairz et al., 2007), *M. tb* (Schaible et al., 2002; Sow et al., 2007, 2009), and *Chlamydia psittaci* (Paradkar et al., 2008). Conversely, innate immune and viral stimuli are known to stimulate the expression of *HAMP* (Sow et al., 2009; Armitage et al., 2011). In this setting suppression, rather than induction, of HAMP by 25D and 1,25D may be beneficial by abrogating HAMP-induced suppression of ferroportin which, in turn, will favor iron export and lower intracellular concentrations of iron. In studies carried out by our group at UCLA, we have shown that 25D and 1,25D suppress transcription of *HAMP* in monocytes and hepatocytes, leading to increased membrane expression of ferroportin, and decreased expression of ferritin (a surrogate biomarker for intracellular iron concentrations) (Bacchetta et al., 2013b). Moreover, in contrast to CAMP and DEFB4, elevated serum 25D levels (but not 1,25D) following vitamin D supplementation of human subjects *in vivo* were associated with potent suppression of circulating concentrations of HAMP (Bacchetta et al., 2013b). It therefore appears that regulation of the HAMP-ferroportin axis is another key facet of vitamin D-mediated innate immune function, complimentary to its reported effects on antibacterial proteins (Liu et al., 2006; Adams and Hewison, 2008; Hewison, 2011), and autophagy (Yuk et al., 2009; Shin et al., 2011) (see **Figure 1**). However, it is important to recognize that the effect of vitamin D on serum levels of hepcidin may have additional consequences that are both positive (suppression of anemia) and negative (decreased hepcidin for systemic infection). This may be particularly important for patients with chronic kidney disease (CKD) who commonly present with impaired circulating levels of 25D and 1,25D, and who are at higher risk of infection. In CKD, low serum 25D has been shown to correlate inversely with anemia (Lac et al., 2010) and directly with blood hemoglobin levels (Kiss et al., 2011). These effects may be due to dysregulation of normal HAMP-ferroportin function under conditions of vitamin D-deficiency, further emphasizing the importance of vitamin D supplementation in these patients.

Vitamin D may also target other innate immunity mechanisms. For example, studies *in vitro* have shown that 1,25D promotes hyporesponsiveness to PAMPs by downregulating expression of TLR2 and TLR4 on monocytes (Sadeghi et al., 2006). In this way, vitamin D appears to promote feedback control pathways that limit antibacterial activity and other innate immune responses, thereby preventing potential inflammatory events that arise from an over-elaboration of immune responses, notably inflammatory T cell responses. Paradoxically, vitamin D can also promote responses that amplify innate immune function. Recent studies have described 1,25D-mediated induction of the triggering receptor on myeloid cells-1 (TREM-1) (Kim et al., 2013), a cell surface protein associated with cytokine and chemokine production (Bouchon et al., 2000) that can also act to amplify TLR signaling (Bouchon et al., 2001). The biological significance of this is still not clear and this mechanism may be more important for cells such as neutrophils which are the principal source of circulating CAMP. Neutrophils express VDR but, unlike monocytes/macrophages, they do not appear to express a functional 1α-hydroxylase and are therefore not subject to intracrine activation of innate immune function. In this setting, activation of proteins such as TREM-1 may help to promote neutrophil responses to circulating 1,25D rather than 25D through enhanced TLR-signaling. This, in turn, would stimulate expression of VDR and sensitivity to 1,25D.

A similar cooperative TLR response has also been described for epithelial keratinocytes, where basal expression of *CYP27B1* is insufficient to facilitate intracrine induction of antibacterial proteins by serum 25D. However, following skin wounding, locally generated transforming growth factor β (TGFβ) enhances expression of CYP27B1 (Schauber et al., 2007). The resulting TGFβ-driven *CYP27B1* expression is then able to stimulate intracrine generation of antibacterial proteins such as CAMP to combat potential infections associated with epidermal injury (Schauber et al., 2007). Interestingly, the TGFβ-induced 1α-hydroxylase activity was also associated with increased keratinocyte expression of TLR2 which further enhances surveillance of infectious bacteria, but also suggests that the effects of vitamin D on TLR expression are likely to be cell-specific. TGFβ and 1,25D may also cooperate to promote expression of other pathways linked to enhanced innate immune responses to infection such as induction of the enzyme 5-lipoxygenase (5-LO) that catalyzes synthesis of leukotrienes. Expression of 5-LO in human monocytes is induced by both 1,25D and TGFβ (Harle et al., 1998), with 1,25D enhancing expression of 5-LO through novel promoter-independent VDRE within exons 10 and 12 and intron M of the 5-LO gene (Stoffers et al., 2010). Although commonly associated with bronchial dilation and asthma, leukotrienes are also known to participate in leukocyte accumulation at sites of infection and phagocytosis of bacteria (Peters-Golden et al., 2005). Leukotrienes have also been shown to trigger the processing of antibacterial CAMP by neutophils (Wan et al., 2007).

Vitamin D-mediated innate immune responses may also be species-specific. VDR-mediated induction of *CAMP* and *DEFB4*, as well as suppression of *HAMP*, appears to be primate-specific; other mammals may therefore utilize alternative innate immunity targets for intracrine 1,25D. For example reactive oxygen species (ROS) can be bacteriocidal; previous studies have shown that macrophages infected with *M. tb* in the presence of 1,25D produce high levels of the superoxide anion ROS via the NADPH oxidase system (Sly et al., 2001). More recent studies have shown that another ROS, nitric oxide (NO), is produced by mouse macrophages as part of innate immune responses to infection, with bacteriocidal consequences (Kohchi et al., 2009). The NO pathway appears to play a pivotal role in mouse responses to *M. tb* infection (Chan et al., 1992), but its importance to human *M. tb* infection is less clear. Moreover, one study using 1,25D and mouse macrophages has reported decreased expression of the enzyme inducible nitric oxide synthase (iNOS) and its NO

product, suggesting that the link between vitamin D and NO in innate immune function is more complex than originally thought (Chang et al., 2004). Irrespective of the antibacterial mechanism that is utilized by animals such as mice, it is generally assumed that vitamin D-mediated induction of these responses will occur via the same intracrine monocyte mechanism that has been described for humans. Although expression of CYP27B1 and 1α-hydroxylase activity has been described for murine macrophages *in vitro* (Esteban et al., 2004; Stoffels et al., 2007), the relative importance of this *in vivo* is still unclear. Indeed recent studies using the CYP27B1 KO mouse have suggested that $CD8^+$ cytotoxic T cells are the predominant source of extra-renal 1,25D within the murine immune system (Ooi et al., 2014). Further studies are required to fully clarify the physiological importance of this observation.

Genome-wide analyses and associated *ex vivo* and *in vitro* experiments have clearly demonstrated the potential importance of vitamin D in maintaining optimal innate antibacterial responses in humans. However, these studies have also prompted three further crucially important questions: (1) how important is vitamin D for the adaptive immune system? (2) can vitamin D supplementation *in vivo* enhance these antibacterial responses? (3) what happens to the vitamin D system in human immune diseases? Each of these questions will be considered in the remaining sections of this review.

VITAMIN D AND ANTIGEN PRESENTATION

In the seminal DNA array analysis of monocyte TLR2/1 responses by Liu et al that highlighted induction of *CYP27B1* and *VDR* by *M. tb*, it was notable that DCs did not produce the same response when challenged with 19 kDa lipoprotein, despite expressing TLR2/1 (Liu et al., 2006). Monocytes/macrophages belong to the same hematopoietic lineage as DCs, and both types of cells are able to act as antigen-presenting cells (APCs) to promote T cell and B cells responses. Furthermore like, monocytes, DCs express *VDR* and *CYP27B1*, and exhibit an active intracrine vitamin D system (Brennan et al., 1987; Fritsche et al., 2003; Hewison et al., 2003). However, in contrast to monocytes/macrophages, the primary function on intracrine vitamin D in DCs appears to be as a regulator of cell maturation, and ability of DCs to present antigen to T cells (Hewison et al., 2003). Differentiation of DCs toward a mature APC is associated with increased expression of *CYP27B1* but, paradoxically, a reciprocal decrease in *VDR* (Hewison et al., 2003). It therefore seems likely that DCs will utilize a paracrine vitamin D system, with immature DCs expressing VDR and responding to 1,25D produced by mature DCs with lower VDR expression. Such a mechanism may be biologically advantageous in that it allows some DCs to mature and promote T cell activation as part of normal adaptive immune responses, whilst preventing an over-elaboration of this response that could lead to inflammatory complications. A similar pattern of differential regulation of CYP27B1 and VDR has also been described for monocytes differentiating toward macrophages (Kreutz et al., 1993). The importance of 1,25D as a modulator of DC function is endorsed by studies of *VDR* and *CYP27B1* knockout mice, which present with lymphatic abnormalities consistent with increased numbers of mature DCs (Griffin et al., 2001; Panda et al., 2001) and dysregulated DC trafficking (Enioutina et al., 2009).

In a similar fashion to macrophages, DCs can be divided into distinct sub-types, specifically myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). These cells exhibit different cytokine and chemokine profiles and exert complementary effects on T cells; mDCs are efficient promoters of naïve T cell function (Liu, 2005), whilst pDCs are more closely associated with attenuation of T cell function (Steinman et al., 2003). *In vitro*, 1,25D preferentially regulates mDCs, with associated suppression of naïve T cell activation (Penna et al., 2007). However mDC and pDC express similar levels of VDR, so tolerogenic pDC may also respond to 1,25D, possibly via local, intracrine mechanisms (Penna et al., 2007). Alternatively, 1,25D generated by pDCs may not act to regulate pDC maturation but may, instead, act in a paracrine fashion on VDR-expressing T-cells. The ability of vitamin D to influence the differentiation and function of DCs provides another layer of innate immune function that complements its antibacterial properties. However, this interaction between 1,25D and DC will also have downstream effects on cells that interact with APCs, namely cells from the adaptive immune system.

Consistent with the DNA array analyses that shed light on the antibacterial function of vitamin D in monocytes and macrophages (Liu et al., 2006), genome-wide analysis of DCs has revealed diverse responses to vitamin D in these cells. Proteomic analyses using matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF strategies has defined the key proteins associated with tolerogenic responses to 1,25D (Ferreira et al., 2012). Intriguingly, the dominant effect of 1,25D treatment of monocyte-derived DCs described in this study was the alteration of proteins associated with the cytoskeleton and metabolic function. The induction of cytoskeletal proteins was shown to be consistent with DC responses to other tolerogenic steroid hormones, such as glucococorticoids (Ferreira et al., 2012). However, by contrast, the potent effects of 1,25D on metabolic pathways in DCs appear to be distinct from the effects of glucocorticoids. In particular, 1,25D induced significantly more proteins associated with carbohydrate metabolism, gluconeogenesis and the TCA cycle relative to the glucocorticoid dexamethasone, whilst 1,25D and dexamethasone shared induction of other groups of proteins such as those associated with glycolysis (Ferreira et al., 2012). This particular study also illustrates a key advantage of genome/proteome-wide analyses, which is the ability to group changes in gene/protein expression according to specific properties such as metabolism or cytoskeletal function. Moreover, more recent developments allow researchers to utilize tools such as Ingenuity Pathway Analysis (IPA) or DAVID to cluster altered genes and show protein or gene interaction networks (Hong et al., 2009). These "interactomes" provide a picture of the cooperativity of responses to a particular cell treatment. For example, recent proteomic analysis of responses to the synthetic 1,25D analog TX527 in immature and mature DCs showed that 65–75% of the proteins identified as TX527 responsive made up a statistically significant interactome, with some commonality between the two DC types (Ferreira et al., 2009). In this particular study the authors used multiple sets of data including the Biomolecular Interaction Network Database (BIND) (Bader et al.,

2003), and the Molecular Interaction Database (MINT) (Zanzoni et al., 2002) to maximize potential interactions. The increasing availability of gene/protein expression databases for different cell types means that this type of strategy is likely to become a more prominent feature of genome-wide expression analyses in the future.

VITAMIN D AND ADAPTIVE IMMUNITY

As outlined above, one of the initial observations linking vitamin D with the immune system was the presence of VDR in activated lymphocytes (Bhalla et al., 1983; Provvedini et al., 1983). The development of lymphocytes takes place in the thymus with VDR being expressed in medullary thymocytes but not in the less mature cortical thymocytes (Ravid et al., 1984). However, once cells leave the thymus and enter the circulation as T or B cells VDR expression is lost until these cells are activated to proliferate by mitogens (Bhalla et al., 1983; Provvedini et al., 1983). Indeed, 1,25D is a potent inhibitor of T-cell proliferation, blocking the transition from early G1 phase to late G1 phase (Bhalla et al., 1984; Nunn et al., 1986), but having no effect on transition from Go (resting) to early G1 or from late G1 to S phase (Rigby et al., 1985). Studies using T cells isolated from lymphatic tissue have shown that expression of VDR and responsiveness to 1,25D is proportional to the rate of cell proliferation (Karmali et al., 1991). Although these early studies have highlighted a role for 1,25D as a regulator of T and B cell proliferation, it has become increasingly clear that the predominant effects of vitamin D on adaptive immune function involve the modulation of T cell phenotype.

T cells consists of several sub-groups including cytotoxic $CD8^+$ T cells, natural killer cells, γδ T cells, memory cells, $CD4^+$ helper T cells (Th cells), and regulatory T cells (Treg). The best characterized vitamin D responses have been described for Th cells, with 1,25D regulating T cell proliferation and cytokine production (Lemire et al., 1985). Activation of naïve Th cells by antigen and APCs generates pluripotent Th_0 cells which can then differentiate into further Th sub-groups based on distinct cytokine profiles. Two of these sub-groups, Th_1 (IL-2, IFNγ, tumor necrosis factor alpha) and Th_2 (IL-3, IL-4, IL-5, IL-10) T cells, respectively support cell-mediated and humoral immunity (Abbas et al., 1996; Romagnani, 2006). *In vitro* 1,25D inhibits expression of Th_1 cytokines (Lemire et al., 1995), whilst promoting Th_2 cytokines (Boonstra et al., 2001). More recently, other Th cell sub-groups have been identified, including interleukin-17 (IL-17)-secreting T-cells (Th_{17} cells) and these cells are also targets for vitamin D. In the autoimmune disease-susceptible non-obese diabetic (NOD) mouse treatment with 1,25D decreased expression of IL-17 (Penna et al., 2006). In a similar fashion, 1,25D suppression of murine retinal autoimmunity involves inhibition of Th17 activity (Tang et al., 2009).

In addition to its effects on Th cells, vitamin D may also act on $CD8^+$ cytotoxic T cells which express relatively high levels of VDR (Rigby et al., 1987; Provvedini and Manolagas, 1989; Veldman et al., 2000). As outlined above, $CD8^+$ cells in mice have also been reported to express the vitamin D-activating enzyme 1α-hydroxylase (Ooi et al., 2014). $CD8^+$ T cells are known to be involved in autoimmune disease such as multiple sclerosis (MS) (Babbe et al., 2000), but do not mediate the effects of 1,25D in suppressing the murine form of MS, experimental autoimmune encepholmyelitis (EAE) (Meehan and DeLuca, 2002). More recent studies have reported a link between vitamin D and a variant of $CD8^+$ T cells, CD8αα cells. Unlike $CD8^+$ T cells, CD8αα cells are not cytotoxic and may play a role in suppressing gastrointestinal inflammation (Cheroutre and Lambolez, 2008). VDR knockout mice exhibit decreased numbers of CD8αα cells (Yu et al., 2008), due to decreased T cell expression of the chemokine receptor CCR9 preventing T cell homing to the gastrointestinal tract. T cell homing defects provides a potential explanation for the increased colonic inflammation observed in VDR knockout mice when crossed with colitis disease-susceptible mice (Froicu et al., 2003). Vitamin D metabolites may also influence T cell homing in other tissues. In the skin, 1,25D stimulates expression of the chemokine receptor 10 (CCR10) which recognizes the chemokine CCL27 secreted by keratinocytes (Sigmundsdottir et al., 2007).

As well as acting as a modulator of Th cell phenotype and function, vitamin D can also influence adaptive immunity by promoting suppressor T cells known as regulatory T cells (Treg) (Barrat et al., 2002). The precise mechanism by which vitamin D regulates Tregs is still somewhat controversial. Initial studies *in vitro* suggested that the ability of 1,25D to promote $CD4^+$ $CD25^+$ Treg was due to indirect effects on antigen-presenting DCs, specifically suppression of DC maturation and increased expression of DC cytokines such as CCL22 (Penna et al., 2007). However, subsequent studies have also described direct effects of 1,25D on T cells to generate CTLA4-positive Treg (Jeffery et al., 2009). Significantly, these studies were focused on the use of active 1,25D as the immunomodulator, and it is only in more recent studies that the role of pro-hormone 25D in Treg development has been investigated (Jeffery et al., 2012). Data from this study demonstrated the ability of 25D to promote the generation of Treg through intracrine/paracrine effects on CYP27B1/VDR-expressing DCs. Notably, this report also highlighted the impact of vitamin D binding protein (DBP) on DC responses to 25D, and concluded that non-DBP-bound (free) 25D is the form of 25D that is biologically active for generation of Tregs (Jeffery et al., 2012). The importance of Treg as a facet of vitamin D immunomodulation is illustrated by various studies *in vivo*. In patients with CKD, systemic administration of 1,25D has been shown to increase numbers of circulating Treg (Ardalan et al., 2007). Conversely, in patients with MS, serum concentrations of 25D correlate with Treg activity (Royal et al., 2009; Smolders et al., 2009), underlining the importance of intracrine pathways in mediating effects of vitamin D on adaptive, as well as innate immunity. In mice, topical application of 1,25D (Gorman et al., 2007) or its synthetic analog calcipotriol (Ghoreishi et al., 2009) have been shown to increase numbers of Treg.

The effects of vitamin D on adaptive immunity have to date been very much focused on its ability to modulate T cell proliferation and phenotype. Nevertheless, early studies reported that 1,25D could also suppress the development of immunoglobulin (Ig)-secreting B cells following mitogenic stimulation (Shiozawa et al., 1985; Iho et al., 1986). Initial experiments suggested that the most likely mechanism for this was an indirect effect through inhibition of Th cells (Lemire et al., 1985), but more recent work

has shown that 1,25D can suppress the differentiation of two types of B cell, plasma cells and class-switched memory cells, through apparent direct effects (Chen et al., 2007). Other reports have shown that 1,25D can regulate B cell IL-10 (Heine et al., 2008) and CCR10 (Shirakawa et al., 2008), suggesting that the effects of 1,25D on these cells is not restricted to their capacity to produce immunoglobulin.

Although genome-wide screening has played a pivotal role in identifying pivotal mechanisms for the interaction between vitamin D and innate immunity, the same cannot be said for vitamin D and adaptive immunity, where genome-wide analyses have complemented an already well-established field of research. Nevertheless, it is interesting to note reports where this strategy has been applied. In some cases these analyses have revealed a role for the vitamin D system, similar to the seminal studies of *M. tb* induction of CYP27B1 and VDR. For example, transcriptional profiling of γδT cells reported induction of VDR following activation of these cells with non-peptidic monoalkyl phosphate ligands (Chen et al., 2005). This small sub-set of T cells plays an important role in inflammatory diseases, and it was therefore speculated that 1,25D may act to suppress these cells as part of a more generalized anti-inflammatory response. Further array analyses have also identified VDR as one of a discrete number of genes involved in the formation of B cell germinal centers (Nakayama et al., 2006).

Array analyses have also been used to characterize the gene regulatory effects associated with immunomodulatory responses to 1,25D. These studies have focused primarily on the effects of 1,25D and its synthetic analogs on DCs, with results underlining the ability of 1,25D to promote decreased antigen presentation and a tolerogenic phenotype in these cells (Griffin et al., 2004; Shen and Zheng, 2004; Pedersen et al., 2009; Szeles et al., 2009). Notably, one of these array studies showed that the effects of 1,25D on DC gene expression were independent of DC differentiation status, suggesting a specific role for 1,25D as a regulator of DC function (Szeles et al., 2009). This particular study also reported that key changes in DC gene expression could be achieved using either 1,25D or 25D, further emphasizing the functional importance of the intracrine vitamin D system in these cells. DNA microarray analyses have also been used to assess 1,25D-mediated regulation of gene expression in $CD4^+$ Th cells following activation of these cells by phorbol myristate acetate and a calcium ionophore to induce VDR (Mahon et al., 2003). The diverse array of gene targets regulated by 1,25D in this particular array analysis suggests that 1,25D can influence Th cells both directly, as well as via effects on antigen-presenting DCs.

VITAMIN D STATUS AND IMMUNE FUNCTION

It is important to recognize that most of the genome-wide analyses that have explored the immunomodulatory effects of vitamin D *in vitro* have focused on treatments using active 1,25D or one of its synthetic analogs. However, as outlined above, pathogen-induction of an intracrine system in cells such as monocytes/macrophages strongly suggests that regulation of immunity *in vivo* is independent of endocrine, systemic 1,25D. Instead it is likely to be primarily driven by local activation of 25D, the major circulating form of vitamin D and determinant of vitamin D status in any given individual. Thus, it is not surprising that translational studies have focused on the relationship between serum 25D and human immune function, including effects on both innate and adaptive immunity.

Epidemiology has shown that vitamin D-insufficiency (serum 25D <30 ng/ml) is associated with increased risk of TB (Wilkinson et al., 2000; Ustianowski et al., 2005; Williams et al., 2008; Wejse et al., 2009). Several clinical trials of vitamin supplementation, as an adjunct to conventional antibiotic therapy, have also been reported with varying success (Nursyam et al., 2006; Martineau et al., 2007; Wejse et al., 2009). Supplementation using 4 × 100,000 IU vitamin D was successful in raising serum concentrations of 25D in TB patients, but this resulted in no overall improvement in sputum conversion time between vitamin D- and placebo-treated patients (Martineau et al., 2011). However, improved sputum conversion time was observed in a specific subset of TB patients with a *Taq1* single nucleotide polymorphism (SNP) within the VDR gene (Martineau et al., 2011), suggesting that genetic factors may influence immune responses to vitamin D supplementation. In a follow-up report to this TB vitamin D supplementation trial, it was shown that raised serum 25D was associated with improved resolution of TB disease (Coussens et al., 2012). Thus, in situations where infectious disease has already become established, it is possible that the role of 25D is primarily focused on anti-inflammatory adaptive immune responses. The link between vitamin D and infection is not restricted to TB. In patients with sepsis, circulating 25D levels have been shown to correlate directly with serum concentrations of CAMP, and inversely with critical illness in these patients (Jeng et al., 2009). Low serum 25D has also been linked to upper respiratory infections such as influenza (Cannell et al., 2006), and in patients with CKD low serum is associated with increased risk of infection and mortality (Gombart et al., 2009a).

To date, the application of genome-wide analyses to further elucidate the impact of serum vitamin D (25D) status on immune function has been limited. In a recent study by Holick and colleagues, array analysis of gene expression in peripheral blood mononuclear cells from vitamin D-sufficient (serum 25D >20 ng/ml, $n = 4$ subjects) and vitamin D-deficient (serum 25D <20 ng/ml, $n = 4$ subjects) revealed 66 differentially expressed genes (>1.5-fold change, $p < 0.01$) (Hossein-nezhad et al., 2013). However, after vitamin D supplementation (2000 IU vitamin D/day for 2 months), there was no significant change in the expression of these genes, even though serum 25D levels were increased in both sufficient and deficient subjects (Hossein-nezhad et al., 2013). Nevertheless, 291 additional genes were found to be differentially expressed in peripheral blood mononuclear cells following vitamin D supplementation (>1.5-fold, $p < 0.01$) (Hossein-nezhad et al., 2013). Similar array analyses carried out by our group using peripheral blood mononuclear cells from elderly vitamin D-deficient (18.8 ± 0.6 ng/ml serum 25D) and vitamin D–sufficient (58 ± 1.7 ng/ml serum 25D) patients, revealed 30 differentially regulated genes (**Figure 2**). These variations in gene expression occurred against a backdrop of no difference in serum 1,25D concentrations between vitamin D-sufficient and -deficient groups, underlining the importance of 25D, and the intracrine vitamin D system as regulators of immune cell

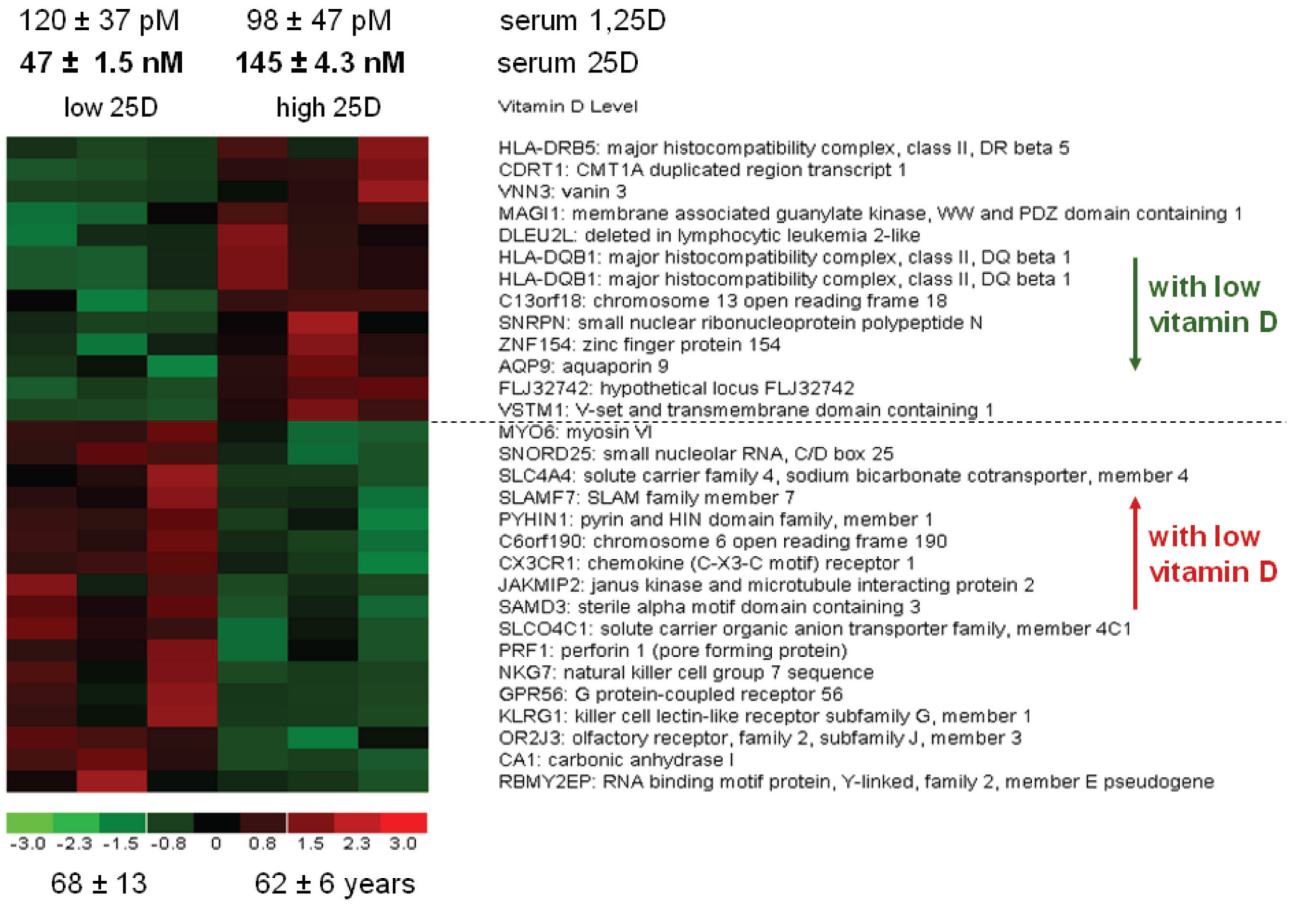

FIGURE 2 | Altered gene expression in leukocytes from vitamin D-deficient donors. Peripheral blood mononuclear cells (PBMC) were obtained from 6 healthy donors who had serum 25D levels that were either low (47 nM, 18.8 ng/ml) or high (145 nM, 58 ng/ml). After isolation of RNA and generation of cDNA, gene expression analyses in the PBMC samples was carried out by DNA array analyses (Affymetrix Exon Expr Chip.HuGene-1_0-st-v1). Data are shown as genes that were either increased (red) or decreased (green) in low 25D donors relative to high 25D donors (>1.5-fold change, $p < 0.05$, unpaired *t*-test).

function. In both this study and the Holick report, array analyses were carried out using mixed populations of systemic immune cells including both innate immunity APCs and lymphocytes of the adaptive immune system. The array analyses will therefore encapsulate both intracrine and paracrine activity of 25D, but will also reflect inherent donor to donor variations in immune cell composition.

Genome-wide analyses of immune responses to altered vitamin D status in mice are also very limited. Data from our group using colon tissue from vitamin D-deficient (serum 25D = 2.5 ± 0.1 ng/ml) vs. vitamin D-sufficient (serum 25D = 24.4 ± 1.8 ng/ml) identified 31 genes that were differentially expressed >2-fold ($p < 0.01$) (Lagishetty et al., 2010). Amongst these, vitamin D-deficient mice showed decreased expression of angiogenin-4 (Ang4), an antimicrobial protein which acts to minimize tissue invasion by enteric bacteria (Hooper et al., 2003). Further studies showed that decreased Ang4 in vitamin D-deficient mice was associated with increased levels of bacteria in the colon epithelium, consistent with compromised innate immune surveillance. Given that dysregulation of innate immune responses to enteric bacteria has been linked to the initiation of tissue inflammation associated with some types of inflammatory bowel disease (Packey and Sartor, 2009), it is possible that vitamin D plays a role in protecting against this disease via the induction of antibacterial Ang4.

Another genome-wide strategy with implications for vitamin D and the immune system, arose from studies aimed at determining the genetic component of vitamin D-deficiency. A recent Genome-Wide Association Study of almost 34,000 individuals showed that SNPs within the *DBP* gene are a key inherited determinant of low vitamin D status (serum 25D <75 nM or 30 ng/ml). Gene variations in *DBP* appear to act by influencing the serum concentrations of DBP protein (Lauridsen et al., 2001) which are known to be linked to serum levels of total 25D and 1,25D (Lauridsen et al., 2005; Wang et al., 2010a). Studies of other *DBP* SNPs suggest that genetic variants of *DBP* are linked to different binding affinities of 25D for DBP protein (Arnaud and Constans, 1993). Both the concentration and binding affinity of DBP protein are important for the serum transport of vitamin D metabolites (notably 25D which binds to DBP with a higher

affinity than 1,25D). However, DBP concentration and affinity also define the amount of 25D that is *not* bound to DBP. This "free" or "bioavailable" fraction of circulating 25D appears to be the form that accesses target cells such as monocytes (Chun et al., 2012), presumably via passive diffusion of lipid-soluble 25D through cell membranes—the so-called "free hormone hypothesis." Studies by our group have shown that antibacterial responses to 25D *in vitro* are more pronounced with low affinity forms of DBP that support higher levels of free 25D (Chun et al., 2010). Studies to date have been based on mathematical estimations of free 25D from total serum concentrations of 25D and DBP (Chun et al., 2012). However, future strategies using actual physical measurement of free 25D will greatly help to clarify the precise importance of total vs. free 25D in determining immune responses to vitamin D.

IMMUNE DISEASE AND THE DYSREGULATION OF VITAMIN D

Genome-wide strategies have played a pivotal role in elucidating the core mechanisms that trigger the intracrine vitamin D system and associated immune responses in cells such as monocytes/macrophages and DCs. Whether these studies have been carried out using freshly isolated preparations of immune cells, or cultured immune cells the resulting data have reflected the potential vitamin D-mediated responses that may occur following a pathogen challenge. What is less clear is how these responses function under conditions of actual human immune disease. An illuminating example of this strategy is provided by the disease leprosy which, like TB, involves a mycobacterial infection—in this case *Mycobacterium leprae* (*M. lep*) or *Mycobacterium lepromatis*. Similar to TB, vitamin D was at one time considered to be a putative therapy for leprosy (Herrera, 1949). However, unlike TB, leprosy can be divided into different disease sub-types, notably tuberculoid leprosy (T-lep) and lepromatous leprosy (L-lep). These two forms of leprosy have very different immune profiles and prognoses (Britton and Lockwood, 2004). DNA array analyses to define the gene expression profiles associated with the T-lep and L-lep forms of leprosy, highlighted elevated expression of CYP27B1, CYP24A1, and VDR in T-lep vs. L-lep lesions (Montoya et al., 2009). The over-arching conclusion from these studies is that the less aggressive form of leprosy, T-lep, is manifested by an intact vitamin D intracrine system that is able to support antibacterial responses to vitamin D. By contrast, L-lep, which is characterized by a high level of macrophage *M. lep* infection, and has a poor prognosis, exhibits an impaired vitamin D intracrine system. Thus, for patients with L-lep, successful elevation of serum 25D concentrations may be less effective in promoting intracrine-mediated regulation of antibacterial responses.

Several questions have arisen from the studies of vitamin D and leprosy. The first concerns the mechanism by which the vitamin D intracrine system is corrupted in L-lep patients. One possibility is that the T cell cytokine profiles that are characteristic of L-lep (e.g., increased IL-4, IL-10, and IFNα/β) exert a detrimental adjunct effect on the underlying TLR2/1-induced intracrine vitamin D system. At the same time, cytokine profiles associated with T-lep (e.g., increased IFNγ) may have more beneficial adjunct effects. Subsequent experiments *in vitro* support this hypothesis, with the Th1 cytokine IFNγ enhancing TLR2/1-induced vitamin D-activation and associated antibacterial activity (Edfeldt et al., 2010; Fabri et al., 2011). Conversely, the L-lep cytokines IL-4 (Edfeldt et al., 2010), IFNβ (Teles et al., 2013), and IL-10 (Teles et al., 2013) suppress antibacterial production (**Figure 3**). The effect of IFNβ appears to be mediated via IL-10 which acts to suppress expression of CYP27B1 (Teles et al., 2013), whereas IL-4 appears to act by stimulating activity of the vitamin D catabolic enzyme CYP24A1 (Edfeldt et al., 2010). The collective conclusion from these studies is that specific human diseases are characterized by T cell cytokines that act to either promote or corrupt the underlying pathogen-PRR-driven vitamin D intracrine system. Cytokine profiles such as this have also been described for active and inactive TB (Berry et al., 2010; Maertzdorf et al., 2012), providing an additional perspective on the varying success of vitamin D supplementation trials with this disease. Disease itself may therefore play a fundamental role in determining the efficacy of immunomodulatory vitamin D for any given patient; for example, it is possible that for diseases such as L-lep, higher levels of serum 25D will be required to achieve a specific antibacterial response. This is clearly an important topic for future research.

The differential regulation of the intracrine vitamin D pathway in T-lep and L-lep has also provided a platform for genome-wide analyses aimed at identifying factors other than T cell cytokines that may be involved in corrupting monocyte vitamin D responses. In a follow-up to the previous DNA array studies for

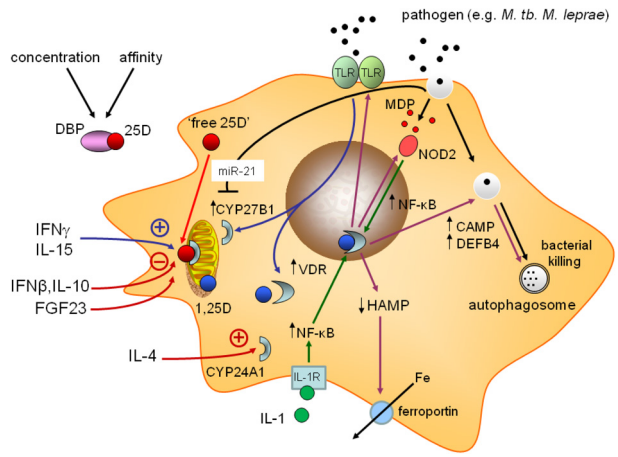

FIGURE 3 | Mechanisms for corruption of vitamin D-mediated antibacterial responses in monocytes. Schematic representation of monocyte/macrophage responses to infection with a pathogen such as *Mycobacterium tuberculosis* (*M. tb*), and associated adjunct signals. Positive effects on the vitamin D intracrine system include: induction of CYP27B1 expression by cytokines such as interferon γ (IFNγ) and interleukin-15 (IL-15). Negative effects include suppression of CYP27B1 by IL-10 and IFNβ, and induction of 24-hydroxylase activity by IL-4. Fibroblast growth factor 23 (FGF23) also suppresses CYP27B1 and microRNA-21 (miR-21) associated with some pathogen infections (e.g., *M. leprae*) can suppress expression of CYP27B1 by degradation of RNA and/or translation. The serum vitamin D binding protein (DBP) may also attenuate intracrine vitamin D by restricting monocyte bioavailability of free 25D.

leprosy, further array analysis of T-lep and L-lep tissues revealed distinct patterns of microRNA (miRNA) expression in these tissues (Liu et al., 2012). Recent studies have shown that miRNAs play a key role in fine-tuning gene expression by interacting with RNA to silence gene expression either by degrading transcripts, or by blocking their translation (Ketting, 2011). In L-lep, 16 miRNAs were found to be differentially induced relative to T-lep tissue, with miRNA-21 (miR-21) being the most prominent of these (Liu et al., 2012). In the context of innate immunity and leprosy, miR-21 may target several important mechanisms, including suppression of IL-1 expression which may, in turn, attenuate intracrine induction of antibacterial DEFB4 (see **Figure 3**). However, importantly, miR-21 is also predicted to interact with *CYP27B1* mRNA and suppress activity of 1α-hydroxylase and decrease localized synthesis of 1,25D. *In vitro*, siRNA knockdown of miR-21 in *M. lep* infected monocytes, restored CYP27B1 expression and 25D-mediated antibacterial responses (Liu et al., 2012). Despite these observations, relatively little is known about how miRNAs corrupt vitamin D signaling in disease situations. Based on genome-wide *in silico* analysis of miRNA target sequences, multiple miRNAs are predicted to influence the expression of proteins associated with vitamin D metabolism and signaling (reviewed in Lisse et al., 2013a). However, other than studies of miR-21, there have been few studies to validate the predicted effects of miRNAs on the vitamin D system. Analysis of ovarian granulosa and breast cancer cells has demonstrated increased expression of miR-125B in these tissue, and a concomitant dysregulation of two of its targets, *VDR* and *CYP24A1* mRNAs (Mohri et al., 2009). It seems likely that future studies will identify other miRNAs that modulate the vitamin D intracrine system in immune cells under disease conditions. Moreover, it is important to recognize that vitamin D itself is a potent regulator of miRNAs. To date, these studies have focused on cancer (Wang et al., 2009, 2011), and bone cells (Lisse et al., 2013b), but similar future studies of 1,25D-regulated non-coding RNAs in immune cells may provide an entirely new perspective on the immunomodulatory actions of vitamin D.

Some important questions about human disease and the immunomodulatory effects of vitamin D remain unanswered. For example, it is still not clear why there is aberrant synthesis of 1,25D by macrophages in granulomatous diseases (Kallas et al., 2010). It is also unclear what effect, if any, viral pathogens such as hepatitis C or HIV have on innate and adaptive immune actions of vitamin D, although HIV infection of some cells has been shown to suppress expression of *VDR* (Chandel et al., 2013). Future studies of vitamin D and the immune system may also explore non-traditional targets for immune regulation. For example, patients with end-stage kidney disease who routinely use dialysis are at high risk of infection and associated mortalities. These patients are also commonly vitamin D-deficient (Zehnder et al., 2007), and this may impair normal innate immune responses to infection. However, as with TB and leprosy, additional disease factors may act to further compromise the intracrine vitamin D system in these patients. Notably, circulating levels of fibroblast growth factor 23 (FGF23), which plays a key role in the endocrine regulation of phosphate homeostasis, are elevated very early in kidney disease (Danziger, 2008; Isakova et al., 2011). One of the important actions of FGF23 is to suppress renal production of 1,25D through the suppression of CYP27B1 expression (Shimada et al., 2004); in this way FGF23 acts as a counterpoint to parathyroid hormone which stimulates CYP27B1 and renal 1,25D production. Until recently, the effects of FGF23 were thought to be restricted to the mineral homeostasis endocrine system. However, work by our group has shown that FGF23 can also act on monocytes to suppress expression of CYP27B1 and the intracrine induction of antibacterial proteins (Bacchetta et al., 2013a). These data highlight a mechanism by which renal disease may compromise vitamin D-mediated immune function, similar to that observed for cytokines associated with infectious disease (see **Figure 3**). As well as providing an explanation for the increased risk of infection in kidney disease patients, these results also suggest a hitherto unrecognized link between the vitamin D endocrine system and its intracrine immune counterpart.

PERSPECTIVES

For many years, the link between vitamin D and the immune system was considered to be a non-classical response with only a pathophysiological relevance. The advent of genome-wide analyses has enabled a complete change in this perspective by providing an unbiased picture of how the vitamin D system is induced by pathogens, and how the resulting intracrine cellular machinery can promote both innate and adaptive immune responses to the pathogen. A key challenge going forward will be to relate these mechanisms to patient vitamin D status, and this is likely to herald a new wave of genome-wide analyses linked to placebo-controlled vitamin D supplementation trials. Interpretation of these studies is likely to be complex. Recent genome-wide analysis of patient tissues has shown that some immune diseases are characterized by corruption of the vitamin D system, so that conventional notions of vitamin D-sufficiency and vitamin D-deficiency may be very different for patients with specific diseases. A key objective for future studies will be to determine whether vitamin D-mediated-immune function is also applicable to mouse models, where genome-wide screening will help to identify immune targets that are related to, or distinct from, human data. Future studies will also need to better characterize disease corruption of vitamin D responses. Screening for disease-specific microRNAs will be particularly important to identify non-coding RNAs that can target components of the intracrine vitamin D. The current shift away from DNA array technology to RNAseq strategies will help to achieve these new objectives in a single genome-wide screen, at increasingly affordable prices.

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REFERENCES

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* 383, 787–793. doi: 10.1038/383787a0

Adams, J., Gacad, M. A., Baker, A. J., Gonzales, B., and Rude, R. K. (1985). Serum concentrations of 1,25-dihydroxyvitamin D3 in platyrrhini and catarrhini:a phylogenetic appraisal. *Am. J. Primatol.* 28, 263–270.

Adams, J. S., and Hewison, M. (2008). Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat. Clin. Pract. Endocrinol. Metab.* 4, 80–90. doi: 10.1038/ncpendmet0716

Adams, J. S., Sharma, O. P., Gacad, M. A., and Singer, F. R. (1983). Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* 72, 1856–1860. doi: 10.1172/JCI111147

Airey, F. S. (1946). Vitamin D as a remedy for lupus vulgaris. *Med. World* 64, 807–810.

Akutsu, N., Lin, R., Bastien, Y., Bestawros, A., Enepekides, D. J., Black, M. J., et al. (2001). Regulation of gene Expression by 1alpha,25-dihydroxyvitamin D3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. *Mol. Endocrinol.* 15, 1127–1139. doi: 10.1210/mend.15.7.0655

Ardalan, M. R., Maljaei, H., Shoja, M. M., Piri, A. R., Khosroshahi, H. T., Noshad, H., et al. (2007). Calcitriol started in the donor, expands the population of CD4+CD25+ T cells in renal transplant recipients. *Transplant. Proc.* 39, 951–953. doi: 10.1016/j.transproceed.2007.04.012

Armitage, A. E., Eddowes, L. A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T. A., et al. (2011). Hepcidin regulation by innate immune and infectious stimuli. *Blood* 118, 4129–4139. doi: 10.1182/blood-2011-04-351957

Arnaud, J., and Constans, J. (1993). Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). *Hum. Genet.* 92, 183–188. doi: 10.1007/BF00219689

Babbe, H., Roers, A., Waisman, A., Lassmann, H., Goebels, N., Hohlfeld, R., et al. (2000). Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J. Exp. Med.* 192, 393–404. doi: 10.1084/jem.192.3.393

Bacchetta, J., Sea, J. L., Chun, R. F., Lisse, T. S., Wesseling-Perry, K., Gales, B., et al. (2013a). Fibroblast growth factor 23 inhibits extrarenal synthesis of 1,25-dihydroxyvitamin D in human monocytes. *J. Bone Miner. Res.* 28, 46–55. doi: 10.1002/jbmr.1740

Bacchetta, J. Z. J., Sea, J. L., Chun, R. F., Lisse, T. S., Zavala, K., Nayak, A., et al. (2013b). Suppression of iron-regulatory hepcidin by vitamin D. *J. Am. Soc. Nephrol.* 25, 564–572. doi: 10.1681/ASN.2013040355

Bader, G. D., Betel, D., and Hogue, C. W. (2003). BIND: the biomolecular interaction network database. *Nucleic Acids Res.* 31, 248–250. doi: 10.1093/nar/gkg056

Barbour, G. L., Coburn, J. W., Slatopolsky, E., Norman, A. W., and Horst, R. L. (1981). Hypercalcemia in an anephric patient with sarcoidosis: evidence for extrarenal generation of 1,25-dihydroxyvitamin D. *N. Engl. J. Med.* 305, 440–443. doi: 10.1056/NEJM198108203050807

Barlow, P. G., Svoboda, P., Mackellar, A., Nash, A. A., York, I. A., Pohl, J., et al. (2011). Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE* 6:e25333. doi: 10.1371/journal.pone.0025333

Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., et al. (2002). *In vitro* generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195, 603–616. doi: 10.1084/jem.20011629

Berry, M. P., Graham, C. M., McNab, F. W., Xu, Z., Bloch, S. A., Oni, T., et al. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 466, 973–977. doi: 10.1038/nature09247

Bhalla, A. K., Amento, E. P., Clemens, T. L., Holick, M. F., and Krane, S. M. (1983). Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J. Clin. Endocrinol. Metab.* 57, 1308–1310. doi: 10.1210/jcem-57-6-1308

Bhalla, A. K., Amento, E. P., Serog, B., and Glimcher, L. H. (1984). 1,25-Dihydroxyvitamin D3 inhibits antigen-induced T cell activation. *J. Immunol.* 133, 1748–1754.

Boonstra, A., Barrat, F. J., Crain, C., Heath, V. L., Savelkoul, H. F., and O'Garra, A. (2001). 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J. Immunol.* 167, 4974–4980.

Bouchon, A., Dietrich, J., and Colonna, M. (2000). Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164, 4991–4995.

Bouchon, A., Facchetti, F., Weigand, M. A., and Colonna, M. (2001). TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107. doi: 10.1038/35074114

Brennan, A., Katz, D. R., Nunn, J. D., Barker, S., Hewison, M., Fraher, L. J., et al. (1987). Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D3 metabolite, dihydroxycholecalciferol. *Immunology* 61, 457–461.

Britton, W. J., and Lockwood, D. N. (2004). Leprosy. *Lancet* 363, 1209–1219. doi: 10.1016/S0140-6736(04)15952-7

Cannell, J. J., Vieth, R., Umhau, J. C., Holick, M. F., Grant, W. B., Madronich, S., et al. (2006). Epidemic influenza and vitamin D. *Epidemiol. Infect.* 134, 1129–1140. doi: 10.1017/S0950268806007175

Chan, J., Xing, Y., Magliozzo, R. S., and Bloom, B. R. (1992). Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J. Exp. Med.* 175, 1111–1122. doi: 10.1084/jem.175.4.1111

Chandel, N., Sharma, B., Husain, M., Salhan, D., Singh, T., Rai, P., et al. (2013). HIV compromises integrity of the podocyte actin cytoskeleton through downregulation of the vitamin D receptor. *Am. J. Physiol. Renal Physiol.* 304, F1347–F1357. doi: 10.1152/ajprenal.00717.2012

Chang, J. M., Kuo, M. C., Kuo, H. T., Hwang, S. J., Tsai, J. C., Chen, H. C., et al. (2004). 1-alpha,25-Dihydroxyvitamin D3 regulates inducible nitric oxide synthase messenger RNA expression and nitric oxide release in macrophage-like RAW 264.7 cells. *J. Lab. Clin. Med.* 143, 14–22. doi: 10.1016/j.lab.2003.08.002

Chen, L., Cencioni, M. T., Angelini, D. F., Borsellino, G., Battistini, L., and Brosnan, C. F. (2005). Transcriptional profiling of gamma delta T cells identifies a role for vitamin D in the immunoregulation of the V gamma 9V delta 2 response to phosphate-containing ligands. *J. Immunol.* 174, 6144–6152.

Chen, S., Sims, G. P., Chen, X. X., Gu, Y. Y., Chen, S., and Lipsky, P. E. (2007). Modulatory effects of 1,25-dihydroxyvitamin d3 on human B cell differentiation. *J. Immunol.* 179, 1634–1647.

Cheroutre, H., and Lambolez, F. (2008). Doubting the TCR coreceptor function of CD8alphaalpha. *Immunity* 28, 149–159. doi: 10.1016/j.immuni.2008.01.005

Chun, R. F., Lauridsen, A. L., Suon, L., Zella, L. A., Pike, J. W., Modlin, R. L., et al. (2010). Vitamin D- binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D. *J. Clin. Endocrinol. Metab.* 95, 3368–3376. doi: 10.1210/jc.2010-0195

Chun, R. F., Peercy, B. E., Adams, J. S., and Hewison, M. (2012). Vitamin D binding protein and monocyte response to 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling. *PLoS ONE* 7:e30773. doi: 10.1371/journal.pone.0030773

Coussens, A. K., Wilkinson, R. J., Hanifa, Y., Nikolayevskyy, V., Elkington, P. T., Islam, K., et al. (2012). Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15449–15454. doi: 10.1073/pnas.1200072109

Danziger, J. (2008). The bone-renal axis in early chronic kidney disease: an emerging paradigm. *Nephrol. Dial. Transplant* 23, 2733–2737. doi: 10.1093/ndt/gfn260

Deretic, V., and Levine, B. (2009). Autophagy, immunity, and microbial adaptations. *Cell Host Microbe* 5, 527–549. doi: 10.1016/j.chom.2009.05.016

Drakesmith, H., and Prentice, A. M. (2012). Hepcidin and the iron-infection axis. *Science* 338, 768–772. doi: 10.1126/science.1224577

Edfeldt, K., Liu, P. T., Chun, R., Fabri, M., Schenk, M., Wheelwright, M., et al. (2010). T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22593–22598. doi: 10.1073/pnas.1011624108

Enioutina, E. Y., Bareyan, D., and Daynes, R. A. (2009). TLR-induced local metabolism of vitamin D3 plays an important role in the diversification of adaptive immune responses. *J. Immunol.* 182, 4296–4305. doi: 10.4049/jimmunol.0804344

Esteban, L., Vidal, M., and Dusso, A. (2004). 1alpha-Hydroxylase transactivation by gamma-interferon in murine macrophages requires enhanced C/EBPbeta expression and activation. *J. Steroid Biochem. Mol. Biol.* 89–90, 131–137. doi: 10.1016/j.jsbmb.2004.03.092

Fabri, M., Stenger, S., Shin, D. M., Yuk, J. M., Liu, P. T., Realegeno, S., et al. (2011). Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. *Sci. Transl. Med.* 3, 104ra102. doi: 10.1126/scitranslmed.3003045

Ferreira, G. B., Kleijwegt, F. S., Waelkens, E., Lage, K., Nikolic, T., Hansen, D. A., et al. (2012). Differential protein pathways in 1,25-dihydroxyvitamin d(3) and dexamethasone modulated tolerogenic human dendritic cells. *J. Proteome Res.* 11, 941–971. doi: 10.1021/pr200724e

Ferreira, G. B., van Etten, E., Lage, K., Hansen, D. A., Moreau, Y., Workman, C. T., et al. (2009). Proteome analysis demonstrates profound alterations in human dendritic cell nature by TX527, an analogue of vitamin D. *Proteomics* 9, 3752–3764. doi: 10.1002/pmic.200800848

Fritsche, J., Mondal, K., Ehrnsperger, A., Andreesen, R., and Kreutz, M. (2003). Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells. *Blood* 102, 3314–3316. doi: 10.1182/blood-2002-11-3521

Froicu, M., Weaver, V., Wynn, T. A., McDowell, M. A., Welsh, J. E., and Cantorna, M. T. (2003). A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases. *Mol. Endocrinol.* 17, 2386–2392. doi: 10.1210/me.2003-0281

Ganz, T. (2009). Iron in innate immunity: starve the invaders. *Curr. Opin. Immunol.* 21, 63–67. doi: 10.1016/j.coi.2009.01.011

Ganz, T. (2011). Hepcidin and iron regulation, 10 years later. *Blood* 117, 4425–4433. doi: 10.1182/blood-2011-01-258467

Ghoreishi, M., Bach, P., Obst, J., Komba, M., Fleet, J. C., and Dutz, J. P. (2009). Expansion of antigen-specific regulatory T cells with the topical vitamin d analog calcipotriol. *J. Immunol.* 182, 6071–6078. doi: 10.4049/jimmunol.0804064

Gombart, A. F., Bhan, I., Borregaard, N., Tamez, H., Camargo, C. A., Jr., Koeffler, H. P., et al. (2009a). Low plasma level of cathelicidin antimicrobial peptide (hCAP18) predicts increased infectious disease mortality in patients undergoing hemodialysis. *Clin. Infect. Dis.* 48, 418–424. doi: 10.1086/596314

Gombart, A. F., Borregaard, N., and Koeffler, H. P. (2005). Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* 19, 1067–1077. doi: 10.1096/fj.04-3284com

Gombart, A. F., Saito, T., and Koeffler, H. P. (2009b). Exaptation of an ancient Alu short interspersed element provides a highly conserved vitamin D- mediated innate immune response in humans and primates. *BMC Genomics* 10:321. doi: 10.1186/1471-2164-10-321

Gorman, S., Kuritzky, L. A., Judge, M. A., Dixon, K. M., McGlade, J. P., Mason, R. S., et al. (2007). Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. *J. Immunol.* 179, 6273–6283.

Griffin, M. D., Lutz, W., Phan, V. A., Bachman, L. A., McKean, D. J., and Kumar, R. (2001). Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6800–6805. doi: 10.1073/pnas.121172198

Griffin, M. D., Xing, N., and Kumar, R. (2004). Gene expression profiles in dendritic cells conditioned by 1alpha,25-dihydroxyvitamin D3 analog. *J. Steroid Biochem. Mol. Biol.* 89–90, 443–448. doi: 10.1016/j.jsbmb.2004.03.039

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 119, 753–766. doi: 10.1016/j.cell.2004.11.038

Harle, D., Radmark, O., Samuelsson, B., and Steinhilber, D. (1998). Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur. J. Biochem.* 254, 275–281. doi: 10.1046/j.1432-1327.1998.2540275.x

Heine, G., Niesner, U., Chang, H. D., Steinmeyer, A., Zugel, U., Zuberbier, T., et al. (2008). 1,25-dihydroxyvitamin D(3) promotes IL-10 production in human B cells. *Eur. J. Immunol.* 38, 2210–2218. doi: 10.1002/eji.200838216

Herrera, G. (1949). Vitamin D in massive doses as an adjuvant to the sulfones in the treatment of tuberculoid leprosy. *Int. J. Lepr.* 17, 35–42.

Hewison, M. (2011). Antibacterial effects of vitamin D. *Nat. Rev. Endocrinol.* 7, 337–345. doi: 10.1038/nrendo.2010.226

Hewison, M., Freeman, L., Hughes, S. V., Evans, K. N., Bland, R., Eliopoulos, A. G., et al. (2003). Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol.* 170, 5382–5390.

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., et al. (2011). Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J. Clin. Endocrinol. Metab.* 96, 1911–1930. doi: 10.1210/jc.2011-0385

Hong, M. G., Pawitan, Y., Magnusson, P. K., and Prince, J. A. (2009). Strategies and issues in the detection of pathway enrichment in genome-wide association studies. *Hum. Genet.* 126, 289–301. doi: 10.1007/s00439-009-0676-z

Hooper, L. V., Stappenbeck, T. S., Hong, C. V., and Gordon, J. I. (2003). Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat. Immunol.* 4, 269–273. doi: 10.1038/ni888

Hossein-nezhad, A., Spira, A., and Holick, M. F. (2013). Influence of vitamin D status and vitamin D3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. *PLoS ONE* 8:e58725. doi: 10.1371/journal.pone.0058725

Hoyer-Hansen, M., Bastholm, L., Mathiasen, I. S., Elling, F., and Jaattela, M. (2005). Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. *Cell Death Differ.* 12, 1297–1309. doi: 10.1038/sj.cdd.4401651

Iho, S., Takahashi, T., Kura, F., Sugiyama, H., and Hoshino, T. (1986). The effect of 1,25-dihydroxyvitamin D3 on *in vitro* immunoglobulin production in human B cells. *J. Immunol.* 136, 4427–4431.

Isakova, T., Wahl, P., Vargas, G. S., Gutierrez, O. M., Scialla, J., Xie, H., et al. (2011). Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int.* 79, 1370–1378. doi: 10.1038/ki.2011.47

Jeffery, L. E., Burke, F., Mura, M., Zheng, Y., Qureshi, O. S., Hewison, M., et al. (2009). 1,25-Dihydroxyvitamin D(3) and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J. Immunol.* 183, 5458–5467. doi: 10.4049/jimmunol.0803217

Jeffery, L. E., Wood, A. M., Qureshi, O. S., Hou, T. Z., Gardner, D., Briggs, Z., et al. (2012). Availability of 25-Hydroxyvitamin D3 to APCs controls the balance between regulatory and inflammatory T cell responses. *J. Immunol.* 189, 5155–5164. doi: 10.4049/jimmunol.1200786

Jeng, L., Yamshchikov, A. V., Judd, S. E., Blumberg, H. M., Martin, G. S., Ziegler, T. R., et al. (2009). Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. *J. Transl. Med.* 7, 28. doi: 10.1186/1479-5876-7-28

Kallas, M., Green, F., Hewison, M., White, C., and Kline, G. (2010). Rare causes of calcitriol-mediated hypercalcemia: a case report and literature review. *J. Clin. Endocrinol. Metab.* 95, 3111–3117. doi: 10.1210/jc.2009-2673

Karmali, R., Hewison, M., Rayment, N., Farrow, S. M., Brennan, A., Katz, D. R., et al. (1991). 1,25(OH)2D3 regulates c-myc mRNA levels in tonsillar T lymphocytes. *Immunology* 74, 589–593.

Ketting, R. F. (2011). microRNA biogenesis and function : an overview. *Adv. Exp. Med. Biol.* 700, 1–14. doi: 10.1007/978-1-4419-7823-3_1

Kim, T. H., Lee, B., Kwon, E., Choi, S. J., Lee, Y. H., Song, G. G., et al. (2013). Regulation of TREM-1 expression by 1,25-dihydroxyvitamin D3 in human monocytes/macrophages. *Immunol. Lett.* 154, 80–85. doi: 10.1016/j.imlet.2013.08.012

Kiss, Z., Ambrus, C., Almasi, C., Berta, K., Deak, G., Horonyi, P., et al. (2011). Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoietin resistance in patients on maintenance hemodialysis. *Nephron Clin. Pract.* 117, c373–c378. doi: 10.1159/000321521

Klionsky, D. J., and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* 290, 1717–1721. doi: 10.1126/science.290.5497.1717

Koeffler, H. P., Reichel, H., Bishop, J. E., and Norman, A. W. (1985). gamma-Interferon stimulates production of 1,25-dihydroxyvitamin D3 by normal human macrophages. *Biochem. Biophys. Res. Commun.* 127, 596–603. doi: 10.1016/S0006-291X(85)80202-3

Kohchi, C., Inagawa, H., Nishizawa, T., and Soma, G. (2009). ROS and innate immunity. *Anticancer Res.* 29, 817–821.

Kreutz, M., Andreesen, R., Krause, S. W., Szabo, A., Ritz, E., and Reichel, H. (1993). 1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. *Blood* 82, 1300–1307.

Lac, P. T., Choi, K., Liu, I. A., Meguerditchian, S., Rasgon, S. A., and Sim, J. J. (2010). The effects of changing vitamin D levels on anemia in chronic kidney disease patients: a retrospective cohort review. *Clin. Nephrol.* 74, 25–32. doi: 10.5414/CNP74025

Lagishetty, V., Misharin, A. V., Liu, N. Q., Lisse, T. S., Chun, R. F., Ouyang, Y., et al. (2010). Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* 151, 2423–2432. doi: 10.1210/en.2010-0089

Lauridsen, A. L., Vestergaard, P., Hermann, A. P., Brot, C., Heickendorff, L., Mosekilde, L., et al. (2005). Plasma concentrations of 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D are related to the phenotype of Gc (vitamin D-binding protein): a cross-sectional study on 595 early postmenopausal women. *Calcif. Tissue Int.* 77, 15–22. doi: 10.1007/s00223-004-0227-5

Lauridsen, A. L., Vestergaard, P., and Nexo, E. (2001). Mean serum concentration of vitamin D- binding protein (Gc globulin) is related to the Gc phenotype in women. *Clin. Chem.* 47, 753–756.

Lemire, J. M., Adams, J. S., Kermani-Arab, V., Bakke, A. C., Sakai, R., and Jordan, S. C. (1985). 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity *in vitro*. *J. Immunol.* 134, 3032–3035.

Lemire, J. M., Archer, D. C., Beck, L., and Spiegelberg, H. L. (1995). Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J. Nutr.* 125, 1704S–1708S.

Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., et al. (2002). Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. *Mol. Endocrinol.* 16, 1243–1256. doi: 10.1210/mend.16.6.0874

Lisse, T. S., Adams, J. S., and Hewison, M. (2013a). Vitamin D and MicroRNAs in Bone. *Crit. Rev. Eukaryot. Gene Expr.* 23, 195–214. doi: 10.1615/CritRevEukaryotGeneExpr.2013007147

Lisse, T. S., Chun, R. F., Rieger, S., Adams, J. S., and Hewison, M. (2013b). Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts. *J. Bone Miner. Res.* 28, 1478–1488. doi: 10.1002/jbmr.1882

Lisse, T. S., Liu, T., Irmler, M., Beckers, J., Chen, H., Adams, J. S., et al. (2011). Gene targeting by the vitamin D response element binding protein reveals a role for vitamin D in osteoblast mTOR signaling. *FASEB J.* 25, 937–947. doi: 10.1096/fj.10-172577

Liu, P. T., Schenk, M., Walker, V. P., Dempsey, P. W., Kanchanapoomi, M., Wheelwright, M., et al. (2009). Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS ONE* 4:e5810. doi: 10.1371/journal.pone.0005810

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., et al. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311, 1770–1773. doi: 10.1126/science.1123933

Liu, P. T., Wheelwright, M., Teles, R., Komisopoulou, E., Edfeldt, K., Ferguson, B., et al. (2012). MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy. *Nat. Med.* 18, 267–273. doi: 10.1038/nm.2584

Liu, Y. J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23, 275–306. doi: 10.1146/annurev.immunol.23.021704.115633

Maertzdorf, J., Weiner, J. 3rd., Mollenkopf, H. J., Network, T. B., Bauer, T., Prasse, A., et al. (2012). Common patterns and disease-related signatures in tuberculosis and sarcoidosis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7853–7858. doi: 10.1073/pnas.1121072109

Mahon, B. D., Wittke, A., Weaver, V., and Cantorna, M. T. (2003). The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells. *J. Cell. Biochem.* 89, 922–932. doi: 10.1002/jcb.10580

Mangelsdorf, D. J., Koeffler, H. P., Donaldson, C. A., Pike, J. W., and Haussler, M. R. (1984). 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *J. Cell Biol.* 98, 391–398. doi: 10.1083/jcb.98.2.391

Martineau, A. R., Timms, P. M., Bothamley, G. H., Hanifa, Y., Islam, K., Claxton, A. P., et al. (2011). High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377, 242–250. doi: 10.1016/S0140-6736(10)61889-2

Martineau, A. R., Wilkinson, R. J., Wilkinson, K. A., Newton, S. M., Kampmann, B., Hall, B. M., et al. (2007). A single dose of vitamin d enhances immunity to mycobacteria. *Am. J. Respir. Crit. Care Med.* 176, 208–213. doi: 10.1164/rccm.200701-007OC

Meehan, T. F., and DeLuca, H. F. (2002). CD8(+) T cells are not necessary for 1 alpha,25-dihydroxyvitamin D(3) to suppress experimental autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5557–5560. doi: 10.1073/pnas.082100699

Mohri, T., Nakajima, M., Takagi, S., Komagata, S., and Yokoi, T. (2009). MicroRNA regulates human vitamin D receptor. *Int. J. Cancer* 125, 1328–1333. doi: 10.1002/ijc.24459

Moller, K. I., Kongshoj, B., Philipsen, P. A., Thomsen, V. O., and Wulf, H. C. (2005). How Finsen's light cured lupus vulgaris. *Photodermatol. Photoimmunol. Photomed.* 21, 118–124. doi: 10.1111/j.1600-0781.2005.00159.x

Montoya, D., Cruz, D., Teles, R. M., Lee, D. J., Ochoa, M. T., Krutzik, S. R., et al. (2009). Divergence of macrophage phagocytic and antimicrobial programs in leprosy. *Cell Host Microbe* 6, 343–353. doi: 10.1016/j.chom.2009.09.002

Moresco, E. M., LaVine, D., and Beutler, B. (2011). Toll-like receptors. *Curr. Biol.* 21, R488–R493. doi: 10.1016/j.cub.2011.05.039

Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S. M., Fritsche, G., et al. (2007). The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular *Salmonella typhimurium*. *Cell. Microbiol.* 9, 2126–2140. doi: 10.1111/j.1462-5822.2007.00942.x

Nakayama, Y., Stabach, P., Maher, S. E., Mahajan, M. C., Masiar, P., Liao, C., et al. (2006). A limited number of genes are involved in the differentiation of germinal center B cells. *J. Cell. Biochem.* 99, 1308–1325. doi: 10.1002/jcb.20952

Nunn, J. D., Katz, D. R., Barker, S., Fraher, L. J., Hewison, M., Hendy, G. N., et al. (1986). Regulation of human tonsillar T-cell proliferation by the active metabolite of vitamin D3. *Immunology* 59, 479–484.

Nursyam, E. W., Amin, Z., and Rumende, C. M. (2006). The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta Med. Indones.* 38, 3–5.

O'Kelly, J., Uskokovic, M., Lemp, N., Vadgama, J., and Koeffler, H. P. (2006). Novel Gemini-vitamin D3 analog inhibits tumor cell growth and modulates the Akt/mTOR signaling pathway. *J. Steroid Biochem. Mol. Biol.* 100, 107–116. doi: 10.1016/j.jsbmb.2006.04.003

Ooi, J. H., McDaniel, K. L., Weaver, V., and Cantorna, M. T. (2014). Murine CD8+T cells but not macrophages express the vitamin D 1alpha-hydroxylase. *J. Nutr. Biochem.* 25, 58–65. doi: 10.1016/j.jnutbio.2013.09.003

Packey, C. D., and Sartor, R. B. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Curr. Opin. Infect. Dis.* 22, 292–301. doi: 10.1097/QCO.0b013e32832a8a5d

Panda, D. K., Miao, D., Tremblay, M. L., Sirois, J., Farookhi, R., Hendy, G. N., et al. (2001). Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7498–7503. doi: 10.1073/pnas.131029498

Paradkar, P. N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J., and Ward, D. M. (2008). Iron depletion limits intracellular bacterial growth in macrophages. *Blood* 112, 866–874. doi: 10.1182/blood-2007-12-126854

Pedersen, A. W., Holmstrom, K., Jensen, S. S., Fuchs, D., Rasmussen, S., Kvistborg, P., et al. (2009). Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells. *Clin. Exp. Immunol.* 157, 48–59. doi: 10.1111/j.1365-2249.2009.03961.x

Penna, G., Amuchastegui, S., Cossetti, C., Aquilano, F., Mariani, R., Sanvito, F., et al. (2006). Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. *J. Immunol.* 177, 8504–8511.

Penna, G., Amuchastegui, S., Giarratana, N., Daniel, K. C., Vulcano, M., Sozzani, S., et al. (2007). 1,25-dihydroxyvitamin d3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J. Immunol.* 178, 145–153.

Peters-Golden, M., Canetti, C., Mancuso, P., and Coffey, M. J. (2005). Leukotrienes: underappreciated mediators of innate immune responses. *J. Immunol.* 174, 589–594.

Provvedini, D. M., and Manolagas, S. C. (1989). 1 Alpha,25-dihydroxyvitamin D3 receptor distribution and effects in subpopulations of normal human T lymphocytes. *J. Clin. Endocrinol. Metab.* 68, 774–779. doi: 10.1210/jcem-68-4-774

Provvedini, D. M., Tsoukas, C. D., Deftos, L. J., and Manolagas, S. C. (1983). 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science* 221, 1181–1183. doi: 10.1126/science.6310748

Ravid, A., Koren, R., Novogrodsky, A., and Liberman, U. A. (1984). 1,25-Dihydroxyvitamin D3 inhibits selectively the mitogenic stimulation of mouse medullary thymocytes. *Biochem. Biophys. Res. Commun.* 123, 163–169. doi: 10.1016/0006-291X(84)90394-2

Reichel, H., Koeffler, H. P., and Norman, A. W. (1986). Regulation of 25-hydroxyvitamin D3 metabolism in a human promyelocytic leukemia cell line (HL-60): 1,25-dihydroxyvitamin D3 stimulates the synthesis of 24,25-dihydroxyvitamin D3. *Arch. Biochem. Biophys.* 251, 222–231. doi: 10.1016/0003-9861(86)90069-X

Rigby, W. F., Noelle, R. J., Krause, K., and Fanger, M. W. (1985). The effects of 1,25-dihydroxyvitamin D3 on human T lymphocyte activation and proliferation: a cell cycle analysis. *J. Immunol.* 135, 2279–2286.

Rigby, W. F., Yirinec, B., Oldershaw, R. L., and Fanger, M. W. (1987). Comparison of the effects of 1,25-dihydroxyvitamin D3 on T lymphocyte subpopulations. *Eur. J. Immunol.* 17, 563–566. doi: 10.1002/eji.1830170420

Romagnani, S. (2006). Regulation of the T cell response. *Clin. Exp. Allergy* 36, 1357–1366. doi: 10.1111/j.1365-2222.2006.02606.x

Ross, A. C., Manson, J. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., et al. (2011). The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J. Clin. Endocrinol. Metab.* 96, 53–58. doi: 10.1210/jc.2010-2704

Royal, W. 3rd., Mia, Y., Li, H., and Naunton, K. (2009). Peripheral blood regulatory T cell measurements correlate with serum vitamin D levels in patients with multiple sclerosis. *J. Neuroimmunol.* 213, 135–141. doi: 10.1016/j.jneuroim.2009.05.012

Sadeghi, K., Wessner, B., Laggner, U., Ploder, M., Tamandl, D., Friedl, J., et al. (2006). Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. *Eur. J. Immunol.* 36, 361–370. doi: 10.1002/eji.200425995

Sanjuan, M. A., Milasta, S., and Green, D. R. (2009). Toll-like receptor signaling in the lysosomal pathways. *Immunol. Rev.* 227, 203–220. doi: 10.1111/j.1600-065X.2008.00732.x

Schaible, U. E., Collins, H. L., Priem, F., and Kaufmann, S. H. (2002). Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *J. Exp. Med.* 196, 1507–1513. doi: 10.1084/jem.20020897

Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., et al. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* 117, 803–811. doi: 10.1172/JCI30142

Shen, Q. Y., and Zheng, S. S. (2004). Identification of genes differentially expressed in monocyte-derived dendritic cells with 1alpha,25-dihydroxyvitamin D3 using cDNA arrays. *J. Zhejiang Univ. Sci.* 5, 222–225. doi: 10.1631/jzus.2004.0222

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., et al. (2004). FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.* 19, 429–435. doi: 10.1359/JBMR.0301264

Shin, D. M., Yuk, J. M., Lee, H. M., Lee, S. H., Son, J. W., Harding, C. V., et al. (2011). Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cell. Microbiol.* 12, 1648–1665. doi: 10.1111/j.1462-5822.2010.01497.x

Shiozawa, K., Shiozawa, S., Shimizu, S., and Fujita, T. (1985). 1 alpha,25-dihydroxyvitamin D3 inhibits pokeweed mitogen-stimulated human B-cell activation: an analysis using serum-free culture conditions. *Immunology* 56, 161–167.

Shirakawa, A. K., Nagakubo, D., Hieshima, K., Nakayama, T., Jin, Z., and Yoshie, O. (2008). 1,25-dihydroxyvitamin D3 induces CCR10 expression in terminally differentiating human B cells. *J. Immunol.* 180, 2786–2795.

Sigmundsdottir, H., Pan, J., Debes, G. F., Alt, C., Habtezion, A., Soler, D., et al. (2007). DCs metabolize sunlight-induced vitamin D3 to "program" T cell attraction to the epidermal chemokine CCL27. *Nat. Immunol.* 8, 285–293. doi: 10.1038/ni1433

Sly, L. M., Lopez, M., Nauseef, W. M., and Reiner, N. E. (2001). 1alpha,25-Dihydroxyvitamin D3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. *J. Biol. Chem.* 276, 35482–35493. doi: 10.1074/jbc.M102876200

Smolders, J., Thewissen, M., Peelen, E., Menheere, P., Cohen Tervaert, J. W., Damoiseaux, J., et al. (2009). Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. *PLoS ONE* 4:e6635. doi: 10.1371/journal.pone.0006635

Sow, F. B., Alvarez, G. R., Gross, R. P., Satoskar, A. R., Schlesinger, L. S., Zwilling, B. S., et al. (2009). Role of STAT1, NF-kappaB, and C/EBPbeta in the macrophage transcriptional regulation of hepcidin by mycobacterial infection and IFN-gamma. *J. Leukoc. Biol.* 86, 1247–1258. doi: 10.1189/jlb.1208719

Sow, F. B., Florence, W. C., Satoskar, A. R., Schlesinger, L. S., Zwilling, B. S., and Lafuse, W. P. (2007). Expression and localization of hepcidin in macrophages: a role in host defense against tuberculosis. *J. Leukoc. Biol.* 82, 934–945. doi: 10.1189/jlb.0407216

Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21, 685–711. doi: 10.1146/annurev.immunol.21.120601.141040

Stoffels, K., Overbergh, L., Bouillon, R., and Mathieu, C. (2007). Immune regulation of 1alpha-hydroxylase in murine peritoneal macrophages: unravelling the IFNgamma pathway. *J. Steroid Biochem. Mol. Biol.* 103, 567–571. doi: 10.1016/j.jsbmb.2006.12.091

Stoffers, K. L., Sorg, B. L., Seuter, S., Rau, O., Radmark, O., and Steinhilber, D. (2010). Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. *J. Mol. Biol.* 395, 884–896. doi: 10.1016/j.jmb.2009.10.022

Szeles, L., Keresztes, G., Torocsik, D., Balajthy, Z., Krenacs, L., Poliska, S., et al. (2009). 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. *J. Immunol.* 182, 2074–2083. doi: 10.4049/jimmunol.0803345

Takahashi, K., Nakayama, Y., Horiuchi, H., Ohta, T., Komoriya, K., Ohmori, H., et al. (2002). Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3. *Immunopharmacol. Immunotoxicol.* 24, 335–347. doi: 10.1081/IPH-120014721

Tang, J., Zhou, R., Luger, D., Zhu, W., Silver, P. B., Grajewski, R. S., et al. (2009). Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. *J. Immunol.* 182, 4624–4632. doi: 10.4049/jimmunol.0801543

Teles, R. M., Graeber, T. G., Krutzik, S. R., Montoya, D., Schenk, M., Lee, D. J., et al. (2013). Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* 339, 1448–1453. doi: 10.1126/science.1233665

Tripathi, S., Tecle, T., Verma, A., Crouch, E., White, M., and Hartshorn, K. L. (2013). The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins. *J. Gen. Virol.* 94, 40–49. doi: 10.1099/vir.0.045013-0

Ustianowski, A., Shaffer, R., Collin, S., Wilkinson, R. J., and Davidson, R. N. (2005). Prevalence and associations of vitamin D deficiency in foreign-born persons with tuberculosis in London. *J. Infect.* 50, 432–437. doi: 10.1016/j.jinf.2004.07.006

Veldman, C. M., Cantorna, M. T., and DeLuca, H. F. (2000). Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch. Biochem. Biophys.* 374, 334–338. doi: 10.1006/abbi.1999.1605

Vergne, I., Chua, J., Singh, S. B., and Deretic, V. (2004). Cell biology of mycobacterium tuberculosis phagosome. *Annu. Rev. Cell Dev. Biol.* 20, 367–394. doi: 10.1146/annurev.cellbio.20.010403.114015

Wan, M., Sabirsh, A., Wetterholm, A., Agerberth, B., and Haeggstrom, J. Z. (2007). Leukotriene B4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses. *FASEB J.* 21, 2897–2905. doi: 10.1096/fj.06-7974com

Wang, J., Lian, H., Zhao, Y., Kauss, M. A., and Spindel, S. (2008). Vitamin D3 induces autophagy of human myeloid leukemia cells. *J. Biol. Chem.* 283, 25596–25605. doi: 10.1074/jbc.M801716200

Wang, T. J., Zhang, F., Richards, J. B., Kestenbaum, B., van Meurs, J. B., Berry, D., et al. (2010a). Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 376, 180–188. doi: 10.1016/S0140-6736(10)60588-0

Wang, T. T., Dabbas, B., Laperriere, D., Bitton, A. J., Soualhine, H., Tavera-Mendoza, L. E., et al. (2010b). Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. *J. Biol. Chem.* 285, 2227–2231. doi: 10.1074/jbc.C109.071225

Wang, T. T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., et al. (2004). Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* 173, 2909–2912.

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., et al. (2005). Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol. Endocrinol.* 19, 2685–2695. doi: 10.1210/me.2005-0106

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011). Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10:58. doi: 10.1186/1476-4598-10-58

Wang, X., Gocek, E., Liu, C. G., and Studzinski, G. P. (2009). MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced

to differentiate by 1,25-dihydroxyvitamin D3. *Cell Cycle* 8, 736–741. doi: 10.4161/cc.8.5.7870

Wejse, C., Gomes, V. F., Rabna, P., Gustafson, P., Aaby, P., Lisse, I. M., et al. (2009). Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. *Am. J. Respir. Crit. Care Med.* 179, 843–850. doi: 10.1164/rccm.200804-567OC

Wilkinson, R. J., Llewelyn, M., Toossi, Z., Patel, P., Pasvol, G., Lalvani, A., et al. (2000). Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *Lancet* 355, 618–621. doi: 10.1016/S0140-6736(99)02301-6

Williams, B., Williams, A. J., and Anderson, S. T. (2008). Vitamin D deficiency and insufficiency in children with tuberculosis. *Pediatr. Infect. Dis. J.* 27, 941–942. doi: 10.1097/INF.0b013e31817525df

Yu, S., Bruce, D., Froicu, M., Weaver, V., and Cantorna, M. T. (2008). Failure of T cell homing, reduced CD4/CD8alphaalpha intraepithelial lymphocytes, and inflammation in the gut of vitamin D receptor KO mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20834–20839. doi: 10.1073/pnas.0808700106

Yuk, J. M., Shin, D. M., Lee, H. M., Yang, C. S., Jin, H. S., Kim, K. K., et al. (2009). Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* 6, 231–243. doi: 10.1016/j.chom.2009.08.004

Zanzoni, A., Montecchi-Palazzi, L., Quondam, M., Ausiello, G., Helmer-Citterich, M., and Cesareni, G. (2002). MINT: a Molecular INTeraction database. *FEBS Lett.* 513, 135–140. doi: 10.1016/S0014-5793(01)03293-8

Zehnder, D., Landray, M. J., Wheeler, D. C., Fraser, W., Blackwell, L., Nuttall, S., et al. (2007). Cross-sectional analysis of abnormalities of mineral homeostasis, vitamin D and parathyroid hormone in a cohort of pre-dialysis patients. The chronic renal impairment in Birmingham (CRIB) study. *Nephron Clin. Pract.* 107, c109–c116. doi: 10.1159/000108652

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Vitamin D and gene networks in human osteoblasts

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Bone formation is indirectly influenced by 1,25-dihydroxyvitamin D3 (1,25D3) through the stimulation of calcium uptake in the intestine and re-absorption in the kidneys. Direct effects on osteoblasts and bone formation have also been established. The vitamin D receptor (VDR) is expressed in osteoblasts and 1,25D3 modifies gene expression of various osteoblast differentiation and mineralization-related genes, such as alkaline phosphatase (ALPL), osteocalcin (BGLAP), and osteopontin (SPP1). 1,25D3 is known to stimulate mineralization of human osteoblasts *in vitro*, and recently it was shown that 1,25D3 induces mineralization via effects in the period preceding mineralization during the pre-mineralization period. For a full understanding of the action of 1,25D3 in osteoblasts it is important to get an integrated network view of the 1,25D3-regulated genes during osteoblast differentiation and mineralization. The current data will be presented and discussed alluding to future studies to fully delineate the 1,25D3 action in osteoblast. Describing and understanding the vitamin D regulatory networks and identifying the dominant players in these networks may help develop novel (personalized) vitamin D-based treatments. The following topics will be discussed in this overview: (1) Bone metabolism and osteoblasts, (2) Vitamin D, bone metabolism and osteoblast function, (3) Vitamin D induced transcriptional networks in the context of osteoblast differentiation and bone formation.

Keywords: vitamin D, osteoblast, differentiation, mineralization, autocrine/paracrine mechanisms, immune system

BONE METABOLISM AND OSTEOBLASTS

Bone is formed during fetal development by two processes: endochondral and intramembranous ossification (for review Bilezikian et al., 2002). Skull and flat bones are formed by intramembranous ossification where there is direct bone formation by condensation of the mesenchyme without a preformed cartilaginous scaffold. Long bones and most of the remaining bones are formed by endochondral ossification (Mackie et al., 2008, 2011; Nishimura et al., 2012). This type of bone formation is characterized by the transition of cartilage into mineralized bone tissue.

Two major processes occur in bone: bone modeling and bone remodeling. While bone modeling drives the growth of the skeleton, bone remodeling is responsible for the maintenance of healthy bone in the adulthood (Teti, 2011). Bone remodeling takes place throughout live and maintains the structural integrity and strength of the bone by removing old or damaged bone and replacing it by new, strong bone. Remodeling is a local process that can take place anywhere on the bone surface throughout the lifespan of a bone. Remodeling occurs in a temporary anatomic unit of osteoclasts and osteoblasts called a bone multicellular unit (BMU) (Martinello et al., 2012; Sims and Martin, 2014). The BMU is a sealed compartment in which bone resorption and subsequent formation are regulated. This coupled resorption and formation characterizes and differentiates bone remodeling from bone modeling, in which bone resorption and formation do not have to occur at the same time and site. For growth and for the maintenance of healthy bone, multiple cell types are of importance: mesenchymal stem cells (MSC), osteoblasts, osteocytes, and hematopoietic stem cells and osteoclasts. The osteoblasts play a pivotal role in bone metabolism by forming bone but also by controlling and regulating the formation and activity of the bone resorbing cell the osteoclast.

Osteoblasts originate from MSC. MSCs are located in the bone marrow but also in almost all other tissues undergoing continuous tissue homeostasis. MSCs can differentiate into osteoblasts, chondrocytes, fibroblasts, adipocytes or myocytes (Friedenstein et al., 1974; Minguell et al., 2001; Yin, 2006). During osteoblast differentiation several functional phases can be identified: proliferation, production and maturation of extracellular matrix (ECM) and ECM mineralization (Owen et al., 1991). Osteoblast differentiation can start by a trigger of certain growth factors (Wang, 1993) as well as hormones and other factors (Eijken et al., 2006). Mature osteoblasts produce and secrete ECM molecules (Owen et al., 1991). Osteoblasts synthesize the most abundant bone ECM protein collagen type I but also a broad range of non-collagenous ECM proteins. Mineralization of the ECM is likely induced by matrix vesicles which derive from osteoblasts (Anderson et al., 2005). When mature osteoblasts initiate mineralization of mature ECM, its fate may vary. Osteoblasts can further differentiate into osteocytes, become a bone lining cell or undergo apoptosis (Jilka et al., 1998; Weinstein et al., 1998). Osteoblasts become osteocytes by being entrapped in self-produced ECM, in which they may survive for decades. Osteocytes form a star-shaped network of cytoplasmic extensions. Osteocytes are thought to function as orchestrators of bone by

sensing and communicating mechanical stress (i.e., bone damage) via these extensions (Bonewald, 2011). It has become evident by genetic approaches that osteocytes play a role in regulation of bone turn-over (resorption and formation) (Nakashima et al., 2011; Atkins and Findlay, 2012). Bone lining cells are less well understood. They are covering the bone surface and prevent it from being in direct contact with the bone marrow. It has been reported that these cells "clean" resorption pits after osteoclasts retreated (Everts et al., 2002). Bone lining cells are considered as inactive osteoblasts. It has been suggested that these cells can be activated to become osteoblasts (Dobnig and Turner, 1995; Chow et al., 1998) but they also may represent the osteoblastic part of the stem cell niche and interact with the hematopoietic stem cells.

The osteoblasts/osteocytes guarantee the close coupling between bone formation and resorption in healthy bone remodeling. Osteoblasts and osteocytes produce the soluble osteoclast stimulating factors RANKL and M-CSF which upon binding to its receptors (RANK and c-Fms, respectively) induce differentiation of osteoclast progenitors and fusion of mononuclear cells into multinucleated tartrate-resistant acid phosphatase positive osteoclasts (Boyle et al., 2003). Besides RANKL, osteoblasts also produce a soluble decoy-receptor: osteoprotegerin (OPG). OPG binds RANKL with high affinity leading to inhibition of osteoclast stimulation and thus leading to less bone resorption (Lacey et al., 1998; Kostenuik and Shalhoub, 2001).

VITAMIN D, BONE METABOLISM, AND OSTEOBLAST

The biologically most active form of vitamin D, 1α,25-dihydroxyvitamin D3 [calcitriol or 1,25(OH)2D3 (1,25D3)] is formed by a stepwise process starting in the skin and involving the liver and kidney. Upon ultraviolet B exposure, 7-dehydrocholesterol (pro-vitamin D3) is transformed into (pre)vitamin D3 (cholecalciferol) in the skin. Subsequent hydroxylation at the C25 and 1α position in liver and kidney, respectively, produce 1,25D3 (Holick, 1995). In bone diseases, vitamin D is used as an anti-rickets agent (Kitanaka et al., 1998; McCollum et al., 2002; Tatsumi et al., 2007), which improves bone mineralization and is often prescribed in combination with other osteoporosis drugs to secure a positive calcium balance. However, a recent metaanalyes by Reid et al suggests that the supplementation of vitamin D for the prevention of osteoporosis is inappropriate without specific risk factors for vitamin D deficiency (Reid et al., 2014).

Up to today it is still in debate whether 1,25D3 effects on bone formation are indirect via intestinal and renal regulation of calcium levels or also via a direct effect on osteoblasts. It has been demonstrated that mice lacking the vitamin D receptor (VDR) gene display retarded growth, severe bone impairment, immune abnormalities, and premature death at only 15 weeks of age due to hypocalcemia (Li et al., 1997; Yoshizawa et al., 1997; Mathieu et al., 2001). A rescue diet restored all pathological effects suggesting that as long as calcium homeostasis is under control, bone itself does not seem to be affected by impaired VDR signaling. The importance of physiological 1,25D3 levels for bone is demonstrated by the mutation of the CYP27B1 gene. Subjects with a mutation in that gene develop vitamin-D-dependent rickets (Li et al., 1997). In a mouse model for rickets, greater extensibility and lower stiffness of fibrils resulted from a decreased grade of mineral deposition (Karunaratne et al., 2012). This further supports the importance of an optimal grade of mineralization for healthy bones (Kitanaka et al., 1998) and points to a role for 1,25D3 herein.

A direct positive effect an 1,25D3 analog on bone formation in ovariectomized rats with only slight changes in serum calcium points to the existence of a direct effect on bone formation (Shevde et al., 2002). This is supported by *in vitro* studies demonstrating direct effects on osteoblasts. The VDR is present in osteoblasts and its expression can be regulated by 1,25D3 itself and by other factors such as parathyroid hormone (PTH), glucocorticoids, transforming growth factor-β, and epidermal growth factor (Pols et al., 1988a,b; Reinhardt and Horst, 1990; van Leeuwen et al., 1991, 1992a,b; Godschalk et al., 1992). The expression of VDR allows 1,25D3 to directly affect osteoblast growth and differentiation. 1,25D3 has been shown to stimulate bone formation and mineralization in all studies using human osteoblasts and stimulate osteogenic differentiation from human mesenchymal stem/stromal cells (MSC) (Ueno et al., 1992; Prince et al., 2001; Jørgensen et al., 2004; Van Driel et al., 2006a,b; Zhou et al., 2006, 2012). 1,25D3 enhanced mineralization by effects on human osteoblasts prior to the onset of mineralization (Woeckel et al., 2010). Thus, 1,25D3 is not directly involved in the process of mineral deposition but more likely in a process preparing the environment/ECM for mineralization. 1,25D3 regulates the osteoblast differentiation marker ALPL and various bone ECM proteins such as COL1A1. Procollagen type I by human osteoblasts was stimulated (Franceschi et al., 1988; Hicok et al., 1998) as well as unaffected (Ingram et al., 1994; Hicok et al., 1998; Siggelkow et al., 1999) by vitamin D. However, gene expression profiling studies demonstrated that the 1,25D3 effect in the pre-mineralization phase is not likely primarily due to changes in expression of ECM proteins and thereby composition of the ECM (Woeckel et al., 2010). Production of alkaline phosphatase (ALPL) positive matrix vesicles was significantly induced by 1,25D3 in this period of osteoblast differentiation (Anderson, 1995) providing a means to enhance mineralization (Woeckel et al., 2010). In addition, previous studies have shown the importance of other factors like TGFβ, IGF-I, bone morphogenetic protein, interferon, PTH, hepatocyte growth factor, epidermal growth factor, and peroxisome proliferator-activated receptor ligands and Wnt signaling for the eventual effect of 1,25D3 on osteoblasts (Petkovich et al., 1987; Pols et al., 1988b; Scharla et al., 1991; Bonewald et al., 1992; Godschalk et al., 1992; van Leeuwen et al., 1992a,b; Ingram et al., 1994; Staal et al., 1994, 1996, 1998; Haussler et al., 1998; Yanagisawa et al., 1999; Sammons et al., 2004; Yarram et al., 2004; Fretz et al., 2007; Chen et al., 2012a, 2013; Woeckel et al., 2012; Yamaguchi and Weitzmann, 2012). These data stress the importance of studying and interpreting the effects of 1,25D3 on bone in a systems biological approach encompassing the different layers of regulation and interactions.

In contrast to human and rat studies, 1,25D3 inhibits differentiation and mineralization in cultures of murine osteoblasts (Shi et al., 2007; Chen et al., 2012a,b, 2013) and murine VDR deficient osteoblasts have increased osteogenic potential (Sooy et al., 2004). 1,25D3 increases in a VDR-dependent manner the expression of

progressive ankylosis (ANK) and ectonucleotide pyrophosphatase phosphodiesterase (ENPP1) in murine osteoblasts. This leads to an increase in the level of pyrophosphate (PPi) that inhibits mineralization (Lieben et al., 2012). 1,25D3 also increases osteopontin shown to inhibit mineralization (Staal et al., 1996). However, transgenic murine models with osteoblast-specific VDR overexpression show increased bone formation and mineralization (Gardiner et al., 2000; Misof et al., 2003; Xue et al., 2006). An 1,25D3 analog had a positive effect on bone nodule formation and mineralization in murine calvarial osteoblast cultures of wild type but not VDR null mice (Shevde et al., 2002) while one study showed increased mineralization in MC3T3 cell cultures (Matsumoto et al., 1991). In a recent study, Yamamoto et al. (2013) illustrated that mice lacking VDR in osteoblasts had an increased bone mass, due to decreased bone resorption.

Overall the present data show variation in effects of 1,25D3 on differentiation and mineralization with overall stimulatory effects in human and rat osteoblasts while overall an inhibitory effect in murine osteoblasts (Van Driel et al., 2006a). Following this, 1,25D3 has been shown to increase RUNX2 expression in human osteoblasts (Prince et al., 2001; Viereck et al., 2002; Maehata et al., 2006) while 1,25D3 suppresses RUNX2 promoter and reduces RUNX2 expression in murine osteoblasts (Prince et al., 2001; Drissi et al., 2002). Osteocalcin (BGLAP) is an interesting gene considering differences in 1,25D3 effects in human and murine osteoblasts (Thomas, 2000). 1,25D3 stimulates BGLAP expression in human and rat osteoblasts while it inhibits BGLAP expression in murine osteoblasts (Lian et al., 1997; Zhang et al., 1997), supporting differences between human/rat osteoblasts and murine with respect to 1,25D3 responsiveness and mineralization.

A full explanation for this apparent discrepancy between human and murine osteoblasts is absent. Both the extracellular milieu (i.e., presence/absence of growth factors, cytokines and other signaling molecules) and the intracellular milieu (e.g., the insulin-like growth factor binding protein-6 that can bind to the VDR and inhibit 1,25D3 induction of ALPL activity) of the cell is important for the eventual effect of 1,25D3 (Cui et al., 2011). Also the extracellular phosphate concentration may affect the 1,25D3 action (Ito et al., 2013). These characteristics may contribute to the differences in 1,25D3 effects observed in human and murine osteoblasts.

Besides stimulation of bone formation /mineralization by osteoblasts 1,25D3 has certain protective control mechanisms in place to avoid pathological over-mineralization. For example, 1,25D3 induces BGLAP and SPP1, established inhibitors of mineralization (Noda et al., 1990; MacDonald et al., 1993) and a stimulator of mineralization, bone sialoprotein (IBSP), is inhibited by 1,25D3 (Li and Sodek, 1993). As mentioned above also the presence or absence of other growth factors, cytokines or signaling molecules may limit the 1,25D3 effect. Examples of this in relation to mineralization are Activin A and follistatin. Activin A inhibits osteoblast differentiation and mineralization (Eijken et al., 2007). Activin A expression in human osteoblasts is stimulated by 1,25D3 (Woeckel et al., 2013), implicating that 1,25D3 as stimulator of human osteoblast differentiation and mineralization also stimulates the production of a mineralization inhibitor. A function in the prevention of over-mineralization is supported by the data that the activin A blocker follistatin enhances 1,25D3 stimulated mineralization (Woeckel et al., 2013). The above mentioned induction of carboxylated osteocalcin by 1,25D3 may fit this hypothesis on preventing over-mineralization. Accumulation of osteocalcin in the ECM of human osteoblast cultures stimulated by 1,25D3 is inhibited by warfarin (antagonist of vitamin K) while vitamin K2 (cofactor of γ-carboxylase) enhanced the 1,25 D3 effect (Koshihara and Hoshi, 1997). 1,25D3 stimulated mineralization was significantly augmented by warfarin (Woeckel et al., 2013). These data on activin A, follistatin, warfarin, and vitamin K put forward a 1,25D3 induced regulatory mechanism to guarantee optimal mineralization (Woeckel et al., 2013). Differences in these regulatory loops may also be part of the differences in 1,25D3 effects in human and murine osteoblast studies.

The most well-known mechanism to limit the biological activity of 1,25D3 is its degradation via 24-hydroxylation. 1,25D3 potently induces CYP24A1, which encodes for the enzyme 24-hydroxylase, in osteoblasts. 24-Hydroxylation is the first step in the degradation cascade of active 1,25D3 (Ohyama et al., 1994). However, hydroxylation at the C-24 position doesn't directly lead to an inactive vitamin D molecule. Henry and Norman demonstrated the significance of 24,25-dihydroxyvitamin D3 (24,25D3) for normal chicken egg hatchability and calcium and phosphorus homeostasis (Henry and Norman, 1978; Norman et al., 1980). Already in 1980 it was shown that 24,25D3 directly stimulates calcification of bone in interaction with PTH and that the number and size of resorption sites in bone is decreased by 24,25D3 (Endo et al., 1980; Galus et al., 1980). Several other studies supported a positive effect of 24,25D3 on bone metabolism (Matsumoto et al., 1985; Tam et al., 1986; Kato et al., 1998) while one study showed no effect of 24,25D3 on histomorphometric parameters in ovariectomized rats (Erben et al., 1992). Administration of 24,25D3 in combination with 1,25D3 improved fracture healing in chickens (Seo et al., 1997) and interestingly, 24,25D3 serum levels correlated to fracture healing (Seo and Norman, 1997). Studies with the CYP24A knockout mouse supported a role for 24,25D3 in fracture repair (St-Arnaud, 2010). Albeit in a human study no positive association with femoral fracture was observed (Weisman et al., 1978). However, a study in pre-dialysis renal insufficiency patients supported a direct, i.e., PTH-independent, functional role of 24,25D3 in bone (Birkenhäger-Frenkel et al., 1995). These data suggest a direct effect on osteoblasts. *In vitro* studies with human osteoblasts have shown that indeed 24,25D3 has direct effects similar to that of 1,25D3 (Van Driel et al., 2006b). A recent comparative gene expression profiling study of 1,25D3, 24,25D3, and 25D3 in primary human and mouse fibroblasts suggested induction of metabolite specific sets of genes and pathways (Tuohimaa et al., 2013). It is important to note that the fact whether biological active levels of 24,25D3 or 1,24,25-trihydroxyvitamin D3 (1,24,25D3) can be reached fully depends on the velocity of the subsequent steps in the degradation pathway after the initial 24-hydroxylation step.

We have shown that osteoblasts besides degradation of active 1,25D3, are able to convert 25-hydroxyvitamin D3 (25D3) into the biologically most active form 1,25D3, suggesting a direct relationship between 1,25D3 synthesis and bone (Van Driel et al.,

2006a). This study showed functionality of 1α-hydroxylation in human osteoblast differentiation. 25D3 induced expression of CYP24, osteocalcin and stimulated ALPL activity and mineralization, which was blocked by inhibition of 1α-hydroxylase by ketoconazole. Downregulation of CYP27B1 in human osteoblasts or perturbation of CYP27B1 supported the requirement of 1α-hydroxylase for the effect on human MSC proliferation and osteogenic differentiation (Atkins et al., 2007; Geng et al., 2011a). CYP27B1 expression is reduced in MSC of older subjects and resistance to 25D3 induced osteoblast formation points to an aging effect (Geng et al., 2011b). The 1α-hydroxylase-dependent 25D3 stimulation of ALPL activity in human MSC was blocked by histone deacytylase inhibition (Zhou et al., 2013). Of interest, 25D3 has been shown to regulate gene expression in a gene expression profiling study with CYP27B1 deficient fibroblasts (Tuohimaa et al., 2013). This suggests that 25D3 may act independent of 1α-hydroxylation.

Up to now the data on 1,25D3 production by osteoblasts are derived from *in vitro* studies. *In vivo* significance of CYP27B1 and 1,25D3 formation in osteoblasts needs yet to be proven, for example by knocking out CYP27B1 specifically in osteoblasts. However, the observed discrepancies in effects on human-murine osteoblasts may hamper this approach. Although yet *in vivo* proof is lacking, the principal of local synthesis of 1,25D3 in bone may explain the observed associations of 25D3 and not of 1,25D3 with bone as well as other parameters (Hewison et al., 2004; Anderson et al., 2013). Besides CYP27B1, osteoblasts also express the receptors megalin and cubulin that are involved in cellular uptake of 25D3 via endocytosis of the vitamin D binding protein (DBP) (Van Driel et al., 2006a; Atkins et al., 2007). Linking back to the above discussed interaction between locally produced growth factors and 1,25D3 is the regulation of CYP27B1 in osteoblasts. Albeit 1,25D3 itself inhibits CYP27B1 expression in MSC as well as in the kidney (Zhou et al., 2010), the regulation appears to be different and more complex than in the kidney involving local regulators. Several locally in bone produced factors affects CYP27B1 expression: TGFβ suppresses 5′-flanking region of CYP27B1 (Turner et al., 2007) and interferon-β reduces while interleukin-1 and IGF-I increase CYP27B1 expression in mature human osteoblasts (Van Driel et al., 2006a; Zhou et al., 2010; Woeckel et al., 2012). The effect of interleukin-1 points to the involvement of NF-κB in stimulation of CYP27B1 expression in human osteoblasts. This is supported by the interferon-β inhibition of NF-κB in synoviocytes (Van Holten et al., 2004) and CYP27B1 regulation in human dendritic cells (Hewison et al., 2003).

1,25D3 plays an important role in maintaining bone health either via controlling calcium and phosphate homeostasis or via direct effects on osteoblasts. This latter is supported by the direct effects of 1,25D3 on osteoblast differentiation, expression and activity of bone formation related proteins and enzymes, and mineralization. The complete vitamin D endocrine system, from receptor to enzymes involved in 1,25D3 synthesis and breakdown, is present in the osteoblast, pointing to an autocrine/paracrine 1,25D3 function in bone. This is the more so interesting as over the past decade it has become clear that osteoblasts are not only involved in bone metabolism but that they also form the hematopoietic stem cell (HSC) niche controlling renewal of HSCs and differentiation of the immune cells (Calvi et al., 2003). Moreover, these HSC niches are also the sites of bone metastasis (Shiozawa et al., 2011). Considering the 1,25D3 effect on the immune system and tumor cell growth it is tempting to speculate that autocrine/paracrine action of 1,25D3 is also beyond bone metabolism and important for other regulatory functions of osteoblasts. It is therefore of critical importance to understand the full picture of 1,25D3 effects on osteoblasts. One of the approaches to obtain information on the effects of 1,25D3 on osteoblasts and MSC in an unbiased way is by omics approaches in combination with bioinformatics. In the next paragraph the current available 1,25D3 gene expression profiling studies of osteoblasts will be discussed.

VITAMIN D AND GENE TRANSCRIPTION IN THE CONTEXT OF OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

1,25D3 has been shown to regulate the expression of various genes related to osteoblast proliferation and differentiation. BMP-2 induced bone formation has been suggested to be enhanced by 1,25D3 induced c-MYC expression (Piek et al., 2010). Induction of Insulin-like growth factor-binding proteins (IGFBP)-2, -3, and -4 expression by 1,25D3 in human MSC may play a role in stimulation of osteogenic differentiation (Kveiborg et al., 2001). Recently, Li and coworkers (Li et al., 2013) demonstrated that IGFBP-3 interacts with the VDR and negatively regulates CYP24 and BGLAP expression. Overexpression of IGFBP-3 inhibited the 1,25D3 activation of ALP in MG-63 human osteosarcoma cells.

1,25D3 also regulated Forkhead Box O (FoxO) transcription factors in murine MC3T3 osteoblasts with FoxO3a being up-regulated while FoxO1 was down-regulated, and FoxO4 not affected. Knockdown of the FoxO's didn't change 1,25D3 inhibition of cell growth but led to increased accumulation of reactive oxygen species after 1,25D3 treatment (Eelen et al., 2013). This may be linked to cellular metabolism and the high energy demanding process of bone formation (Komarova et al., 2000; Chen et al., 2008; Bruedigam et al., 2010). Unfortunately, the effect of FoxO's knockdown on mineralization in these murine MC3T3 osteoblast cultures was not reported. 1,25D3 increased vascular endothelial growth factor (VEGF) expression in human and rat osteoblasts is interesting considering the relationship between bone formation and angiogenesis (Wang et al., 1996; Schlaeppi et al., 1997; Corrado et al., 2013). VEGF has been shown to be involved in the 1,25D3 bone anabolic effect (Wang et al., 1997).

Recent studies placed miRNAs in the 1,25D3 mechanism of action spectrum in osteoblasts. Five miRNAs were found to be differentially expressed in primary human osteoblast after 6 h of treatment with 1,25D3 (Lisse et al., 2013a,b). Interestingly, miR-637 and miR-1228 are two miRNAs located intergenic in DAPK3 and LRP1, respectively. miR-1228 was upregulated and coexpressed with its host gene LRP1 suggesting a conventional VDRE- mediated transactivation upon 1,25D3 treatment. Since LRP1 is known to mediate the canonical Wnt pathway in fibroblasts (Terrand et al., 2009), this suggests an indirect regulation of Wnt signaling by 1,25D3 adding to other data on 1,25D3 and Wnt signaling interaction (Fretz et al., 2007; Haussler et al., 2010).

The target of miR-1228, BMP2K, was previously identified to be increased in mouse osteoblasts upon treatment with BMP2 (Kearns et al., 2001). Stable expression of BMP2K in mouse osteoprogenitor cells decreased ALPL activity and osteocalcin mRNA levels. This suggests that 1,25D3 induced expression of miR-1228 may affect osteoblast differentiation via down-regulation of BMP2K.

On the contrary, 1,25D3 upregulated miR-637 while its host gene was downregulated suggesting a different way of regulation of the two transcripts. miR-637 stimulated the degradation of COL4A mRNA levels that is expressed in the basement membrane and is downregulated during early differentiation of mouse MC3T3-E1 osteoblasts (Hong et al., 2010). It is becoming evident that miRNAs play an important role in osteoblast differentiation and bone formation (Lian et al., 2012) and in the near future more data on their role in 1,25D3 action in osteoblasts will come forward (Lisse et al., 2013a).

IDENTIFICATION OF 1,25D3 TARGET GENES IN OSTEOBLASTS

In the past various studies have investigated the effects of 1,25D3 on target gene expression and VDR binding to DNA response elements. Only a few of these genome-wide studies have investigated the effects of 1,25D3 in the context of osteoblasts (**Table 1**). The studies that carried out are very heterogenic with regard to the differentiation stage of the cells (MSC vs. primary osteoblasts vs. Cell line), time points of treatment (2–6 h after treatment) and the 1,25D3 concentration that is used (1–100 nM). Together this makes it difficult to compare the different studies. Systematic analyses of both mRNA gene expression profiling and VDR binding experiments at early time points after induction with 1,25D3 will uncover direct target genes. Below we will address a few of these studies and the results obtained.

ChIP ANALYSES IN OSTEOBLASTS

Upon binding of 1,25D3 to the VDR, the VDR binds with its heterodimeric partner retinoid X receptor (RXR) on the vitamin D receptor response elements (VDRE). The VDRE consists of the hexameric sequence AGGTCAxxxAGGTCA (Ozono et al., 1990) but variants to the conserved sequence have been identified (Meyer, 2005). Due to the diversity of VDRE, bioinformatics approaches are limited in identifying whole genome VDR binding sites. To identify direct target genes of VDR, genome-wide approaches such as ChIP-chip or ChIP-seq approaches have to be performed. Systematic analyses of VDR binding upon activation by 1,25D3 combined with bioinformatics approaches identifies VDRE (VDR response elements) and subsequently direct targets of Vitamin D signaling. A few studies have started to identify 1,25D3 target genes is various cell types such as a human derived lymphoblastoid cell line (Ramagopalan et al., 2010) and monocytes (Heikkinen et al., 2011). Recently, the first VDR binding experiments in osteoblasts were published (Meyer et al., 2010). Meyer et al. analyzed the genomic locations that bind VDR, RXR, RNA polymerase II and acetylated H4 after 3 h treatment with 1,25D3 in mouse MCT3T-E1 osteoblasts. Interestingly, only 13% of the identified sites was located in classical promoter regions upstream vitamin D target genes. The majority of sites that were found to bind VDR, RXR and acetylated H4 were located distal (43%) and within intronic and exonic regions (44%). This demonstrates that distal transcriptional control contributes to the majority of vitamin D3-mediated transcription. Genome wide ChIP-seq analyses with human osteoblasts should illustrate whether binding of VDR at distal locations is conserved.

Pilot analysis of our gene expression profiles of osteogenic and adipogenic MSCs illustrated that many known 1,25D3 responsive genes (on basis of Ingenuity database; www.ingenuity.com) are dynamically expressed during adipogenic as well as osteogenic differentiation (data not published). This data does not directly show that these genes are regulated by 1,25D3 but it suggests that 1,25D3-responsive genes can have a role during the differentiation of mesenchymal precursors. Many of the two-fold regulated genes during osteogenic differentiation and those that were identified previously to be regulated by 1,25D3 are involved in Cell Cycle (41/162; GO:0007049), response to steroid hormone (21/162; GO:0048545), regulation of phosphate metabolic process (26/162, GO:0019220), regulation of apoptosis (31/162, GO:0042981), extracellular region part (36/162; GO:0044421). ChIP analyses using VDR and expression profiling of 1,25D3

Table 1 | Genome-wide studies of vitamin D and osteoblasts.

Publication	Experiment	Species	Cell type	Treatment
Lisse et al., 2013a,b	Expression profiling miRNA	Homo sapiens	Primary osteoblasts	1,25D3 10^{-8} M 6 h
Woeckel et al., 2012	Expression profiling mRNA	Homo sapiens	Pre-osteoblasts svHFO	1,25D3 10^{-8} M 2 and 24 h
Tarroni et al., 2012	Expression profiling mRNA	Homo sapiens	Primary osteoblasts	1,25D3 10^{-7} M 24 h
Grundberg et al., 2011	Expression profiling mRNA	Homo sapiens	Trabecular bone	1,25D3 10^{-7} M 2 and 24 h
Piek et al., 2010	Expression profiling mRNA	Homo sapiens	MSCs	1,25D3 10^{-8} M 0, 1, 3, 6, 12, 24, 48, 72, 120, 192, and 288 h
Meyer et al., 2010	VDR localization ChIP-chip	Mus musculus	Pre-osteoblasts MC3T3-E1	1,25D3 10^{-7} M 3 h
Woeckel et al., 2010	Expression profiling mRNA	Homo sapiens	Pre-osteoblasts svHFO	1,25D3 10^{-8} M 3, 7, 12, and 19 days
Eelen et al., 2004	Expression profiling mRNA	Mus musculus	Pre-osteoblasts MC3T3-E1	1,25D3 10^{-8} M 6 and 12 h
Farach-Carson and Xu, 2002	Expression profiling mRNA	Rattus norvegicus	Osteosarcoma ROS 17/2.8	1,25D3 10^{-9} M 0, 6 and 24 h

Database searches were performed using Bone[Title/Abstract] OR osteoblast[Title/Abstract] AND vitamin D AND microarray in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) and GEO (http://www.ncbi.nlm.nih.gov/geo/).

transcriptional activity against the backdrop of osteogenic MSC will be needed to demonstrate the importance of VDR—1,25D3 binding in osteoblast function either in bone formation, regulation of osteoclast formation and activity or in the stem cell niche.

GENE EXPRESSION PROFILING IN OSTEOBLASTS

Besides binding of the VDR to gene regulatory elements, important information on the effect of 1,25D3 on osteoblasts comes from expression profiling studies upon 1,25D3 treatment. Several gene expression profiling studies have been performed to examine the effect of 1,25D3 on RNA expression in osteoblasts. Gene expression profiling in murine MC3T3 cells showed down-regulation of DNA replication genes (Eelen et al., 2004) which fits the earlier observed inhibition of proliferation in these cells. Gene profiling of 1,25D3 treated human osteoblasts at multiple days during the differentiation phase before mineralization did not show regulation a specific set of DNA replication genes (Woeckel et al., 2010). Cell death, RNA splicing translation, and cell cycle genes were identified by Gene Ontology analyses as being most significantly overrepresented (Woeckel et al., 2010). Only 0.6 % (3 genes) of the genes changed in expression during the mineralizing period were also changed prior to mineralization (Woeckel et al., 2010). This study demonstrated that 1,25D3 has different effects on gene expression dependent on the differentiation stage of the cells and should be carefully addressed when investigating the effects of 1,25D3 on mesenchymal stem/stromal cells and differentiated osteoblasts.

Tarroni et al. found that upon 24 h treatment of human osteoblasts with 1,25D3 most genes were upregulated (136 up vs. 20 down) indicating the transcriptional activation of 1,25D3 (Tarroni et al., 2012). Pathway analyses identified various biological functions and/or diseases related to bone metabolism and cellular processes/molecular functions related to skeletal development. The link with skeletal development is supported by another study showing 1,25D3 induced expression in human and mouse osteoblasts of the odd-skipped related genes Osr1 and Osr2, known from expression in the developing limb (Verlinden et al., 2013).

Tarroni et al. also showed strong change in expression of genes linked to inflammation or immune and lymphatic system development (Tarroni et al., 2012). In line with this, is the observation of a gene profiling study showing interferon-related genes being overrepresented after 1,25D3 treatment of human osteoblasts. The interferon signaling related genes were down-regulated by 1,25D3 (Woeckel et al., 2012). The observations on processes related to the immune system are interesting from at least two points of view. Firstly, because of the link between the immune system and bone and the effect of immune cells-derived cytokines on bone metabolism, e.g., in conditions like rheumatoid arthritis. Secondly, considering the above mentioned role of the osteoblasts in the stem cell niche and control of hematopoietic stem cell renewal and differentiation. The expression profiling data and the identification of functions and processes related to the immune system may support a role of vitamin D in osteoblasts control the stem cell niche (Kawamori et al., 2010).

CONCLUSION

Vitamin D can regulate bone metabolism in an indirect way via controlling calcium and phosphate homeostasis but also via direct effects on osteoblasts. In fact, the complete vitamin D endocrine system is present in osteoblasts. This enables osteoblasts to respond not only to vitamin D via the VDR but also to synthesize the biological most active vitamin D metabolite 1,25D3 and to act in an autocrine/paracrine manner. Vitamin D directly regulates gene expression and stimulates mineralization in *ex vivo* cultures of human and rat osteoblasts. The effect on mineralization may depend on species and/or environmental context that can alter the eventual vitamin D effect. Besides effects on bone metabolism, vitamin D effects on osteoblasts may be related to additional functions of osteoblasts such as the hematopoietic stem cell niche. Interesting in this respect is that gene expression profiling studies on vitamin D-treated osteoblasts revealed genes and processes related to the immune system. Further studies are needed to delineate these non-bone metabolism related effects of vitamin D in osteoblasts in greater detail at cellular and molecular level. A future challenge will be to construct networks representing the effects of vitamin D, either in bone metabolism- or in non-bone metabolism-related processes, against the backdrop of osteoblast differentiation by systems biological approaches.

REFERENCES

Anderson, H. C. (1995). Molecular biology of matrix vesicles. *Clin. Orthop. Relat. Res.* 266–280. doi: 10.1097/00003086-199505000-00034

Anderson, H. C., Garimella, R., and Tague, S. E. (2005). The role of matrix vesicles in growth plate development and biomineralization. *Front. Biosci.* 10:1576. doi: 10.2741/1576

Anderson, P. H., Lam, N. N., Turner, A. G., Davey, R. A., Kogawa, M., Atkins, G. J., et al. (2013). The pleiotropic effects of vitamin D in bone. *J. Steroid Biochem. Mol. Biol.* 136, 190–194. doi: 10.1016/j.jsbmb.2012.08.008

Atkins, G. J., Anderson, P. H., Findlay, D. M., Welldon, K. J., Vincent, C., Zannettino, A. C. W., et al. (2007). Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1α, 25-Dihydroxyvitamin D3. *Bone* 40, 1517–1528. doi: 10.1016/j.bone.2007.02.024

Atkins, G. J., and Findlay, D. M. (2012). Osteocyte regulation of bone mineral: a little give and take. *Osteoporos Int.* 23, 2067–2079. doi: 10.1007/s00198-012-1915-z

Bilezikian, J. P., Raisz, L. G., Rodan, G. A., and Karaplis, A. C. (2002). "Embryonic development of bone and the molecular regulation of intramembranous and endochondral bone formation," in *Principles of Bone Biology*, ed A. C. Karaplis (San Diego, CA: Academic Press), 53–84. doi: 10.1016/B978-0-12-373884-4.00025-2

Birkenhäger-Frenkel, D. H., Pols, H. A., Zeelenberg, J., Eijgelsheim, J. J., Schot, R., Nigg, A. L., et al. (1995). Effects of 24R,25-dihydroxyvitamin D3 in combination with 1 alpha-hydroxyvitamin D3 in predialysis renal insufficiency: biochemistry and histomorphometry of cancellous bone. *J. Bone Miner. Res.* 10, 197–204. doi: 10.1002/jbmr.5650100205

Bonewald, L. F. (2011). The amazing osteocyte. *J. Bone Miner. Res.* 26, 229–238. doi: 10.1002/jbmr.320

Bonewald, L. F., Kester, M. B., Schwartz, Z., Swain, L. D., Khare, A., Johnson, T. L., et al. (1992). Effects of combining transforming growth factor beta and 1,25-dihydroxyvitamin D3 on differentiation of a human osteosarcoma (MG-63). *J. Biol. Chem.* 267, 8943–8949.

Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* 423, 337–342. doi: 10.1038/nature01658

Bruedigam, C., Eijken, M., Koedam, M., van de Peppel, J., Drabek, K., Chiba, H., et al. (2010). A new concept underlying stem cell lineage skewing that explains the detrimental effects of thiazolidinediones on bone. *Stem Cells* 28, 916–927. doi: 10.1002/stem.405

Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841–846. doi: 10.1038/nature02040

Chen, C. T., Shih, Y. R., Kuo, T. K., Lee, O. K., and Wei, Y. H. (2008). Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells* 26, 960–968. doi: 10.1634/stemcells.2007-0509

Chen, J., Dosier, C. R., Park, J. H., De, S., Guldberg, R. E., Boyan, B. D., et al. (2013). Mineralization of three-dimensional osteoblast cultures is enhanced by the interaction of 1α,25-dihydroxyvitamin D3 and BMP2 via two specific vitamin D receptors. *J. Tissue Eng. Regen. Med.* doi: 10.1002/term.1770. [Epub ahead of print].

Chen, K., Aenlle, K. K., Curtis, K. M., Roos, B. A., and Howard, G. A. (2012a). Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. *Bone* 51, 69–77. doi: 10.1016/j.bone.2012.04.002

Chen, Y.-C., Ninomiya, T., Hosoya, A., Hiraga, T., Miyazawa, H., and Nakamura, H. (2012b). 1α,25-dihydroxyvitamin D3 inhibits osteoblastic differentiation of mouse periodontal fibroblasts. *Arch. Oral Biol.* 57, 453–459. doi: 10.1016/j.archoralbio.2011.10.005

Chow, J. W., Wilson, A. J., Chambers, T. J., and Fox, S. W. (1998). Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-Week-Old Rats. *J. Bone Miner. Res.* 13, 1760–1767. doi: 10.1359/jbmr.1998.13.11.1760

Corrado, A., Neve, A., and Cantatore, F. P. (2013). Expression of vascular endothelial growth factor in normal, osteoarthritic and osteoporotic osteoblasts. *Clin. Exp. Med.* 13, 81–84. doi: 10.1007/s10238-011-0170-5

Cui, J., Ma, C., Qiu, J., Ma, X., Wang, X., Chen, H., et al. (2011). A novel interaction between insulin-like growth factor binding protein-6 and the vitamin D receptor inhibits the role of vitamin D3 in osteoblast differentiation. *Mol. Cell. Endocrinol.* 338, 84–92. doi: 10.1016/j.mce.2011.03.011

Dobnig, H., and Turner, R. T. (1995). Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology* 136, 3632–3638. doi: 10.1210/en.136.8.3632

Drissi, H., Pouliot, A., Koolloos, C., Stein, J. L., Lian, J. B., Stein, G. S., et al. (2002). 1, 25-(OH)2-vitamin D3 suppresses the bone-related Runx2/Cbfa1 gene promoter. *Exp. Cell Res.* 274, 323–333. doi: 10.1006/excr.2002.5474

Eelen, G., Verlinden, L., Meyer, M. B., Gijsbers, R., Pike, J. W., Bouillon, R., et al. (2013). 1,25-dihydroxyvitamin D3 and the aging-related forkhead box O and sestrin proteins in osteoblasts. *J. Steroid Biochem. Mol. Biol.* 136, 112–119. doi: 10.1016/j.jsbmb.2012.09.011

Eelen, G., Verlinden, L., Van Camp, M., Mathieu, C., Carmeliet, G., Bouillon, R., et al. (2004). Microarray analysis of 1α,25-dihydroxyvitamin D3-treated MC3T3-E1 cells. *J. Steroid Biochem. Mol. Biol.* 89–90, 405–407. doi: 10.1016/j.jsbmb.2004.03.008

Eijken, M., Koedam, M., van Driel, M., Buurman, C. J., Pols, H. A. P., and van Leeuwen, J. P. T. M. (2006). The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. *Mol. Cell. Endocrinol.* 248, 87–93. doi: 10.1016/j.mce.2005.11.034

Eijken, M., Swagemakers, S., Koedam, M., Steenbergen, C., Derkx, P., Uitterlinden, A. G., et al. (2007). The activin A-follistatin system: potent regulator of human extracellular matrix mineralization. *FASEB J.* 21, 2949–2960. doi: 10.1096/fj.07-8080com

Endo, H., Kiyoki, M., Kawashima, K., Naruchi, T., and Hashimoto, Y. (1980). Vitamin D3 metabolites and PTH synergistically stimulate bone formation of chick embryonic femur *in vitro*. *Nature* 286, 262–264. doi: 10.1038/286262a0

Erben, R. G., Weiser, H., Sinowatz, F., Rambeck, W. A., and Zucker, H. (1992). Vitamin D metabolites prevent vertebral osteopenia in ovariectomized rats. *Calcif. Tissue Int.* 50, 228–236. doi: 10.1007/BF00296287

Everts, V., Delaisse, J. M., Korper, W., Jansen, D. C., Tigchelaar-Gutter, W., Saftig, P., et al. (2002). The bone lining cell: its role in cleaning lacunae and initiating bone formation. *J. Bone Miner. Res.* 17, 77–90. doi: 10.1359/jbmr.2002.17.1.77

Farach-Carson, M. C., and Xu, Y. (2002). Microarray detection of gene expression changes induced by 1,25(OH)(2)D(3) and a Ca(2+) influx-activating analog in osteoblastic ROS 17/2.8 cells. *Steroids* 67, 467–470. doi: 10.1016/S0039-128X(01)00168-4

Franceschi, R. T., Romano, P. R., and Park, K. Y. (1988). Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells. *J. Biol. Chem.* 263, 18938–18945.

Fretz, J. A., Zella, L. A., Kim, S., Shevde, N. K., and Pike, J. W. (2007). 1,25-dihydroxyvitamin D3 induces expression of the Wnt signaling co-regulator LRP5 via regulatory elements located significantly downstream of the genes transcriptional start site. *J. Steroid Biochem. Mol. Biol.* 103, 440–445. doi: 10.1016/j.jsbmb.2006.11.018

Friedenstein, A. J., Deriglasova, U. F., Kulagina, N. N., Panasuk, A. F., Rudakowa, S. F., Luriá, E. A., et al. (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp. Hematol.* 2, 83–92.

Galus, K., Szymendera, J., Zaleski, A., and Schreyer, K. (1980). Effects of 1α-hydroxyvitamin D3 and 24R, 25-dihydroxyvitamin D3 on bone remodeling. *Calcif. Tissue Int.* 31, 209–213. doi: 10.1007/BF02407183

Gardiner, E. M., Baldock, P. A., Thomas, G. P., Sims, N. A., Henderson, N. K., Hollis, B., et al. (2000). Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage. *FASEB J.* 14, 1908–1916. doi: 10.1096/fj.99-1075com

Geng, S., Zhou, S., and Glowacki, J. (2011a). Effects of 25-hydroxyvitamin D(3) on proliferation and osteoblast differentiation of human marrow stromal cells require CYP27B1/1α-hydroxylase. *J. Bone Miner. Res.* 26, 1145–1153. doi: 10.1002/jbmr.298

Geng, S., Zhou, S., and Glowacki, J. (2011b). Age-related decline in osteoblastogenesis and 1α-hydroxylase/CYP27B1 in human mesenchymal stem cells: stimulation by parathyroid hormone. *Aging Cell* 10, 962–971. doi: 10.1111/j.1474-9726.2011.00735.x

Godschalk, M., Levy, J. R., and Downs, R. W. (1992). Glucocorticoids decrease vitamin D receptor number and gene expression in human osteosarcoma cells. *J. Bone Miner. Res.* 7, 21–27. doi: 10.1002/jbmr.5650070105

Grundberg, E., Adoue, V., Kwan, T., Ge, B., Duan, Q. L., Lam, K. C. L., et al. (2011). Global analysis of the impact of environmental perturbation on cis-regulation of gene expression. *PLoS Genet.* 7:e1001279. doi: 10.1371/journal.pgen.1001279

Haussler, M. R., Haussler, C. A., Whitfield, G. K., Hsieh, J.-C., Thompson, P. D., Barthel, T. K., et al. (2010). The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. *J. Steroid Biochem. Mol. Biol.* 121, 88–97. doi: 10.1016/j.jsbmb.2010.03.019

Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., et al. (1998). The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone Miner. Res.* 13, 325–349. doi: 10.1359/jbmr.1998.13.3.325

Heikkinen, S., Väisänen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1,25-Dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Henry, H., and Norman, A. (1978). Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. *Science* 201, 835–837. doi: 10.1126/science.684411

Hewison, M., Freeman, L., Hughes, S. V., Evans, K. N., Bland, R., Eliopoulos, A. G., et al. (2003). Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol.* 170, 5382–5390.

Hewison, M., Zehnder, D., Chakraverty, R., and Adams, J. S. (2004). Vitamin D and barrier function: a novel role for extra-renal 1α-hydroxylase. *Mol. Cell. Endocrinol.* 215, 31–38. doi: 10.1016/j.mce.2003.11.017

Hicok, K. C., Thomas, T., Gori, F., Rickard, D. J., Spelsberg, T. C., and Riggs, B. L. (1998). Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma. *J. Bone Miner. Res.* 13, 205–217. doi: 10.1359/jbmr.1998.13.2.205

Holick, M. F. (1995). Defects in the synthesis and metabolism of vitamin D. *Exp. Clin. Endocrinol. Diabetes* 103, 219–227. doi: 10.1055/s-0029-1211354

Hong, D., Chen, H.-X., Yu, H.-Q., Liang, Y., Wang, C., Lian, Q.-Q., et al. (2010). Morphological and proteomic analysis of early stage of osteoblast differentiation in osteoblastic progenitor cells. *Exp. Cell Res.* 316, 2291–2300. doi: 10.1016/j.yexcr.2010.05.011

Ingram, R. T., Bonde, S. K., Riggs, B. L., and Fitzpatrick, L. A. (1994). Effects of transforming growth factor beta (TGFβ) and 1,25 dihydroxyvitamin D3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. *Differentiation* 55, 153–163. doi: 10.1046/j.1432-0436.1994.5520153.x

Ito, N., Findlay, D. M., Anderson, P. H., Bonewald, L. F., and Atkins, G. J. (2013). Extracellular phosphate modulates the effect of 1α,25-dihydroxy vitamin D3

(1,25D) on osteocyte like cells. *J. Steroid Biochem. Mol. Biol.* 136, 1–4. doi: 10.1016/j.jsbmb.2012.09.029

Jilka, R. L., Weinstein, R. S., Bellido, T., Parfitt, A. M., and Manolagas, S. C. (1998). Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J. Bone Miner. Res.* 13, 793–802. doi: 10.1359/jbmr.1998.13.5.793

Jørgensen, N. R., Henriksen, Z., Sørensen, O. H., and Civitelli, R. (2004). Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an *in vitro* model for human bone marrow-derived primary osteoblasts. *Steroids* 69, 219–226. doi: 10.1016/j.steroids.2003.12.005

Karunaratne, A., Esapa, C. T., Hiller, J., Boyde, A., Head, R., Bassett, J. H. D., et al. (2012). Significant deterioration in nanomechanical quality occurs through incomplete extrafibrillar mineralization in rachitic bone: evidence from *in-situ* synchrotron X-ray scattering and backscattered electron imaging. *J. Bone Min. Res.* 27, 876–890. doi: 10.1002/jbmr.1495

Kato, A., Seo, E. G., Einhorn, T. A., Bishop, J. E., and Norman, A. W. (1998). Studies on 24R,25-dihydroxyvitamin D3: evidence for a nonnuclear membrane receptor in the chick tibial fracture-healing callus. *Bone* 23, 141–146. doi: 10.1016/S8756-3282(98)00085-4

Kawamori, Y., Katayama, Y., Asada, N., Minagawa, K., Sato, M., Okamura, A., et al. (2010). Role for vitamin D receptor in the neuronal control of the hematopoietic stem cell niche. *Blood* 116, 5528–5535. doi: 10.1182/blood-2010-04-279216

Kearns, A. E., Donohue, M. M., Sanyal, B., and Demay, M. B. (2001). Cloning and characterization of a novel protein kinase that impairs osteoblast differentiation *in vitro*. *J. Biol. Chem.* 276, 42213–42218. doi: 10.1074/jbc.M106163200

Kitanaka, S., Takeyama, K., Murayama, A., Sato, T., Okumura, K., Nogami, M., et al. (1998). Inactivating mutations in the 25-hydroxyvitamin D3 1alpha-hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *N. Engl. J. Med.* 338, 653–661. doi: 10.1056/NEJM199803053381004

Komarova, S. V., Ataullakhanov, F. I., and Globus, R. K. (2000). Bioenergetics and mitochondrial transmembrane potential during differentiation of cultured osteoblasts. *Am. J. Physiol. Cell Physiol.* 279, C1220–C1229.

Koshihara, Y., and Hoshi, K. (1997). Vitamin K2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts *in vitro*. *J. Bone Miner. Res.* 12, 431–438. doi: 10.1359/jbmr.1997.12.3.431

Kostenuik, P. J., and Shalhoub, V. (2001). Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. *Curr. Pharm. Des.* 7, 613–635. doi: 10.2174/1381612013397807

Kveiborg, M., Flyvbjerg, A., Eriksen, E. F., and Kassem, M. (2001). 1,25-dihydroxyvitamin D3 stimulates the production of insulin-like growth factor-binding proteins-2, -3 and -4 in human bone marrow stromal cells. *Eur. J. Endocrinol.* 144, 549–557. doi: 10.1530/eje.0.1440549

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93, 165–176. doi: 10.1016/S0092-8674(00)81569-X

Li, J., Jin, D., Fu, S., Mei, G., Zhou, J., Lei, L., et al. (2013). Insulin-like growth factor binding protein-3 modulates osteoblast differentiation via interaction with vitamin D receptor. *Biochem. Biophys. Res. Commun.* 436, 632–637. doi: 10.1016/j.bbrc.2013.04.111

Li, J. J., and Sodek, J. (1993). Cloning and characterization of the rat bone sialoprotein gene promoter. *Biochem. J.* 289(Pt 3), 625–629.

Li, Y. C., Pirro, A. E., Amling, M., Delling, G., Baron, R., Bronson, R., et al. (1997). Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc. Natl. Acad. Sci. U.S.A.* 94, 9831–9835. doi: 10.1073/pnas.94.18.9831

Lian, J. B., Shalhoub, V., Aslam, F., Frenkel, B., Green, J., Hamrah, M., et al. (1997). Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. *Endocrinology* 138, 2117–2127.

Lian, J. B., Stein, G. S., van Wijnen, A. J., Stein, J. L., Hassan, M. Q., Gaur, T., et al. (2012). MicroRNA control of bone formation and homeostasis. *Nat. Revi. Endocrinol.* 8, 212–227. doi: 10.1038/nrendo.2011.234

Lieben, L., Masuyama, R., Torrekens, S., Van Looveren, R., Schrooten, J., Baatsen, P., et al. (2012). Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D–induced Inhibition of Bone Mineralization. *J. Clin. Invest.* 122, 1803–1815. doi: 10.1172/JCI45890

Lisse, T. S., Adams, J. S., and Hewison, M. (2013a). Vitamin D and microRNAs in bone. *Crit. Rev. Eukaryot. Gene Expr.* 23, 195–214. doi: 10.1615/CritRevEukaryotGeneExpr.2013007147

Lisse, T. S., Chun, R. F., Rieger, S., Adams, J. S., and Hewison, M. (2013b). Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts. *J. Bone Miner. Res.* 28, 1478–1488. doi: 10.1002/jbmr.1882

MacDonald, P. N., Dowd, D. R., Nakajima, S., Galligan, M. A., Reeder, M. C., Haussler, C. A., et al. (1993). Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. *Mol. Cell. Biol.* 13, 5907–5917.

Mackie, E. J., Ahmed, Y. A., Tatarczuch, L., Chen, K.-S., and Mirams, M. (2008). Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int. J. Biochem. Cell Biol.* 40, 46–62. doi: 10.1016/j.biocel.2007.06.009

Mackie, E. J., Tatarczuch, L., and Mirams, M. (2011). The skeleton: a multi-functional complex organ. The growth plate chondrocyte and endochondral ossification. *J. Endocrinol.* 211, 109–121. doi: 10.1530/JOE-11-0048

Maehata, Y., Takamizawa, S., Ozawa, S., Kato, Y., Sato, S., Kubota, E., et al. (2006). Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: roles of osterix, an osteoblast-related transcription factor. *Matrix Biol.* 25, 47–58. doi: 10.1016/j.matbio.2005.09.001

Martinello, T., Bronzini, I., Volpin, A., Vindigni, V., Maccatrozzo, L., Caporale, G., et al. (2012). Successful recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel. *J. Tissue Eng. Regen. Med.* doi: 10.1002/term.1557. [Epub ahead of print].

Mathieu, C., Van Etten, E., Gysemans, C., Decallonne, B., Kato, S., Laureys, J., et al. (2001). *In vitro* and *in vivo* analysis of the immune system of vitamin D receptor knockout mice. *J. Bone Miner. Res.* 16, 2057–2065. doi: 10.1359/jbmr.2001.16.11.2057

Matsumoto, T., Ezawa, I., Morita, K., Kawanobe, Y., and Ogata, E. (1985). Effect of vitamin D metabolites on bone metabolism in a rat model of postmenopausal osteoporosis. *J. Nutr. Sci. Vitaminol.* 31, S61–S65. doi: 10.3177/jnsv.31.Supplement_S61

Matsumoto, T., Igarashi, C., Takeuchi, Y., Harada, S., Kikuchi, T., Yamato, H., et al. (1991). Stimulation by 1, 25-dihydroxyvitamin D3 of *in vitro* mineralization induced by osteoblast-like MC3T3-E1 cells. *Bone* 12, 27–32. doi: 10.1016/8756-3282(91)90051-J

McCollum, E. V., Pitz, W., Simmonds, N., Becker, J. E., Shipley, P. G., and Bunting, R. W. (2002). The effect of additions of fluorine to the diet of the rat on the quality of the teeth. 1925. Studies on experimental rickets. XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. 1922. The effect of addition. *J. Biol. Chem.* 277:E8.

Meyer, M. B. (2005). The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. *Mol. Endocrinol.* 20, 1447–1461. doi: 10.1210/me.2006-0031

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2010). Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. *J. Steroid Biochem. Mol. Biol.* 121, 136–141. doi: 10.1016/j.jsbmb.2010.02.011

Minguell, J. J., Erices, A., and Conget, P. (2001). Mesenchymal stem cells. *Exp. Biol. Med. (Maywood).* 226, 507–520.

Misof, B. M., Roschger, P., Tesch, W., Baldock, P. A., Valenta, A., Messmer, P., et al. (2003). Targeted overexpression of vitamin D receptor in osteoblasts increases calcium concentration without affecting structural properties of bone mineral crystals. *Calcif. Tissue Int.* 73, 251–257. doi: 10.1007/s00223-002-2139-6

Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-hora, M., Feng, J. Q., et al. (2011). Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat. Med.* 17, 1231–1234. doi: 10.1038/nm.2452

Nishimura, R., Hata, K., Ono, K., Amano, K., Takigawa, Y., Wakabayashi, M., et al. (2012). Regulation of endochondral ossification by transcription factors. *Front. Biosci.* 17:4076. doi: 10.2741/4076

Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., DeLuca, H. F., and Denhardt, D. T. (1990). Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or Osteopontin) gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9995–9999. doi: 10.1073/pnas.87.24.9995

Norman, A. W., Henry, H. L., and Malluche, H. H. (1980). 24R, 25-dihydroxyvitamin D3 and 1α, 25-dihydroxyvitamin D3; are both indispensable for calcium and phosphorus homeostasis. *Life Sci.* 27, 229–237. doi: 10.1016/0024-3205(80)90142-3

Ohyama, Y., Ozono, K., Uchida, M., Shinki, T., Kato, S., Suda, T., et al. (1994). Identification of a vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D3 24-hydroxylase gene. *J. Biol. Chem.* 269, 10545–10550.

Owen, T. A., Aronow, M. S., Barone, L. M., Bettencourt, B., Stein, G. S., and Lian, J. B. (1991). Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in norma. *Endocrinology* 128, 1496–1504. doi: 10.1210/endo-128-3-1496

Ozono, K., Liao, J., Kerner, S. A., Scott, R. A., and Pike, J. W. (1990). The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. *J. Biol. Chem.* 265, 21881–21888.

Petkovich, P. M., Wrana, J. L., Grigoriadis, A. E., Heersche, J. N., and Sodek, J. (1987). 1,25-dihydroxyvitamin D3 increases epidermal growth factor receptors and transforming growth factor beta-like activity in a bone-derived cell line. *J. Biol. Chem.* 262, 13424–13428.

Piek, E., Sleumer, L. S., van Someren, E. P., Heuver, L., de Haan, J. R., de Grijs, I., et al. (2010). Osteo-transcriptomics of human mesenchymal stem cells: accelerated gene expression and osteoblast differentiation induced by vitamin D reveals c-MYC as an enhancer of BMP2-induced osteogenesis. *Bone* 46, 613–627. doi: 10.1016/j.bone.2009.10.024

Pols, H. A., Birkenhäger, J. C., Schilte, J. P., and Visser, T. J. (1988a). Evidence that the self-induced metabolism of 1,25-dihydroxyvitamin D-3 limits the homologous up-regulation of its receptor in rat osteosarcoma cells. *Biochim. Biophys. Acta* 970, 122–129. doi: 10.1016/0167-4889(88)90170-X

Pols, H. A. P., van Leeuwen, J. P. T. M., Schilte, J. P., Visser, T. J., and Birkenhäger, J. C. (1988b). Heterologous up-regulation of the 1, 25-dihydroxyvitamin D3 receptor by parathyroid hormone (PTH) and PTH-like peptide in osteoblast-like cells. *Biochem. Biophys. Res. Commun.* 156, 588–594. doi: 10.1016/S0006-291X(88)80883-0

Prince, M., Banerjee, C., Javed, A., Green, J., Lian, J. B., Stein, G. S., et al. (2001). Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. *J. Cell. Biochem.* 80, 424–440. doi: 10.1002/1097-4644(20010301)80:3%3C424::AID-JCB160%3E3.0.CO;2-6

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Reid, I. R., Bolland, M. J., and Grey, A. (2014). Effects of vitamin D supplements on bone mineral density: a systematic review and meta-analysis. *Lancet* 383, 146–155. doi: 10.1016/S0140-6736(13)61647-5

Reinhardt, T. A., and Horst, R. L. (1990). Parathyroid hormone down-regulates 1,25-dihydroxyvitamin D receptors (VDR) and VDR messenger ribonucleic acid *in vitro* and blocks homologous up-regulation of VDR *in vivo*. *Endocrinology* 127, 942–948. doi: 10.1210/endo-127-2-942

Sammons, J., Ahmed, N., El-Sheemy, M., and Hassan, H. T. (2004). The role of BMP-6, IL-6, and BMP-4 in mesenchymal stem cell-dependent bone development: effects on osteoblastic differentiation induced by parathyroid hormone and vitamin D(3). *Stem Cells Dev.* 13, 273–280. doi: 10.1089/154732804323099208

Scharla, S. H., Strong, D. D., Mohan, S., Baylink, D. J., and Linkhart, T. A. (1991). 1,25-dihydroxyvitamin D3 differentially regulates the production of insulin-like growth factor I (IGF-I) and IGF-binding protein-4 in mouse osteoblasts. *Endocrinology* 129, 3139–3146. doi: 10.1210/endo-129-6-3139

Schlaeppi, J. M., Gutzwiller, S., Finkenzeller, G., and Fournier, B. (1997). 1,25-dihydroxyvitamin D3 induces the expression of vascular endothelial growth factor in osteoblastic cells. *Endocr. Res.* 23, 213–229. doi: 10.3109/07435809709031855

Seo, E. G., Einhorn, T. A., and Norman, A. W. (1997). 24R, 25-dihydroxyvitamin D3: an essential vitamin D3 metabolite for both normal bone integrity and healing of tibial fracture in chicks. *Endocrinology* 138, 3864–3872.

Seo, E. G., and Norman, A. W. (1997). Three-fold induction of renal 25-hydroxyvitamin D3-24-hydroxylase activity and increased serum 24,25-dihydroxyvitamin D3 levels are correlated with the healing process after chick tibial fracture. *J. Bone Miner. Res.* 12, 598–606. doi: 10.1359/jbmr.1997.12.4.598

Shevde, N. K., Plum, L. A., Clagett-Dame, M., Yamamoto, H., Pike, J. W., and DeLuca, H. F. (2002). A potent analog of 1α, 25-dihydroxyvitamin D3 selectively induces bone formation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13487–13491. doi: 10.1073/pnas.202471299

Shi, Y.-C., Worton, L., Esteban, L., Baldock, P., Fong, C., and Eisman, J. A. (2007). Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation. *Bone* 41, 87–96. doi: 10.1016/j.bone.2007.04.174

Shiozawa, Y., Pedersen, E. A., Havens, A. M., Jung, Y., Mishra, A., Joseph, J., et al. (2011). Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow. *J. Clin. Invest.* 121, 1298–1312. doi: 10.1172/JCI43414

Siggelkow, H., Schulz, H., Kaesler, S., Benzler, K., Atkinson, M. J., and Hüfner, M. (1999). 1,25 dihydroxyvitamin-D3 attenuates the confluence-dependent differences in the osteoblast characteristic proteins alkaline phosphatase, procollagen I peptide, and osteocalcin. *Calcif. Tissue Int.* 64, 414–421. doi: 10.1007/PL00005823

Sims, N. A., and Martin, T. J. (2014). Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. *Bonekey Rep.* 3, 481. doi: 10.1038/bonekey.2013.215

Sooy, K., Sabbagh, Y., and Demay, M. B. (2004). Osteoblasts lacking the vitamin D receptor display enhanced osteogenic potential *in vitro*. *J. Cell. Biochem.* 94, 81–87. doi: 10.1002/jcb.20313

Staal, A., Birkenhäger, J. C., Pols, H. A., Buurman, C. J., Vink-van Wijngaarden, T., Kleinekoort, W. M. C., et al. (1994). Transforming growth factor β-induced dissociation between vitamin D receptor level and 1, 25-dihydroxyvitamin D3 action in osteoblast-like cells. *Bone Miner.* 26, 27–42. doi: 10.1016/S0169-6009(08)80160-2

Staal, A., Geertsma-Kleinekoort, W. M., van den Bemd, G. J., Buurman, C. J., Birkenhäger, J. C., Pols, H. A., et al. (1998). Regulation of osteocalcin production and bone resorption by 1,25-dihydroxyvitamin D3 in mouse long bones: interaction with the bone-derived growth factors TGF-beta and IGF-I. *J. Bone Miner. Res.* 13, 36–43. doi: 10.1359/jbmr.1998.13.1.36

Staal, A., Lian, J. B., van Wijnen, A. J., Desai, R. K., Pols, H. A. P., Birkenhäger, J. C., et al. (1996). Antagonistic effects of transforming growth factor-beta on vitamin D3 enhancement of osteocalcin and osteopontin transcription: reduced interactions of vitamin D receptor/retinoid X receptor complexes with vitamin E response elements. *Endocrinology* 137, 2001–2011. doi: 10.1210/endo.137.5.8612541

St-Arnaud, R. (2010). CYP24A1-deficient mice as a tool to uncover a biological activity for vitamin D metabolites hydroxylated at position 24. *J. Steroid Biochem. Mol. Biol.* 121, 254–256. doi: 10.1016/j.jsbmb.2010.02.002

Tam, C. S., Heersche, J. N., Jones, G., Murray, T. M., and Rasmussen, H. (1986). The effect of vitamin D on bone *in vivo*. *Endocrinology* 118, 2217–2224. doi: 10.1210/endo-118-6-2217

Tarroni, P., Villa, I., Mrak, E., Zolezzi, F., Mattioli, M., Gattuso, C., et al. (2012). Microarray analysis of 1,25(OH)2D3 regulated gene expression in human primary osteoblasts. *J. Cell. Biochem.* 113, 640–649. doi: 10.1002/jcb.23392

Tatsumi, S., Ishii, K., Amizuka, N., Li, M., Kobayashi, T., Kohno, K., et al. (2007). Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.* 5, 464–475. doi: 10.1016/j.cmet.2007.05.001

Terrand, J., Bruban, V., Zhou, L., Gong, W., El Asmar, Z., May, P., et al. (2009). LRP1 controls intracellular cholesterol storage and fatty acid synthesis through modulation of Wnt signaling. *J. Biol. Chem.* 284, 381–388. doi: 10.1074/jbc.M806538200

Teti, A. (2011). Bone development: overview of bone cells and signaling. *Curr. Osteoporos. Rep.* 9, 264–273. doi: 10.1007/s11914-011-0078-8

Thomas, G. (2000). Species-divergent regulation of human and mouse osteocalcin genes by calciotropic hormones. *Exp. Cell Res.* 258, 395–402. doi: 10.1006/excr.2000.4912

Tuohimaa, P., Wang, J.-H., Khan, S., Kuuslahti, M., Qian, K., Manninen, T., et al. (2013). Gene expression profiles in human and mouse primary cells provide new insights into the differential actions of vitamin D3 metabolites. Edited by Andrzej T Slominski. *PLoS ONE* 8:e75338. doi: 10.1371/journal.pone.0075338

Turner, A. G., Dwivedi, P. P., May, B. K., and Morris, H. A. (2007). Regulation of the CYP27B1 5'-flanking region by transforming growth factor-beta in ROS

17/2.8 osteoblast-like cells. *J. Steroid Biochem. Mol. Biol.* 103, 322–325. doi: 10.1016/j.jsbmb.2006.12.041

Ueno, K., Katayama, T., Miyamoto, T., and Koshihara, Y. (1992). Interleukin-4 enhances *In vitro*; mineralization in human osteoblast-like cells. *Biochem. Biophys. Res. Commun.* 189, 1521–1526. doi: 10.1016/0006-291X(92)90248-J

Van Driel, M., Koedam, M., Buurman, C. J., Hewison, M., Chiba, H., Uitterlinden, A. G., et al. (2006a). Evidence for auto/paracrine actions of vitamin D in bone: 1 -hydroxylase expression and activity in human bone cells. *FASEB J.* 20, 2417–2419. doi: 10.1096/fj.06-6374fje

Van Driel, M., Koedam, M., Buurman, C. J., Roelse, M., Weyts, F., Chiba, H., et al. (2006b). Evidence that both 1α,25-dihydroxyvitamin D3 and 24-hydroxylated D3 enhance human osteoblast differentiation and mineralization. *J. Cell. Biochem.* 99, 922–935. doi: 10.1002/jcb.20875

Van Holten, J., Reedquist, K., Sattonet-Roche, P., Smeets, T. J. M., Plater-Zyberk, C., Vervoordeldonk, M. J., et al. (2004). Treatment with recombinant interferon-beta reduces inflammation and slows cartilage destruction in the collagen-induced arthritis model of rheumatoid arthritis. *Arthritis Res. Ther.* 6, R239–R249. doi: 10.1186/ar1165

van Leeuwen, J. P., Birkenhäger, J. C., Buurman, C. J., Van den Bemd, G. J., Bos, M. P., and Pols, H. A. (1992a). Bidirectional regulation of the 1, 25-dihydroxyvitamin D3 receptor by phorbol ester-activated protein kinase-C in osteoblast-like cells: interaction with adenosine 3′,5′-monophosphate-induced up-regulation of the 1, 25-dihydroxyvitamin D3 Recep. *Endocrinology* 130, 2259–2266.

van Leeuwen, J. P., Birkenhäger, J. C., Vink-van Wijngaarden, T., van den Bemd, G. J., and Pols, H. A. (1992b). Regulation of 1,25-dihydroxyvitamin D3 receptor gene expression by parathyroid hormone and cAMP-agonists. *Biochem. Biophys. Res. Commun.* 185, 881–886. doi: 10.1016/0006-291X(92)91709-Y

van Leeuwen, J. P., Pols, H. A., Schilte, J. P., Visser, T. J., and Birkenhäger, J. C. (1991). Modulation by epidermal growth factor of the basal 1,25(OH)2D3 receptor level and the heterologous up-regulation of the 1,25(OH)2D3 receptor in clonal osteoblast-like cells. *Calcif. Tissue Int.* 49, 35–42. doi: 10.1007/BF02555900

Verlinden, L., Kriebitzsch, C., Eelen, G., Van Camp, M., Leyssens, C., Tan, B. K., et al. (2013). The odd-skipped related genes Osr1 and Osr2 are induced by 1,25-dihydroxyvitamin D3. *J. Steroid Biochem. Mol. Biol.* 136, 94–97. doi: 10.1016/j.jsbmb.2012.12.001

Viereck, V., Siggelkow, H., Tauber, S., Raddatz, D., Schutze, N., and Hüfner, M. (2002). Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. *J. Cell. Biochem.* 86, 348–356. doi: 10.1002/jcb.10220

Wang, D. S., Miura, M., Demura, H., and Sato, K. (1997). Anabolic effects of 1, 25-dihydroxyvitamin D3 on osteoblasts are enhanced by vascular endothelial growth factor produced by osteoblasts and by growth factors produced by endothelial cells. *Endocrinology* 138, 2953–2962.

Wang, D. S., Yamazaki, K., Nohtomi, K., Shizume, K., Ohsumi, K., Shibuya, M., et al. (1996). Increase of vascular endothelial growth factor mRNA expression by 1, 25−dihydroxyvitamin D3 in human osteoblast−like cells. *J. Bone Miner. Res.* 11, 472–479. doi: 10.1002/jbmr.5650110408

Wang, E. A. (1993). Bone morphogenetic proteins (BMPs): therapeutic potential in healing bony defects. *Trends Biotechnol.* 11, 379–383. doi: 10.1016/0167-7799(93)90096-R

Weinstein, R. S., Jilka, R. L., Parfitt, A. M., and Manolagas, S. C. (1998). Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J. Clin. Invest.* 102, 274–282. doi: 10.1172/JCI2799

Weisman, Y., Salama, R., Harell, A., and Edelstein, S. (1978). Serum 24,25-Dihydroxyvitamin D and 25-Hydroxyvitamin D Concentrations in Femoral Neck Fracture. *Br. Med. J.* 2, 1196. doi: 10.1136/bmj.2.6146.1196-a

Woeckel, V. J., Alves, R. D. A. M., Swagemakers, S. M. A., Eijken, M., Chiba, H., van der Eerden, B. C. J., et al. (2010). 1Alpha,25-(OH)2D3 acts in the early phase of osteoblast differentiation to enhance mineralization via accelerated production of mature matrix vesicles. *J. Cell. Physiol.* 225, 593–600. doi: 10.1002/jcp.22244

Woeckel, V. J., Koedam, M., van de Peppel, J., Chiba, H., van der Eerden, B. C. J., and van Leeuwen, J. P. T. M. (2012). Evidence of vitamin d and interferon-β cross-talk in human osteoblasts with 1α,25-dihydroxyvitamin D3 being dominant over interferon-β in stimulating mineralization. *J. Cell. Physiol.* 227, 3258–3266. doi: 10.1002/jcp.24020

Woeckel, V. J., van der Eerden, B. C. J., Schreuders-Koedam, M., Eijken, M., and van Leeuwen, J. P. T. M. (2013). 1α,25-dihydroxyvitamin D3 stimulates activin a production to fine-tune osteoblast-induced mineralization. *J. Cell. Physiol.* 228, 2167–2174. doi: 10.1002/jcp.24388

Xue, Y., Karaplis, A. C., Hendy, G. N., Goltzman, D., and Miao, D. (2006). Exogenous 1,25-dihydroxyvitamin D3 exerts a skeletal anabolic effect and improves mineral ion homeostasis in mice that are homozygous for both the 1alpha-hydroxylase and parathyroid hormone null alleles. *Endocrinology* 147, 4801–4810. doi: 10.1210/en.2006-0403

Yamaguchi, M., and Weitzmann, M. N. (2012). High dose 1,25(OH)2D3 inhibits osteoblast mineralization *in vitro*. *Int. J. Mol. Med.* 29, 934–938. doi: 10.3892/ijmm.2012.900

Yamamoto, Y., Yoshizawa, T., Fukuda, T., Shirode-Fukuda, Y., Yu, T., Sekine, K., et al. (2013). Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. *Endocrinology* 154, 1008–1020. doi: 10.1210/en.2012-1542

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., et al. (1999). Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321. doi: 10.1126/science.283.5406.1317

Yarram, S. J., Tasman, C., Gidley, J., Clare, M., Sandy, J. R., and Mansell, J. P. (2004). Epidermal growth factor and calcitriol synergistically induce osteoblast maturation. *Mol. Cell. Endocrinol.* 220, 9–20. doi: 10.1016/j.mce.2004.04.005

Yin, T. (2006). The stem cell niches in bone. *J. Clin. Invest.* 116, 1195–1201. doi: 10.1172/JCI28568

Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., et al. (1997). Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat. Genet.* 16, 391–396. doi: 10.1038/ng0897-391

Zhang, R., Ducy, P., and Karsenty, G. (1997). 1,25-dihydroxyvitamin D3 inhibits osteocalcin expression in mouse through an indirect mechanism. *J. Biol. Chem.* 272, 110–116. doi: 10.1074/jbc.272.1.110

Zhou, S., Geng, S., and Glowacki, J. (2013). Histone deacetylation mediates the rejuvenation of osteoblastogenesis by the combination of 25(OH)D3 and parathyroid hormone in MSCs from elders. *J. Steroid Biochem. Mol. Biol.* 136, 156–159. doi: 10.1016/j.jsbmb.2012.09.002

Zhou, S., Glowacki, J., Kim, S. W., Hahne, J., Geng, S., Mueller, S. M., et al. (2012). Clinical characteristics influence *in vitro* action of 1,25-dihydroxyvitamin D 3in human marrow stromal cells. *J. Bone Miner. Res.* 27, 1992–2000. doi: 10.1002/jbmr.1655

Zhou, S., LeBoff, M. S., and Glowacki, J. (2010). Vitamin D metabolism and action in human bone marrow stromal cells. *Endocrinology* 151, 14–22. doi: 10.1210/en.2009-0969

Zhou, Y., Liu, Y., and Tan, J. (2006). Is 1, 25-dihydroxyvitamin D3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells *in vitro*? *Chin. Med. J. (Engl.)* 119, 1278–1286.

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The role of vitamin D in skeletal and cardiac muscle function

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Myopathy is a feature of many inflammatory syndromes. Chronic inflammation has been linked to pathophysiological mechanisms which implicate 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)-mediated signaling pathways with emerging evidence supporting a role for the vitamin D receptor (VDR) in contractile and metabolic function of both skeletal and cardiac muscle. Altered VDR expression in skeletal and cardiac muscle has been reported to result in significant effects on metabolism, calcium signaling and fibrosis in these tissues. Elevated levels of serum inflammatory cytokines, such as IL-6, TNF-α and IFNγ, have been shown to impact myogenic and nuclear receptor signaling pathways in cancer-induced cachexia. The dysregulation of nuclear receptors, such as VDR and RXRα in muscle cells, has also been postulated to result in myopathy via their effects on muscle structural integrity and metabolism. Future research directions include generating transcriptome-wide information incorporating VDR and its gene targets and using systems biology approaches to identify altered molecular networks in human tissues such as muscle. These approaches will aid in the development of novel therapeutic targeting strategies for inflammation-induced myopathies.

Keywords: cytokines, cancer cachexia, skeletal muscle, cardiac muscle and transcriptome

VITAMIN D AND MUSCLE FUNCTION

Vitamin D is necessary for the maintenance of structural integrity and function of the musculoskeletal system (Pfeifer et al., 2002). Severe deficiency results in impaired bone strength and deformation i.e., rickets in children and osteomalacia in adults. Vitamin D plays a significant role in calcium homeostasis and bone metabolism through its actions on target tissues (DeLuca, 2004). Serum vitamin D levels have been correlated to muscle cell contractility, muscle strength, and postural stability (Rodman and Baker, 1978; Marcinkowska, 2001; Grimaldi et al., 2013; Girgis et al., 2014). Low serum levels have been related to proximal muscle weakness, gait disturbance, paresthesia, and discomfort within the muscles (Skaria et al., 1975; Schott and Wills, 1976; Glerup and Eriksen, 1999; Glerup et al., 2000; Pfeifer et al., 2002; Ahmed et al., 2009).

The clinical features of myopathy associated with severe vitamin D deficiency are supported by findings of abnormal histological and electrophysiological changes in muscle. Histological analysis of skeletal muscle biopsied from adults with vitamin D deficiency reveal enlarged inter-fibrillar spaces, infiltration of fat, presence of glycogen granules, fibrosis, and type II muscle fiber (fast-twitch) atrophy (Sorensen et al., 1979; Yoshikawa et al., 1979; Boland, 1986; Sato et al., 2005). Recent interest has been in reversal of some of these pathological effects of this clinical syndrome. Meta-analysis of randomized controlled trials in the elderly with low serum levels of vitamin D, demonstrated a decrease in the risk of falls following supplementation with vitamin D (Rejnmark, 2011). This outcome has been attributed to the ability of vitamin D to impact muscle fiber composition hence skeletal muscle structure. Studies in vitamin D deficient patients revealed an increase in percentage of type II fibers, a significant increase in mean type II muscle fiber diameter and area particularly of type IIa muscle fibers following treatment with 1-α-hydroxyvitaminD_3 and calcium. However, it is still unclear if vitamin D supplementation induced formation of new type II fibers or increased transition of existing type I fibers from to type II (Sorensen et al., 1979; Sato et al., 2005). Vitamin D has also been demonstrated to increase cell proliferation and inhibit apoptosis in injured rat soleus skeletal muscle, with positive functional outcomes such as faster recovery of contraction forces (Stratos et al., 2013). The therapeutic potential of vitamin D supplementation has also recently been tested on dysferlin gene regulation and dysferlinopathies (autosomal recessive neuromuscular disorder characterized by progressive muscle wasting due to dysferlin gene mutations and a deficiency of functional dysferlin protein). Vitamin D increased dysferlin gene expression in both HL60 monocytes and skeletal muscle cells via the activation of vitamin D receptor (VDR) which binds to the dysferlin promoter; and non-genomic MEK/ERK signaling and classical genomic effects. 1,25$(OH)_2D_3$ has also been reported to suppress myotube formation by decreasing Myf5 and myogenin gene expression resulting in increased myotube diameters but reduced myostatin expression potentially alleviating the myopathic effects of muscle weakness and reduced contractile function (Luna et al., 2012).

Experiments in C2C12 cells highlight some key molecular regulatory effects of 1,25$(OH)_2D_3$ including: (1) increased expression and nuclear translocation of the VDR, (2) decreased cell proliferation, (3) decreased IGF-I expression, and (4) increased IGF-II and follistatin expression and decreasing the expression of myostatin which appeared to promote myogenic differentiation and (5) altered differentiation and myotube size. Hence, vitamin D may also be considered for use in intervention studies for muscle conditions that involve these mechanisms (Garcia et al., 2011; Girgis et al., 2014).

THE ROLE OF VITAMIN D RECEPTOR IN MUSCLE FUNCTION

The effects of vitamin D are modulated by its receptor, therefore the expression and distribution of VDR is of significant importance. Early studies demonstrated the presence of the VDR in cultured human myoblasts and myotubes which showed a response to physiological concentrations of 1,25-$(OH)_2D_3$. VDR is also present in human skeletal muscle cells within the nuclei and has been shown to play a role in skeletal muscle development, my fiber size and morphology (Simpson et al., 1985; Costa et al., 1986; Bischoff et al., 2001; Bischoff-Ferrari et al., 2006). Skeletal muscle development requires a co-ordinated series of transcription factor and growth factor events that enable progenitor cells to undergo myoblast determination (requiring Pax3, Pax7, MyoD, and Myf5) then myoblast to myotube determination (requiring $p21^{Cip1}$, myogenin, MEF2C and Rb) then further myotube maturation requiring innervation, MRF4, MLP) (Ludolph and Konieczny, 1995; Perry and Rudnicki, 2000; Ryhänen et al., 2003; Miyazawa et al., 2005; Washington et al., 2011). VDR and myosin heavy chain isoform was shown to co-localize in skeletal muscle biopsies in older female subjects (Ceglia et al., 2010). VDR has also been shown to impact the expression of myogenic transcriptional regulators, in particular Myf5, myogenin, E2A, and early myosin heavy chain isoforms (Endo et al., 2003; Girgis et al., 2014). C2C12 myoblasts treated with 1,25-$(OH)_2D_3$ showed increased VDR and CYP24A1 expression above endogenous levels which resulted in inhibition in cell proliferation (Srikuea et al., 2012; Girgis et al., 2014). Furthermore, inhibition of myogenic differentiation of C2C12 and G8 cell lines was also achieved with suppression of VDR expression, suggesting that myoblasts require signals transmitted through VDR for differentiation into myocytes. Myogenic differentiation likely involves the orchestration of myogenic transcription factors in skeletal muscle (Girgis et al., 2013). Vitamin D signaling may modulate $p21^{CIP1}$ and Rb as well as myogenin, which are important in myogenic differentiation of myoblasts to myotubes (Ludolph and Konieczny, 1995; Perry and Rudnicki, 2000). Autocrine vitamin D signaling has also been reported to regulate functional effects such as contraction and remodeling in smooth muscle cells although the autocrine effects in skeletal and cardiac muscles still require characterization (Weisman et al., 2005; Maghni et al., 2007; Eggersdorfer and Stöcklin, 2013).

Effects in VDR-null mutant mice further highlight the importance of the VDR in muscle biology. Apart from the observed growth retardation, osteomalacia and systemic metabolic changes such as secondary hyperparathyroidism and hypocalcemia, these mutant mice also had abnormal muscle structure and function (Burne et al., 2005). VDR-null mutant mice displayed a progressive decrease in their muscle fiber diameters compared to those of wild-type mice, which was evident early in the postnatal period (prior to weaning) and associated with an abnormally high expression of myogenic differentiation factors. These observations suggest alterations in muscle cell differentiation pathways and thus abnormal muscle fiber development and maturation (Endo et al., 2003). Interestingly, the muscle fiber abnormalities described were diffuse without any preference for type I or II fibers, which was different to myopathy due to vitamin D deficiency where there was a predominance of type II fiber loss. Additionally, the mutant mice had a total 33% body weight reduction compared to controls at maturity; implying a post-natal role for VDR in maintaining weight (Song et al., 2003). Increased VDR expression is also correlated with regeneration (Srikuea et al., 2012), but levels of VDR appear to decrease with increasing age, which has been proposed as a potential mechanism contributing to reduced muscle strength in the Bischoff-Ferrari et al. (2004).

In the context of muscle biology, VDR mediates both non-genomic and genomic effects of vitamin D (Buitrago et al., 2001; Capiati et al., 2002). VDR knock-down experiments demonstrated that 1,25-$(OH)_2D_3$-induced p38 MAPK activity occurs through Src phosphorylation, while also reducing ERK1/2 and Akt activity. These non-genomic effects include the stimulation of transmembrane second messenger systems involving adenylyl cyclase/cAMP/PKA and PLC/DAG+IP_3/PKC to affect contractile function and myogenesis. Furthermore, 1,25−$(OH)_2D_3$has also been reported to mediate Ca^{2+}release through voltage and store dependent calcium channels (SOC, CEE) in avian muscle cells (Santillan et al., 2004). Caveolae have also been shown to be involved in 1,25−$(OH)_2D_3$activation of in c-Src-MAPKs in C2C12 cells. Ca^{2+} influx in caveolae is triggered by the interaction between VDR with TRCP3, an integral protein of capacitative Ca^{2+} entry (CCE) (Buitrago and Boland, 2010; Buitrago et al., 2011).

New perspectives on vitamin D, chronic inflammation, and muscle physiology

Valuable insights into the role of vitamin D and muscle function have arisen from the study of certain pathological settings such as chronic inflammatory conditions. These conditions share phenotypic characteristics to vitamin D deficiency states and VDR-null mice. Myopathy is a feature of a number of chronic inflammatory syndromes. Chronic inflammation has been linked to pathophysiological mechanisms which implicate non-genomic and genomic 1,25-$(OH)_2D_3$-mediated signaling pathways. Skeletal muscle may be considered as having a level of plasticity, allowing it to respond to environmental, physiological and pathophysiological stimuli that elicit alterations in size, fiber-type and metabolism. Molecular factors such as insulin-like growth factors, calcineurin, desmin, Myf5, Mrf4, MyoD and myogenin have been identified as positive regulators of muscle size, while tumor necrosis factor (TNF)-α, myostatin and components of the ubiquitin pathway, have been recognized as regulators of muscle wasting. Emerging evidence supports a role for VDR in the contractile and metabolic function of both skeletal and cardiac muscle in health and disease (**Figure 1**). The expression of VDR and its interaction at

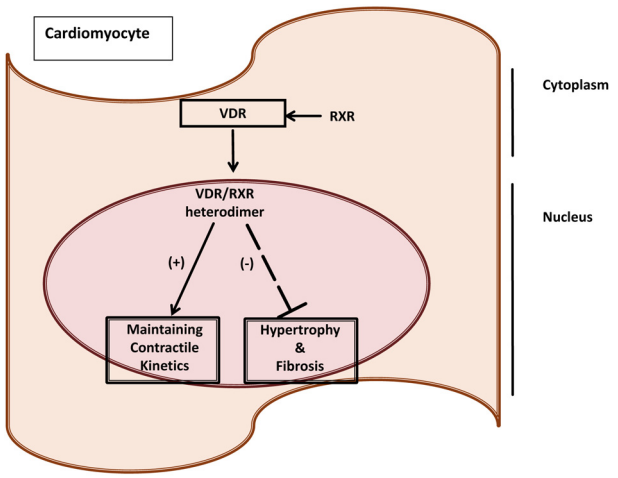

FIGURE 1 | VDR as a marker of cardiac dysfunction induced by cancer cachexia. Vitamin D Receptor (VDR) as a molecular marker of maladaptive responses due to cancer cachexia in the heart. VDR has been linked to having cardioprotective mechanisms by maintaining contractile kinetics, and regulating fibrosis and hypertrophy (Yuan et al., 2007; Tishkoff et al., 2008; Koleganova et al., 2009).

the molecular level with proteins that are involved in inflammation, signaling and ultimately contractile function of both skeletal and cardiac muscle is of importance. *In vitro* cell culture models, *in vivo* rodent models as well as clinical studies in humans are starting to clarify the mechanisms of vitamin D action mediated via the VDR in muscle in order to enhance our understanding of their role in inflammatory mediated myopathy and muscle weakness (Girgis et al., 2012, 2013, 2014).

Exercise-induced muscle damage has been shown to increase the expression of VDR while altering gene expression of inflammatory cytokines such as interleukin (IL)-6 and TNF-α and alterations in signaling molecules involved with vitamin D signaling pathways such as phosphorylation of AMPK, p38, ERK1/2, IKK, and IκB simultaneously (Choi et al., 2013). An inverse relationship is generally reported for vitamin D, cancer and muscle structure and function. Alterations in metabolic status and physical activity play a role, however paraneoplastic syndromes such as cancer cachexia integrate many metabolic and catabolic molecular mechanisms which result in pathophysiological skeletal and more recently cardiac muscle effects (Choi et al., 2013). Low serum vitamin D levels are highly prevalent in advanced cancer patients with cachexia or fatigue (Dev et al., 2011). Elevated levels of inflammatory circulating factors, include C-reactive protein (CRP), a currently utilized clinical marker. The VDR axis is reported to play a fundamental role with possible association between CRP and VDR gene polymorphisms, in cancer patients with cachexia. This suggests the notion of cachexia-prone genotypes or to cachexia-resistant genotypes (Punzi et al., 2012). It has been suggested that tumor associated effects such as these may in part be addressed by nutraceutical vitamin D supplemented diets to improve vitamin D status (Endo et al., 1998; Morley, 2009; Morley et al., 2009; Strohle et al., 2010).

Data arising from the study of muscle structure and function in cancer cachexia has revealed new insights into vitamin D. Cancer cachexia is a debilitating clinical syndrome which causes up to 30% of cancer related deaths by either immobility, respiratory and/or cardiac failure (Fearon, 2008) and is characterized by weight loss; up-regulation of inflammatory markers such as IL-6, IL-1, TNF-α and interferon gamma (IFN)γ; hypercalcemia; and insulin resistance (Argiles et al., 2003; Sato et al., 2003; Jackman and Kandarian, 2004; Evans et al., 2008; Tisdale, 2009; Asp et al., 2010). The interaction between host factors and tumor cells is proposed to cause an excess production of cytokines and improper stimulation of downstream signaling molecules which results in weakness and decreased physical activity; thus highlighting the detrimental effects of cachexia on quality of life (Dahele et al., 2007). Of these cytokines, IL-6 is thought to be a key mediator of skeletal and cardiac muscle wasting in the pathogenesis of CC (Argiles et al., 2003; Haddad et al., 2005; Baltgalvis et al., 2008; Tisdale, 2009; Carson and Baltgalvis, 2010). Current treatment strategies are limited and do little to improve survival (Michael and Tannock, 1998; Mantovani et al., 2008).

More recently, we have identified a link between IL-6, the myogenic transcriptional regulator MEF2C and muscle breakdown due to CC (Shum et al., 2012). Different underlying molecular effects may also underlie the pathological changes in skeletal vs. cardiac muscle due to cancer (Shum et al., 2012; Tan et al., 2013; Shum et al., 2013; Falconer et al., in press) "Exercise genes" have now been identified in humans, which now opens the gateway for analyses that focus on the genetic basis of performance. These include the genes encoding for: the angiotensin converting enzyme, alpha-actinin 3, bradykinin, ciliary neurotrophic factor, interleukin-15, insulin-like growth factor II, myostatin and the VDR which have been proposed to play a role in inter-subject variability in muscle strength or size. Current data is only available from healthy subjects, hence genetic variability that may account for these effects still requires further analysis particularly in the context of muscle disease (Stewart and Rittweger, 2006). Furthermore, conversion toward a fatigue prone, type II skeletal myofiber phenotype has been observed due to cancer, which potentially makes this condition treatable with vitamin D.

Cardiac muscle effects due to cancer cachexia. Cardiac weight loss is a relatively unreported feature in cancer cachexia although autopsy studies revealed that "cardiac atrophy" is a prominent feature in advanced cancer patients (Hellerstein and Santiago-Stevenson, 1950). Recent studies have demonstrated that the reversal of cardiac and skeletal muscle weight loss increased longevity in mouse models of cancer cachexia, implying that these effects on the heart may contribute to poor prognosis in cancer patients (Zhou et al., 2010). The molecular basis of this cardiomyopathy induced by cancer cachexia is unclear. We and others have established the IL-6 driven, colon 26 (C26) carcinoma cachexia mouse model to study cancer cachexia (Tanaka et al., 1990; Asp et al., 2010; Zhou et al., 2010; Shum et al., 2012). The C26 model demonstrates significant body wasting, has no metastases to the heart, thus effects seen are largely due to the tumor or the host-tumor response (Matsumoto et al., 1999; Schwarzkopf et al., 2006; Strassmann et al., 1992). Features of

cardiac wasting in the end stages of cachexia (i.e., 20–25% body weight loss) observed in C26 and other cachectic animal models include: heart weight loss; marked fibrosis; oxidative modifications; reduced expression of contractile apparatus proteins; no increase of apoptosis; and lower ejection fraction (Fukuda et al., 2009; Springer et al., 2009; Marin-Corral et al., 2010; Tian et al., 2010; Shum et al., Unplublished Data). Genes that mediate muscle atrophy such as atrogin-1 and Murf-1, were unaltered in the heart unlike skeletal muscle; implying that cardiac wasting occurs via different molecular pathways (Zhou et al., 2010; Shum et al., 2013; Unplublished Data). Vitamin D and its gene effects in the context of functional consequences have been described in skeletal muscle cell culture models, cardiac muscle and smooth muscle (Meems et al., 2011; Girgis et al., 2014). However, the roles of VDR and 1,25-$(OH)_2D_3$ need further characterization in the context of muscle wasting due to cancer cachexia **(Figure 1)**.

Vitamin D and cardiac pathology. Vitamin D and its analogs may potentially have palliative effects in the cardiovascular system. Long term exposure to angiotensin II has been shown to induce hypertension, cardiac hypertrophy, activation of the hypertrophic fetal gene program atrial natriuretic peptide (ANP), B-type natriuretic peptide and alpha skeletal actin gene expression), increased expression of the pro-hypertrophic modulatory calcineurin inhibitor protein 1 (MCIP 1), and increased fibrosis with augmented procollagen 1 and 3 gene expression. Co-administration of paricalcitol (a vitamin D analog with agonist properties) in an animal model of non-renin-dependent cardiac hypertrophy partially reversed the reported AII-dependent effects. Interestingly, the effects of agonist-bound vitamin D receptor appeared to elicit potent anti-hypertrophic activity in this model of cardiac hypertrophy. The anti-hypertrophic activity appears to be at least partially intrinsic to the cardiac myocyte and may involve suppression of the MCIP 1 protein (Chen and Gardner, 2013). Though the cardiovascular system is not thought to represent a classical target for 1,25-$(OH)_2D_3$ and retinoic acid (RA), it is clear that both cardiomyocytes and vascular smooth muscle cells respond to these nuclear receptor hormones (NRHs) with changes in growth characteristics and gene expression (**Figure 1**). These NRHs suppress many of the phenotypic correlates of endothelin-induced hypertrophy in a cultured neonatal rat cardiac ventriculocyte model. Each of these NRHs reduced endothelin-stimulated ANP secretion in a dose-dependent manner and when the two were used in combination, they proved to be more effective than when either NRH was used alone. 1,25-$(OH)_2D_3$ abrogated the increase in cell size seen after endothelin treatment. These findings suggest that liganded vitamin D and retinoid receptors are capable of modulating the hypertrophic process *in vitro* and that agents acting through these or similar signaling pathways may be of value in probing the molecular mechanisms underlying hypertrophy (Wu et al., 1996) **(Figure 1)**.

Transcriptome-wide effects and muscle

Recently, transcriptome-wide approaches have been applied to muscle in order to get a global view of changes that occur due to various stimuli, for example structural vs. metabolic. The transcriptional profile of VDR mRNA isoforms has been examined for differences in bone, cartilage and paravertebral muscles between tissues from curve concavity and convexity. VDR was differentially expressed in paravertebral muscles in patients with juvenile idiopathic scoliosis (JIS) and adult idiopathic scoliosis (AIS). The VDRl isoform appears to contribute to curve concavity in paravertebral muscles. Furthermore, muscular transcriptome differentiation was evident between curve concavity and convexity in JIS patients. Tob2 and MED13 gene expression in paravertebral muscles appear to differentiate the two types of idiopathic scoliosis (Nowak et al., 2012).

Gene expression has been examined in skeletal muscle tissue of obese insulin-resistant subjects before and after a euglycemic-hyperinsulinemic clamp to determine the pathogenesis of insulin resistance. Differential gene expression was demonstrated for enzymes, transcription, and translation regulators, transporters, G protein-coupled receptors, cytokines, and ligand-dependent nuclear receptors. Metabolic pathways that incorporated, inflammatory signaling and nuclear receptors were also significantly different. These included LXR/RXR activation, VDR/RXR activation, interleukin IL-8, acute phase response, IL-10, triggering receptor expressed on myeloid cells 1, peroxisome proliferator-activated receptor, G-beta/gamma and hepatocyte growth factor and IL6 signaling (Rudkowska et al., 2013).

Comparisons between transcriptomes and proteomes in muscle tissues and activated CD4+ and CD8+ T lymphocytes (T-cells) analyzed using Affymetrix microarrays and mass spectrometry, from type 2 diabetes (T2DM) subjects and matched non-diabetic controls, demonstrated reduced gene expression for insulin receptor (INSR), VDR, insulin degrading enzyme, Akt, insulin receptor substrate-1 (IRS-1), IRS-2, glucose transporter 4 (GLUT4), and enzymes of the glycolytic pathway in the T2DM subjects compared controls. Increased gene expression was shown for plasma cell glycoprotein-1, TNFα, and gluconeogenic enzymes in T2DM subjects. Observed alterations in transcriptomes and proteomes between muscle and activated T-cells of T2DM were comparable suggesting a more global molecular basis for insulin resistance (Stentz and Kitabchi, 2007).

Conclusion and perspectives

There is now clear evidence supporting a significant role for vitamin D in the biology and function of skeletal and cardiac muscle. Current evidence outlines a number of effects of vitamin D on these muscle types including intracellular calcium handling, differentiation and contractile protein composition. However further study using novel investigative strategies is still warranted to better delineate the role and functions of vitamin D in muscle. The molecular interplay between cytokine signaling, VDR expression, genetic variability in patients with myopathy due to chronic inflammatory conditions such as cancer cachexia may reveal the molecular basis for changes that have been observed in skeletal and cardiac muscle. Early transcriptomic studies on the effects of cytokines in muscle wasting due to cancer cachexia have provided clues regarding potential molecular mechanisms induced by cytokines that drive muscle wasting which may potentially also implicate vitamin D mediated transcriptional mechanisms although this still remains to

be defined. A better characterization of the role of VDR in the context of inflammation-mediated muscle wasting and weakness may also potentially translate into significant clinical applications by informing nutraceutical approaches using vitamin D supplementation as a potential strategy for reversing muscle wasting.

REFERENCES

Ahmed, W., Khan, N., Glueck, C. J., Pandey, S., Wang, P., Goldenberg, N., et al. (2009). Low serum 25 (OH) vitamin D levels (<32 ng/mL) are associated with reversible myositis-myalgia in statin-treated patients. *Transl. Res.* 153, 11–16. doi: 10.1016/j.trsl.2008.11.002

Argiles, J. M., Moore-Carrasco, R., Fuster, G., Busquets, S., and Lopez-Soriano, F. J. (2003). Cancer cachexia: the molecular mechanisms. *Int. J. Biochem. Cell Biol.* 35, 405–409. doi: 10.1016/S1357-2725(02)00251-0

Asp, M. L., Tian, M., Wendel, A. A., and Belury, M. A. (2010). Evidence for the contribution of insulin resistance to the development of cachexia in tumor-bearing mice. *Int. J. Cancer* 126, 756–763. doi: 10.1002/ijc.24784

Baltgalvis, K. A., Berger, F. G., Pena, M. M., Davis, J. M., Muga, S. J., and Carson, J. A. (2008). Interleukin-6 and cachexia in ApcMin/+ mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R393–R401. doi: 10.1152/ajpregu.00716.2007

Bischoff-Ferrari, H. A., Borchers, M., Gudat, F., Durmuller, U., Stahelin, H. B., and Dick, W. (2004). Vitamin D receptor expression in human muscle tissue decreases with age. *J. Bone Miner. Res.* 19, 265–269. doi: 10.1359/jbmr.2004.19.2.265

Bischoff-Ferrari, H. A., Conzelmann, M., Stahelin, H. B., Dick, W., Carpenter, M. G., Adkin, A. L., et al. (2006). Is fall prevention by vitamin D mediated by a change in postural or dynamic balance? *Osteoporos. Int.* 17, 656–663. doi: 10.1007/s00198-005-0030-9

Bischoff, H. A., Borchers, M., Gudat, F., Duermueller, U., Theiler, R., Stahelin, H. B., et al. (2001). *In situ* detection of 1,25-dihydroxyvitamin D3 receptor in human skeletal muscle tissue. *Histochem. J.* 33, 19–24. doi: 10.1023/A:1017535728844

Boland, R. (1986). Role of vitamin D in skeletal muscle function. *Endo. Rev.* 7, 434–448. doi: 10.1210/edrv-7-4-434

Buitrago, C., and Boland, R. (2010). Caveolae and caveolin-1 are implicated in 1alpha,25(OH)2-vitamin D3-dependent modulation of Src, MAPK cascades and VDR localization in skeletal muscle cells. *J. Steroid Biochem. Mol. Biol.* 121, 169–175. doi: 10.1016/j.jsbmb.2010.03.002

Buitrago, C., Costabel, M., and Boland, R. (2011). PKC and PTPalpha participate in Src activation by 1alpha,25OH2 vitamin D3 in C2C12 skeletal muscle cells. *Mol. Cell. Endocrinol.* 339, 81–89. doi: 10.1016/j.mce.2011.03.022

Buitrago, C., Vazquez, G., De Boland, A. R., and Boland, R. (2001). The vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by 1,25(OH)(2)D(3). *Biochem. Biophys. Res. Commun.* 289, 1150–1156. doi: 10.1006/bbrc.2001.6072

Burne, T. H., McGrath, J. J., Eyles, D. W., and Mackay-Sim, A. (2005). Behavioural characterization of vitamin D receptor knockout mice. *Behav. Brain Res.* 157, 299–308. doi: 10.1016/j.bbr.2004.07.008

Capiati, D., Benassati, S., and Boland, R. L. (2002). 1,25(OH)2-vitamin D3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. *J. Cell. Biochem.* 86, 128–135. doi: 10.1002/jcb.10191

Carson, J. A., and Baltgalvis, K. A. (2010). Interleukin 6 as a key regulator of muscle mass during cachexia. *Exerc. Sport Sci. Rev.* 38, 168–76. doi: 10.1097/JES.0b013e3181f44f11

Ceglia, L., da Silva Morais, M., Park, L. K., Morris, E., Harris, S. S., Bischoff-Ferrari, H. A., et al. (2010). Multi-step immunofluorescent analysis of vitamin D receptor loci and myosin heavy chain isoforms in human skeletal muscle. *J. Mol. Histol.* 41, 137–142. doi: 10.1007/s10735-010-9270-x

Chen, S., and Gardner, D. G. (2013). Liganded vitamin D receptor displays anti-hypertrophic activity in the murine heart. *J. Steroid Biochem. Mol. Biol.* 136, 150–155. doi: 10.1016/j.jsbmb.2012.09.007

Choi, M., Park, H., Cho, S., and Lee, M. (2013). Vitamin D3 supplementation modulates inflammatory responses from the muscle damage induced by high-intensity exercise in SD rats. *Cytokine* 63, 27–35. doi: 10.1016/j.cyto.2013.03.018

Costa, E. M., Blau, H. M., and Feldman, D. (1986). 1,25-dihydroxyvitamin D3 receptors and hormonal responses in cloned human skeletal muscle cells. *Endocrinology* 119, 2214–2220. doi: 10.1210/endo-119-5-2214

Dahele, M., Skipworth, R. J. E., Wall, L., Voss, A., Preston, T., and Fearon, K. C. H. (2007). Objective physical activity and self-reported quality of life in patients receiving palliative chemotherapy. *J. Pain Symptom Manage.* 33, 676–685. doi: 10.1016/j.jpainsymman.2006.09.024

DeLuca, H. F. (2004). Overview of general physiologic features and functions of vitamin D. *Am. J. Clin. Nutr.* 80, 1689S–1696S.

Dev, R., Del Fabbro, E., Schwartz, G. G., Hui, D., Palla, S. L., Gutierrez, N., et al. (2011). Preliminary report: vitamin D deficiency in advanced cancer patients with symptoms of fatigue or anorexia. *Oncologist* 16, 1637–1641. doi: 10.1634/theoncologist.2011-0151

Eggersdorfer, M., and Stöcklin, E. (2013). Vitamin D, an essential nutrient with versatile functions in nearly all organs. *Int. J. Vitam Nutr. Res.* 83, 92–100. doi: 10.1024/0300-9831/a000151

Endo, I., Inoue, D., Mitsui, T., Umaki, Y., Akaike, M., Yoshizawa, T., et al. (2003). Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors. *Endocrinology* 144, 5138–5144. doi: 10.1210/en.2003-0502

Endo, K., Katsumata, K., Iguchi, H., Kubodera, N., Teramoto, T., Ikeda, K., et al. (1998). Effect of combination treatment with a vitamin D analog (OCT) and a bisphosphonate (AHPrBP) in a nude mouse model of cancer-associated hypercalcemia. *J. Bone Miner. Res.* 13, 1378–1383. doi: 10.1359/jbmr.1998.13.9.1378

Evans, W. J., Morley, J. E., Argilés, J., Bales, C., Baracos, V., Guttridge, D., et al. (2008). Cachexia: a new definition. *Clin. Nutr.* 27, 793–799. doi: 10.1016/j.clnu.2008.06.013

Falconer, R., Tan, T. C., and Polly, P. (in press). Autophagy and cardiac dysfunction in cancer cachexia. *World J. Can. Res.*

Fearon, K. C. H. (2008). Cancer cachexia: developing multimodal therapy for a multidimensional problem. *Eur. J. Cancer* 44, 1124–1132. doi: 10.1016/j.ejca.2008.02.033

Fukuda, T., Sumi, T., Nobeyama, H., Yoshida, H., Matsumoto, Y., Yasui, T., et al. (2009). Multiple organ failure of tumur-bearing rabbits in cancer cachexia is caused by apoptosis of normal organ cells. *Int. J. Oncol.* 34, 61–67.

Garcia, L. A., King, K. K., Ferrini, M. G., Norris, K. C., and Artaza, J. N. (2011). 1,25(OH)2vitamin D3 stimulates myogenic differentiation by inhibiting cell proliferation and modulating the expression of promyogenic growth factors and myostatin in C2C12 skeletal muscle cells. *Endocrinology* 152, 2976–2986. doi: 10.1210/en.2011-0159

Girgis, C. M., Cheng, K., Scott, C. H., and Gunton, J. E. (2012). Novel links between HIFs, type 2 diabetes, and metabolic syndrome. *Trends Endocrinol. Metab.* 23372–23380. doi: 10.1016/j.tem.2012.05.003

Girgis, C. M., Clifton-Bligh, R. J., Hamrick, M. W., Holick, M. F., and Gunton, J. E. (2013). The roles of vitamin D in skeletal muscle: form, function, and metabolism. *Endocrine Rev.* 34, 33–83. doi: 10.1210/er.2012-1012

Girgis, C. M., Clifton-Bligh, R. J., Mokbel, N., Cheng, K., and Gunton, J. E. (2014). Vitamin D Signaling Regulates Proliferation, Differentiation, and Myotube Size in C2C12 Skeletal Muscle Cells. *Endocrinology* 155, 347–357. doi: 10.1210/en.2013-1205

Girgis, C. M., Clifton-Bligh, R. J., Turner, N., Lau, S. L., and Gunton, J. E. (2014). Effects of vitamin D in skeletal muscle: falls, strength, athletic performance and insulin sensitivity. *Clin. Endocrinol.* 80, 169–181. doi: 10.1111/cen.12368

Glerup, H., and Eriksen, E. F. (1999). Acroparaesthesia–a typical finding in vitamin D deficiency. *Rheumatology* 38, 482. doi: 10.1093/rheumatology/38.5.482

Glerup, H., Mikkelsen, K., Poulsen, L., Hass, E., Overbeck, S., Andersen, H., et al. (2000). Hypovitaminosis D myopathy without biochemical signs of osteomalacic bone involvement. *Calcified Tissue Int.* 66, 419–424. doi: 10.1007/s002230010085

Grimaldi, A. S., Parker, B. A., Capizzi, J. A., Clarkson, P. M., and Pescatello, L. S., White, M. C., et al. (2013). 25(OH) vitamin D is associated with greater muscle strength in healthy men and women. *Med. Sci. Sports Exerc.* 45, 157–162. doi: 10.1249/MSS.0b013e31826c9a78

Haddad, F., Zaldivar, F., Cooper, D. M., and Adams, G. R. (2005). IL-6-induced skeletal muscle atrophy. *J. Appl. Physiol.* 98, 911–917. doi: 10.1152/japplphysiol.01026.2004

Hellerstein, H. K., and Santiago-Stevenson, D. (1950). Atrophy of the heart: a correlative study of eighty-five proved cases. *Circulation* 1, 93–126. doi: 10.1161/01.CIR.1.1.93

Jackman, R. W., and Kandarian, S. C. (2004). The molecular basis of skeletal muscle atrophy. *Am. J. Physiol. Cell Physiol.* 287, C834–C843. doi: 10.1152/ajpcell.00579.2003

Koleganova, N., Piecha, G., Ritz, E., and Gross, M.-L. (2009). Calcitriol ameliorates capillary deficit and fibrosis of the heart in subtotally nephrectomized rats. *Nephrol. Dial. Transplant.* 24, 778–787. doi: 10.1093/ndt/gfn549

Ludolph, D. C., and Konieczny, S. F. (1995). Transcription factor families: muscling in on the myogenic program. *FASEB J.* 9, 1595–1604.

Luna, N. D., Diaz-Manera, J., Paradas, C., Iturriaga, C., Rojas-Garcia, R., Araque, J., et al. (2012). 1alpha,25(OH)(2)-Vitamin D3 increases dysferlin expression in vitro and in a human clinical trial. *Mol. Ther.* 20, 1988–1997. doi: 10.1038/mt.2012.156

Maghni, K., Bossé, Y., and Hudson, T. J. (2007). 1alpha,25-dihydroxy-vitamin D3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. *Physiol. Genom.* 29, 161–168.

Mantovani, G., Macciò, A., Madeddu, C., Gramignano, G., Serpe, R., Massa, E., et al. (2008). Randomized phase III clinical trial of five different arms of treatment for patients with cancer cachexia: interim results. *Nutrition* 24, 305–313. doi: 10.1016/j.nut.2007.12.010

Marcinkowska, E. (2001). A run for a membrane vitamin D receptor. *Biol. Signals Recept.* 10, 341–349. doi: 10.1159/000046902

Marin-Corral, J., Fontes, C. C., Pascual-Guardia, S., Sanchez, F., Olivan, M., Argilés, J. M., et al. (2010). Redox balance and carbonylated proteins in limb and heart muscles of cachectic rats. *Antioxid. Redox Signal.* 12, 365–380. doi: 10.1089/ars.2009.2818

Matsumoto, T., Fujimoto-Ouchi, K., Tamura, S., Tanaka, Y., and Ishitsuka, H. (1999). Tumour inoculation site-dependent induction of cachexia in mice bearing colon 26 carcinoma. *Br. J. Cancer* 79, 764–769. doi: 10.1038/sj.bjc.6690123

Meems, L. M., van der Harst, P., van Gilst, W. H., de Boer, R. A. (2011). Vitamin D biology in heart failure: molecular mechanisms and systematic review. *Curr. Drug Targets* 12, 29–41. doi: 10.2174/138945011793591554

Michael, M., and Tannock, I. F. (1998). Measuring health-related quality of life in clinical trials that evaluate the role of chemotherapy in cancer treatment. *CMAJ* 158, 1727–1734.

Miyazawa, K., Iguchi, T., Asada, M., Gotoh, A., Mizutani, S., and Ohyashiki, K. (2005). Combined treatment of leukemia cells with vitamin K2 and 1alpha,25-dihydroxyvitamin D3 enhances monocytic differentiation along with becoming resistant to apoptosis by induction of cytoplasmic p21CIP1. *Int. J. Oncol.* 27, 893–900.

Morley, J. E. (2009). Calories and cachexia. *Curr. Opin. Clin. Nutr. Metab. Care* 12, 607–610. doi: 10.1097/MCO.0b013e328331e9ce

Morley, J. E., Anker, S. D., and Evans, W. J. (2009). Cachexia and aging: an update based on the Fourth International Cachexia Meeting. *J. Nutr. Health Aging* 13, 47–55. doi: 10.1007/s12603-009-0009-x

Nowak, R., Szota, J., and Mazurek, U. (2012). Vitamin D receptor gene (VDR) transcripts in bone, cartilage, muscles and blood and microarray analysis of vitamin D responsive genes expression in paravertebral muscles of juvenile and adolescent idiopathic scoliosis patients. *BMC Musculoskelet. Disord.* 13, 259. doi: 10.1186/1471-2474-13-259

Perry, R. L. S., and Rudnicki, M. A. (2000). Molecular mechanisms regulating myogenic determination and differentiation. *Front. Biosci.* 5, D750–D767.

Pfeifer, M., Begerow, B., and Minne, H. W. (2002). Vitamin D and muscle function. *Osteoporosis Int.* 13, 187–194. doi: 10.1007/s001980200012

Punzi, T., Fabris, A., Morucci, G., Biagioni, P., Gulisano, M., Ruggiero, M., et al. (2012). C-reactive protein levels and vitamin d receptor polymorphisms as markers in predicting cachectic syndrome in cancer patients. *Mol. Diagnosis Ther.* 16, 115–124. doi: 10.2165/11632380-000000000-00000

Rejnmark, L. (2011). Effects of vitamin d on muscle function and performance: a review of evidence from randomized controlled trials. *Ther. Adv. Chronic Dis.* 2, 25–37. doi: 10.1177/2040622310381934

Rodman, J. S., and Baker, T. (1978). Changes in the kinetics of muscle contraction in vitamin D-depleted rats. *Kidney Int.* 13, 189–193. doi: 10.1038/ki.1978.28

Rudkowska, I., Jacques, H., Weisnagel, S. J., Marette, A., and Vohl, M. C. (2013). Transcriptomic profiles of skeletal muscle tissue following an euglycemic-hyperinsulinemic clamp in insulin-resistant obese subjects. *Genes Nutr.* 8, 91–98. doi: 10.1007/s12263-012-0298-2

Ryhänen, S., Jääskeläinen, T., Mahonen, A., and Mäenpää, P. H. (2003). Inhibition of MG-63 cell cycle progression by synthetic vitamin D3 analogs mediated by p27, Cdk2, cyclin E, and the retinoblastoma protein. *Biochem. Pharmacol.* 66, 495–504.

Santillan, G., Katz, S., Vazquez, G., and Boland, R. L. (2004). TRPC3-like protein and vitamin D receptor mediate 1alpha,25(OH)2D3-induced SOC influx in muscle cells. *Int. J. Biochem. cell Biol.* 36, 1910–1918. doi: 10.1016/j.biocel.2004.01.027

Sato, K., Onuma, E., Yocum, R. C., and Ogata, E. (2003). Treatment of malignancy-associated hypercalcemia and cachexia with humanized anti-parathyroid hormone-related protein antibody. *Semin. Oncol.* 30, 167–173. doi: 10.1053/j.seminoncol.2003.08.019

Sato, Y., Iwamoto, J., Kanoko, T., and Satoh, K. (2005). Low-dose vitamin D prevents muscular atrophy and reduces falls and hip fractures in women after stroke: a randomized controlled trial. *Cerebrovasc. Dis.* 20, 187–192. doi: 10.1159/000087203

Schott, G. D., and Wills, M. R. (1976). Muscle weakness in osteomalacia. *Lancet* 1, 626–629. doi: 10.1016/S0140-6736(76)90428-1

Schwarzkopf, M., Coletti, D., Sassoon, D., and Marazzi, G. (2006). Muscle cachexia is regulated by a p53–PW1/Peg3-dependent pathway. *Genes Dev.* 20, 3440–3452. doi: 10.1101/gad.412606

Shum, A. M., Mahendradatta, T., Taylor, R. J., Painter, A. B., Moore, M. M., Tsoli, M., et al. (2012). Disruption of MEF2C signaling and loss of sarcomeric and mitochondrial integrity in cancer-induced skeletal muscle wasting. *Aging* 4, 133–143.

Shum, A. M., Tan, T. C., and Polly, P. (2013). A comparative study of gene expression changes in skeletal and cardiac muscle from the colon 26 carcinoma mouse model of cachexia:identification of common molecular targets for future intervention studies. *World J. Cancer Res.* 1, 69–77.

Simpson, R. U., Thomas, G. A., and Arnold, A. J. (1985). Identification of 1,25-dihydroxyvitamin D3 receptors and activities in muscle. *J. Biol. Chem.* 260, 8882–8891.

Skaria, J., Katiyar, B. C., Srivastava, T. P., and Dube, B. (1975). Myopathy and neuropathy associated with osteomalacia. *Acta Neurol. Scand.* 51, 37–58. doi: 10.1111/j.1600-0404.1975.tb01358.x

Song, Y., Kato, S., and Fleet, J. C. (2003). Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECaC2 and calbindin D9k mRNA. *J. Nutr.* 133, 374–380.

Sorensen, O. H., Lund, B., Saltin, B., Lund, B., Andersen, R. B., Hjorth, L., et al. (1979). Myopathy in bone loss of ageing: improvement by treatment with 1 alpha-hydroxycholecalciferol and calcium. *Clin. Sci.* 56, 157–161.

Springer, J., Hartmann, A., Palus, S., Grzesiak, A., Aaschia, E., and Anker, S. D. (2009). Development of severe progressive heart failure in experimental cancer cachexia. *J. Card. Fail.* 15, S25–S25. doi: 10.1016/j.cardfail.2009.06.391

Srikuea, R., Zhang, X., Park-Sarge, O. K., and Esser, K. A. (2012). VDR and CYP27B1 are expressed in C2C12 cells and regenerating skeletal muscle: potential role in suppression of myoblast proliferation. *Am. J. Physiol. Cell Physiol.* 303, C396–C405. doi: 10.1152/ajpcell.00014.2012

Stentz, F. B., and Kitabchi, A. E. (2007). Transcriptome and proteome expressions involved in insulin resistance in muscle and activated T-lymphocytes of patients with type 2 diabetes. *Genomics Proteomics Bioinformatics* 5, 216–235. doi: 10.1016/S1672-0229(08)60009-1

Stewart, C. E., and Rittweger, J. (2006). Adaptive processes in skeletal muscle: molecular regulators and genetic influences. *J. Musculoskelet. Neuronal Interact.* 6, 73–86.

Strassmann, G., Fong, M., Kenney, J. S., and Jacob, C. O. (1992). Evidence for the involvement of interleukin 6 in experimental cancer cachexia. *J. Clin. Invest.* 89, 1681–1684. doi: 10.1172/JCI115767

Stratos, I., Li, Z., Herlyn, P., Rotter, R., Behrendt, A. K., Mittlmeier, T., et al. (2013). Vitamin D increases cellular turnover and functionally restores the skeletal muscle after crush injury in rats. *Am. J. Pathol.* 182, 895–904. doi: 10.1016/j.ajpath.2012.11.006

Strohle, A., Zanker, K., and Hahn, A. (2010). Nutrition in oncology: the case of micronutrients (review). *Oncol. Rep.* 24, 815–828. doi: 10.3892/or.2010.815

Tanaka, Y., Eda, H., Tanaka, T., Udagawa, T., Ishikawa, T., Horii, I., et al. (1990). Experimental cancer cachexia induced by transplantable colon 26 Adenocarcinoma in mice. *Cancer Res.* 50, 2290–2295.

Tan, T. C., Gupta, S., Shum, A. M. Y., and Polly, P. (2013). The Role of IL-6 and Myogenic transcription factors in skeletal muscle wasting and dysfunction due to cancer cachexi. *World J. Cancer Res.* 1, 15–23.

Tian, M., Nishijima, Y., Asp, M. L., Stout, M. B., Reiser, P. J., and Belury, M. A. (2010). Cardiac alterations in cancer-induced cachexia in mice. *Int. J. Oncol.* 37, 347–353.

Tisdale, M. J. (2009). Mechanisms of cancer cachexia. *Physiol. Rev*. 89, 381–410. doi: 10.1152/physrev.00016.2008

Tishkoff, D. X., Nibbelink, K. A., Holmberg, K. H., Dandu, L., and Simpson, R. U. (2008). Functional Vitamin D Receptor (VDR) in the T-tubules of cardiac myocytes: VDR Knockout cardiomyocyte contractility. *Endocrinology* 149, 558–564. doi: 10.1210/en.2007-0805

Washington, M. N., Kim, J. S., and Weigel, N. L. (2011). 1α,25-dihydroxyvitamin D3 inhibits C4-2 prostate cancer cell growth via a retinoblastoma protein (Rb)-independent G1 arrest. *Prostate* 71, 98–110. doi: 10.1002/pros.21226

Weisman, Y., Somjen, D., Kohen, F., Gayer, B., Limor, R., Sharon, O., et al. (2005). 25-hydroxyvitamin D3-1alpha-hydroxylase is expressed in human vascular smooth muscle cells and is upregulated by parathyroid hormone and estrogenic compounds. *Circulation* 111, 1666–1671. doi: 10.1161/01.CIR.0000160353.27927.70

Wu, J., Garami, M., Cheng, T., and Gardner, D. G. (1996). 1,25(OH)2 vitamin D3, and retinoic acid antagonize endothelin-stimulated hypertrophy of neonatal rat cardiac myocytes. *J. Clin. Invest.* 97, 1577–1588. doi: 10.1172/JCI118582

Yoshikawa, S., Nakamura, T., Tanabe, H., and Imamura, T. (1979). Osteomalacic myopathy. *Endocrinol. Japonica* 26, 65–72. doi: 10.1507/endocrj1954.26.Supplement_65

Yuan, W., Pan, W., Kong, J., Zheng, W., Szeto, F. L., Wong, K. E., et al. (2007). 1,25-Dihydroxyvitamin D3 Suppresses Renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J. Biol. Chem.* 282, 29821–29830. doi: 10.1074/jbc.M705495200

Zhou, X., Wang, J. L., Lu, J., Song, Y., Kwak, K. S., Jiao, Q., et al. (2010). Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell* 142, 531–543. doi: 10.1016/j.cell.2010.07.011

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Vitamin D and adipose tissue—more than storage

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The pandemic increase in obesity is inversely associated with vitamin D levels. While a higher BMI was causally related to lower 25-hydroxyvitamin D (25(OH)D), no evidence was obtained for a BMI lowering effect by higher 25(OH)D. Some of the physiological functions of $1,25(OH)_2D_3$ (1,25-dihydroxycholecalciferol or calcitriol) via its receptor within the adipose tissue have been investigated such as its effect on energy balance, adipogenesis, adipokine, and cytokine secretion. Adipose tissue inflammation has been recognized as the key component of metabolic disorders, e.g., in the metabolic syndrome. The adipose organ secretes more than 260 different proteins/peptides. However, the molecular basis of the interactions of $1,25(OH)_2D_3$, vitamin D binding proteins (VDBPs) and nuclear vitamin D receptor (VDR) after sequestration in adipose tissue and their regulations are still unclear. $1,25(OH)_2D_3$ and its inactive metabolites are known to inhibit the formation of adipocytes in mouse 3T3-L1 cell line. In humans, $1,25(OH)_2D_3$ promotes preadipocyte differentiation under cell culture conditions. Further evidence of its important functions is given by VDR knock out ($VDR^{-/-}$) and CYP27B1 knock out ($CYP27B1^{-/-}$) mouse models: Both $VDR^{-/-}$ and $CYP27B1^{-/-}$ models are highly resistant to the diet induced weight gain, while the specific overexpression of human VDR in adipose tissue leads to increased adipose tissue mass. The analysis of microarray datasets from human adipocytes treated with macrophage-secreted products up-regulated VDR and CYP27B1 genes indicating the capacity of adipocytes to even produce active $1,25(OH)_2D_3$. Experimental studies demonstrate that $1,25(OH)_2D_3$ has an active role in adipose tissue by modulating inflammation, adipogenesis and adipocyte secretion. Yet, further *in vivo* studies are needed to address the effects and the effective dosages of vitamin D in human adipose tissue and its relevance in the associated diseases.

Keywords: 1,25-dihydroxycholecalciferol or calcitriol, vitamin D binding protein, gene regulation, adipose tissue, adipogenesis, secretion, adipokines

INTRODUCTION

Adipose tissue is no longer regarded as a simple storage organ since it has been convincingly shown that it secretes more than 260 different proteins/peptides (Lehr et al., 2012). Lean people have about 5 kg of adipose tissue, while in obese and severely obese individuals the adipose tissue/organ could amount to 50 kg

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D3, 1,25-dihydroxycholecalciferol; VDBPs, Vitamin D binding proteins; VDR, Vitamin D receptor; DKK1, dickkopf 1; SFRP2, Frizzled-related protein 2; BMSCs, bone marrow stromal cells; PPARγ, peroxisome proliferator-activated receptor gamma; RXRα, retinoid X receptor alpha; WNT10, wingless-type MMTV integration site family member 10; C/EBP (α, β, and γ), CCAAT/enhancer-binding proteins (α, β, and γ); ETO, C/EBPβ corepressor eight twenty-one; FABP4, fatty acid binding protein 4; LPL, lipoprotein lipase; FASN, fatty acid synthase; SCD1, stearoyl-coA desaturase 1; GLUT4, glucose transporter type 4; PEPCK, phosphoenolpyruvate carboxykinase; LPS, lipopolysaccharide; TLR, toll like receptor; IL-6R, IL-6 receptors; NFκB, nuclear factor kappa-B; P38MAPK, p38 mitogen-activated protein kinase; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IκBα, inhibitor kappa-B; UCPs, uncoupling proteins; $VDR^{-/-}$, VDR knock out; $CYP27B1^{-/-}$, CYP27B1 knock out; BMI, Body mass index; BMPs, bone morphogenetic proteins; FGFs, fibroblast growth factors; TGFβ, transforming-growth factor β; IGF1, insulin like growth factor 1; JAK-STAT3, janus kinase-signal transducer and activator of transcription 3; S6K1, ribosomal protein S6 kinase 1; WNT, wingless family; Rb, protein of retinoblastoma family; Pref1, preadipocyte factor 1; Necdin, melanoma-associated antigen family of proteins member; SREBP1, sterol regulatory binding protein 1; MSCs, mesenchymal stem cells; AP2, adipocyte-binding protein 2; MCP1, monocyte chemoattractant protein 1; CYP27B1,(25(OH)D)-1α-hydroxylase; CPTII, carnitine palmitoyltransferase II; WAT, White adipose tissue; VDREs, vitamin D response elements; ChIP-seq, chromatin immunoprecipitation—sequencing; LCLs, lymphoblastoid cell lines.

or more (Frankenfield et al., 2001). Excess in adipose tissue has been attributed to a variety of diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases and decrease in life expectancy (Adams et al., 2006; Despres and Lemieux, 2006; Kahn et al., 2006; Van Gaal et al., 2006). Adiposity is one of the most serious public health problems, associated with vitamin D insufficiency due to the decreased bioavailability of vitamin D_3 (Wortsman et al., 2000). The Institute of Medicine (IOM) recommended 25-hydroxyvitamin D (25(OH)D) levels as reliable biomarker for assessment of Vitamin D status; currently values $\leq$50 nmol/l or $\leq$20 ng/ml are considered inadequate or not sufficient and values $\geq$50 nmol/l or $\geq$20 ng/ml as adequate or sufficient (Ross et al., 2011) (**Figure 1**). 25(OH)D levels have been determined by a variety of methods yielding different results. The National Institutes of Health's Office of Dietary Supplements together with National Institute of Standards and Technology (NIST) therefore developed a standard reference material-972 (SRM-972) for accuracy of laboratory vitamin D measurements (Phinney et al., 2012). A recent study by the D-CarDia consortium employed a Mendelian randomization (MR) approach to establish causality and direction of the association between vitamin D status and obesity measured by body mass index (BMI) using information from 21 adult cohorts (up to 42,024 participants) (Vimaleswaran et al., 2013). The consortium found that a higher BMI was causally related to lower 25(OH)D; no evidence was obtained for a BMI lowering effect of higher 25(OH)D. However, the study did not provide insights into the cellular action of $1,25(OH)_2D_3$ (1,25-dihydroxycholecalciferol or calcitriol). While the knowledge of the effects of $1,25(OH)_2D_3$ as an essential hormone and transcription factor is further emerging, it is increasingly acknowledged that $1,25(OH)_2D_3$ down regulates inflammatory responses in the adipose tissue. The anti-inflammatory effects of $1,25(OH)_2D_3$ might have notable influences on population health and disease prevention, since inflammation is thought to be the underlying cause of a range of metabolic disorders (Hotamisligil, 2006; Huotari and Herzig, 2008; Vlasova et al., 2010).

VITAMIN D AND ADIPOGENESIS

Adipose tissue expansion is a remarkable process characterized by the enlargement of adipocyte size known as hypertrophy and by the increase in the number of adipocytes known as hyperplasia, which is more strongly associated with severity of obesity (Arner and Spalding, 2010). Both processes emerge through sequential stages of differentiation to form mature adipocytes; this process is called adipogenesis. Mesodermal cells are influenced by various signals like bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), transforming-growth factor β(TGFβ) and insulin like growth factor 1 (IGF1) to form preadipocytes (Lowe et al., 2011). Furthermore, preadipocytes undergo differentiation to mature adipocytes by several intracellular signaling molecules (**Figure 2**) including janus kinase-signal transducer and activator

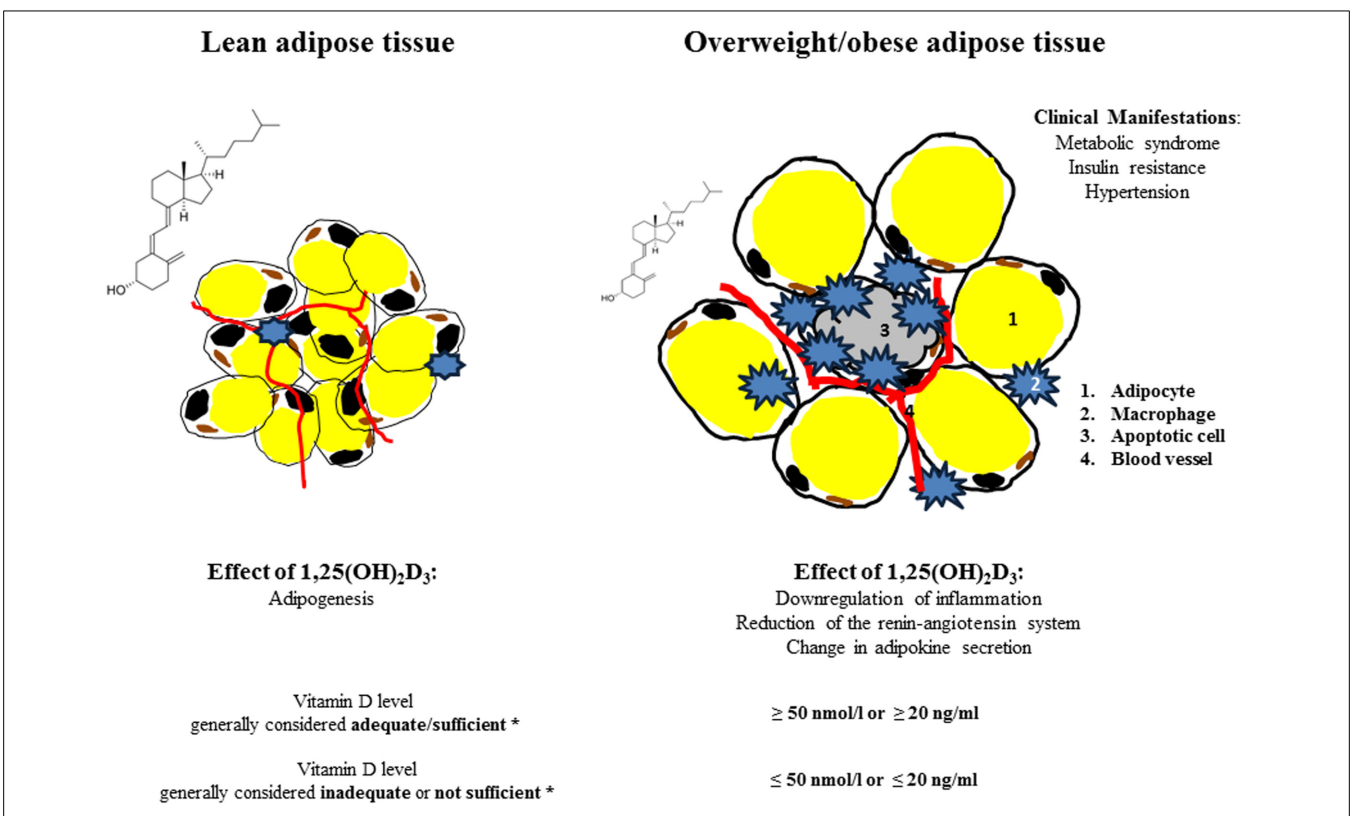

FIGURE 1 | Effect of $1,25(OH)_2D_3$ on adipose tissue/lean and obese state. *Vitamin D levels (25(OH)D) according to the Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2011.

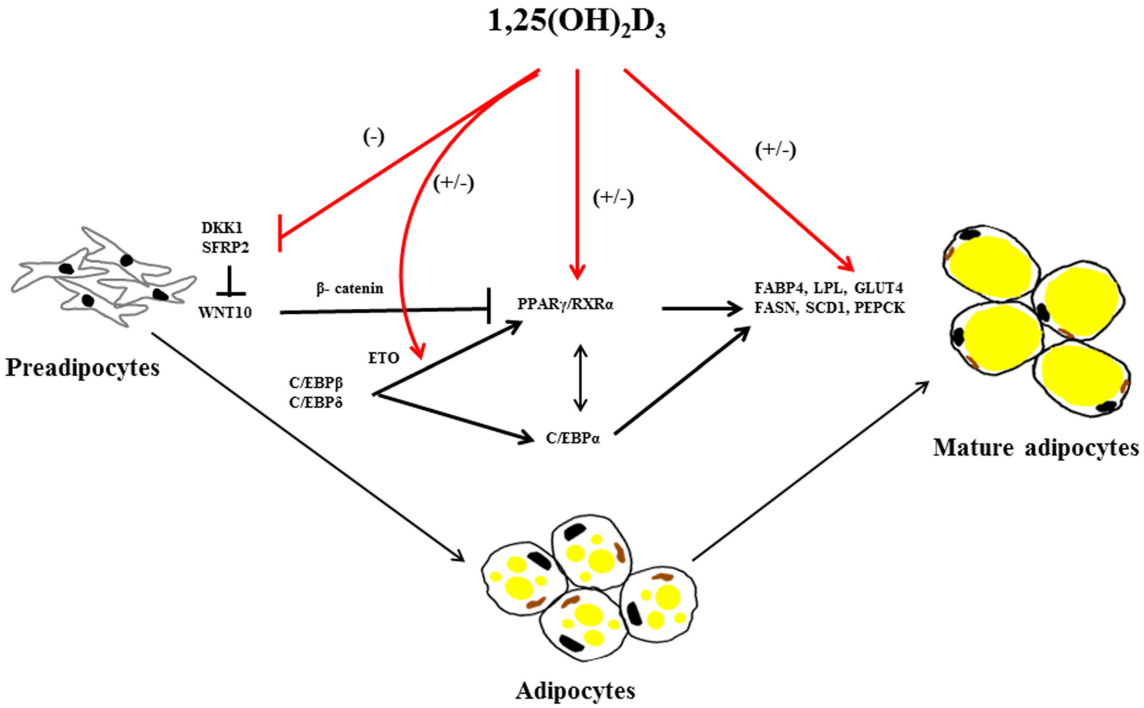

FIGURE 2 | Overview of $1,25(OH)_2D_3$ on regulating factors in adipogenesis. $1,25(OH)_2D_3$ has both stimulating and inhibition effect on adipogenesis. $1,25(OH)_2D_3$ suppress dickkopf1(DKK1) and secreted frizzled-related protein 2 (SFRP2) expression in mouse bone marrow stromal cells (BMSCs) there by suppressing adipogenic peroxisome proliferator-activated receptor γ/retinoid X receptor complex (PPARγ/RXRα) mediated by stabilization of β-catenin through wingless-type MMTV integration site family, member 10 (WNT10) inhibition. Furthermore, $1,25(OH)_2D_3$ regulates several adipogenic mediators during differentiation, proliferation and maturation of mesodermal cells into adipocytes including *CCAAT/enhancer-binding proteins α, β, and γ (C/EBPα, β, and γ), C/EBPβ corepressor eight twenty-one (ETO), PPARγ/RXRα, fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl-coA desaturase-1 (SCD1), glucose transporter type 4 (GLUT4) and phosphoenolpyruvate carboxykinase (PEPCK).* The arrows in the figure indicate activation and blunted line indicate inhibition. The red lines and (+/−) indicate stimulatory or inhibitory effect of $1,25(OH)_2D_3$ on adipogenesis dependent on the species or cellular systems studied.

of transcription 3 (JAK-STAT3) (Zhang et al., 2011), glutathione (Vigilanza et al., 2011), SMAD proteins (Jin et al., 2006) and ribosomal protein S6 kinase 1 (S6K1) (Carnevalli et al., 2010) affecting adipogenic transcription factors. In preadipocytes, differentiation factors need to be released from their suppressive signaling molecules such as members of wingless (WNT) family (Ross et al., 2000), protein of the retinoblastoma (Rb) family (Scime et al., 2005), preadipocyte factor 1 (Pref1) (Smas and Sul, 1993) and Necdin, member of the melanoma-associated antigen family of proteins (Fujiwara et al., 2012) to undergo differentiation. The terminal differentiation to mature adipocytes is regulated by a number of transcriptional factors including early key regulator CAAT/enhancer binding proteins (C/EBPβ followed by C/EBPα, C/EBPδ), the master regulator PPARγ and sterol regulatory binding protein 1 (SREBP1) (Payne et al., 2009; White and Stephens, 2010). These transcriptional factors induce expression of various genes related to lipogenesis, lipolysis and insulin sensitivity including fatty acid binding protein (*FABP4*), lipoprotein lipase (*LPL*), glucose transporter (*GLUT4*) and fatty acid synthase (*FASN*) (Lefterova et al., 2008; Nielsen et al., 2008; Madsen et al., 2014).

Investigations of the molecular regulation of $1,25(OH)_2D_3$ on adipogenesis have been conducted *in vitro*. In mouse 3T3-L1 preadipocytes, $1,25(OH)_2D_3$ inhibits adipogenesis by acting on multiple targets suppressing C/EBPα and PPARγ expression, specifically antagonizing the transacting activity of PPARγ, and sequestering the nuclear receptor retinoic X receptor (RXR), a member nuclear receptor superfamily and down regulating both C/EBP β mRNA expression and C/EBP β nuclear protein levels (Kong and Li, 2006) (**Figure 2**). $1,25(OH)_2D_3$ stimulates expression of the C/EBP β corepressor, eight twenty-one (ETO), and thus further inhibits the action of any remaining C/EBP β transcriptional effects required for adipogenesis (Blumberg et al., 2006).

Although early studies have established an inhibitory action of $1,25(OH)_2D_3$ in 3T3-L1 preadipocytes differentiation, recently, a more specific effect of $1,25(OH)_2D_3$ on WNT signaling emerged. WNT/β-catenin maintain preadipocytes in their undifferentiated state and thus preventing adipogenesis (Ross et al., 2000). The anti-adipogenic effect of $1,25(OH)_2D_3$ is mediated by maintenance of WNT10B and nuclear β-catenin levels expression levels in 3T3-L1 preadipocytes, thereby suppressing transcription factor PPARγ (Lee et al., 2012). In addition, $1,25(OH)_2D_3$ also inhibited mouse bone marrow stromal cells(BMSCs) differentiation into adipocytes by suppression of dickkopf1 (DKK1) and secreted frizzled-related protein 2 (SFRP2) expression levels

via VDR mediated WNT signaling (Cianferotti and Demay, 2007).

In contrast, $1,25(OH)_2D_3$ treatment of porcine mesenchymal stem cells (MSCs) stimulated both proliferation and differentiation in a dose dependent manner toward adipocytic phenotype by increasing PPARγ, LPL and adipocyte-binding protein 2 (AP2) mRNA levels (Mahajan and Stahl, 2009). In human tissue, $1,25(OH)_2D_3$ promotes differentiation of already committed subcutaneous preadipocytes through increased expression of adipogenic markers *FABP4* and *LPL* (Nimitphong et al., 2012). Narvaez et al. (2013) demonstrated that mesenchymal cells differentiate in the presence of $1,25(OH)_2D_3$ toward adipocytes with an enhanced lipid accumulation and increased expression of adipogenic marker genes (*FASN*, *FABP4*, and *PPARγ*).

In conclusion, $1,25(OH)_2D_3$ regulates adipogenesis at various levels of the entire differentiation process (**Figure 2**). However, there are significant differences summarized in **Table 1**; the reasons for these differences are not clear at the moment—methodological differences as well as physiological roles of the adipose tissue in different species in their environments might affect these processes. Further studies are needed to address the effects of vitamin D in adipose tissue and its relevance in the associated diseases.

VITAMIN D AND ADIPOSE TISSUE INFLAMMATION

In obesity, adipose tissue undergoes hypertrophic enlargement, which results in an imbalanced blood flow leading to hypoxia, inflammation and macrophage infiltration (Goossens, 2008; Trayhurn, 2013). The hypertrophied adipocytes are characterized by a reduced secretion of adiponectin and increased secretion of several proinflammatory cytokines such as interleukin IL-6, IL-8, TNF-α, resistin and MCP1 (Wellen and Hotamisligil, 2003; Maury and Brichard, 2010; Vlasova et al., 2010).

$1,25(OH)_2D_3$ acts at several levels to modulate the function of the immune system (Lemire, 2000). Several *in vitro* studies in the mouse 3T3-L1 cell line and human adipocytes have demonstrated that $1,25(OH)_2D_3$ inhibits chronic inflammation in adipose tissue (**Table 2**). However, earlier studies performed in 3T3-L1 and human adipocytes demonstrate contradictory results favoring inflammatory cytokine expression (Sun and Zemel, 2008); the reasons for the contradictory findings are unclear. Recent evidence focuses on the involvement of $1,25(OH)_2D_3$ in the regulation of adipose tissue inflammation by reducing the proinflammatory cytokines secreted from adipose tissue.

In differentiated adipocytes from human subcutaneous white adipose tissue $1,25(OH)_2D_3$ attenuates TNF-α induced MCP-1 secretion, while it inhibited secretion of adiponectin without affecting its mRNA levels (Lorente-Cebrian et al., 2012). In human subcutaneous adipose tissue fragments $1,25(OH)_2D_3$ reduced IL-1β induced expression of the inflammatory genes MCP-1, IL-6 and IL-8. However, results from the cell culture experiments have not been consistent with the *in vivo* findings. In a randomized controlled trial including fifty-five obese subjects, oral supplementation of vitamin D 7000 IU per day over 26

Table 1 | Effect of $1,25(OH)_2D_3$ on adipogenesis in different species.

Species and cell type	Effect on adipogenesis	References
MOUSE		
3T3-L1 preadipocytes	**Inhibition** - *VDR* and *RXR* mediated suppression of *C/EBPα*, *PPARγ*, *and C/EBPβ* (increased C/EBPβ corepressor ETO) - Through maintenance of WNT10B and β-catenin levels	Blumberg et al., 2006; Kong and Li, 2006; Lee et al., 2012
Primary preadipocytes	**Promotion** - Increasing *FABP4, adiponectin and PPARγ*	Nimitphong et al., 2012
Mouse bone marrow stromal cells(BMSCs)	**Inhibition** - Suppression of DKK1 and SFRP2 (WNT suppressors)	Cianferotti and Demay, 2007
PORCINE		
Porcine preadipocytes	**Inhibition** - Inhibition of *PPARγ and RXR*, down regulated *LPL, PEPCK, GPDH, SCD1, and GLUT4*	Zhuang et al., 2007
Porcine mesenchymal stem cells (MSCs)	**Promotion** - Increased adipogenic markers (*PPARγ, LPL* AP_2)	Mahajan and Stahl, 2009
HUMAN		
Subcutaneous preadipocytes	**Promotion** - Increasing expression (*FABP4 and LPL*)	Nimitphong et al., 2012
Mesenchymal progenitor cells from human adipose tissue	**Promotion** - Increase of adipogenic marker genes (*FASN, FABP, and PPARγ*)	Narvaez et al., 2013

Table 2 | $1,25(OH)_2D_3$ and inflammation.

Cell type	$1,25(OH)_2D_3$ Mechanism of action	References
Mouse 3T3-L1 and human adipocytes (differentiated from subcutaneous preadipocytes)	Increased IL-6 and TNFα in mouse 3T3-L1 Increased IL-6 and IL-8 in human adipocytes	Sun and Zemel, 2007
Mouse 3T3-L1 and human adipocytes (differentiated from subcutaneous preadipocytes)	Increased CD14, MIF, M-CSF, MIP, TNFα, IL-6, and MCP-1	Sun and Zemel, 2008
Human adipocytes (differentiated from subcutaneous preadipocytes)	Regulated nearly 140 genes favoring inflammation and oxidative stress	Sun et al., 2008
Mouse 3T3-L1 and Swiss mice on HFD supplemented with $1,25(OH)_2D_3$	Reduction of IL-6 in both cell culture medium and tissue EFP	Lira et al., 2011
Preadipocytes isolated from human subcutaneous WAT	Reduction in MCP-1 and adiponectin	Lorente-Cebrian et al., 2012
Bone marrow-derived human mesenchymal stem cells and mature adipocytes from subcutaneous adipose tissue	Reduction in IL-6 and inhibited NF-κB nuclear translocation	Mutt et al., 2012
Mouse 3T3-L1 and human preadipocytes	Decreased IL-6, MCP-1, IL-1β and inactivation of NF-κB by inducing IκBα, decreased p38 phosphorylation	Marcotorchino et al., 2012
Human subcutaneous adipose tissue fragments	Reduction in MCP-1, IL-6, and IL-8.	Wamberg et al., 2013
Human preadipocytes	Reduction in MCP-1, IL-8 and IL-6 and inactivation of NF-κB by upregulation of IκBα	Gao et al., 2013
Human preadipocytes differentiated to mature adipocytes	Reduction in MCP1, IL-8, RANTES, IL-6 and IL-1β Increased IκBα levels and reduced NF-κB p65 phosphorylation results in inhibition of NF-κB Decreased phosphorylated p38 MAPK	Ding et al., 2013a,b

weeks did neither affect inflammation markers in the circulation nor in the adipose tissue (Wamberg et al., 2013). In mice on high fat diet, dietary supplementation of $1,25(OH)_2D_3$ (0.05 mg/kg of diet) reduced their IL-6 protein content in epididymal adipose tissue and in the 3T3-L1 cell line stimulated by LPS (Lira et al., 2011).

Signal transduction of inflammatory pathways in adipose tissue involves activation of NF-κB and translocation of p65 to nucleus mediated by degradation of IκBα (Tourniaire et al., 2013). Mutt et al. (2012) have demonstrated that, $1,25(OH)_2D_3$ suppressed LPS-stimulated IL-6 secretion in human isolated mature and MSC differentiated adipocytes. This was confirmed by Marcotorchino et al. (2012), who demonstrated that $1,25(OH)_2D_3$ inhibits the inflammatory markers in both mouse and human adipocytes via the involvement of p38 MAP kinase and NF-κB classical inflammatory pathway and later by Gao et al. (2013) and Ding et al. (2013a).

In summary, the presence of $1,25(OH)_2D_3$ inhibited chemokine and cytokine secretion in human adipocytes. $1,25(OH)_2D_3$ strongly inhibited the activation of the NF-κB and MAPK signaling pathways, which prevent gene transcription of the proinflammatory factors (**Figure 3**). $1,25(OH)_2D_3$ has been shown by different groups in different models to significantly reduce inflammation in the adipose tissue. However, further studies are needed to provide more evidence for the physiological relevance and the concentration levels of active $1,25(OH)_2D_3$ in lean and obese subjects required to ameliorate the inflammation and associated complications.

VITAMIN D AND ADIPOSE TISSUE ENERGY HOMEOSTASIS

The discovery of VDR expression in adipocytes was the cornerstone for the investigations of the effect of vitamin D on adipose tissue beyond its role in bone metabolism (Stumpf, 1995; Ding et al., 2012). Recent findings in genetically modified mouse models highlighted a new role for vitamin D and its receptor VDR in adipose tissue energy homeostasis. VDR knockout ($VDR^{-/-}$) mice had reduced body weight and lower serum leptin concentrations, despite of an increased compensatory food intake compared to wild type mice of different genetic background C57BL6 and CD1. $VDR^{-/-}$ mice were highly resistant to high fat diet induced weight gain (Narvaez et al., 2009). In addition, these mice are characterized by a relatively short lifespan, alopecia, osteoporosis, ectopic calcification, progressive loss of hearing and balance (Keisala et al., 2009; Tuohimaa, 2009). Mice lacking CYP27B1 [the (25(OH)D)-1α-hydroxylase enzyme, converts $25(OH)D_3$ in to $1,25(OH)_2D_3$], displayed features similar to $VDR^{-/-}$ with reduced body weight, hypoleptinemia and hyperphagia. Interestingly, uncoupling protein 1 (UCP-1) expression in white adipose tissue of the $VDR^{-/-}$ mice was increased 25-fold.

In addition to reduced body weight, $VDR^{-/-}$ mice had less body fat and lower levels of plasma triglycerides and cholesterol in comparison to the wild type counterparts even though mice were challenged with a high fat diet (Wong et al., 2009; Weber and Erben, 2013). The depletion of adipose tissue in younger $VDR^{-/-}$ mice progresses with aging and resulted in severe mammary adipose tissue atrophy, along with the increased respiration and energy expenditure (Welsh et al., 2011). The effect on plasma lipid profile and unaltered food intake in these mice was confirmed by an increased β-oxidation rate in isolated adipocytes mediated by the induction of carnitine palmitoyltransferase II (CPTII) (**Figure 3**). $VDR^{-/-}$ mice had an increased basal metabolism demonstrated by the total energy expenditure, oxygen consumption and CO_2 production in comparison with the wild type mice (Wong et al., 2009). In addition, UCP1, UCP2, and UCP3 mRNAs were upregulated in brown adipose tissue of the $VDR^{-/-}$ mice fed high fat diet. In contrast to VDR knock out models with the

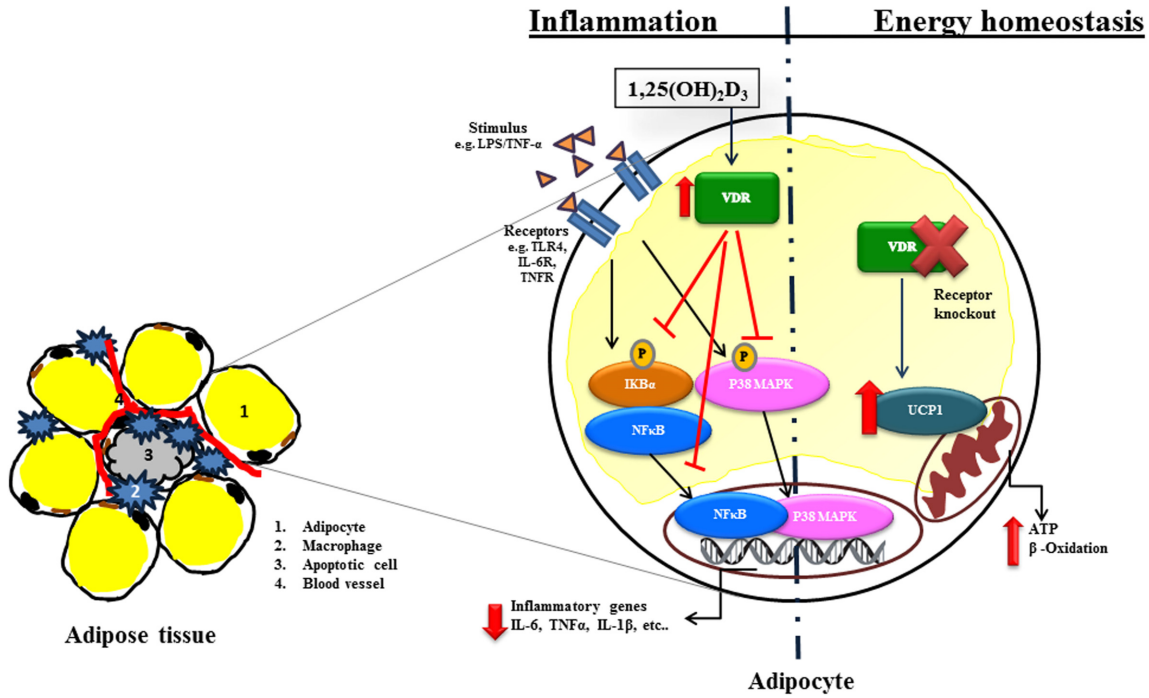

FIGURE 3 | Molecular actions of $1,25(OH)_2D_3$ in inflammation and energy homeostasis in adipocytes. Stimulation via e.g., lipopolysaccharide (LPS), TNF-α via specific receptors e.g., Toll like receptor (TLR), IL-6 receptors (IL-6R) activate Nuclear factor kappa-B (NFκB) or p38 mitogen-activated protein kinase (P38MAPK) signaling dependent transcription of inflammatory genes such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). $1,25(OH)_2D_3$ inhibits inflammation by inhibiting Inhibitor kappa-B (IκBα) phosphorylation and translocation of NFκB as well P38MAPK into the nucleus. Furthermore, $1,25(OH)_2D_3$ affects energy homeostasis. $VDR^{-/-}$ mice increases energy expenditure through uncoupling proteins (UCPs). The arrows in the figure indicate activation and blunted line indicate inhibition. The red lines and arrows indicate the effect of $1,25(OH)_2D_3$ on inflammatory signaling pathway.

ablation of the receptor in the whole animal, adipose tissue specific overexpression of human VDR via the adipocyte fatty acid binding protein (aP2) promoter/enhancer element resulted in a decreased energy expenditure and oxygen consumption and thus the mice had an increased body weight and fat mass (Wong et al., 2011).

In conclusion, these transgenic animal models indicate a critical and complex role for $1,25(OH)_2D_3$ and VDR signaling in energy homeostasis. However, notwithstanding the cell and mouse studies, further studies need to explore the role of vitamin D on human adipose tissue metabolism *in vivo*.

GENETIC VIEW ON THE ACTIONS OF VDR IN ADIPOCYTES: INTEGRATION WITH OTHER TISSUES

The VDR genomic interactions in different types of cells and tissues have been mapped by *in vitro* experiments where target cells (primary or secondary) have been treated with $1,25(OH)_2D_3$. Upon stimulation of VDR by its ligand, it forms a heterodimer with RXR and subsequently binds to the vitamin D response elements (VDREs) within the regulatory regions of target genes. The abundance of VDR binding sites and the regulation of changes in gene expressions are analyzed using array technology and the combination of chromatin immunoprecipitation (ChIP) with massive parallel sequencing (ChIP-seq). These advanced techniques have provided novel mechanistic insights of $1,25(OH)_2D_3$ action via VDR in the regulation of cellular metabolism and disease states. However, studies on genome-wide actions of VDR in adipocytes are sparse.

Recent microarray studies of human adipocytes and preadipocytes incubated with macrophage-conditioned medium derived from U937 monocytes, confirmed the induction of genes associated with the metabolism and action of $1,25(OH)_2D_3$, including CYP27B1 and VDR (Trayhurn et al., 2011). An earlier single microarray study in human subcutaneous adipose tissue derived preadipocytes differentiated to adipocytes demonstrated 237 $1,25(OH)_2D_3$ responsive genes (cell proliferation, angiogenesis, cell cycle, inflammation and response to oxidative stress) (Sun et al., 2008).

Most recent studies in the other cell types such as monocytes, primary $CD4^+$ T-lymphocytes, adenocarcinoma, hepatic stellate and lymphoblastoid cell lines (LCLs) (Ramagopalan et al., 2010; Heikkinen et al., 2011; Meyer et al., 2012; Ding et al., 2013b; Handel et al., 2013; Tuoresmäki et al., 2014) contribute to a systemic understanding of $1,25(OH)_2D_3$ induced gene regulation. Depending on the cell type, concentration and length of $1,25(OH)_2D_3$ incubation approximately 2000 VDR genomic binding sites have been found in these studies. Yet, alterations in DNA accessibility in cell lines after short-term stimulation with $1,25(OH)_2D_3$ may not reflect the physiological $1,25(OH)_2D_3$ levels *in vivo* due to the different tissue environment and

sympathetic influence. In primary CD4+ lymphocyte cells, isolated from nine healthy individuals with measured serum 25(OH)D levels, VDR binding sites ranged from 200 to 7118 across the genome and the corresponding 25(OH)D levels directly correlated with the number of VDR binding sites, suggesting far greater number of VDR binding sites in $1,25(OH)_2D_3$ sufficient than the insufficient subjects (Handel et al., 2013).

Genome-wide VDR cistromes are not available in adipocytes, but recent VDR binding sites in other cell types has been mapped with ChIP-seq from both upstream and downstream of the transcription start site. Further genome wide view actions of VDR in adipocytes as well as integration of other tissue specific cell types are warranted.

CONCLUSION AND FUTURE DIRECTIONS

Adipose tissue acts in addition to nutrient storage as an active endocrine organ. In the obese state, sub-clinical inflammation increases the risk of a variety of chronic diseases. Vitamin D deficiency is common in overweight and obese individuals, and it is possible that lower circulating concentrations may contribute to increases in metabolic risk. A genome-wide association study of 25(OH)D concentrations in 33996 individuals of European descent from 15 cohorts found variants near genes involved in cholesterol synthesis, hydroxylation, and vitamin D transport affect vitamin D status (Wang et al., 2010). Genetic variation at these loci identifies individuals who have a substantially increased risk of vitamin D insufficiency.

On the cellular level, $1,25(OH)_2D_3$ has a significant role in adipogenesis and inflammation which might be species dependent. Holick et al. (1989) demonstrated that the peak circulating concentrations of 25(OH)D in the elderly are about 30% of that of the young. These findings suggest that there will be significant challenges in the translation of the finding from models and non-human primates to the targeted human populations (healthy, diseased, black, white, age, BMI, geographical latitude, race). More evidence accumulates that one dose does not fit all (Powe et al., 2014). Powe and colleagues evaluated vitamin D binding proteins (VDBP) and 25(OH)D levels in black and white Americans. Black adult Americans had low 25(OH)D levels and with the threshold of 20 or 30 ng/ml, 77–96% of them would be classified as vitamin D deficient. Surprisingly, the black study participants had higher bone mineral density, higher calcium levels and only slightly higher parathyroid levels than the white study participants due to VDBP gene polymorphisms (rs7041 and rs4588). The authors speculated that the low levels of VDBP might protect against the adverse effects of vitamin D deficiency. Sufficient levels of this essential hormone and the development of potent novel vitamin D receptor analogs (Peräkylä et al., 2005; Leyssens et al., 2014), which could be easily and cheaply substituted, are beneficial in the maintenance of health and prevention of a number of diseases associated with vitamin D deficiency. Recent systemic review and meta-analysis summary of observational studies and randomized interventions investigated the association between the circulating 25(OH)D concentrations and cause specific mortality in 900,000 subjects in 26 countries (Chowdhury et al., 2014). There was an inverse association of mortality risk and vitamin D levels, yet the observed association could be direct [suboptimal 25(OH)D concentrations] or indirect through higher BMI or disadvantageous social circumstances. Thus, prospective intervention studies are needed to establish potential causal associations between vitamin D levels and disease outcomes.

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REFERENCES

Adams, K. F., Schatzkin, A., Harris, T. B., Kipnis, V., Mouw, T., Ballard-Barbash, R., et al. (2006). Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old. *N. Engl. J. Med.* 355, 763–778. doi: 10.1056/NEJMoa055643

Arner, P., and Spalding, K. L. (2010). Fat cell turnover in humans. *Biochem. Biophys. Res. Commun.* 396, 101–104. doi: 10.1016/j.bbrc.2010.02.165

Blumberg, J. M., Tzameli, I., Astapova, I., Lam, F. S., Flier, J. S., and Hollenberg, A. N. (2006). Complex role of the vitamin D receptor and its ligand in adipogenesis in 3T3-L1 cells. *J. Biol. Chem.* 281, 11205–11213. doi: 10.1074/jbc.M510343200

Carnevalli, L. S., Masuda, K., Frigerio, F., Le Bacquer, O., Um, S. H., Gandin, V., et al. (2010). S6K1 plays a critical role in early adipocyte differentiation. *Dev. Cell.* 18, 763–774. doi: 10.1016/j.devcel.2010.02.018

Chowdhury, R., Kunutsor, S., Vitezova, A., Oliver-Williams, C., Chowdhury, S., Kiefte-de-Jong, J. C., et al. (2014). Vitamin D and risk of cause specific death: systematic review and meta-analysis of observational cohort and randomised intervention studies. *BMJ* 348:g1903. doi: 10.1136/bmj.g1903

Cianferotti, L., and Demay, M. B. (2007). VDR-mediated inhibition of DKK1 and SFRP2 suppresses adipogenic differentiation of murine bone marrow stromal cells. *J. Cell. Biochem.* 101, 80–88. doi: 10.1002/jcb.21151

Despres, J. P., and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. *Nature* 444, 881–887. doi: 10.1038/nature05488

Ding, C., Gao, D., Wilding, J., Trayhurn, P., and Bing, C. (2012). Vitamin D signalling in adipose tissue. *Br. J. Nutr.* 108, 1915–1923. doi: 10.1017/S0007114512003285

Ding, C., Wilding, J. P., and Bing, C. (2013a). 1,25-dihydroxyvitamin D3 protects against macrophage-induced activation of NFkappaB and MAPK signalling and chemokine release in human adipocytes. *PLoS ONE* 8:e61707. doi: 10.1371/journal.pone.0061707

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013b). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Frankenfield, D. C., Rowe, W. A., Cooney, R. N., Smith, J. S., and Becker, D. (2001). Limits of body mass index to detect obesity and predict body composition. *Nutrition* 17, 26–30. doi: 10.1016/S0899-9007(00)00471-8

Fujiwara, K., Hasegawa, K., Ohkumo, T., Miyoshi, H., Tseng, Y. H., and Yoshikawa, K. (2012). Necdin controls proliferation of white adipocyte progenitor cells. *PLoS ONE* 7:e30948. doi: 10.1371/journal.pone.0030948

Gao, D., Trayhurn, P., and Bing, C. (2013). 1,25-Dihydroxyvitamin D3 inhibits the cytokine-induced secretion of MCP-1 and reduces monocyte recruitment by human preadipocytes. *Int. J. Obes. (Lond)* 37, 357–365. doi: 10.1038/ijo.2012.53

Goossens, G. H. (2008). The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiol. Behav.* 94, 206–218 doi: 10.1016/j.physbeh.2007.10.010

Handel, A. E., Sandve, G. K., Disanto, G., Berlanga-Taylor, A. J., Gallone, G., Hanwell, H., et al. (2013). Vitamin D receptor ChIP-seq in primary CD4+ cells: relationship to serum 25-hydroxyvitamin D levels and autoimmune disease. *BMC Med.* 11:163. doi: 10.1186/1741-7015-11-163

Heikkinen, S., Vaisanen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1alpha,25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Holick, M. F., Matsuoka, L. Y., and Wortsman, J. (1989). Age, vitamin D, and solar ultraviolet. *Lancet* 2, 1104–1105. doi: 10.1016/S0140-6736(89)91124-0

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* 444, 860–867. doi: 10.1038/nature05485

Huotari, A., and Herzig, K. H. (2008). Vitamin D and living in northern latitudes–an endemic risk area for vitamin D deficiency. *Int. J. Circumpolar Health* 67, 164–178. doi: 10.3402/ijch.v67i2-3.18258

Jin, W., Takagi, T., Kanesashi, S. N., Kurahashi, T., Nomura, T., Harada, J., et al. (2006). Schnurri-2 controls BMP-dependent adipogenesis via interaction with Smad proteins. *Dev. Cell.* 10, 461–471. doi: 10.1016/j.devcel.2006.02.016

Kahn, S. E., Hull, R. L., and Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444, 840–846. doi: 10.1038/nature05482

Keisala, T., Minasyan, A., Lou, Y. R., Zou, J., Kalueff, A. V., Pyykkö, I., et al. (2009). Premature aging in vitamin D receptor mutant mice. *J. Steroid Biochem. Mol. Biol.* 115, 91–97. doi: 10.1016/j.jsbmb.2009.03.007

Kong, J., and Li, Y. C. (2006). Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. *Am. J. Physiol. Endocrinol. Metab.* 290, E916–E924. doi: 10.1152/ajpendo.00410.2005

Lee, H., Bae, S., and Yoon, Y. (2012). Anti-adipogenic effects of 1,25-dihydroxyvitamin D3 are mediated by the maintenance of the wingless-type MMTV integration site/beta-catenin pathway. *Int. J. Mol. Med.* 30, 1219–1224. doi: 10.3892/ijmm.2012.1101

Lefterova, M. I., Zhang, Y., Steger, D. J., Schupp, M., Schug, J., Cristancho, A., et al. (2008). PPARgamma and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev.* 22, 2941–2952. doi: 10.1101/gad.1709008

Lehr, S., Hartwig, S., Lamers, D., Famulla, S., Muller, S., Hanisch, F. G., et al. (2012). Identification and validation of novel adipokines released from primary human adipocytes. *Mol. Cell. Proteomics* 11:M111.010504. doi: 10.1074/mcp.M111.010504

Lemire, J. (2000). 1,25-Dihydroxyvitamin D3–a hormone with immunomodulatory properties. *Z. Rheumatol.* 59, 24–27. doi: 10.1007/s003930070034

Leyssens, C., Verlinden, L., and Verstuyf, A. (2014). The future of vitamin D analogs. *Front. Physiol.* 5:122. doi: 10.3389/fphys.2014.00122

Lira, F. S., Rosa, J. C., Cunha, C. A., Ribeiro, E. B., do Nascimento, C. O., Oyama, L. M., et al. (2011). Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. *Lipids Health Dis.* 10:37. doi: 10.1186/1476-511X-10-37

Lorente-Cebrian, S., Eriksson, A., Dunlop, T., Mejhert, N., Dahlman, I., Astrom, G., et al. (2012). Differential effects of 1alpha,25-dihydroxycholecalciferol on MCP-1 and adiponectin production in human white adipocytes. *Eur. J. Nutr.* 51, 335–342. doi: 10.1007/s00394-011-0218-z

Lowe, C. E., O'Rahilly, S., and Rochford, J. J. (2011). Adipogenesis at a glance. *J. Cell. Sci.* 124, 2681–2686. doi: 10.1242/jcs.079699

Madsen, M. S., Siersbaek, R., Boergesen, M., Nielsen, R., and Mandrup, S. (2014). Peroxisome proliferator-activated receptor gamma and c/ebpalpha synergistically activate key metabolic adipocyte genes by assisted loading. *Mol. Cell. Biol.* 34, 939–954. doi: 10.1128/MCB.01344-13

Mahajan, A., and Stahl, C. H. (2009). Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. *J. Nutr. Biochem.* 20, 512–520. doi: 10.1016/j.jnutbio.2008.05.010

Marcotorchino, J., Gouranton, E., Romier, B., Tourniaire, F., Astier, J., Malezet, C., et al. (2012). Vitamin D reduces the inflammatory response and restores glucose uptake in adipocytes. *Mol. Nutr. Food Res.* 56, 1771–1782. doi: 10.1002/mnfr.201200383

Maury, E., and Brichard, S. M. (2010). Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol. Cell. Endocrinol.* 314, 1–16. doi: 10.1016/j.mce.2009.07.031

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2012). VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol. Endocrinol.* 26, 37–51. doi: 10.1210/me.2011-1109

Mutt, S. J., Karhu, T., Lehtonen, S., Lehenkari, P., Carlberg, C., Saarnio, J., et al. (2012). Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D(3) via the NF-kappaB pathway. *FASEB J.* 26, 4400–4407. doi: 10.1096/fj.12-210808

Narvaez, C. J., Matthews, D., Broun, E., Chan, M., and Welsh, J. (2009). Lean phenotype and resistance to diet-induced obesity in vitamin D receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue. *Endocrinology* 150, 651–661. doi: 10.1210/en.2008-1118

Narvaez, C. J., Simmons, K. M., Brunton, J., Salinero, A., Chittur, S. V., and Welsh, J. E. (2013). Induction of STEAP4 correlates with 1,25-dihydroxyvitamin D3 stimulation of adipogenesis in mesenchymal progenitor cells derived from human adipose tissue. *J. Cell. Physiol.* 228, 2024–2036. doi: 10.1002/jcp.24371

Nielsen, R., Pedersen, T. A., Hagenbeek, D., Moulos, P., Siersbaek, R., Megens, E., et al. (2008). Genome-wide profiling of PPARgamma: RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes Dev.* 22, 2953–2967. doi: 10.1101/gad.501108

Nimitphong, H., Holick, M. F., Fried, S. K., and Lee, M. J. (2012). 25-hydroxyvitamin D(3) and 1,25-dihydroxyvitamin D(3) promote the differentiation of human subcutaneous preadipocytes. *PLoS ONE* 7:e52171. doi: 10.1371/journal.pone.0052171

Payne, V. A., Au, W. S., Lowe, C. E., Rahman, S. M., Friedman, J. E., O'Rahilly, S., et al. (2009). C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. *Biochem. J.* 425, 215–223. doi: 10.1042/BJ20091112

Peräkylä, M., Malinen, M., Herzig, K. H., and Carlberg, C. (2005). Gene regulatory potential of nonsteroidal vitamin D receptor ligands. *Mol. Endocrinol.* 19, 2060–2073. doi: 10.1210/me.2004-0417

Phinney, K. W., Bedner, M., Tai, S. S., Vamathevan, V. V., Sander, L. C., Sharpless, K. E., et al. (2012). Development and certification of a standard reference material for vitamin D metabolites in human serum. *Anal. Chem.* 84, 956–962. doi: 10.1021/ac202047n

Powe, C. E., Karumanchi, S. A., and Thadhani, R. (2014). Vitamin D-binding protein and vitamin D in blacks and whites. *N. Engl. J. Med.* 370, 880–881. doi: 10.1056/NEJMoa1306357

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Ross, A., Taylor, C., Yaktine, A., and Del Valle, H. (2011). *Dietary Reference Intakes for Calcium and Vitamin D. Institute of Medicine Report*. Washington, DC: The National Academies Press.

Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., et al. (2000). Inhibition of adipogenesis by Wnt signaling. *Science* 289, 950–953. doi: 10.1126/science.289.5481.950

Scime, A., Grenier, G., Huh, M. S., Gillespie, M. A., Bevilacqua, L., Harper, M. E., et al. (2005). Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. *Cell. Metab.* 2, 283–295. doi: 10.1016/j.cmet.2005.10.002

Smas, C. M., and Sul, H. S. (1993). Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell* 73, 725–734. doi: 10.1016/0092-8674(93)90252-L

Stumpf, W. E. (1995). Vitamin D sites and mechanisms of action: a histochemical perspective. reflections on the utility of autoradiography and cytopharmacology for drug targeting. *Histochem. Cell Biol.* 104, 417–427. doi: 10.1007/BF01464331

Sun, X., Morris, K. L., and Zemel, M. B. (2008). Role of calcitriol and cortisol on human adipocyte proliferation and oxidative and inflammatory stress: a microarray study. *J. Nutrigenet. Nutrigenomics* 1, 30–48. doi: 10.1159/000109873

Sun, X., and Zemel, M. B. (2007). Calcium and 1,25-dihydroxyvitamin D3 regulation of adipokine expression. *Obesity (Silver Spring)* 15, 340–348. doi: 10.1038/oby.2007.540

Sun, X., and Zemel, M. B. (2008). Calcitriol and calcium regulate cytokine production and adipocyte-macrophage cross-talk. *J. Nutr. Biochem.* 19, 392–399. doi: 10.1016/j.jnutbio.2007.05.013

Tourniaire, F., Romier-Crouzet, B., Lee, J. H., Marcotorchino, J., Gouranton, E., Salles, J., et al. (2013). Chemokine expression in inflamed adipose tissue is mainly mediated by NF-kappaB. *PLoS ONE* 8:e66515. doi: 10.1371/journal.pone.0066515

Trayhurn, P., O'Hara, A., and Bing, C. (2011). Interrogation of microarray datasets indicates that macrophage-secreted factors stimulate the expression of genes associated with vitamin D metabolism (VDR and CYP27B1) in human adipocytes. *Adipobiology* 3, 29–34.

Trayhurn, P. (2013). Hypoxia and adipose tissue function and dysfunction in obesity. *Physiol. Rev.* 93, 1–21. doi: 10.1152/physrev.00017.2012

Tuohimaa, P. (2009). Vitamin D and aging. *J. Steroid Biochem. Mol. Biol.* 114, 78–84. doi: 10.1016/j.jsbmb.2008.12.020

Tuoresmäki, P., Väisänen, S., Neme, A., Heikkinen, S., and Carlberg, C. (2014). Patterns of genome-wide VDR locations. *PLoS ONE* 9:e96105. doi: 10.1371/journal.pone.0096105

Van Gaal, L. F., Mertens, I. L., and De Block, C. E. (2006). Mechanisms linking obesity with cardiovascular disease. *Nature* 444, 875–880. doi: 10.1038/nature05487

Vigilanza, P., Aquilano, K., Baldelli, S., Rotilio, G., and Ciriolo, M. R. (2011). Modulation of intracellular glutathione affects adipogenesis in 3T3-L1 cells. *J. Cell. Physiol.* 226, 2016–2024. doi: 10.1002/jcp.22542

Vimaleswaran, K. S., Berry, D. J., Lu, C., Tikkanen, E., Pilz, S., Hiraki, L. T., et al. (2013). Causal relationship between obesity and vitamin D status: bi-directional mendelian randomization analysis of multiple cohorts. *PLoS Med.* 10:e1001383. doi: 10.1371/journal.pmed.1001383

Vlasova, M., Purhonen, A. K., Jarvelin, M. R., Rodilla, E., Pascual, J., and Herzig, K. H. (2010). Role of adipokines in obesity-associated hypertension. *Acta Physiol. (Oxf).* 200, 107–127. doi: 10.1111/j.1748-1716.2010.02171.x

Wamberg, L., Cullberg, K. B., Rejnmark, L., Richelsen, B., and Pedersen, S. B. (2013). Investigations of the anti-inflammatory effects of vitamin D in adipose tissue: results from an *in vitro* study and a randomized controlled trial. *Horm. Metab. Res.* 45, 456–462. doi: 10.1055/s-0032-1331746

Wang, T. J., Zhang, F., Richards, J. B., Kestenbaum, B., van Meurs, J. B., Berry, D., et al. (2010). Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 376, 180–188. doi: 10.1016/S0140-6736(10)60588-0

Weber, K., and Erben, R. G. (2013). Differences in triglyceride and cholesterol metabolism and resistance to obesity in male and female vitamin D receptor knockout mice. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 97, 675–683. doi: 10.1111/j.1439-0396.2012.01308.x

Wellen, K. E., and Hotamisligil, G. S. (2003). Obesity-induced inflammatory changes in adipose tissue. *J. Clin. Invest.* 112, 1785–1788. doi: 10.1172/JCI20514

Welsh, J., Zinser, L. N., Mianecki-Morton, L., Martin, J., Waltz, S. E., James, H., et al. (2011). Age-related changes in the epithelial and stromal compartments of the mammary gland in normocalcemic mice lacking the vitamin D3 receptor. *PLoS ONE* 6:e16479. doi: 10.1371/journal.pone.0016479

White, U. A., and Stephens, J. M. (2010). Transcriptional factors that promote formation of white adipose tissue. *Mol. Cell. Endocrinol.* 318, 10–14. doi: 10.1016/j.mce.2009.08.023

Wong, K. E., Kong, J., Zhang, W., Szeto, F. L., Ye, H., Deb, D. K., et al. (2011). Targeted expression of human vitamin D receptor in adipocytes decreases energy expenditure and induces obesity in mice. *J. Biol. Chem.* 286, 33804–33810. doi: 10.1074/jbc.M111.257568

Wong, K. E., Szeto, F. L., Zhang, W., Ye, H., Kong, J., Zhang, Z., et al. (2009). Involvement of the vitamin D receptor in energy metabolism: regulation of uncoupling proteins. *Am. J. Physiol. Endocrinol. Metab.* 296, E820–E828. doi: 10.1152/ajpendo.90763.2008

Wortsman, J., Matsuoka, L. Y., Chen, T. C., Lu, Z., and Holick, M. F. (2000). Decreased bioavailability of vitamin D in obesity. *Am. J. Clin. Nutr.* 72, 690–693.

Zhang, K., Guo, W., Yang, Y., and Wu, J. (2011). JAK2/STAT3 pathway is involved in the early stage of adipogenesis through regulating C/EBPbeta transcription. *J. Cell. Biochem.* 112, 488–497. doi: 10.1002/jcb.22936

Zhuang, H., Lin, Y., and Yang, G. (2007). Effects of 1,25-dihydroxyvitamin D3 on proliferation and differentiation of porcine preadipocyte *in vitro*. *Chem. Biol. Interact.* 170, 114–123. doi: 10.1016/j.cbi.2007.07.012

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Vitamin D in inflammatory diseases

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Changes in vitamin D serum levels have been associated with inflammatory diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis (MS), atherosclerosis, or asthma. Genome- and transcriptome-wide studies indicate that vitamin D signaling modulates many inflammatory responses on several levels. This includes (i) the regulation of the expression of genes which generate pro-inflammatory mediators, such as cyclooxygenases or 5-lipoxygenase, (ii) the interference with transcription factors, such as NF-κB, which regulate the expression of inflammatory genes and (iii) the activation of signaling cascades, such as MAP kinases which mediate inflammatory responses. Vitamin D targets various tissues and cell types, a number of which belong to the immune system, such as monocytes/macrophages, dendritic cells (DCs) as well as B- and T cells, leading to individual responses of each cell type. One hallmark of these specific vitamin D effects is the cell-type specific regulation of genes involved in the regulation of inflammatory processes and the interplay between vitamin D signaling and other signaling cascades involved in inflammation. An important task in the near future will be the elucidation of the regulatory mechanisms that are involved in the regulation of inflammatory responses by vitamin D on the molecular level by the use of techniques such as chromatin immunoprecipitation (ChIP), ChIP-seq, and FAIRE-seq.

Keywords: $1\alpha,25(OH)_2D_3$, VDR, cyclooxygenase, NFκB, NFAT, MKP1, interleukins, innate immune system

INTRODUCTION: $1\alpha,25(OH)_2D_3$ AND INFLAMMATORY DISEASES

It is now well established that the physiological importance of the vitamin D status extends far beyond the regulation of bone metabolism. According to its manifold functions in immune homeostasis, increasing evidence relates serum vitamin D levels as well as polymorphisms in enzymes involved in vitamin D metabolism to the incidence of chronic inflammatory diseases like asthma, atherosclerosis and autoimmune diseases (Stojanovic et al., 2011; Summerday et al., 2012; Szekely and Pataki, 2012). However, whether vitamin D exerts a salutatory or deteriorating role in such diseases is still under debate. This review will focus on the knowledge regarding the role of vitamin D in inflammatory diseases by the examples of asthma, atherosclerosis and autoimmune diseases.

$1\alpha,25(OH)_2D_3$ AND ASTHMA

According to the World Health Organization (WHO), asthma is the most common chronic disease among children (http://www.who.int/mediacentre/factsheets/fs307/en/index.html). In this context, several studies addressed the interrelationship of the maternal as well as infant vitamin D status and the prevalence and severity of asthma. Three studies by Brehm et al. analyzed the relationship between vitamin D levels and asthma severity in Costa Rican, North American and Puerto Rican children, respectively (Brehm et al., 2009, 2010, 2012). Collectively, they found high prevalences of vitamin D insufficiency in asthmatic children and vitamin D insufficiency was correlated with severe asthma exacerbations. However, the prevalence of vitamin D insufficiency was high in Puerto Rican children irrespective of the indisposition from asthma, with roughly comparable percentages between asthma patients and otherwise healthy children (Brehm et al., 2012). Although few studies showed no correlation between serum vitamin D levels and the presence of asthma (Menon et al., 2012; Gergen et al., 2013), many studies state a higher prevalence of vitamin D deficiency in asthmatic children (Freishtat et al., 2010; Chinellato et al., 2011a,b; Ehlayel et al., 2011; Hollams et al., 2011; Bener et al., 2012; Krobtrakulchai et al., 2013) and adults (Li et al., 2011b). Additionally, in many cases a relation between low vitamin D levels and reduced asthma control is found. Furthermore, metabolomic analysis of breath condensates revealed reduced levels of vitamin D metabolites in children with asthma (Carraro et al., 2013). Similarly, enhanced vitamin D binding protein levels were found in bronchoalveolar lavage fluid of asthmatic children (Gupta et al., 2012b). Interestingly, one study describes an age-dependent association between serum vitamin D level and asthma prevalence in children (Van Oeffelen et al., 2011).

A different relationship between the vitamin D status and asthma has been brought up by a northern Finland birth cohort study, which revealed an increased risk of asthma in adults who received high dose vitamin D supplementation in their childhood (Hypponen et al., 2004). In accordance with these findings, a prospective study by Tolppanen et al. revealed an increased risk of wheezing in association with higher vitamin D levels, but no correlation of lower vitamin D levels to respiratory sicknesses

(Tolppanen et al., 2013). Another study reinforces the finding of increased susceptibility to asthma after vitamin D supplementation, yet only regarding supplementation of water soluble formulations and not in connection with vitamin D supplementation in peanut oil (Kull et al., 2006).

There is debate as to whether maternal vitamin D levels during the pregnancy influence the susceptibility to asthma of the progeny. Whereas some reports showed no correlation between maternal or cord blood vitamin D levels and an increased risk of childhood asthma (Camargo et al., 2011; Rothers et al., 2011; Morales et al., 2012; Pike et al., 2012; Magnus et al., 2013), another report indicates that high maternal vitamin D levels correlate with enhanced probability of asthma development in children (Gale et al., 2008). In contrast, some reports associate higher vitamin D intake during pregnancy with reduced risk of childhood wheezing and asthma (Camargo et al., 2007; Devereux et al., 2007; Erkkola et al., 2009).

Mechanistically, vitamin D induced protection against airway inflammation has been related to a modulated T cell response to allergens as well as induction of the immunoglobulin-like anti-inflammatory cell surface protein CD200 on T cells, that acts on target immune cells which express the CD200 receptor (CD200R) (Dimeloe et al., 2012; Gorman et al., 2012; Urry et al., 2012). Many authors suggest that the beneficial effect of sufficient vitamin D levels on asthma development results from the immune enhancing effect of vitamin D and the simultaneous prevention of respiratory infections (Ginde et al., 2009; Camargo et al., 2011; Majak et al., 2011; Morales et al., 2012).

Furthermore, there is evidence that the serum vitamin D level has also an influence on asthma therapy, as vitamin D has been demonstrated to enhance glucocorticoid (GC) action and lower serum vitamin D levels are associated with higher corticosteroid requirement, at least in children, or even therapy-resistance (Searing et al., 2010; Goleva et al., 2012; Gupta et al., 2012a; Wu et al., 2012). Additionally, the therapeutic effect of specific allergen immunotherapy has been correlated to serum vitamin D levels (Majak et al., 2012).

Besides serum vitamin D levels also polymorphisms of genes of the vitamin D pathway such as the vitamin D receptor (VDR) have been associated with asthma (Poon et al., 2004; Raby et al., 2004; Saadi et al., 2009; Li et al., 2011a; Pillai et al., 2011; Maalmi et al., 2013), yet, not all studies revealed a correlation between vitamin D pathway polymorphisms and asthma prevalence (Vollmert et al., 2004; Fang et al., 2009).

1α,25(OH)$_2$D$_3$ AND ATHEROSCLEROSIS

Another chronic inflammatory disease that is more prevalent in the elderly population is atherosclerosis. Early studies on atherosclerosis development in several animal models revealed an accelerating effect of high doses of vitamin D. Vascular calcification was observed in some of these studies, but not all (Zemplenyi and Mrhova, 1965; Kudejko, 1968; Taura et al., 1979; Kunitomo et al., 1981; Toda et al., 1983, 1985). Moreover, 1α,25-dihydroxyvitamin D$_3$, the active form of vitamin D, stimulated vascular calcification by *in vitro* by reducing the expression of parathyroid hormone-related peptide as well as stimulating alkaline phosphatase activity in bovine vascular smooth muscle cells (Jono et al., 1998). On the other hand, there is a large body of research from clinical studies in humans indicating that low levels of serum 25-hydroxy vitamin D are associated with atherosclerosis (Reis et al., 2009; Carrelli et al., 2011; Shanker et al., 2011; Cheraghi et al., 2012). In line with this, the incidence of osteoporosis, a disease known to be related to vitamin D inadequacy, correlates with the incidence of atherosclerosis (Stojanovic et al., 2011). Therefore, different mechanisms may account for the promotion of atherogenesis by high and low vitamin D levels, respectively, and calcification may be crucial in the case of hypervitaminosis. Moreover, differences between the animal and human system may account for the conflicting results.

With respect to atherogenesis, 1α,25-dihydroxyvitamin D$_3$ has been demonstrated to reduce macrophage adhesion and migration as well as foam cell formation in monocytes isolated from type 2 diabetic patients (Oh et al., 2012; Riek et al., 2013a,b). Mechanistic investigations in the context of these studies attributed the beneficial effects of vitamin D to a reduction of endoplasmatic reticulum stress in macrophages. This has been investigated in two mouse models, where vitamin D deficiency facilitated atherosclerosis, which could be reversed in the course of macrophage endoplasmatic reticulum stress suppression (Weng et al., 2013). Further evidence on beneficial effects of calcitriol treatment on atherosclerosis development has been obtained from an investigation with apolipoprotein E knock-out mice. In this study, oral calcitriol treatment decreased the production of proinflammatory chemokines, led to a reduced amount of inflammatory effector cells in atherosclerotic plaques and simultaneously increased amounts of regulatory T cells (Takeda et al., 2010). A similar link between vitamin D, T cell modulation, and atherosclerosis has also been established in humans with chronic kidney disease (CKD) (Yadav et al., 2012).

The renin-angiotensin-system is known for its detrimental effects on the cardiovascular system and has been shown to play an important role in the development of atherosclerosis. Interestingly, numerous studies in mice document that vitamin D signaling suppresses the renin-angiotensin-system and that vitamin D deficiency is associated with an increased activity of the renin-angiotensin-system (Li et al., 2002; Zhou et al., 2008; Szeto et al., 2012; Weng et al., 2013). Moreover, the inverse associations which are described for vitamin D and the occurrence of inflammatory cytokines, C-reactive protein, and adhesion molecules suggest a inhibitory role for vitamin D in the genesis of atherosclerosis (Brewer et al., 2011). Additionally, there is experimental evidence that vitamin D reduces the expression of matrix metalloproteinases that are involved in vascular calcification (Nakagawa et al., 2005; Qin et al., 2006).

However, there are also studies that found no evidence for an association between low vitamin D and atherosclerosis in patients suffering from different autoimmune diseases (Mok et al., 2012; Sachs et al., 2013). Similarly, there was no evidence for an association of *Bsm*I polymorphism, an intronic single nucleotide variation of the VDR gene, with atherosclerosis (El-Shehaby et al., 2013). Yet, it has been shown that atherosclerosis in monkeys is associated with low levels of VDR expression in coronary arteries even in the presence of higher plasma vitamin D concentrations (Schnatz et al., 2012a,b). Moreover, the activation of vitamin D

can occur locally in macrophages that infiltrate atherosclerotic lesions and local vitamin D response might thus not necessarily correlate with serum vitamin D levels (Richart et al., 2007).

$1\alpha,25(OH)_2D_3$ AND AUTOIMMUNE DISEASES

It is well established that vitamin D plays an important role in the regulation of immune functions (Schwalfenberg, 2011; Zhang et al., 2013a). Accordingly, several inflammatory autoimmune diseases like rheumatic disorders and type 1 diabetes have been associated with vitamin D deficiency (Adorini and Penna, 2008; Shapira et al., 2010). Inflammatory processes in the central nervous system are a hallmark of the autoimmune disease multiple sclerosis (MS) (Deckx et al., 2013). Several studies indicate that MS patients have lower levels of vitamin D and that higher vitamin D levels as well as vitamin D supplementation have a protective effect against MS (Munger et al., 2004, 2006; Ozgocmen et al., 2005). Moreover, vitamin D levels have been shown to vary in concordance with MS exacerbations (Correale et al., 2009) and it is possible that low vitamin D levels are rather a consequence of the sun avoidance of MS patients and not a direct cause of the disease (Munger et al., 2006). Regarding the effectiveness of vitamin D supplementation in the course of MS treatment, there are studies in mice and humans that suggest a beneficial effect of treatment (Goldberg et al., 1986; Wingerchuk et al., 2005; Pedersen et al., 2007; Burton et al., 2010). Interestingly, a gender specific effect of vitamin D has been demonstrated in mice and humans, which points to greater effects of vitamin D in females (Spach and Hayes, 2005; Correale et al., 2010).

Overall, there have been only a few controlled trials documenting the outcome of vitamin D supplementation on disease activity in rheumatic conditions, and the role of vitamin D in rheumatoid arthritis is therefore controversially discussed (Gatenby et al., 2013). Yet, a metaanalysis of observational studies on the vitamin D intake and vitamin D serum levels suggests an inverse association with rheumatoid arthritis (Song et al., 2012). Additionally, *in vitro* experiments with macrophages from healthy donors and rheumatoid arthritis patients indicate an enhanced anti-inflammatory potential of vitamin D in macrophages from the latter group (Neve et al., 2013).

It has been shown that the onset of autoimmunity in type 1 diabetes is preceded by a proinflammatory metabolic serum profile (Knip and Simell, 2012). Concurrently, a study in Italian children revealed reduced vitamin D serum levels in children at the onset of type 1 diabetes compared to children hospitalized for other reasons (Franchi et al., 2013). In conformity with these findings, metaanalyses suggest an association between vitamin D intake in early life and susceptibility for type 1 diabetes (Zipitis and Akobeng, 2008; Dong et al., 2013).

For inflammatory bowel disease (IBD), another autoimmune disorder, similar associations to that described above regarding vitamin D status and sunlight exposure have been reported (Garg et al., 2012; Ananthakrishnan, 2013). Animal studies in vitamin D deficient and VDR knockout (KO) mice reveal a dysregulation of T cells that might be of importance in the pathogenesis of IBD (Ooi et al., 2012).

In summary, there is considerable evidence for an association between vitamin D deficiency and inflammatory diseases. However, regarding the causality of this association and the benefit of vitamin D supplementation, only limited information is available and the existing data are still inconsistent.

INTERFERENCE OF $1\alpha,25(OH)_2D_3$ WITH PRO-INFLAMMATORY TRANSCRIPTION FACTORS AND SIGNALING PATHWAYS

Cell type specific up-regulation of proinflammatory genes and down-regulation of anti-inflammatory genes is a hallmark of the onset of an inflammatory reaction. Depending on the cell type, up-regulation of certain cytokines or enzymes which generate mediators of inflammation can occur at the transcriptional or posttranscriptional level. In addition, there is considerable crosstalk between various pathways which allows adaptation of the host defense reactions to the environment. According to their functions, the regulators of inflammatory reactions can be receptors such as toll like receptors, signal transducers as well as transcription factors which translate the activation of certain signal cascades into gene transcription. Additionally, regulation of gene expression during inflammatory processes can also occur on posttranslational level which is not focus of this review.

At the level of intracellular signal transduction, MAP kinases such as JNK or p38 have been identified as central signal transducers of inflammatory signals. Interestingly, it has been observed that there is a cross talk between VDR/RXR and MAP kinase signaling on many levels and the outcome, e.g., stimulation or inhibition, depends on the stimulus, cell type and the response (Miodovnik et al., 2012). Regarding inflammation, it has become obvious that vitamin D inhibits production of proinflammatory cytokines like IL-6 or TNFα in monocytes via the inhibition of p38 MAP kinase (Zhang et al., 2012). Inhibition of p38 in monocytes was found to be due to induction of MAPK phosphatase-1 (MKP1) which dephosphorylates p38 and thus reduces p38 activation (**Figure 1**). A similar mechanism was found in prostate cells where induction of MKP5 by $1\alpha,25(OH)_2D_3$ was responsible for down-regulation of IL-6 mRNA expression (Nonn et al., 2006). $1\alpha,25(OH)_2D_3$ increases MKP5 transcription by induction of VDR/RXR binding to a VDRE in the MKP5 promoter. Beside this indirect modulation of signaling cascades, $1\alpha,25(OH)_2D_3$ and its receptor complex VDR/RXR can interact with other transcription factors such as NF-κB, nuclear factor of activated T-cells (NFAT), or the glucocorticoid receptor (GCR) which leads to anti-inflammatory effects (**Figure 2**). Activation of VDR inhibits NF-κB activation and signaling. NFκB is a ubiquitously expressed transcription factor which represents a heterodimer. In the inactive state it interacts with IκB which keeps it in the cytosol (Karin and Lin, 2002). Upon cell activation by proinflammatory stimuli, IκB is phosphorylated and subsequently ubiquitinylated, which leads to proteasomal degradation of the IκB protein. Free NFκB translocates to the nucleus where it activates transcription of proinflammatory cytokines, antiapoptotic factors as well as of enzymes involved in the generation of proinflammatory mediators such as COX-2 (Karin and Lin, 2002; Tsatsanis et al., 2006). It has been shown that $1\alpha,25(OH)_2D_3$ down-regulates NF-κB levels in lymphocytes (Yu et al., 1995) and that the vitamin D analog TX 527 prevents NF-κB activation in monocytes (Stio et al., 2007). Inhibition of NFκB activation

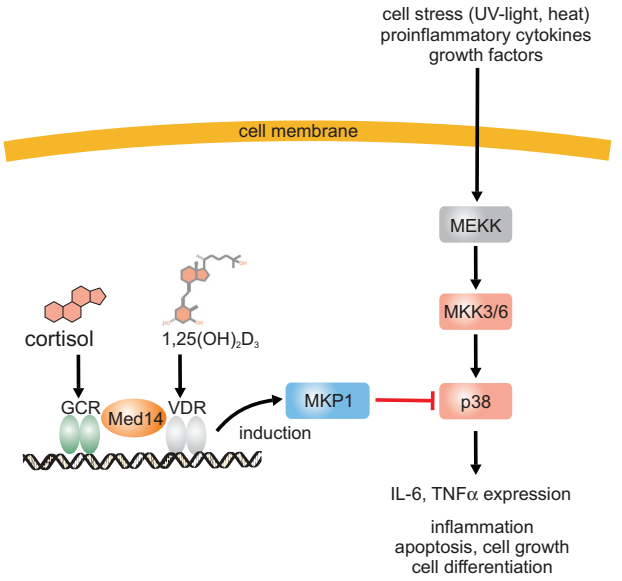

FIGURE 1 | Inhibition of the p38 MAP kinase pathway by 1α,25(OH)$_2$D$_3$ and a mechanism for the synergistic anti-inflammatory effects of 1α,25(OH)$_2$D$_3$ and glucocorticoids. Proinflammatory stimuli lead to p38 MAP kinase phosphorylation and activation which subsequently induces expression of many proinflammatory proteins such as IL-6 and TNFα. 1α,25(OH)$_2$D$_3$ induces MKP1 expression which dephosphorylates and inactivates p38 MAP kinase. 1α,25(OH)$_2$D$_3$ stimulates glucocorticoid-induced MKP1 expression via enhanced expression of Med14.

by 1α,25(OH)$_2$D$_3$-mediated up-regulation of IκB expression was reported in human peritoneal macrophages (Cohen-Lahav et al., 2006) (**Figure 2**). Additionally, interference of vitamin D signaling with DNA binding of NFκB was found (Harant et al., 1998). It was shown that 1α,25(OH)$_2$D$_3$ inhibits NF-κB activity in human MRC-5 fibroblasts but not translocation of its subunits p50 and p65. The partial inhibition of NFκB DNA binding by 1α,25(OH)$_2$D$_3$ was dependent on de novo protein synthesis, suggesting that 1α,25(OH)$_2$D$_3$ may regulate expression of cellular factors which contribute to reduced DNA binding of NFκB (Harant et al., 1998). Thus, it seems that vitamin D is able to inhibit NFκB activation as well DNA binding (**Figure 2**).

Another interesting target for the anti-inflammatory signaling of vitamin D is NFAT (**Figure 2**). This transcription factor is activated by dephosphorylation by calcineurin which leads to translocation of this protein and transcriptional activation of proinflammatory genes such as interleukin 2 and cyclooxygenase-2 (Duque et al., 2005; Muller and Rao, 2010). In T-lymphocytes, it was shown for the interleukin 2 promoter that VDR-RXR heterodimers bind to an NFAT binding site and thus inhibit NFAT activity (Takeuchi et al., 1998). Similar data were obtained for interleukin 17 where 1α,25(OH)$_2$D$_3$ blocked NFAT activity which contributed to repression of interleukin17A expression in inflammatory CD4$^+$ T cells by the hormone (Joshi et al., 2011).

Another interesting finding was that vitamin D enhances the anti-inflammatory activities of GCs (**Figure 1**). The strong anti-inflammatory activities of GCs are mediated by the GCR. It belongs to the nuclear receptor family. Upon ligand binding the receptor dimerizes and translocates into the nucleus where it binds to GC-responsive elements (GRE) and modulates gene expression (Barnes, 1998). In general, GCs down-regulate expression of pro-inflammatory genes and up-regulate anti-inflammatory genes. It was found in asthmatics that dexamethasone-induced MKP-1 expression as a marker for GC responsiveness is significantly increased when serum vitamin D levels increase suggesting that vitamin D may enhance GC responsiveness (Sutherland et al., 2010). It is interesting to note that MKP-1 is also a vitamin D target gene as mentioned above (Zhang et al., 2012). Vitamin D enhancement of GC-induction of MKP1 was abolished both in purified CD14$^+$ and CD14$^-$ cells and it was found that the synergism depends on vitamin D-induced GM-CSF release from CD14$^-$ cells and GM-CSF-dependent MED14 induction in CD14$^+$ cells (Zhang et al., 2013b). MED14 is part of the mediator complex involved in the regulation of transcriptional initiation and it was found to form a complex with VDR and mediate ligand-dependent enhancement of transcription by the VDR (Rachez et al., 1999) (**Figure 1**). Interestingly, MED14 also enhances gene activation by the GCR in a gene-specific manner (Chen et al., 2006). For MKP1 it was found in human monocytes that VDR and GCR bind to a corresponding VDRE and two GREs after ligand stimulation (**Figure 1**). After GM-CSF treatment, MED14 was recruited to the promoter after addition of 1α,25(OH)$_2$D$_3$ but not dexamethasone indicating that MED14 recruitment depends on the VDR (Zhang et al., 2013b). 1α,25(OH)$_2$D$_3$ enhanced the binding of the GCR to the GRE in close proximity to the VDRE in the presence of GM-CSF and ChIP analysis suggest a MED14-VDR-GCR complex at the MKP1 promoter with bridges the crosstalk between vitamin D and GCs (Zhang et al., 2013b). The data from single gene analyses such as MKP1 suggest that the VDR interacts with other signaling pathways.

At present there are genome-wide data available from immortalized lymphoblastoid cell lines (Ramagopalan et al., 2010), undifferentiated and LPS stimulated THP-1 cells (Heikkinen et al., 2011; Tuoresmäki et al., 2014), LS180 colorectal cancer cells (Meyer et al., 2012) and LX2 hepatic stellate cells (Ding et al., 2013). These six ChIP-seq data sets showed 21,776 non-overlapping VDR binding sites whereas only 54 sites were common in all six data sets. The data suggest that, apart from a few sites, VDR binding is strongly cell and stimulus specific. In the non-overlapping binding sites, only 17.5% contain a DR3-type VDRE whereas the percentage of DR3-type response elements is enriched in highly ligand-responsive loci. All these data suggest that the VDR interacts with other transcription factors and that these interactions might only be in part ligand dependent. Regarding inflammation, the genome-wide effects of LPS on VDR location in THP-1 cells are of special interest (Tuoresmäki et al., 2014). From the 805 VDR binding sites, only 462 overlap in untreated and LPS-treated THP cells which were stimulated with 1α,25(OH)$_2$D$_3$. Thus, LPS treatment leads to a considerable change in VDR location. In THP-1 cells, bioinformatic searches for shared binding sites revealed motifs for CEBP1, PU.1 in stimulated THP-1 cells whereas NFYA, LHX3-like and NANOG were found for unstimulated cells but no transcription factor has been identified in conjunction with LPS treatment. Of note, binding

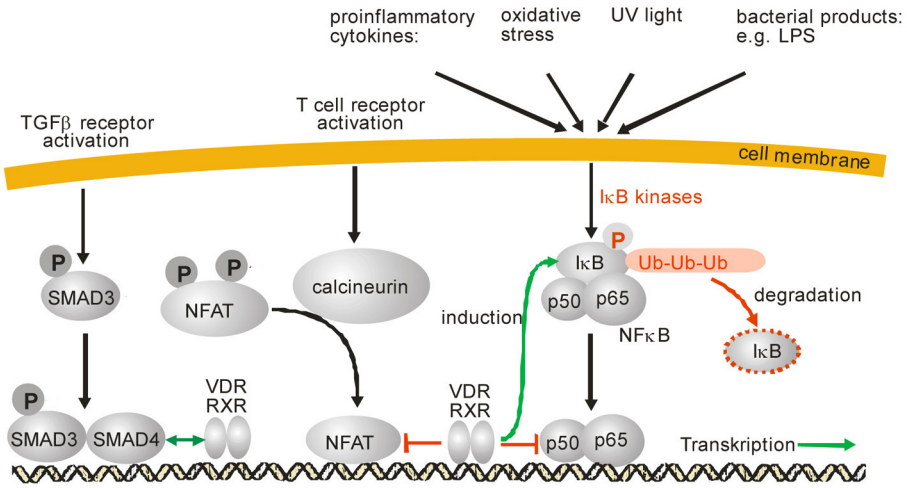

FIGURE 2 | SMAD, NFAT and NFκB signaling and modulation of these signaling pathways by 1α,25(OH)$_2$D$_3$, respective VDR/RXR. IκB phosphorylation after various cell stress signals leads to its ubiquitinylation and subsequent proteosomal degradation. After IκB degradation, NFκB is released and translocates into the nucleus where it binds to DNA and modulates gene expression. Activation of NFAT is mediated by the protein phosphatase calcineurin which dephosphorylates NFAT. After dephosphorylation, NFAT translocates into the nucleus, interacts with a variety of other transcription factors and modulates gene expression. Activation of TGFβ receptors leads to phosphorylation of SMAD2 and SMAD3 as well as subsequent translocation into the nucleus. SMAD3 forms a complex with SMAD4 and modulates gene expression of its target genes. After activation by 1α,25(OH)$_2$D$_3$ the VDR/RXR heterodimer can inhibit NFκB signaling either by induction of IκB or by interference with NFκB DNA binding. Also, inhibition of NFAT signaling was reported by prevention of NFAT binding to its response elements.

sites for JUN, a component of the AP1 transcription factor, were found to be enriched at VDR loci in LX2 hepatic stellate cells. This is of interest regarding inflammation as AP1 is known to be a transcription factor that regulates expression of many proinflammatory genes. At present, there are many data available on single gene levels but there is still a missing link between these data and the genome-wide observations. Since VDR signaling seems to be strongly cell type and stimulus-dependent, more genome-wide data with different cell types and stimuli are required to understand the mechanisms how 1α,25(OH)$_2$D$_3$ modulates gene expression under inflammatory conditions.

REGULATION OF THE EXPRESSION OF PROINFLAMMATORY ENZYMES BY 1α,25(OH)$_2$D$_3$

Arachidonic acid derived eicosanoids which comprise prostaglandins and leukotrienes play an important role in inflammatory processes (Harizi et al., 2008). Of the enzymes involved in prostaglandin synthesis, cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase 1 (mPGES-1) have been shown to be induced in many inflammatory conditions (Tomasoni et al., 1998; Murakami et al., 2000; Cipollone and Fazia, 2006; Petrovic et al., 2006) and inhibition of both enzymes is a common approach in the treatment of inflammatory diseases (Fahmi, 2004; Ramalho et al., 2009; Dallaporta et al., 2010).

In prostate cancer cells it has been demonstrated that 1α,25(OH)$_2$D$_3$ inhibits the expression of COX-2 on mRNA and protein level as well as the expression of prostaglandin receptors on mRNA level and simultaneously upregulates prostaglandin catabolism via 15-hydroxyprostaglandin dehydrogenase (Moreno et al., 2005). In addition, the combination of calcitriol with COX-inhibitors led to synergistic growth inhibition (Moreno et al., 2005). Similar results were obtained with the combination of 1α,25(OH)$_2$D$_3$ and COX-inhibitors in different leukemia cells (Jamshidi et al., 2008). In accordance with the previous findings, treatment with the vitamin D analog elocalcitol resulted in decreased COX-2 expression and diminished PGE$_2$ synthesis in prostate cells (Penna et al., 2009). The COX-2/PGE$_2$-pathway was also identified as the mediator of the growth inhibitory effect of calcitriol in breast cancer cells (Yuan et al., 2012). Furthermore, COX-2 upregulation in placental trophoblasts in response to oxidative stress and in myometrial cells in response to interleukin-1β was inhibited by 1α,25(OH)$_2$D$_3$ (Sun et al., 2013; Thota et al., 2013).

Thill et al. found correlations between VDR expression and expression of COX-2 as well as 15-hydroxy PG dehydrogenase in malignant breast cells and in cells from female reproductive tissues (Thill et al., 2009, 2010, 2012).

In human lung fibroblasts inhibition of PGE$_2$-production by vitamin D was found which was not due to altered COX-expression. Yet, vitamin D inhibited IL-1β-induced mPGES-1 expression and simultaneously stimulated 15-hydroxy PG dehydrogenase (Liu et al., 2014).

5-lipoxygenase (5-LO) accounts for the first two steps in leukotriene biosynthesis. Leukotrienes exert potent proinflammatory actions and have been associated with several chronic inflammatory diseases (Haeggstrom and Funk, 2011).

In the myeloid cell line HL-60, treatment with 1α,25(OH)$_2$D$_3$ triggers differentiation into monocytic cells. Simultaneously, 1α,25(OH)$_2$D$_3$ has been shown to induce 5-LO expression on mRNA and protein level as well as to increase 5-LO enzyme activity (Bennett et al., 1993; Brungs et al., 1994). A similar effect was also observed in the monocytic cell line Mono Mac

6. Additionally, this effect was strongly enhanced by the combination of $1\alpha,25(OH)_2D_3$ with transforming growth factor β (TGF-β) (Brungs et al., 1995; Harle et al., 1998). Mechanistically, the effect of $1\alpha,25(OH)_2D_3$ on 5-LO expression was related to VDR binding sites in the 5-LO promoter and distal parts of the 5-LO gene (Sorg et al., 2006; Stoffers et al., 2010) and is due to stimulation of 5-LO transcript elongation (Stoffers et al., 2010).

Previous results suggest a modulatory role of vitamin D in the inflammatory response of cells of the monocyte/macrophage lineage, which is again modulated by TGF-β. In this context, it is interesting that macrophages contain 1α-hydroxylase and therefore are capable of autocrine or paracrine activation of vitamin D (Lagishetty et al., 2011). Moreover, in keratinocytes autocrine TGF-β production is induced by vitamin D (Kim et al., 1992). Crucial participation of monocytes/macrophages in diverse inflammatory processes has been demonstrated (Cutolo, 1999; Yoon and Jun, 1999; Moore et al., 2013). Besides induction of 5-lipoxygenase, the combination of TGF-β and $1\alpha,25(OH)_2D_3$ has been shown to induce the differentiation antigen CD69 in monocytic cells (Wobke et al., 2013). Overexpression of CD69 again, has been shown in the context of local dermal inflammation, systemic lupus erythematosus, hyperthyroid Graves' disease and autoimmune thyroiditis (Fernandez-Herrera et al., 1996; Portales-Perez et al., 1997; Crispin et al., 1998; Gessl and Waldhausl, 1998).

$1\alpha,25(OH)_2D_3$ AS REGULATOR OF CYTOKINE GENE EXPRESSION, PROTEIN PRODUCTION/RELEASE AND SIGNALING

TGF-β AND Smad SIGNALING IN INFLAMMATION AND THE INFLUENCE OF $1\alpha, 25(OH)_2D_3$

TGF-β is a pleiotropic cytokine with a broad range of biologic effects, which is involved in the regulation of inflammatory processes on several levels. A main mechanism in this respect is the maintenance of T cell tolerance to self or innocuous antigens (Li and Flavell, 2008). In cancer-associated inflammation, TGF-β suppresses the anti-tumor activity of diverse immune cells, including T-cells, natural killer (NK) cells, neutrophils, monocytes and macrophages (Bierie and Moses, 2010). A great number of studies focused on the role of TGF-β in fibrosis and associated inflammation. In these diseases, TGF-β regulates influx and activation of immune cells, as well as the actual fibrotic process, and thus the delicate balance between an appropriate inflammatory response and the development of pathologic fibrosis (Flanders, 2004; Sheppard, 2006; Lan, 2011). Several mechanistic links between inflammation and fibrosis are known, but the complete picture remains to be established (Lee and Kalluri, 2010). TGF-β signaling in these processes has been attributed both to canonical TGF-β signaling via the Smad proteins (signal-dependent transcription factors) as well as non-Smad signaling pathways (e.g., via MAPK pathways) (**Figure 2**).

Independent of inflammatory model systems, $1\alpha,25(OH)_2D_3$ and TGF-β/Smad signaling pathways have been found to be interrelated through three mechanisms: (i) the existence of a common regulator protein, the oncoprotein Ski, which can repress both pathways (Ueki and Hayman, 2003), (ii) the possibility of joint gene regulation via VDR and Smad recognition elements that are located in close proximity to a target promoter (Subramaniam et al., 2001) (**Figure 2**) or (ii) direct interaction of Smad3 and vitamin D signaling, whereby Smad acts as a coregulator specific for ligand-induced VDR transactivation (Yanagisawa et al., 1999).

The influence of vitamin D on inflammation-related signaling via TGF-β and Smad has mainly been investigated in models of fibrosis, and distinct mechanisms have been elucidated. Activation of $1\alpha,25(OH)_2D_3$ signaling by the natural ligand itself or its synthetic analogs reduces TGF-β expression (Kim et al., 2013) and interferes with the downstream signaling. The latter occurs via several mechanisms: downregulation of phosphorylated activatory Smads (Smad2/3 and 4) accompanied by upregulation of inhibitory Smad6 (Kim et al., 2013) (**Figure 2**); an inhibitory interaction between $1\alpha,25(OH)_2D_3$-bound VDR and Smad3 (Ito et al., 2013) or inhibition of Smad2 phosphorylation and nuclear translocation of Smad2/3, coincident with inhibited protein expression from TGF-β target genes (Halder et al., 2011). Similar findings have been made in studies with nephropathy models where suppression of TGF-β and p-Smad2/3 expression (Xiao et al., 2009) or a decrease in Smad2 and an increase in inhibitory Smad7 (Hullett et al., 2005) have been detected. In a large-scale study using hepatic stellate cells, TGF-β has been shown to cause chromatin remodeling events that led to a redistribution of genome-wide VDR binding sites (the VDR cistrome) with a shift toward VDR binding to Smad3-dependent, profibrotic target genes. In this study, VDR ligands led to a reduced Smad3 occupancy at these genes and thus antifibrotic effects (Ding et al., 2013). Although hepatic stellate cells do not belong to the immune system, and the interplay between VDR and TGF-β/Smad signaling may be dependent on the cell type, key aspects of this elaborate study deserve mention. More than 10^4 genomic sites were found to be co-occupied by both VDR and SMAD3 in these cells, and an analysis of the spatial relationships between the two transcription factors revealed that the respective response elements were located within a range of 200 base pairs (one nucleosomal window). Mechanistically, TGF-β signaling seems to deplete nucleosomes from the co-occupied sites and thus allow access of VDR to these sites. Vitamin D signaling on the other hand seems to limit TGF-β activation by inhibited coactivator recruitment. Spatiotemporal analysis revealed that $1\alpha,25(OH)_2D_3$/TGF-β-induced VDR and SMAD3 binding to the co-occupied sites were inversely correlated. The maximum of SMAD3 binding occurred 1 h after treatment and was reduced by 70% after 4 h, when VDR binding was maximal. Therefore, TGF-β signaling seems to change the chromatin architecture in a way in which liganded VDR can reverse Smad activation.

THE INFLUENCE OF $1\alpha,25(OH)_2D_3$ ON INTERLEUKIN (IL) GENE EXPRESSION AND SIGNALING

The finding that $1\alpha,25(OH)_2D_3$ interacts with the production of interleukins (Tsoukas et al., 1984) is of certain interest in the history of vitamin D research, as a crucial finding that expanded the view to roles beyond calcium homeostasis and crucially contributed to establish an immunoregulatory function of vitamin D (Tsoukas et al., 1984).

The interleukins are a large group of cytokines of central importance for the intercellular communication between the

different cells generally involved in inflammatory responses. These cells mainly encompass the leukocytes in their various stages of differentiation (distinct T-cells subsets, monocytes, macrophages, dendritic cells (DCs), granulocytes and B-lymphocytes) and cells of the connective tissue and vasculature (fibroblasts, endothelial cells). Furthermore, in specific organ-related diseases with inflammatory components (psoriasis, CKD, placental infection/inflammation, obesity, and others), further cell types are involved, e.g., keratinocytes, endothelial cells, trophoblasts, and adipocytes. All of them are capable of synthesizing interleukins, and the influence of $1\alpha,25(OH)_2D_3$ on IL gene expression has been investigated. The influence of $1\alpha,25(OH)_2D_3$ on IL gene expression and signaling in the different cell types will be outlined in the following.

Leukocytes

Several studies, especially the early ones, included *ex vivo* experiments with cellular samples from healthy individuals, mainly with PBMC (Rigby et al., 1984; Tsoukas et al., 1984; Saggese et al., 1989; Muller and Bendtzen, 1992), (partly) isolated T-cells (Bhalla et al., 1986), (partly) isolated monocytes (Bhalla et al., 1986; Muller et al., 1992; Zarrabeitia et al., 1992; Lemire et al., 1995; Lyakh et al., 2005), or cocultures of T-cells and monocytes (Tsoukas et al., 1989).

PBMC and T-cells

In stimulated PBMC, as a preparation that includes different cell types, $1\alpha,25(OH)_2D_3$ caused suppression of IL-2 production (Rigby et al., 1984; Tsoukas et al., 1984; Saggese et al., 1989) and reduced release of IL-1β, IL-6, and IL-10 (Joshi et al., 2011). Furthermore, the vitamin D analog paracalcitol led to reduced IL-8 production in stimulated PBMC (Eleftheriadis et al., 2010).

In more cell specific experiments with (partly) isolated T-cells, $1\alpha,25(OH)_2D_3$-mediated inhibition of IL-2 mRNA synthesis induced by lectin/phorbol ester (Matsui et al., 1986) or protein production induced by lectin (Bhalla et al., 1986), was confirmed. This was also observed for the two subsets of $CD4^+$ and $CD8^+$ T-cells (Jordan et al., 1989), which however displayed stimulus-dependency for IL-2 protein production. In a more detailed analysis, IL production by $CD4^+$ and $CD8^+$ cells was studied by flow cytometry on single cell level. In both populations, a decrease in IL-2 production was found. Conversely, regarding other IL class members analyzed in the same study, $1\alpha,25(OH)_2D_3$ increased the low percentage of IL-13-producing cells in both subsets and IL-6 producing $CD4^+$ and $CD8^+$ T-cells could only be detected after incubation with $1\alpha,25(OH)_2D_3$ (Willheim et al., 1999) (**Figure 3**).

The finding that IL-2 gene expression is reduced by $1\alpha,25(OH)_2D_3$ in T-cells has moreover been confirmed in two studies using the human T-cell line Jurkat, and the mechanisms have been studied. It has been found that the VDR seems to cause direct transcriptional repression of IL-2 gene expression via blockage of a positive regulatory element recognized by the transcription factor NFAT within the IL-2 promoter (Alroy et al., 1995). In a later study, the repression has been kinetically classified as a primary response to $1\alpha,25(OH)_2D_3$, and ligand-dependent VDR binding at the IL-2 gene locus was detected using ChIP assays (Matilainen et al., 2010b) (**Figure 2**). It has to be mentioned, however, that long term pretreatment of Jurkat cells with $1\alpha,25(OH)_2D_3$ before stimulation with mitogen and phorbol ester seems to enhance IL-2 mRNA expression (Prehn and Jordan, 1989). Studies using T-cells from other species confirmed the inhibitory effect of $1\alpha,25(OH)_2D_3$ on IL-2 production (Hodler et al., 1985).

Similar findings as for IL-2 have been made regarding the inhibition of IL-17 production by $1\alpha,25(OH)_2D_3$ from T-cells in a more recent report. It has been found that (i) the VDR competes for binding with NFAT and recruits histone deacetylase (HDAC) to the human IL-17 promoter, thus inhibiting its activation, (ii) binding of the activatory transcription factor Runx1 to the mouse IL-17A promoter was inhibited through sequestration of Runx1 by the VDR in the presence of $1\alpha,25(OH)_2D_3$ and (iii) $1\alpha,25(OH)_2D_3$ induced the IL-17 inhibiting transcription factor Foxp3 (Joshi et al., 2011). Other studies suggest a post-transcriptional mechanism of IL-17 inhibition by VDR via induction of the translation inhibitor C/EBP homologous protein (CHOP) (Chang et al., 2010).

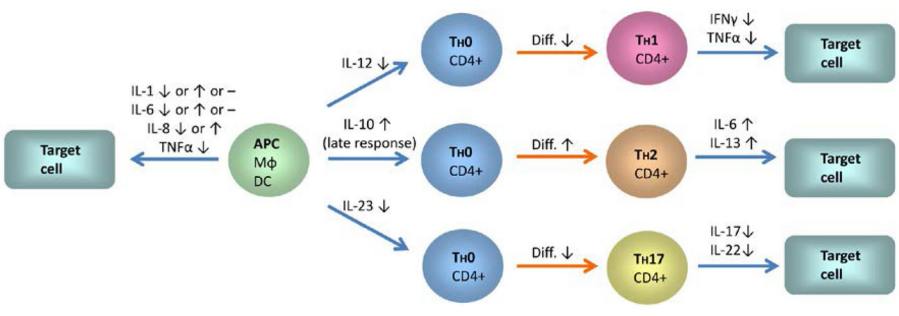

FIGURE 3 | The influence of $1\alpha,25(OH)_2D_3$ on the expression of interleukins, TNFα and IFNγ in monocytes, dendritic cells, and different T-cell subsets. Blue arrows indicate IL signaling between the different cell types and red arrows indicate differentiation processes. IL-12 and IL-23 expression is downregulated in monocytes and dendritic cells by $1\alpha,25(OH)_2D_3$. In contrast, IL-10 expression is enhanced. A shift from a Th1 profile toward the Th2 type and a decrease in Th17 responses is to be anticipated from these changes. Inhibition of T-cell autoregulation by $1\alpha,25(OH)_2D_3$-mediated suppression of IL-2 expression is not shown. Abbreviations and symbols: APC, antigen presenting cell; MΦ, macrophage; DC, dendritic cell; ↑, upregulation; ↓, downregulation; -, no changes.

Apart from studies with PBMC or T-cells from healthy individuals or experiments with cell lines, a few studies exist with cell samples from patients suffering from inflammatory diseases. In contrast to the findings with cells from healthy individuals after 1α,25$(OH)_2D_3$ treatment, PBMC isolated from hemodialysis patients responded to treatment with 1α$(OH)D_3$ by enhanced IL-2 protein production, however, starting from a significantly lower level of IL-2 production compared to healthy controls (Tabata et al., 1988). The capacity of PBMC from Crohn's disease patients to produce IL-6 has been elevated by 1α,25$(OH)_2D_3$ treatment of the patients (Bendix-Struve et al., 2010). IL-6 and IL-8 production and mRNA expression have been found to be decreased by 1α,25$(OH)_2D_3$ in stimulated PBMC of psoriatic patients (Inoue et al., 1998). In PBMC from treatment-naive patients with early rheumatoid arthritis (RA), reduced IL-17A and increased IL-4 levels have been observed in the presence of 1α,25$(OH)_2D_3$. In the FACS-separated subpopulation of memory T-cells (CD45RO+), 1α,25$(OH)_2D_3$ suppressed IL-17A, IL-17F and IL-22 (Colin et al., 2010) (**Figure 3**).

Monocytes

In an early report, IL-1 production by human monocytes/macrophages enriched from PBMC has been found to be elevated by single 1α,25$(OH)_2D_3$ treatment (Bhalla et al., 1986). In subsequent studies with stimulated, monocyte-enriched cultures from PBMC, either no 1α,25$(OH)_2D_3$ effect has been detected (Zarrabeitia et al., 1992) or a reduction of IL-1 (and IL-6) production has been found, which seemed to be based on post-transcriptional events (Muller et al., 1992). The decrease in IL-1 production has been confirmed for co-cultures of T-cells and monocyte-enriched PBMC (Tsoukas et al., 1989). However, it has to be pointed out that different stimuli to elicit IL-1 production had been used in these studies. In human monocytic cell lines, (U937, HL-60 or THP-1), no induction (THP-1), or upregulation of IL-1β mRNA (U937, HL-60) by 1α,25$(OH)_2D_3$ has been detected, which varied with the presence or absence and the type of the co-stimulus that was used (phorbol ester, lipopolysaccharide) (Bhalla et al., 1991; Blifeld et al., 1991; Fagan et al., 1991). Further it is noteworthy that conflicting data exist for studies with U937 cells regarding the actual secretion of IL-1β protein (Blifeld et al., 1991; Fagan et al., 1991; Taimi et al., 1993). In THP-1 cells stimulated with agonists for Toll-like receptor 8, IL-1β mRNA was induced and could be suppressed by 1α,25$(OH)_2D_3$ (Li et al., 2013).

In a more recent study, expression of IL-1 and IL-6 mRNA in freshly isolated monocytes and macrophages cultured for 7 days has been investigated. Interestingly, IL-1 and IL-6 gene expression has been regulated differently in these two distinct stages of monocyte/macrophage maturation. In the monocytes, basal IL-1 and IL-6 mRNA expression has been found to be slightly upregulated by 1α,25$(OH)_2D_3$ treatment compared to untreated controls. For 1α,25$(OH)_2D_3$ treated monocytes that were additionally stimulated with LPS or TNFα, no or only marginal differences have been found compared to LPS or TNFα treatment without 1α,25$(OH)_2D_3$ preincubation. In contrast, 1α,25$(OH)_2D_3$ treatment reduced basal IL-1 and IL-6 levels in macrophages. In 1α,25$(OH)_2D_3$ treated macrophages that were additionally stimulated with LPS or TNFα, only TNFα-stimulated IL-6 mRNA expression was influenced, whereas no significant changes were observed for IL-1 and IL-6 after 1α,25$(OH)_2D_3$/LPS-treatment. These findings show that in monocytes/macrophages, the influence of 1α,25$(OH)_2D_3$ on IL expression depends on the type of IL under consideration, the degree of maturation, and the stimulus that is employed (Di Rosa et al., 2012). In a second recent investigation, significant inhibition of IL-6 mRNA expression and protein secretion was observed in PBMC, and subsequent FACS-based analysis revealed a concomitant decrease in CD14+ IL-6-producing monocytes (Zhang et al., 2012) (**Figure 3**).

Apart from the two prominent monokines IL-1 and IL-6, the synthesis of IL-3 has been found to be influenced by 1α,25$(OH)_2D_3$ in the murine monocytic cell line WEHI-3. However, whereas one report describes dose-dependent inhibition of IL-3 production in this cell line (Abe et al., 1986), the second finds concentration-dependent stimulation or inhibition of IL-3 production (Hodler et al., 1985). Furthermore, the interleukin family members IL-8, IL10, and IL-12 have been studied more intensely on mechanistic level.

IL-10 and IL-12-production by stimulated primary human monocytes has been found to be negatively regulated by 1α,25$(OH)_2D_3$ (Lemire et al., 1995; Lyakh et al., 2005). These two genes have been identified as primary 1α,25$(OH)_2D_3$ target genes as judged by rapid VDR recruitment detected via ChIP assays in the monocytic cell line THP-1 (Matilainen et al., 2010b). Further studies with this cell line include extensive mechanistic analyses regarding the influence of 1α,25$(OH)_2D_3$ on the expression of IL-8, IL-10, and IL-12B. The IL-8 gene has been shown to be an up-regulated, primary target gene, located within an insulated cluster of CXC motif ligand (CXCL) genes. IL-8 and its neighboring genes CXCL1 and CXCL6 seem to be under the control of a consensus VDR binding motif located 22 kb downstream of the IL-8 transcription start site, which mediates 1α,25$(OH)_2D_3$–dependent chromatin opening (Ryynanen and Carlberg, 2013). As discussed in this report, this finding is seemingly in contradiction with other studies (e.g., Di Rosa et al., 2012). These studies used different cells and foremost, cells were stimulated with agents like LPS that activate transcription factors, e.g., NF-κB, that are themselves regulated by 1α,25$(OH)_2D_3$. As described above, NF-κB activity is inhibited by 1α,25$(OH)_2D_3$ (Harant et al., 1998) (**Figure 2**). It has been put forward that 1α,25$(OH)_2D_3$ may have a dual effect: primary up-regulation of genes like IL-8, which supports the inflammatory response in the early phase of inflammation, e.g., by IL-8 production, and secondary effects which would help to shut down the inflammatory process, e.g., by inhibition of NF-κB-mediated pro-inflammatory responses (Ryynanen and Carlberg, 2013). This could explain that in another study in which THP-1 cells were used, no significant effect of 1α,25$(OH)_2D_3$ on IL-8 expression was found on protein level. In this study, the cells have been stimulated with LPS after only 2 h of 1α,25$(OH)_2D_3$ treatment before IL-8 protein was analyzed after 24 and 48 h (Kuo et al., 2010). Similarly, U937 cells exposed to high glucose (a condition which leads to different stress responses like NF-κB or MAPK activation) (Stan et al., 2011; Yang et al., 2013) showed lower IL-8 secretion

after pretreatment with 1α,25$(OH)_2D_3$ (Jain and Micinski, 2013). Therefore, the interference of 1α,25$(OH)_2D_3$ with cell signaling pathways of inflammatory or cell stress responses, like NF-κB or MAPK activation, and differences in treatment schedules may explain the different findings. In contrast to IL-8 as an up-regulated gene, the primary effect of 1α,25$(OH)_2D_3$ on IL-10 expression is down-regulation, followed by up-regulation at a later stage (**Figure 3**). Cyclic binding of VDR to a distal promoter region with conserved VDREs, that loops 1α,25$(OH)_2D_3$-dependently to the transcription start site and induces epigenetic changes and chromatin remodeling, was detected (Matilainen et al., 2010a,b). IL-12B has been identified as a 1α,25$(OH)_2D_3$-dependently down-regulated gene in LPS-treated THP-1 cells. The gene harbors two VDR binding sites within ∼6 kb upstream of the transcription start site to which the VDR and its partner retinoid receptor (RXR) recruit co-repressors and consequently induce epigenetic changes associated with gene repression (Matilainen et al., 2010b; Gynther et al., 2011). An earlier report attributed the down-regulation of IL-12 via interference of 1α,25$(OH)_2D_3$/VDR with NF-κB binding to proximal IL-12 promoter regions (D'Ambrosio et al., 1998). It has been suggested in the more recent report that this suppression of proximal sites is due to epigenetic changes at that location via the distal VDRE binding sites identified in the more recent study (Gynther et al., 2011) (**Figure 3**).

In addition to data from experiments with monocytes, macrophages, and DCs as differentiated members of the monocytic lineage have been investigated.

In macrophages from vitamin D-deficient mice, IL-1, and IL-6 production (evaluated as biological activity) was significantly reduced relative to control mice. Notably, this was paralleled by a decrease in macrophage cytotoxicity. Furthermore, the vitamin D deficient mice had reduced serum levels of IL-1 and IL-6 after challenge with LPS (Kankova et al., 1991). In human monocyte-derived macrophages and PMA-differentiated U937 cells, which were stimulated with LPS or PMA, IL-1β production was strongly stimulated by 1α,25$(OH)_2D_3$. This effect was ascribed to increased IL-1β transcription, but not by RNA stabilization, and seemed to be mediated by Erk1/2. Moreover, 1α,25$(OH)_2D_3$ induced the expression and phosphorylation of CCAAT enhancer-binding protein β as a known IL-1 β-regulating transcription factor (Lee et al., 2011). The upregulation of IL-1β by 1α,25$(OH)_2D_3$ is also relevant for infection-induced inflammation, as in THP-1 cells or primary human macrophages infected with *Mycobacterium tuberculosis* (as well as in non-infected controls), 1α,25$(OH)_2D_3$ increased the expression of IL-1β mRNA. IL-1β is a critical factor for host defense in this disease. Notably, mature intracellular IL-1β protein was only detected in infected, 1α,25$(OH)_2D_3$ treated THP-1 cells, which represents a further level of gene expression control exerted by 1α,25$(OH)_2D_3$. Secretion of IL-1β was only seen in infected cells, and significantly enhanced by 1α,25$(OH)_2D_3$. With respect to the mechanism, the study revealed 1α,25$(OH)_2D_3$–dependent binding of VDR to a promoter-proximal consensus VDRE, which was paralleled by upregulated VDR-expression, and recruitment of RNA polymerase II to the transcription start site (Verway et al., 2013).

In a further study with mouse macrophages, 1α,25$(OH)_2D_3$ led to reduced mRNA expression of the IL-12 subunit p40 in response to LPS/interferon gamma (IFNγ) stimulation (Korf et al., 2012), which is in line with the effects seen in monocytes, as described above (**Figure 3**). Stimulation of the macrophages with 1α,25$(OH)_2D_3$ was accompanied by upregulation of VDR and the 1α,25$(OH)_2D_3$–catabolic enzyme CYP24. Further changes concerned the potential to stimulate T-cells, as assessed by co-culture experiments including FACS analysis of surface markers. These effects could not be observed with IL-10 deficient macrophages. Notably, the effects on IL-12 p40 expression and T-cell stimulation also occurred in monocytes/macrophages from non-obese diabetic (NOD) mice, which have a background of inflammatory features seen in type 1 diabetes (Korf et al., 2012).

Analogous studies have been conducted for DCs from NOD mice or non- obese diabetes-resistant (NOR) control mice. In both cases, 1α,25$(OH)_2D_3$ altered the phenotype of DCs and inhibited the LPS/IFNγ–induced mRNA expression and protein secretion of IL-10 and IL-12 (Van Etten et al., 2004). In general, it has been shown that 1α,25$(OH)_2D_3$ prevents *in vitro* differentiation of human monocytes into immature DCs, associated with decreased capacity to activate T-cells. Furthermore, 1α,25$(OH)_2D_3$ inhibits maturation of DCs. In maturating DCs, 1α,25$(OH)_2D_3$ reduces IL-12p70 and enhances IL-10 secretion upon stimulation of the DCs by CD40-crosslinking (Penna and Adorini, 2000). This has been independently confirmed for IL-12p70 production upon LPS stimulation (Sochorova et al., 2009). Additionally, these findings are in line with a study on the generation of regulatory DCs for therapeutic use from human monocytes, which were differentiated in the presence of 1α,25$(OH)_2D_3$. Apart from reduced LPS-induced IL-12 and enhanced IL-10 secretion of the maturating cells, a major characteristic of these 1α,25$(OH)_2D_3$–treated DCs is their low level of IL-23 secretion, which was apparent with or without stimulation with LPS (Pedersen et al., 2009) (**Figure 3**). A further recent investigation used monocyte-derived DCs from Crohn's disease patients. When the cells were cultured in the presence of 25$(OH)D_3$ or 1α,25$(OH)_2D_3$ and matured with LPS, they exhibited significantly increased IL-6 production, and non-significant reductions in and IL-10 and IL-12p70. IL-1β and Il-8 levels were not affected in this study (Bartels et al., 2013).

B-cells and neutrophils

B-cells and neutrophils have been less intensively studied, but the available data show that IL gene expression in these cells is also targeted by 1α,25$(OH)_2D_3$. In isolated human peripheral B-cells, IL-10 secretion can be induced by stimulation (cross-linking of B-cell receptor/CD40 antibody/IL-4). This production can be enhanced by 1α,25$(OH)_2D_3$. Besides the influence on IL gene expression, 1α,25$(OH)_2D_3$ induces the expression of VDR and Cyp24 mRNA in the stimulated B-cells. These activated cells also express Cyp27b1 mRNA and are able to produce 1α,25$(OH)_2D_3$ from 25$(OH)D_3$. Binding of VDR to a VDRE in the proximal IL-10 promoter has been shown by ChIP assay, and binding of RNA-polymerase II could only be detected in IL-10 secreting B-cells (Heine et al., 2008).

Neutrophils respond to $1\alpha,25(OH)_2D_3$ by a slight reduction of IL-1β mRNA expression. Notably, the abundance of VDR mRNA in neutrophils has been found to be comparable with monocytes (Takahashi et al., 2002).

Fibroblasts, keratinocytes, endothelial cells

In a first study where these cell types were used, IL-1-stimulated normal human dermal fibroblasts, normal human keratinocytes and normal human endothelial cells were investigated regarding changes of IL-8 mRNA and protein expression in dependence of $1\alpha,25(OH)_2D_3$ treatment. IL-8 expression was reduced by $1\alpha,25(OH)_2D_3$ on both levels of gene expression for fibroblasts and keratinocytes, but not for endothelial cells, where no significant changes have been found (Larsen et al., 1991).

For IL-8, and also for IL-6 protein production, this result has been confirmed in studies using phorbol ester stimulated human fibroblast cell lines (Srviastava et al., 1994), and in experiments employing TNF-α-stimulated human dermal fibroblasts (Fukuoka et al., 1998). Similar results have been obtained with fibroblast cultures obtained from surgery of patients suffering from nasal polyposis, which is defined as a chronic inflammatory process. However, rather high concentrations (10–100 μM) of $1\alpha,25(OH)_2D_3$ were necessary to significantly reduce IL-6 and IL-8 production in these cells (Rostkowska-Nadolska et al., 2010).

In cultured normal human keratinocytes, only minor effects were observed for IL-1α and IL-8 production, when the influence of $1\alpha,25(OH)_2D_3$ was investigated for otherwise untreated cells. However, TNF-α-stimulation led to slightly enhanced IL-1α and markedly increased IL-8 secretion, which could be reduced by $1\alpha,25(OH)_2D_3$ (Zhang et al., 1994). This was confirmed for IL-8 (Koizumi et al., 1997). On the other hand, stimulation with phorbol ester plus LPS caused a rise in IL-8 production, but a decrease in IL-1α. $1\alpha,25(OH)_2D_3$ inhibited IL-8 secretion and restored IL-1α production (Zhang et al., 1994). Stimulation of normal human keratinocytes with IL-17A resulted in a pronounced increase in IL-6 mRNA and IL-8 protein secretion, which could be effectively blocked by $1\alpha,25(OH)_2D_3$ treatment (Peric et al., 2008). In a mechanistically insightful study, the effect of $1\alpha,25(OH)_2D_3$ on the expression of IL-1α, the intracellular IL-1 receptor antagonist (icIL-1Ra) and IL-18 was studied in mouse primary keratinocytes. Treatment with $1\alpha,25(OH)_2D_3$ induced IL-1α and icIL-1Ra mRNA and protein, however, the ratio of icIL-1Ra to IL-1, which determines the effect on IL-1 activity, was markedly increased, and indeed reduced IL-1 activity could be detected. The use of keratinocytes from $VDR^{-/-}$ mice confirmed that the effect was mediated by VDR. Regarding the mechanism of gene regulation, increased IL-1α mRNA stability was observed and enhanced icIL-1Ra gene transcription via a secondary mechanism have been suggested to account for the effects on these gene. $1\alpha,25(OH)_2D_3$ markedly suppressed IL-18 mRNA expression, and the effect was dependent on VDR, as no effect of $1\alpha,25(OH)_2D_3$ was seen in $VDR^{-/-}$ mice. These mice exhibit markedly elevated basal levels of IL-18 mRNA and protein, and expression of human VDR in these mice could restore basal levels (Kong et al., 2006).

A further cell type involved in inflammatory responses, especially in infection-mediated inflammation, are epithelial cells. Treatment of human microvessel endothelial cells with $1\alpha,25(OH)_2D_3$ suppresses LPS-induced IL-6 and IL-8 release, whereas $1\alpha,25(OH)_2D_3$ alone does not affect IL production. As assessed by reporter gene assay, this seems to be based on inhibition of LPS-induced NF-κB activation. This activation usually occurs via the MyD88-dependent branch of TLR4-signaling. In contrast, $1\alpha,25(OH)_2D_3$ did not influence the activity of interferon-β-promoter constructs, which has been determined as a measure of MyD88-independent LPS/TLR4 signaling (Equils et al., 2006). Reduced IL-6 and IL-8 production was also seen in $1\alpha,25(OH)_2D_3$-treated cystic fibrosis respiratory epithelial cell lines challenged with LPS. With respect to NF-κB-signaling, reduced IκBα phosphorylation and increased total cellular IκBα upon $1\alpha,25(OH)_2D_3$ treatment have been found in this study (McNally et al., 2011) (**Figure 2**). Similar findings have been made for human umbilical vein cord endothelial cells (HUVEC) incubated cultured in a CKD-like environment (hypocalcemia, advanced glycation end products, parathyroid hormone) and $1\alpha,25(OH)_2D_3$. This environment provoked enhanced IL-6 expression and secretion, increased DNA-binding of NF-κB-p65 and decreased IκBα expression. These changes were counteracted by $1\alpha,25(OH)_2D_3$ (Talmor-Barkan et al., 2011). In TNFα-stimulated human coronary arterial cells, a slight, but significant reduction of IL-8 production has been observed for $1\alpha,25(OH)_2D_3$ treatment in certain concentrations, but IL-6 production could not be influenced (Kudo et al., 2012). An interesting novel mechanism for interference of $1\alpha,25(OH)_2D_3$ and LPS-stimulated IL-8 production from epithelial cells has been proposed in a recent study, where a vitamin D_3 derivative have been found to increase the release of the soluble form of CD14 (sCD14) via ERK1/2 activation. Neutralization of LPS by sCD14 could account for the effect of the vitamin D analog (Hidaka et al., 2013).

Trophoblasts, endometrial cells, myometrial cells

Placental inflammation including release of interleukins is associated with preeclampsia, preterm labor, and abortion. Therefore, cell types involved in this inflammatory condition have been investigated regarding the influence of $1\alpha,25(OH)_2D_3$ on IL secretion. In cultured human trophoblasts, $1\alpha,25(OH)_2D_3$ reduced TNFα-induced IL-6 mRNA expression and protein secretion (Diaz et al., 2009). Mechanistic evidence regarding the influence of vitamin D signaling on IL gene expression in placental tissue was presented in a study with for $Cyp27b1^{-/-}$ (vitamin D-activating 1α-hydroxylase) mice and $VDR^{-/-}$ mice. In these mice, basal expression of IL-10 mRNA was decreased relative to wildtype placentas, and LPS stimulation resulted in higher levels of IL-6 mRNA in the $^{-/-}$ placentas compared to wildtype. PCR array analysis of LPS-stimulated placental tissue from $Cyp27b1^{-/-}$ mice revealed enhanced expression of IL-4, IL-15, and IL-18 mRNA relative to WT and the same experiments with $VDR^{-/-}$ mice yielded higher IL-1α and IL-6 mRNA levels. Further experiments with LPS-stimulated placentas from WT mice showed that treatment with $25(OH)D_3$ as the substrate of CYP27B1 reduces IL-6 mRNA expression. Moreover, LPS challenge of pregnant WT mice led to enhanced expression of Cyp27b1 and VDR. Apart from the mechanistic conclusion that

VDR signaling is a factor that controls IL gene expression, these results show that pro-inflammatory stimuli are able to enhance the expression of crucial vitamin D signaling components which are able to mediate anti-inflammatory responses (Liu et al., 2011).

In line with these findings, experiments using human endometrial cells from women with unexplained recurrent spontaneous abortion (URSA) or in controls, significant down-regulation of IL-6 by $1,25(OH)_2D_3$ was observed in two cell types (whole endometrial cells and endometrial stromal cells), but for IL-8, opposed effects were observed for the two cell types in URSA samples, which highlights the complexity of these responses given the fact that several cell types are involved in inflammatory processes (Tavakoli et al., 2011).

Adipocytes

Obesity is a disease condition which is strongly associated with low-grade inflammation, therefore adipocytes have been used as a further model system regarding the interplay of vitamin D signaling and IL gene expression/production. In a recent report, human adipocytes from biopsies and from differentiated human mesenchymal stromal cells were studied with respect to IL-6 gene expression/release depending on the presence of $1\alpha,25(OH)_2D_3$. LPS-induced IL-6 mRNA and protein were reduced in both systems by cotreatment with $1\alpha,25(OH)_2D_3$. Regarding the underlying signal transduction events, it was shown that $1\alpha,25(OH)_2D_3$ inhibited IκB phosphorylation and thus NF-κB translocation into the nucleus (**Figure 2**). DNA binding of NF-κB complexes upon LPS stimulation was significantly reduced in $1\alpha,25(OH)_2D_3$-pretreated cells compared to controls (Mutt et al., 2012). A further recent investigation addressed the influence of *in vitro* and *in vivo* administered $1\alpha,25(OH)_2D_3$ on IL-6 and IL-8 gene expression from IL-1β-stimulated human adipose tissue. The adipose tissue samples have been either (i) treated *in vitro* with $1\alpha,25(OH)_2D_3$ or have been (ii) obtained from obese subjects with low plasma levels of $25(OH)D_3$ after *in vivo* (oral) treatment with high-dose $1\alpha,25(OH)_2D_3$ or placebo. In the *in vitro* study, reduced mRNA levels of IL-6 and IL-8 and reduced IL-6 and IL-8 protein (significance only shown for IL-8) have been found. However, although the *in vivo* treatment led to a small decrease of IL-6 and IL-8 mRNA expression in the adipose tissue, there were no significant differences between the $1\alpha,25(OH)_2D_3$–treated and the control group. Oral treatment with $1\alpha,25(OH)_2D_3$ did also not significantly change circulating levels of IL protein in the subjects pre- and post-treatment(Wamberg et al., 2013). These findings urge caution about the extrapolation of *in vitro* findings to the *in vivo* situation.

Apart from studies with primary cells, cultures of adipocyte-like murine 3T3-L1 cells have been used, but contradictory results have been reported e.g., regarding IL-6 gene expression (Sun and Zemel, 2008; Marcotorchino et al., 2012).

VDR GENE VARIANTS, VDR GENE SILENCING, AND IL GENE EXPRESSION/PRODUCTION

A further aspect that underscores the importance of vitamin D signal transduction on IL biosynthesis is the effect of the VDR receptor gene variants on IL gene expression. The single-nucleotide polymorphism *Fok*I, which comprises a shorter VDR protein of 424 aa or the long isoform with 427 aa, influences IL-12 expression. In human monocytes and DCs, presence of the short VDR isoform leads to a higher expression of IL-12 compared to the long isoform, a result which was reflected by results from reporter gene assays with IL-12 promoter fragments (Van Etten et al., 2007). Moreover, VDR gene promoter variants have an impact on the expression of IL-10 in blood mononuclear cells (Selvaraj et al., 2008).

Changes in IL production can be observed in VDR KO mice. VDR KO considerably facilitates development of IL-17 secreting T-cells (T_h17 cells) in response to respective *in vitro* stimuli. Further, enhanced IL-17 production was observed in these T_h17 cells compared to wildtype. Conversely, a reduction in regulatory T-cells and tolerogenic DCs was observed. Moreover, IBD can be induced experimentally in these mice by transfer of naive T-cells that develop into specific, IBD-inducing subsets. The severity of IBD was strongly enhanced in VDR KO mice compared to control animals, which was ascribed to the increased propensity for development into T_h17 cells (Bruce et al., 2011).

INFLUENCE OF $1\alpha,25(OH)_2D_3$ ON IL RECEPTOR EXPRESSION

Apart from induction of IL gene expression/protein release, $1\alpha,25(OH)_2D_3$ may also modulate IL signaling via regulation of IL receptor expression. In early reports, moderate downregulation (Matsui et al., 1986) or no changes (Jordan et al., 1989) were found regarding IL-2 receptor expression in $1\alpha,25(OH)_2D_3$ treated, mitogen-stimulated PBMC, or mitogen/phorbol ester-stimulated T-cells, respectively. However, IL-2 mediated expression of IL-2 receptor units was superinduced by $1\alpha,25(OH)_2D_3$ in mitogen-stimulated PBMC (Rigby et al., 1990). The vitamin D_3 upregulated protein 1 (VDUP1), which is expressed in a $1\alpha,25(OH)_2D_3$-dependent manner, has been found to inhibit the activity of the IL-3 receptor promoter (Han et al., 2003). On the other hand, IL-1 and IL-4 receptor densities seem to be upregulated by $1\alpha,25(OH)_2D_3$ on a murine T-cell line and a murine osteoblast cell line, respectively (Lacey et al., 1993a,b). Furthermore, downregulated IL-22 mRNA and protein levels have been detected in cultured epidermis tissue treated with calcipotriol, a vitamin D analog (Moniaga et al., 2013).

THE INFLUENCE OF $1\alpha,25(OH)_2D_3$ ON TNFα mRNA AND PROTEIN EXPRESSION AND RELEASE

The impact of $1\alpha,25(OH)_2D_3$ on TNFα gene expression was primarily studied in PBMC, primary monocytes/macrophages or in monocytic cell lines. Expression was investigated both on mRNA level and/or on the level of protein production, and was sometimes reported in terms of protein release as a secondary readout.

In general, *in vitro* or *in vivo* treatment with $1\alpha,25(OH)_2D_3$ of PBMC caused a decrease in TNFα gene expression and/or TNFα production. This was the case for PBMC from healthy donors that were stimulated with different agents (LPS, Muller et al., 1992; Panichi et al., 1998; Rausch-Fan et al., 2002); live *Mycobacterium tuberculosis* (Prabhu Anand et al., 2009), as well as for PBMC from patients suffering from diseases with inflammatory features [renal disease (Riancho et al., 1993; Panichi et al., 1998); pulmonary tuberculosis (Prabhu Anand et al., 2009)]. Analogous findings

were obtained with monocyte-enriched PBMC after stimulation with LPS (Muller et al., 1992), IFNγ or phorbol ester (Zarrabeitia et al., 1992) (however, not with LPS in this particular report). In one of the latter studies, nuclear run-off analysis did not indicate that TNFα gene transcription was affected by $1\alpha,25(OH)_2D_3$ (Muller et al., 1992).

In contrast to the findings with PBMC or monocyte-enriched PBMC, studies that used human primary monocytes or macrophages often found increased TNFα expression/secretion after $1\alpha,25(OH)_2D_3$ exposure, either regarding basal levels (human monocyte-derived macrophages; Bermudez et al., 1990) or with respect to stimulus-induced mRNA or protein levels (murine alveolar macrophages/LPS- or PMA-stimulation, Higashimoto et al., 1995; peritoneal macrophages from continuous peritoneal dialysis patients/LPS-stimulation, Cohen et al., 2001). In line with this, murine bone-marrow derived macrophages (BMMs) responded to $1\alpha,25(OH)_2D_3$ with an increase in TNFα mRNA abundance, which was synergistically enhanced by LPS stimulation. This study also addressed molecular mechanisms. Treatment with $1\alpha,25(OH)_2D_3$ and stimulation with LPS did not influence TNFα mRNA stability, but the data suggested that $1\alpha,25(OH)_2D_3$ regulates the TNFα gene on transcriptional level, as a VDR-binding sequence could be identified in the TNFα promoter region using electrophoretic mobility shift assays (Hakim and Bar-Shavit, 2003).

When human monocytic cell lines were studied, heterogeneous results were obtained, and the outcome seems to depend on the differentiation status of the cells (e.g., Bhalla et al., 1991). For the three cell lines that were mainly employed, the order of their stage of maturation is known. HL-60 cells are myelomonocytic stem-cells and thus are the least mature cell line; U937 are characterized as monoblasts, and represent an intermediate stage; and THP-1 cells are regarded as promonocytic cells and are therefore the most mature cell line (Frankenberger et al., 1994).

In HL-60 cells, $1\alpha,25(OH)_2D_3$ had no influence on PMA-induced TNFα mRNA expression, but enhanced it in U937 cells (Bhalla et al., 1991). In a second study, $1\alpha,25(OH)_2D_3$ preincubation of U937 cells accelerated LPS-induced TNFα mRNA expression and led to higher steady-state mRNA levels which were associated with enhanced TNFα protein production. Mechanistic analysis pointed to a secondary effect since $1\alpha,25(OH)_2D_3$ pretreatment was needed for more than 6 h in order to achieve enhanced TNFα protein synthesis. The requirement of $1\alpha,25(OH)_2D_3$-driven expression of the LPS co-receptor CD14, was suggested to be the mechanistic basis of his secondary effect (Prehn et al., 1992). In a further investigation, differentiation by $1\alpha,25(OH)_2D_3$ enhanced LPS-induced TNFα secretion in U937 and THP-1 cells. Concomitant increase in TNFα mRNA was confirmed for U937 cells (Taimi et al., 1993). In contrast, $1\alpha,25(OH)_2D_3$ was reported to significantly suppress TNFα release in LPS-stimulated THP-1 cells and human primary monocytes (Kuo et al., 2010), and a further study reported reduced TNFα production and secretion from $1\alpha,25(OH)_2D_3$-treated, IFNγ-activated THP-1 cells (Villaggio et al., 2012).

In one report, TNFα mRNA levels of $1\alpha,25(OH)_2D_3$–treated human PBMC, U937 and THP-1 cells, that were stimulated either with LPS or with phytohemagglutinin (PHA), were compared. Differences occurred between the two sample types and the two stimuli. In PBMC, LPS had no influence on TNFα expression in the presence of $1\alpha,25(OH)_2D_3$, whereas upon PHA-stimulation, reduced TNFα mRNA levels were observed. In contrast, U937 cells (but not THP-1 cells) responded by an increase in TNFα mRNA expression (Blifeld et al., 1991).

Taken together, several studies report an increase in TNFα mRNA and protein expression in $1\alpha,25(OH)_2D_3$-treated, subsequently stimulated U937 cells, but equivocal effects were found with the more mature THP-1 cells. In monocyte-derived DCs from patients that suffer from Crohn's disease, TNFα production was decreased when the cells were differentiated with LPS in the presence of $1\alpha,25(OH)_2D_3$ (Bartels et al., 2013).

T-cells have not been intensively studied, but regulation of TNFα-expression by $1\alpha,25(OH)_2D_3$ has been analyzed in T-cell subsets obtained from normal healthy subjects and pulmonary tuberculosis patients. Here, $1\alpha,25(OH)_2D_3$ reduced the percentage of TNFα-expressing T-cell subsets (CD3+, CD3+CD4+, CD3+CD8+) (Prabhu Anand et al., 2009) (**Figure 3**).

Other cell types that were analyzed are prostate cancer lines, where $1\alpha,25(OH)_2D_3$ reduced basal TNFα mRNA expression (Golovko et al., 2005), or $1\alpha,25(OH)_2D_3$/IL-1β-stimulated synoviocytes, where TNFα mRNA was decreased (Feng et al., 2013).

In summary, $1\alpha,25(OH)_2D_3$-mediated downregulation of TNFα gene expression has been found in cell preparations which contain a high percentage of T-cells (PBMC or monocyte-enriched PBMC). In monocytic cells, upregulation has been reported for cell lines that represent an intermediate monocytic differentiation state, whereas for more mature cells, heterogeneous results have been found. Regarding the mechanism, it has been suggested that primary effects may play a role for $1\alpha,25(OH)_2D_3$ regulation of TNFα gene expression, since a VDR binding element has been found in the TNFα promoter region (Hakim and Bar-Shavit, 2003). On the other hand, kinetic analysis pointed to a secondary effect, where the expression of CD14 could play a role, at least for LPS-induced TNFα expression (Prehn et al., 1992). It has to be noted, however, that cell-type specific mechanisms have been found for T-cell specific expression of the TNFα gene. Cell type-specific DNA-protein-interactions have been identified for the TNFα gene when T-cells and monocytic cells were compared. A highly conserved region in intron 3 seems to be responsible for cell specificity, as this sequence induces specific activity of a TNFα-reporter plasmid in Jurkat T-cells, but not THP-1 cells (Barthel and Goldfeld, 2003). Possibly, cell specific protein complexes within this region interact with $1\alpha,25(OH)_2D_3$ signaling components in T-cells.

THE INFLUENCE OF $1\alpha,25(OH)_2D_3$ ON INTERFERON γ GENE EXPRESSION

IFNγ is a well-established effector in anti-infectious host reactions, autoimmune diseases and inflammation. IFNγ is mainly produced by NK and T-cells. Inhibition of IFNγ mRNA and protein secretion has been described for $1\alpha,25(OH)_2D_3$-treated human PBMC, peripheral blood lymphocytes or T-cells that were stimulated with phytohemagglutinin and phorbol ester (Matsui et al., 1986; Reichel et al., 1987; Rigby et al., 1987; Inoue et al., 1998) (**Figure 3**). Mechanistic insights exist from

experiments using transient transfection of IFNγ promoter constructs in Jurkat T-cells. Here, it could be concluded that two VDR binding regions, one around −200 bp from the transcription start site and the second directly around the transcription start site, are involved in the regulation of IFNγ gene expression by $1\alpha,25(OH)_2D_3$ (Cippitelli and Santoni, 1998).

CONCLUSIONS

It is well established that $1\alpha,25(OH)_2D_3$ influences cytokine gene expression and signaling in several different cell types. Firstly, this is the case for the pleiotropic mediator TGF-β, for which it has been shown that either the expression of the cytokine itself or expression of associated signaling components is downregulated by $1\alpha,25(OH)_2D_3$. In hepatocytes, $1\alpha,25(OH)_2D_3$ has been found to influence TGF-β signaling in a genome wide scale by directing binding of Smad proteins to target genes. These actions of $1\alpha,25(OH)_2D_3$ on TGF-β expression or signaling were able to inhibit fibrosis and associated inflammation. Second, the interleukins are a vast group of inflammatory cytokines that are clearly regulated by $1\alpha,25(OH)_2D_3$ in a cell-specific manner. However, for several members of this family (e.g., IL-1, IL-6, and IL-8), both positive or negative regulation by $1\alpha,25(OH)_2D_3$ has been observed. A closer look at the parameters that determine the outcome of $1\alpha,25(OH)_2D_3$ action on the expression of these genes is warranted. This applies in particular to the time-scale of changes in gene expression, as different responses may occur during separate stages of $1\alpha,25(OH)_2D_3$ action. Regarding the mechanisms, recruitment of VDR to the respective genomic regions, as well as interaction of $1\alpha,25(OH)_2D_3$ signaling with other transcription factors involved in IL expression (NFAT, NF-κB, Runx1), seem to occur. Concerning the p38 MAP kinase phosphatase MKP1, it was found that GCR and VDR/RXR act in a synergistic manner to induce MKP1 expression in monocytes. This results in reduced p38 activation and reduced formation of proinflammatory cytokines. As a further cytokine, the proinflammatory mediator TNFα has been identified as a $1\alpha,25(OH)_2D_3$ target gene. Also in this case, the vitamin D effects are cell-specific: With cell samples that mainly contain T-cells, downregulation of TNFα has been observed, whereas for monocytic cells, either positive or negative regulation occurred depending on the differentiation state. Finally, gene expression of the proinflammatory mediator IFNγ has been described to be suppressed by $1\alpha,25(OH)_2D_3$ in T-cells. Altogether, the influence of $1\alpha,25(OH)_2D_3$ on the expression of interleukins, TNFα, and IFNγ by different cell types, and the consequences for the cellular interplay that are to be anticipated, amounts to a complex picture. In **Figure 3**, the influence of $1\alpha,25(OH)_2D_3$ on the expression of these cytokines is summarized for the major immune cells (monocytes, DCs, and different T-cell subsets). The resulting pattern supports a shift of T-cell responses from a Th1 type toward Th2 reactions and a suppression of Th17 responses. The effect of $1\alpha,25(OH)_2D_3$ on cytokine expression in antigen presenting cells (monocytes, DCs) remains unclear and seems to depend on the time of stimulation, the differentiation state and other factors.

PERSPECTIVES

Modulation of GCR, NFκB, NFAT as well as SMAD signaling plays a central role in the immunomodulatory activities of $1\alpha,25(OH)_2D_3$. Mechanistic studies on individual genes gave some mechanistic insights into the mechanisms involved in the interaction between VDR/RXR and the above mentioned transcription factors. These mechanisms include competitive binding as well as a crosstalk between the signaling pathways on multiple levels including the promoter level. However, by using ChIP seq and other techniques which allow a genome-wide view, we are just starting to understand the signaling network which is responsible for cell-type-specific and locus-dependent gene activation by ligand-regulated transcription factors such as VDR/RXR. For example, intersecting VDR/SMAD regulatory circuits have just been unraveled and it was shown that TGFβ signaling facilitates VDR binding to certain gene loci. More such data are required to increase our understanding of the complex gene regulatory network that is affected by $1\alpha,25(OH)_2D_3$. Especially, genome-wide data on VDR loci in conjunction with analyses of other, inflammation-related key transcription factors in different cell types and various stimuli are necessary to understand the complex regulation of gene transcription during inflammation.

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REFERENCES

Abe, J., Moriya, Y., Saito, M., Sugawara, Y., Suda, T., and Nishii, Y. (1986). Modulation of cell growth, differentiation, and production of interleukin-3 by 1 alpha,25-dihydroxyvitamin D3 in the murine myelomonocytic leukemia cell line WEHI-3. *Cancer Res.* 46, 6316–6321.

Adorini, L., and Penna, G. (2008). Control of autoimmune diseases by the vitamin D endocrine system. *Nat. Clin. Pract. Rheumatol.* 4, 404–412. doi: 10.1038/ncprheum0855

Alroy, I., Towers, T. L., and Freedman, L. P. (1995). Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. *Mol. Cell. Biol.* 15, 5789–5799.

Ananthakrishnan, A. N. (2013). Environmental triggers for inflammatory bowel disease. *Curr. Gastroenterol. Rep.* 15, 302. doi: 10.1007/s11894-012-0302-4

Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. (Lond.)* 94, 557–572.

Bartels, L. E., Jorgensen, S. P., Bendix, M., Hvas, C. L., Agnholt, J., Agger, R., et al. (2013). 25-Hydroxy vitamin D3 modulates dendritic cell phenotype and function in Crohn's disease. *Inflammopharmacology* 21, 177–186. doi: 10.1007/s10787-012-0168-y

Barthel, R., and Goldfeld, A. E. (2003). T cell-specific expression of the human TNF-alpha gene involves a functional and highly conserved chromatin signature in intron 3. *J. Immunol.* 171, 3612–3619. doi: 10.4049/jimmunol.171.7.3612

Bendix-Struve, M., Bartels, L. E., Agnholt, J., Dige, A., Jorgensen, S. P., and Dahlerup, J. F. (2010). Vitamin D3 treatment of Crohn's disease patients increases stimulated T cell IL-6 production and proliferation. *Aliment. Pharmacol. Ther.* 32, 1364–1372. doi: 10.1111/j.1365-2036.2010.04463.x

Bener, A., Ehlayel, M. S., Tulic, M. K., and Hamid, Q. (2012). Vitamin D deficiency as a strong predictor of asthma in children. *Int. Arch. Allergy Immunol.* 157, 168–175. doi: 10.1159/000323941

Bennett, C. F., Chiang, M. Y., Monia, B. P., and Crooke, S. T. (1993). Regulation of 5-lipoxygenase and 5-lipoxygenase-activating protein expression in HL-60 cells. *Biochem. J.* 289 (Pt. 1), 33–39.

Bermudez, L. E., Young, L. S., and Gupta, S. (1990). 1,25 Dihydroxyvitamin D3-dependent inhibition of growth or killing of Mycobacterium avium complex in human macrophages is mediated by TNF and GM-CSF. *Cell. Immunol.* 127, 432–441. doi: 10.1016/0008-8749(90)90144-G

Bhalla, A. K., Amento, E. P., and Krane, S. M. (1986). Differential effects of 1,25-dihydroxyvitamin D3 on human lymphocytes and monocyte/macrophages:

inhibition of interleukin-2 and augmentation of interleukin-1 production. *Cell. Immunol.* 98, 311–322. doi: 10.1016/0008-8749(86)90291-1

Bhalla, A. K., Paavonen, T., Williams, M. M., Delves, P. J., and Lydyard, P. M. (1991). Regulation of interleukin-1 and tumour necrosis factor gene expression in myelomonocytic cell lines by 1,25-dihydroxyvitamin D3. *Immunology* 72, 61–64.

Bierie, B., and Moses, H. L. (2010). Transforming growth factor beta (TGF-beta) and inflammation in cancer. *Cytokine Growth Factor Rev.* 21, 49–59. doi: 10.1016/j.cytogfr.2009.11.008

Blifeld, C., Prehn, J. L., and Jordan, S. C. (1991). Stimulus-specific 1,25(OH)2D3 modulation of TNF and IL-1-beta gene expression in human peripheral blood mononuclear cells and monocytoid cell lines. *Transplantation* 51, 498–503. doi: 10.1097/00007890-199102000-00043

Brehm, J. M., Acosta-Perez, E., Klei, L., Roeder, K., Barmada, M., Boutaoui, N., et al. (2012). Vitamin D insufficiency and severe asthma exacerbations in Puerto Rican children. *Am. J. Respir. Crit. Care Med.* 186, 140–146. doi: 10.1164/rccm.201203-0431OC

Brehm, J. M., Celedon, J. C., Soto-Quiros, M. E., Avila, L., Hunninghake, G. M., Forno, E., et al. (2009). Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *Am. J. Respir. Crit. Care Med.* 179, 765–771. doi: 10.1164/rccm.200808-1361OC

Brehm, J. M., Schuemann, B., Fuhlbrigge, A. L., Hollis, B. W., Strunk, R. C., Zeiger, R. S., et al. (2010). Serum vitamin D levels and severe asthma exacerbations in the Childhood Asthma Management Program study. *J. Allergy Clin. Immunol.* 126, 52–58. doi: 10.1016/j.jaci.2010.03.043

Brewer, L. C., Michos, E. D., and Reis, J. P. (2011). Vitamin D in atherosclerosis, vascular disease, and endothelial function. *Curr. Drug Targets* 12, 54–60. doi: 10.2174/138945011793591617

Bruce, D., Yu, S., Ooi, J. H., and Cantorna, M. T. (2011). Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. *Int. Immunol.* 23, 519–528. doi: 10.1093/intimm/dxr045

Brungs, M., Radmark, O., Samuelsson, B., and Steinhilber, D. (1994). On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGF beta. *Biochem. Biophys. Res. Commun.* 205, 1572–1580. doi: 10.1006/bbrc.1994.2846

Brungs, M., Radmark, O., Samuelsson, B., and Steinhilber, D. (1995). Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U.S.A.* 92, 107–111. doi: 10.1073/pnas.92.1.107

Burton, J. M., Kimball, S., Vieth, R., Bar-Or, A., Dosch, H. M., Cheung, R., et al. (2010). A phase I/II dose-escalation trial of vitamin D3 and calcium in multiple sclerosis. *Neurology* 74, 1852–1859. doi: 10.1212/WNL.0b013e3181e1cec2

Camargo, C. A. Jr., Ingham, T., Wickens, K., Thadhani, R., Silvers, K. M., Epton, M. J., et al. (2011). Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma. *Pediatrics* 127, e180–e187. doi: 10.1542/peds.2010-0442

Camargo, C. A. Jr., Rifas-Shiman, S. L., Litonjua, A. A., Rich-Edwards, J. W., Weiss, S. T., Gold, D. R., et al. (2007). Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *Am. J. Clin. Nutr.* 85, 788–795.

Carraro, S., Giordano, G., Reniero, F., Carpi, D., Stocchero, M., Sterk, P. J., et al. (2013). Asthma severity in childhood and metabolomic profiling of breath condensate. *Allergy* 68, 110–117. doi: 10.1111/all.12063

Carrelli, A. L., Walker, M. D., Lowe, H., McMahon, D. J., Rundek, T., Sacco, R. L., et al. (2011). Vitamin D deficiency is associated with subclinical carotid atherosclerosis: the Northern Manhattan study. *Stroke* 42, 2240–2245. doi: 10.1161/STROKEAHA.110.608539

Chang, S. H., Chung, Y., and Dong, C. (2010). Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression. *J. Biol. Chem.* 285, 38751–38755. doi: 10.1074/jbc.C110.185777

Chen, W., Rogatsky, I., and Garabedian, M. J. (2006). MED14 and MED1 differentially regulate target-specific gene activation by the glucocorticoid receptor. *Mol. Endocrinol.* 20, 560–572. doi: 10.1210/me.2005-0318

Cheraghi, N., Dai, H., and Raghuveer, G. (2012). Vitamin D deficiency is associated with atherosclerosis-promoting risk factor clustering but not vascular damage in children. *Med. Sci. Monit.* 18, CR687–CR692. doi: 10.12659/MSM.883593

Chinellato, I., Piazza, M., Sandri, M., Peroni, D. G., Cardinale, F., Piacentini, G. L., et al. (2011b). Serum vitamin D levels and exercise-induced bronchoconstriction in children with asthma. *Eur. Respir. J.* 37, 1366–1370. doi: 10.1183/09031936.00044710

Chinellato, I., Piazza, M., Sandri, M., Peroni, D., Piacentini, G., and Boner, A. L. (2011a). Vitamin D serum levels and markers of asthma control in Italian children. *J. Pediatr.* 158, 437–441. doi: 10.1016/j.jpeds.2010.08.043

Cipollone, F., and Fazia, M. L. (2006). COX-2 and atherosclerosis. *J. Cardiovasc. Pharmacol.* 47(Suppl. 1), S26–S36. doi: 10.1097/00005344-200605001-00006

Cippitelli, M., and Santoni, A., (1998). Vitamin D3: a transcriptional modulator of the interferon-gamma gene. *Eur. J. Immunol.* 28, 3017-3030.

Cohen, M. L., Douvdevani, A., Chaimovitz, C., and Shany, S. (2001). Regulation of TNF-alpha by 1alpha,25-dihydroxyvitamin D3 in human macrophages from CAPD patients. *Kidney Int.* 59, 69–75. doi: 10.1046/j.1523-1755.2001.00467.x

Cohen-Lahav, M., Shany, S., Tobvin, D., Chaimovitz, C., and Douvdevani, A. (2006). Vitamin D decreases NFkappaB activity by increasing IkappaBalpha levels. *Nephrol. Dial. Transplant.* 21, 889–897. doi: 10.1093/ndt/gfi254

Colin, E. M., Asmawidjaja, P. S., Van Hamburg, J. P., Mus, A. M., Van Driel, M., Hazes, J. M., et al. (2010). 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum.* 62, 132–142. doi: 10.1002/art.25043

Correale, J., Ysrraelit, M. C., and Gaitan, M. I. (2009). Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* 132, 1146–1160. doi: 10.1093/brain/awp033

Correale, J., Ysrraelit, M. C., and Gaitan, M. I. (2010). Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. *J. Immunol.* 185, 4948–4958. doi: 10.4049/jimmunol.1000588

Crispin, J. C., Martinez, A., De Pablo, P., Velasquillo, C., and Alcocer-Varela, J. (1998). Participation of the CD69 antigen in the T-cell activation process of patients with systemic lupus erythematosus. *Scand. J. Immunol.* 48, 196–200. doi: 10.1046/j.1365-3083.1998.00366.x

Cutolo, M. (1999). Macrophages as effectors of the immunoendocrinologic interactions in autoimmune rheumatic diseases. *Ann. N.Y. Acad. Sci.* 876, 32–41. discussion: 41–32.

Dallaporta, M., Pecchi, E., Thirion, S., Jean, A., and Troadec, J. D. (2010). Toward the management of inflammation: recent developments of mPGES-1 inhibitors. *Recent Pat. CNS Drug Discov.* 5, 70–80. doi: 10.2174/157488910789753549

D'Ambrosio, D., Cippitelli, M., Cocciolo, M. G., Mazzeo, D., Di Lucia, P., Lang, R., et al. (1998). Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J. Clin. Invest.* 101, 252–262. doi: 10.1172/JCI1050

Deckx, N., Lee, W. P., Berneman, Z. N., and Cools, N. (2013). Neuroendocrine Immunoregulation in Multiple Sclerosis. *Clin. Dev. Immunol.* 2013:705232. doi: 10.1155/2013/705232

Devereux, G., Litonjua, A. A., Turner, S. W., Craig, L. C., McNeill, G., Martindale, S., et al. (2007). Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am. J. Clin. Nutr.* 85, 853–859.

Diaz, L., Noyola-Martinez, N., Barrera, D., Hernandez, G., Avila, E., Halhali, A., et al. (2009). Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. *J. Reprod. Immunol.* 81, 17–24. doi: 10.1016/j.jri.2009.02.005

Dimeloe, S., Richards, D. F., Urry, Z. L., Gupta, A., Stratigou, V., Farooque, S., et al. (2012). 1alpha,25-dihydroxyvitamin D3 promotes CD200 expression by human peripheral and airway-resident T cells. *Thorax* 67, 574–581. doi: 10.1136/thoraxjnl-2011-200651

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Di Rosa, M., Malaguarnera, G., De Gregorio, C., Palumbo, M., Nunnari, G., and Malaguarnera, L. (2012). Immuno-modulatory effects of vitamin D3 in human monocyte and macrophages. *Cell. Immunol.* 280, 36–43. doi: 10.1016/j.cellimm.2012.10.009

Dong, J. Y., Zhang, W. G., Chen, J. J., Zhang, Z. L., Han, S. F., and Qin, L. Q. (2013). Vitamin D intake and risk of type 1 diabetes: a meta-analysis of observational studies. *Nutrients* 5, 3551–3562. doi: 10.3390/nu5093551

Duque, J., Fresno, M., and Iniguez, M. A. (2005). Expression and function of the nuclear factor of activated T cells in colon carcinoma cells: involvement in the regulation of cyclooxygenase-2. *J. Biol. Chem.* 280, 8686–8693. doi: 10.1074/jbc.M413076200

Ehlayel, M. S., Bener, A., and Sabbah, A. (2011). Is high prevalence of vitamin D deficiency evidence for asthma and allergy risks? *Eur. Ann. Allergy Clin. Immunol.* 43, 81–88.

Eleftheriadis, T., Antoniadi, G., Liakopoulos, V., Kartsios, C., Stefanidis, I., and Galaktidou, G. (2010). Paricalcitol reduces basal and lipopolysaccharide-induced (LPS) TNF-alpha and IL-8 production by human peripheral blood mononuclear cells. *Int. Urol. Nephrol.* 42, 181–185. doi: 10.1007/s11255-009-9541-1

El-Shehaby, A. M., El-Khatib, M. M., Marzouk, S., and Battah, A. A. (2013). Relationship of BsmI polymorphism of vitamin D receptor gene with left ventricular hypertrophy and atherosclerosis in hemodialysis patients. *Scand. J. Clin. Lab. Invest.* 73, 75–81. doi: 10.3109/00365513.2012.743163

Equils, O., Naiki, Y., Shapiro, A. M., Michelsen, K., Lu, D., Adams, J., et al. (2006). 1,25-Dihydroxyvitamin D inhibits lipopolysaccharide-induced immune activation in human endothelial cells. *Clin. Exp. Immunol.* 143, 58–64. doi: 10.1111/j.1365-2249.2005.02961.x

Erkkola, M., Kaila, M., Nwaru, B. I., Kronberg-Kippila, C., Ahonen, S., Nevalainen, J., et al. (2009). Maternal vitamin D intake during pregnancy is inversely associated with asthma and allergic rhinitis in 5-year-old children. *Clin. Exp. Allergy* 39, 875–882. doi: 10.1111/j.1365-2222.2009.03234.x

Fagan, D. L., Prehn, J. L., Adams, J. S., and Jordan, S. C. (1991). The human myelomonocytic cell line U-937 as a model for studying alterations in steroid-induced monokine gene expression: marked enhancement of lipopolysaccharide-stimulated interleukin-1 beta messenger RNA levels by 1,25-dihydroxyvitamin D3. *Mol. Endocrinol.* 5, 179–186. doi: 10.1210/mend-5-2-179

Fahmi, H. (2004). mPGES-1 as a novel target for arthritis. *Curr. Opin. Rheumatol.* 16, 623–627. doi: 10.1097/01.bor.0000129664.81052.8e

Fang, W. L., Gao, L. B., Liang, W. B., Xue, H., Bai, P., Lv, M. L., et al. (2009). Association analysis of vitamin D receptor gene polymorphisms in chinese population with asthma. *Iran. J. Allergy Asthma Immunol.* 8, 141–147.

Feng, X., Lv, C., Wang, F., Gan, K., Zhang, M., and Tan, W. (2013). Modulatory effect of 1,25-dihydroxyvitamin D 3 on IL1 beta -induced RANKL, OPG, TNF alpha, and IL-6 expression in human rheumatoid synoviocyte MH7A. *Clin. Dev. Immunol.* 2013:160123. doi: 10.1155/2013/160123

Fernandez-Herrera, J., Fernandez-Ruiz, E., Lopez-Cabrera, M., Garcia-Diez, A., Sanchez-Madrid, F., and Gonzalez-Amaro, R. (1996). CD69 expression and tumour necrosis factor-alpha immunoreactivity in the inflammatory cell infiltrate of halo naevi. *Br. J. Dermatol.* 134, 388–393. doi: 10.1111/j.1365-2133.1996.tb16219.x

Flanders, K. C. (2004). Smad3 as a mediator of the fibrotic response. *Int. J. Exp. Pathol.* 85, 47–64. doi: 10.1111/j.0959-9673.2004.00377.x

Franchi, B., Piazza, M., Sandri, M., Mazzei, F., Maffeis, C., and Boner, A. L. (2013). Vitamin D at the onset of type 1 diabetes in Italian children. *Eur. J. Pediatr.* 173, 477–482. doi: 10.1007/s00431-013-2204-3

Frankenberger, M., Pforte, A., Sternsdorf, T., Passlick, B., Baeuerle, P. A., and Ziegler-Heitbrock, H. W. (1994). Constitutive nuclear NF-kappa B in cells of the monocyte lineage. *Biochem. J.* 304(Pt 1), 87–94.

Freishtat, R. J., Iqbal, S. F., Pillai, D. K., Klein, C. J., Ryan, L. M., Benton, A. S., et al. (2010). High prevalence of vitamin D deficiency among inner-city African American youth with asthma in Washington, DC. *J. Pediatr.* 156, 948–952. doi: 10.1016/j.jpeds.2009.12.033

Fukuoka, M., Ogino, Y., Sato, H., Ohta, T., and Komoriya, K. (1998). Regulation of RANTES and IL-8 production in normal human dermal fibroblasts by active vitamin D3 (tacalcitol). *Br. J. Pharmacol.* 124, 1433–1438. doi: 10.1038/sj.bjp.0701988

Gale, C. R., Robinson, S. M., Harvey, N. C., Javaid, M. K., Jiang, B., Martyn, C. N., et al. (2008). Maternal vitamin D status during pregnancy and child outcomes. *Eur. J. Clin. Nutr.* 62, 68–77. doi: 10.1038/sj.ejcn.1602680

Garg, M., Lubel, J. S., Sparrow, M. P., Holt, S. G., and Gibson, P. R. (2012). Review article: vitamin D and inflammatory bowel disease–established concepts and future directions. *Aliment. Pharmacol. Ther.* 36, 324–344. doi: 10.1111/j.1365-2036.2012.05181.x

Gatenby, P., Lucas, R., and Swaminathan, A. (2013). Vitamin D deficiency and risk for rheumatic diseases: an update. *Curr. Opin. Rheumatol.* 25, 184–191. doi: 10.1097/BOR.0b013e32835cfc16

Gergen, P. J., Teach, S. J., Mitchell, H. E., Freishtat, R. F., Calatroni, A., Matsui, E., et al. (2013). Lack of a relation between serum 25-hydroxyvitamin D concentrations and asthma in adolescents. *Am. J. Clin. Nutr.* 97, 1228–1234. doi: 10.3945/ajcn.112.046961

Gessl, A., and Waldhausl, W. (1998). Elevated CD69 expression on naive peripheral blood T-cells in hyperthyroid Graves' disease and autoimmune thyroiditis: discordant effect of methimazole on HLA-DR and CD69. *Clin. Immunol. Immunopathol.* 87, 168–175. doi: 10.1006/clin.1998.4524

Ginde, A. A., Mansbach, J. M., and Camargo, C. A. Jr. (2009). Vitamin D, respiratory infections, and asthma. *Curr. Allergy Asthma Rep.* 9, 81–87. doi: 10.1007/s11882-009-0012-7

Goldberg, P., Fleming, M. C., and Picard, E. H. (1986). Multiple sclerosis: decreased relapse rate through dietary supplementation with calcium, magnesium and vitamin D. *Med. Hypotheses* 21, 193–200. doi: 10.1016/0306-9877(86)90010-1

Goleva, E., Searing, D. A., Jackson, L. P., Richers, B. N., and Leung, D. Y. (2012). Steroid requirements and immune associations with vitamin D are stronger in children than adults with asthma. *J. Allergy Clin. Immunol.* 129, 1243–1251. doi: 10.1016/j.jaci.2012.01.044

Golovko, O., Nazarova, N., and Tuohimaa, P. (2005). Vitamin D-induced up-regulation of tumour necrosis factor alpha (TNF-alpha) in prostate cancer cells. *Life Sci.* 77, 562–577. doi: 10.1016/j.lfs.2004.10.072

Gorman, S., Tan, D. H., Lambert, M. J., Scott, N. M., Judge, M. A., and Hart, P. H. (2012). Vitamin D(3) deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic airway disease. *Pediatr. Allergy Immunol.* 23, 83–87. doi: 10.1111/j.1399-3038.2011.01146.x

Gupta, A., Bush, A., Hawrylowicz, C., and Saglani, S. (2012a). Vitamin D and asthma in children. *Paediatr. Respir. Rev.* 13, 236–243. quiz 243. doi: 10.1016/j.prrv.2011.07.003

Gupta, A., Dimeloe, S., Richards, D. F., Bush, A., Saglani, S., and Hawrylowicz, C. M. (2012b). Vitamin D binding protein and asthma severity in children. *J. Allergy Clin. Immunol.* 129, 1669–1671. doi: 10.1016/j.jaci.2012.02.017

Gynther, P., Toropainen, S., Matilainen, J. M., Seuter, S., Carlberg, C., and Vaisanen, S. (2011). Mechanism of 1alpha,25-dihydroxyvitamin D(3)-dependent repression of interleukin-12B. *Biochim. Biophys. Acta* 1813, 810–818. doi: 10.1016/j.bbamcr.2011.01.037

Haeggstrom, J. Z., and Funk, C. D. (2011). Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem. Rev.* 111, 5866–5898. doi: 10.1021/cr200246d

Hakim, I., and Bar-Shavit, Z. (2003). Modulation of TNF-alpha expression in bone marrow macrophages: involvement of vitamin D response element. *J. Cell. Biochem.* 88, 986–998. doi: 10.1002/jcb.10453

Halder, S. K., Goodwin, J. S., and Al-Hendy, A. (2011). 1,25-Dihydroxyvitamin D3 reduces TGF-beta3-induced fibrosis-related gene expression in human uterine leiomyoma cells. *J. Clin. Endocrinol. Metab.* 96, E754–E762. doi: 10.1210/jc.2010-2131

Han, S. H., Jeon, J. H., Ju, H. R., Jung, U., Kim, K. Y., Yoo, H. S., et al. (2003). VDUP1 upregulated by TGF-beta1 and 1,25-dihydorxyvitamin D3 inhibits tumor cell growth by blocking cell-cycle progression. *Oncogene* 22, 4035–4046. doi: 10.1038/sj.onc.1206610

Harant, H., Wolff, B., and Lindley, I. J. (1998). 1Alpha,25-dihydroxyvitamin D3 decreases DNA binding of nuclear factor-kappaB in human fibroblasts. *FEBS Lett.* 436, 329–334. doi: 10.1016/S0014-5793(98)01153-3

Harizi, H., Corcuff, J. B., and Gualde, N. (2008). Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends Mol. Med.* 14, 461–469. doi: 10.1016/j.molmed.2008.08.005

Harle, D., Radmark, O., Samuelsson, B., and Steinhilber, D. (1998). Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur. J. Biochem.* 254, 275–281. doi: 10.1046/j.1432-1327.1998.2540275.x

Heikkinen, S., Vaisanen, S., Pehkonen, P., Seuter, S., Benes, V., and Carlberg, C. (2011). Nuclear hormone 1alpha,25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* 39, 9181–9193. doi: 10.1093/nar/gkr654

Heine, G., Niesner, U., Chang, H. D., Steinmeyer, A., Zugel, U., Zuberbier, T., et al. (2008). 1,25-dihydroxyvitamin D(3) promotes IL-10 production in human B cells. *Eur. J. Immunol.* 38, 2210–2218. doi: 10.1002/eji.200838216

Hidaka, M., Wakabayashi, I., Takeda, Y., and Fukuzawa, K. (2013). Vitamin D(3) derivatives increase soluble CD14 release through ERK1/2 activation and decrease IL-8 production in intestinal epithelial cells. *Eur. J. Pharmacol.* 721, 305–312. doi: 10.1016/j.ejphar.2013.09.014

Higashimoto, Y., Ohata, M., Iwamoto, Y., Fujimoto, H., Uetani, K., Suruda, T., et al. (1995). Stimulatory effect of 1 alpha,25-dihydroxyvitamin D3 on mouse alveolar macrophage tumor necrosis factor-alpha production *in vitro*: involvement

of protein kinase C and Ca2+/calmodulin-dependent kinase. *Respiration* 62, 89–94. doi: 10.1159/000196398

Hodler, B., Evequoz, V., Trechsel, U., Fleisch, H., and Stadler, B. (1985). Influence of vitamin D3 metabolites on the production of interleukins 1,2 and 3. *Immunobiology* 170, 256–269. doi: 10.1016/S0171-2985(85)80075-9

Hollams, E. M., Hart, P. H., Holt, B. J., Serralha, M., Parsons, F., De Klerk, N. H., et al. (2011). Vitamin D and atopy and asthma phenotypes in children: a longitudinal cohort study. *Eur. Respir. J.* 38, 1320–1327. doi: 10.1183/09031936.00029011

Hullett, D. A., Laeseke, P. F., Malin, G., Nessel, R., Sollinger, H. W., and Becker, B. N. (2005). Prevention of chronic allograft nephropathy with vitamin D. *Transpl. Int.* 18, 1175–1186. doi: 10.1111/j.1432-2277.2005.00187.x

Hypponen, E., Sovio, U., Wjst, M., Patel, S., Pekkanen, J., Hartikainen, A. L., et al. (2004). Infant vitamin d supplementation and allergic conditions in adulthood: northern Finland birth cohort 1966. *Ann. N.Y. Acad. Sci.* 1037, 84–95. doi: 10.1196/annals.1337.013

Inoue, M., Matsui, T., Nishibu, A., Nihei, Y., Iwatsuki, K., and Kaneko, F. (1998). Regulatory effects of 1alpha,25-dihydroxyvitamin D3 on inflammatory responses in psoriasis. *Eur. J. Dermatol.* 8, 16–20.

Ito, I., Waku, T., Aoki, M., Abe, R., Nagai, Y., Watanabe, T., et al. (2013). A nonclassical vitamin D receptor pathway suppresses renal fibrosis. *J. Clin. Invest.* 123, 4579–4594. doi: 10.1172/JCI67804

Jain, S. K., and Micinski, D. (2013). Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. *Biochem. Biophys. Res. Commun.* 437, 7–11. doi: 10.1016/j.bbrc.2013.06.004

Jamshidi, F., Zhang, J., Harrison, J. S., Wang, X., and Studzinski, G. P. (2008). Induction of differentiation of human leukemia cells by combinations of COX inhibitors and 1,25-dihydroxyvitamin D3 involves Raf1 but not Erk 1/2 signaling. *Cell Cycle* 7, 917–924. doi: 10.4161/cc.7.7.5620

Jono, S., Nishizawa, Y., Shioi, A., and Morii, H. (1998). 1,25-Dihydroxyvitamin D3 increases *in vitro* vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. *Circulation* 98, 1302–1306. doi: 10.1161/01.CIR.98.13.1302

Jordan, S. C., Toyoda, M., Prehn, J., Lemire, J. M., Sakai, R., and Adams, J. S. (1989). 1,25-Dihydroxyvitamin-D3 regulation of interleukin-2 and interleukin-2 receptor levels and gene expression in human T cells. *Mol. Immunol.* 26, 979–984. doi: 10.1016/0161-5890(89)90116-8

Joshi, S., Pantalena, L. C., Liu, X. K., Gaffen, S. L., Liu, H., Rohowsky-Kochan, C., et al. (2011). 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol. Cell. Biol.* 31, 3653–3669. doi: 10.1128/MCB.05020-11

Kankova, M., Luini, W., Pedrazzoni, M., Riganti, F., Sironi, M., Bottazzi, B., et al. (1991). Impairment of cytokine production in mice fed a vitamin D3-deficient diet. *Immunology* 73, 466–471.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. *Nat. Immunol.* 3, 221–227. doi: 10.1038/ni0302-221

Kim, C. S., Joo, S. Y., Lee, K. E., Choi, J. S., Bae, E. H., Ma, S. K., et al. (2013). Paricalcitol attenuates 4-hydroxy-2-hexenal-induced inflammation and epithelial-mesenchymal transition in human renal proximal tubular epithelial cells. *PLoS ONE* 8:e63186. doi: 10.1371/journal.pone.0063186

Kim, H. J., Abdelkader, N., Katz, M., and McLane, J. A. (1992). 1,25-Dihydroxyvitamin-D3 enhances antiproliferative effect and transcription of TGF-beta1 on human keratinocytes in culture. *J. Cell. Physiol.* 151, 579–587. doi: 10.1002/jcp.1041510318

Knip, M., and Simell, O. (2012). Environmental triggers of type 1 diabetes. *Cold Spring Harb. Perspect. Med.* 2:a007690. doi: 10.1101/cshperspect.a007690

Koizumi, H., Kaplan, A., Shimizu, T., and Ohkawara, A. (1997). 1,25-Dihydroxyvitamin D3 and a new analogue, 22-oxacalcitriol, modulate proliferation and interleukin-8 secretion of normal human keratinocytes. *J. Dermatol. Sci.* 15, 207–213. doi: 10.1016/S0923-1811(97)00609-9

Kong, J., Grando, S. A., and Li, Y. C. (2006). Regulation of IL-1 family cytokines IL-1alpha, IL-1 receptor antagonist, and IL-18 by 1,25-dihydroxyvitamin D3 in primary keratinocytes. *J. Immunol.* 176, 3780–3787. doi: 10.4049/jimmunol.176.6.3780

Korf, H., Wenes, M., Stijlemans, B., Takiishi, T., Robert, S., Miani, M., et al. (2012). 1,25-Dihydroxyvitamin D3 curtails the inflammatory and T cell stimulatory capacity of macrophages through an IL-10-dependent mechanism. *Immunobiology* 217, 1292–1300. doi: 10.1016/j.imbio.2012.07.018

Krobtrakulchai, W., Praikanahok, J., Visitsunthorn, N., Vichyanond, P., Manonukul, K., Pratumvinit, B., et al. (2013). The effect of vitamin d status on pediatric asthma at a university hospital, Thailand. *Allergy Asthma Immunol. Res.* 5, 289–294. doi: 10.4168/aair.2013.5.5.289

Kudejko, J. (1968). Effect of vitamin D 3 and induced calciphylaxis on atherosclerosis of rabbits treated with cholesterol and calcium gluconate. *Gerontologia* 14, 35–44. doi: 10.1159/000211640

Kudo, K., Hasegawa, S., Suzuki, Y., Hirano, R., Wakiguchi, H., Kittaka, S., et al. (2012). 1alpha,25-Dihydroxyvitamin D(3) inhibits vascular cellular adhesion molecule-1 expression and interleukin-8 production in human coronary arterial endothelial cells. *J. Steroid Biochem. Mol. Biol.* 132, 290–294. doi: 10.1016/j.jsbmb.2012.07.003

Kull, I., Bergstrom, A., Melen, E., Lilja, G., Van Hage, M., Pershagen, G., et al. (2006). Early-life supplementation of vitamins A and D, in water-soluble form or in peanut oil, and allergic diseases during childhood. *J. Allergy Clin. Immunol.* 118, 1299–1304. doi: 10.1016/j.jaci.2006.08.022

Kunitomo, M., Kinoshita, K., and Bando, Y. (1981). Experimental atherosclerosis in rats fed a vitamin D, cholesterol-rich diet. *J. Pharmacobiodyn.* 4, 718–723. doi: 10.1248/bpb1978.4.718

Kuo, Y. T., Kuo, C. H., Lam, K. P., Chu, Y. T., Wang, W. L., Huang, C. H., et al. (2010). Effects of vitamin D3 on expression of tumor necrosis factor-alpha and chemokines by monocytes. *J. Food Sci.* 75, H200–H204. doi: 10.1111/j.1750-3841.2010.01704.x

Lacey, D. L., Erdmann, J. M., and Tan, H. L. (1993a). 1,25-Dihydroxyvitamin D3 increases type 1 interleukin-1 receptor expression in a murine T cell line. *J. Cell. Biochem.* 52, 159–170. doi: 10.1002/jcb.240520208

Lacey, D. L., Erdmann, J. M., Tan, H. L., and Ohara, J. (1993b). Murine osteoblast interleukin 4 receptor expression: upregulation by 1,25 dihydroxyvitamin D3. *J. Cell. Biochem.* 53, 122–134. doi: 10.1002/jcb.240530205

Lagishetty, V., Liu, N. Q., and Hewison, M. (2011). Vitamin D metabolism and innate immunity. *Mol. Cell. Endocrinol.* 347, 97–105. doi: 10.1016/j.mce.2011.04.015

Lan, H. Y. (2011). Diverse roles of TGF-beta/Smads in renal fibrosis and inflammation. *Int. J. Biol. Sci.* 7, 1056–1067. doi: 10.7150/ijbs.7.1056

Larsen, C. G., Kristensen, M., Paludan, K., Deleuran, B., Thomsen, M. K., Zachariae, C., et al. (1991). 1,25(OH)2-D3 is a potent regulator of interleukin-1 induced interleukin-8 expression and production. *Biochem. Biophys. Res. Commun.* 176, 1020–1026. doi: 10.1016/0006-291X(91)90384-J

Lee, B. N., Kim, T. H., Jun, J. B., Yoo, D. H., Woo, J. H., Choi, S. J., et al. (2011). Upregulation of interleukin-1beta production by 1,25-dihydroxyvitamin D(3) in activated human macrophages. *Mol. Biol. Rep.* 38, 2193–2201. doi: 10.1007/s11033-010-0348-z

Lee, S. B., and Kalluri, R. (2010). Mechanistic connection between inflammation and fibrosis. *Kidney Int. Suppl.* 78, S22–S26. doi: 10.1038/ki.2010.418

Lemire, J. M., Archer, D. C., Beck, L., and Spiegelberg, H. L. (1995). Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J. Nutr.* 125, 1704s–1708s.

Li, B., Baylink, D. J., Deb, C., Zannetti, C., Rajaallah, F., Xing, W., et al. (2013). 1,25-Dihydroxyvitamin D3 suppresses TLR8 expression and TLR8-mediated inflammatory responses in monocytes *in vitro* and experimental autoimmune encephalomyelitis *in vivo*. *PLoS ONE* 8:e58808. doi: 10.1371/journal.pone.0058808

Li, F., Jiang, L., Willis-Owen, S. A., Zhang, Y., and Gao, J. (2011a). Vitamin D binding protein variants associate with asthma susceptibility in the Chinese Han population. *BMC Med. Genet.* 12:103. doi: 10.1186/1471-2350-12-103

Li, F., Peng, M., Jiang, L., Sun, Q., Zhang, K., Lian, F., et al. (2011b). Vitamin D deficiency is associated with decreased lung function in Chinese adults with asthma. *Respiration* 81, 469–475. doi: 10.1159/000322008

Li, M. O., and Flavell, R. A. (2008). Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* 28, 468–476. doi: 10.1016/j.immuni.2008.03.003

Li, Y. C., Kong, J., Wei, M., Chen, Z. F., Liu, S. Q., and Cao, L. P. (2002). 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J. Clin. Invest.* 110, 229–238. doi: 10.1172/JCI0215219

Liu, N. Q., Kaplan, A. T., Lagishetty, V., Ouyang, Y. B., Ouyang, Y., Simmons, C. F., et al. (2011). Vitamin D and the regulation of placental inflammation. *J. Immunol.* 186, 5968–5974. doi: 10.4049/jimmunol.1003332

Liu, X., Nelson, A., Wang, X., Farid, M., Gunji, Y., Ikari, J., et al. (2014). Vitamin d modulates prostaglandin e2 synthesis and degradation in human lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 50, 40–50. doi: 10.1165/rcmb.2013-0211OC

Lyakh, L. A., Sanford, M., Chekol, S., Young, H. A., and Roberts, A. B. (2005). TGF-beta and vitamin D3 utilize distinct pathways to suppress IL-12 production and modulate rapid differentiation of human monocytes into CD83+ dendritic cells. *J. Immunol.* 174, 2061–2070. doi: 10.4049/jimmunol.174.4.2061

Maalmi, H., Sassi, F. H., Berraies, A., Ammar, J., Hamzaoui, K., and Hamzaoui, A. (2013). Association of vitamin D receptor gene polymorphisms with susceptibility to asthma in Tunisian children: a case control study. *Hum. Immunol.* 74, 234–240. doi: 10.1016/j.humimm.2012.11.005

Magnus, M. C., Stene, L. C., Haberg, S. E., Nafstad, P., Stigum, H., London, S. J., et al. (2013). Prospective study of maternal mid-pregnancy 25-hydroxyvitamin D Level and early childhood respiratory disorders. *Paediatr. Perinat. Epidemiol.* 27, 532–541. doi: 10.1111/ppe.12080

Majak, P., Jerzynska, J., Smejda, K., Stelmach, I., Timler, D., and Stelmach, W. (2012). Correlation of vitamin D with Foxp3 induction and steroid-sparing effect of immunotherapy in asthmatic children. *Ann. Allergy Asthma Immunol.* 109, 329–335. doi: 10.1016/j.anai.2012.08.002

Majak, P., Olszowiec-Chlebna, M., Smejda, K., and Stelmach, I. (2011). Vitamin D supplementation in children may prevent asthma exacerbation triggered by acute respiratory infection. *J. Allergy Clin. Immunol.* 127, 1294–1296. doi: 10.1016/j.jaci.2010.12.016

Marcotorchino, J., Gouranton, E., Romier, B., Tourniaire, F., Astier, J., Malezet, C., et al. (2012). Vitamin D reduces the inflammatory response and restores glucose uptake in adipocytes. *Mol. Nutr. Food Res.* 56, 1771–1782. doi: 10.1002/mnfr.201200383

Matilainen, J. M., Husso, T., Toropainen, S., Seuter, S., Turunen, M. P., Gynther, P., et al. (2010a). Primary effect of 1alpha,25(OH)(2)D(3) on IL-10 expression in monocytes is short-term down-regulation. *Biochim. Biophys. Acta* 1803, 1276–1286. doi: 10.1016/j.bbamcr.2010.07.009

Matilainen, J. M., Rasanen, A., Gynther, P., and Vaisanen, S. (2010b). The genes encoding cytokines IL-2, IL-10 and IL-12B are primary 1alpha,25(OH)2D3 target genes. *J. Steroid Biochem. Mol. Biol.* 121, 142–145. doi: 10.1016/j.jsbmb.2010.03.020

Matsui, T., Takahashi, R., Nakao, Y., Koizumi, T., Katakami, Y., Mihara, K., et al. (1986). 1,25-Dihydroxyvitamin D3-regulated expression of genes involved in human T-lymphocyte proliferation and differentiation. *Cancer Res.* 46, 5827–5831.

McNally, P., Coughlan, C., Bergsson, G., Doyle, M., Taggart, C., Adorini, L., et al. (2011). Vitamin D receptor agonists inhibit pro-inflammatory cytokine production from the respiratory epithelium in cystic fibrosis. *J. Cyst. Fibros.* 10, 428–434. doi: 10.1016/j.jcf.2011.06.013

Menon, J., Maranda, L., and Nwosu, B. U. (2012). Serum 25-hydroxyvitamin D levels do not correlate with asthma severity in a case-controlled study of children and adolescents. *J. Pediatr. Endocrinol. Metab.* 25, 673–679. doi: 10.1515/jpem-2012-0143

Meyer, M. B., Goetsch, P. D., and Pike, J. W. (2012). VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol. Endocrinol.* 26, 37–51. doi: 10.1210/me.2011-1109

Miodovnik, M., Koren, R., Ziv, E., and Ravid, A. (2012). The inflammatory response of keratinocytes and its modulation by vitamin D: the role of MAPK signaling pathways. *J. Cell. Physiol.* 227, 2175–2183. doi: 10.1002/jcp.22951

Mok, C. C., Birmingham, D. J., Leung, H. W., Hebert, L. A., Song, H., and Rovin, B. H. (2012). Vitamin D levels in Chinese patients with systemic lupus erythematosus: relationship with disease activity, vascular risk factors and atherosclerosis. *Rheumatology (Oxf.)* 51, 644–652. doi: 10.1093/rheumatology/ker212

Moniaga, C. S., Egawa, G., Miyachi, Y., and Kabashima, K. (2013). Calcipotriol modulates IL-22 receptor expression and keratinocyte proliferation in IL-22-induced epidermal hyperplasia. *J. Dermatol. Sci.* 71, 76–77. doi: 10.1016/j.jdermsci.2013.03.011

Moore, K. J., Sheedy, F. J., and Fisher, E. A. (2013). Macrophages in atherosclerosis: a dynamic balance. *Nat. Rev. Immunol.* 13, 709–721. doi: 10.1038/nri3520

Morales, E., Romieu, I., Guerra, S., Ballester, F., Rebagliato, M., Vioque, J., et al. (2012). Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. *Epidemiology* 23, 64–71. doi: 10.1097/EDE.0b013e31823a44d3

Moreno, J., Krishnan, A. V., Swami, S., Nonn, L., Peehl, D. M., and Feldman, D. (2005). Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* 65, 7917–7925. doi: 10.1158/0008-5472.can-05-1435

Muller, K., and Bendtzen, K. (1992). Inhibition of human T lymphocyte proliferation and cytokine production by 1,25-dihydroxyvitamin D3. Differential effects on CD45RA+ and CD45R0+ cells. *Autoimmunity* 14, 37–43. doi: 10.3109/08916939309077355

Muller, K., Haahr, P. M., Diamant, M., Rieneck, K., Kharazmi, A., and Bendtzen, K. (1992). 1,25-Dihydroxyvitamin D3 inhibits cytokine production by human blood monocytes at the post-transcriptional level. *Cytokine* 4, 506–512. doi: 10.1016/1043-4666(92)90012-G

Muller, M. R., and Rao, A. (2010). NFAT, immunity and cancer: a transcription factor comes of age. *Nat. Rev. Immunol.* 10, 645–656. doi: 10.1038/nri2818

Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S., and Ascherio, A. (2006). Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* 296, 2832–2838. doi: 10.1001/jama.296.23.2832

Munger, K. L., Zhang, S. M., O'Reilly, E., Hernan, M. A., Olek, M. J., Willett, W. C., et al. (2004). Vitamin D intake and incidence of multiple sclerosis. *Neurology* 62, 60–65. doi: 10.1212/01.WNL.0000101723.79681.38

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., et al. (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J. Biol. Chem.* 275, 32783–32792. doi: 10.1074/jbc.M003505200

Mutt, S. J., Karhu, T., Lehtonen, S., Lehenkari, P., Carlberg, C., Saarnio, J., et al. (2012). Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D(3) via the NF-kappaB pathway. *FASEB J.* 26, 4400–4407. doi: 10.1096/fj.12-210880

Nakagawa, K., Sasaki, Y., Kato, S., Kubodera, N., and Okano, T. (2005). 22-Oxa-1alpha,25-dihydroxyvitamin D3 inhibits metastasis and angiogenesis in lung cancer. *Carcinogenesis* 26, 1044–1054. doi: 10.1093/carcin/bgi049

Neve, A., Corrado, A., and Cantatore, F. P. (2013). Immunomodulatory effects of vitamin D in peripheral blood monocyte-derived macrophages from patients with rheumatoid arthritis. *Clin. Exp. Med.* doi: 10.1007/s10238-013-0249-2. [Epub ahead of print].

Nonn, L., Peng, L., Feldman, D., and Peehl, D. M. (2006). Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. *Cancer Res.* 66, 4516–4524. doi: 10.1158/0008-5472.CAN-05-3796

Oh, J., Riek, A. E., Weng, S., Petty, M., Kim, D., Colonna, M., et al. (2012). Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. *J. Biol. Chem.* 287, 11629–11641. doi: 10.1074/jbc.M111.338673

Ooi, J. H., Chen, J., and Cantorna, M. T. (2012). Vitamin D regulation of immune function in the gut: why do T cells have vitamin D receptors? *Mol. Aspects Med.* 33, 77–82. doi: 10.1016/j.mam.2011.10.014

Ozgocmen, S., Bulut, S., Ilhan, N., Gulkesen, A., Ardicoglu, O., and Ozkan, Y. (2005). Vitamin D deficiency and reduced bone mineral density in multiple sclerosis: effect of ambulatory status and functional capacity. *J. Bone Miner. Metab.* 23, 309–313. doi: 10.1007/s00774-005-0604-9

Panichi, V., De Pietro, S., Andreini, B., Bianchi, A. M., Migliori, M., Taccola, D., et al. (1998). Calcitriol modulates *in vivo* and *in vitro* cytokine production: a role for intracellular calcium. *Kidney Int.* 54, 1463–1469. doi: 10.1046/j.1523-1755.1998.00152.x

Pedersen, A. W., Holmstrom, K., Jensen, S. S., Fuchs, D., Rasmussen, S., Kvistborg, P., et al. (2009). Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells. *Clin. Exp. Immunol.* 157, 48–59. doi: 10.1111/j.1365-2249.2009.03961.x

Pedersen, L. B., Nashold, F. E., Spach, K. M., and Hayes, C. E. (2007). 1,25-dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by inhibiting chemokine synthesis and monocyte trafficking. *J. Neurosci. Res.* 85, 2480–2490. doi: 10.1002/jnr.21382

Penna, G., and Adorini, L. (2000). 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* 164, 2405–2411. doi: 10.4049/jimmunol.164.5.2405

Penna, G., Fibbi, B., Amuchastegui, S., Corsiero, E., Laverny, G., Silvestrini, E., et al. (2009). The vitamin D receptor agonist elocalcitol inhibits IL-8-dependent

benign prostatic hyperplasia stromal cell proliferation and inflammatory response by targeting the RhoA/Rho kinase and NF-kappaB pathways. *Prostate* 69, 480–493. doi: 10.1002/pros.20896

Peric, M., Koglin, S., Kim, S. M., Morizane, S., Besch, R., Prinz, J. C., et al. (2008). IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J. Immunol.* 181, 8504–8512. doi: 10.4049/jimmunol.181.12.8504

Petrovic, N., Knight, D. A., Bomalaski, J. S., Thompson, P. J., and Misso, N. L. (2006). Concomitant activation of extracellular signal-regulated kinase and induction of COX-2 stimulates maximum prostaglandin E2 synthesis in human airway epithelial cells. *Prostaglandins Other Lipid Mediat.* 81, 126–135. doi: 10.1016/j.prostaglandins.2006.08.006

Pike, K. C., Inskip, H. M., Robinson, S., Lucas, J. S., Cooper, C., Harvey, N. C., et al. (2012). Maternal late-pregnancy serum 25-hydroxyvitamin D in relation to childhood wheeze and atopic outcomes. *Thorax* 67, 950–956. doi: 10.1136/thoraxjnl-2012-201888

Pillai, D. K., Iqbal, S. F., Benton, A. S., Lerner, J., Wiles, A., Foerster, M., et al. (2011). Associations between genetic variants in vitamin D metabolism and asthma characteristics in young African Americans: a pilot study. *J. Investig. Med.* 59, 938–946. doi: 10.231/JIM.0b013e318220df41

Poon, A. H., Laprise, C., Lemire, M., Montpetit, A., Sinnett, D., Schurr, E., et al. (2004). Association of vitamin D receptor genetic variants with susceptibility to asthma and atopy. *Am. J. Respir. Crit. Care Med.* 170, 967–973. doi: 10.1164/rccm.200403-412OC

Portales-Perez, D., Gonzalez-Amaro, R., Abud-Mendoza, C., and Sanchez-Armass, S. (1997). Abnormalities in CD69 expression, cytosolic pH and Ca2+ during activation of lymphocytes from patients with systemic lupus erythematosus. *Lupus* 6, 48–56. doi: 10.1177/096120339700600107

Prabhu Anand, S., Selvaraj, P., and Narayanan, P. R. (2009). Effect of 1,25 dihydroxyvitamin D3 on intracellular IFN-gamma and TNF-alpha positive T cell subsets in pulmonary tuberculosis. *Cytokine* 45, 105–110. doi: 10.1016/j.cyto.2008.11.004

Prehn, J. L., Fagan, D. L., Jordan, S. C., and Adams, J. S. (1992). Potentiation of lipopolysaccharide-induced tumor necrosis factor-alpha expression by 1,25-dihydroxyvitamin D3. *Blood* 80, 2811–2816.

Prehn, J. L., and Jordan, S. C. (1989). Incubation of T cell or monocytic cell lines with 1,25-dihydroxyvitamin D3 before mitogen stimulation potentiates IL-2 and IL-1 beta mRNA levels. *Transplant. Proc.* 21, 90–91.

Qin, X., Corriere, M. A., Matrisian, L. M., and Guzman, R. J. (2006). Matrix metalloproteinase inhibition attenuates aortic calcification. *Arterioscler. Thromb. Vasc. Biol.* 26, 1510–1516. doi: 10.1161/01.ATV.0000225807.76419.a7

Raby, B. A., Lazarus, R., Silverman, E. K., Lake, S., Lange, C., Wjst, M., et al. (2004). Association of vitamin D receptor gene polymorphisms with childhood and adult asthma. *Am. J. Respir. Crit. Care Med.* 170, 1057–1065. doi: 10.1164/rccm.200404-447OC

Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., et al. (1999). Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 398, 824–828. doi: 10.1038/19783

Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360. doi: 10.1101/gr.107920.110

Ramalho, T. C., Rocha, M., Da Cunha, E. F., and Freitas, M. P. (2009). The search for new COX-2 inhibitors: a review of 2002 - 2008 patents. *Expert Opin. Ther. Pat.* 19, 1193–1228. doi: 10.1517/13543770903059125

Rausch-Fan, X., Leutmezer, F., Willheim, M., Spittler, A., Bohle, B., Ebner, C., et al. (2002). Regulation of cytokine production in human peripheral blood mononuclear cells and allergen-specific th cell clones by 1alpha,25-dihydroxyvitamin D3. *Int. Arch. Allergy Immunol.* 128, 33–41. doi: 10.1159/000058001

Reichel, H., Koeffler, H. P., Tobler, A., and Norman, A. W. (1987). 1 alpha,25-Dihydroxyvitamin D3 inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 84, 3385–3389. doi: 10.1073/pnas.84.10.3385

Reis, J. P., Von Muhlen, D., Michos, E. D., Miller, E. R. 3rd, Appel, L. J., Araneta, M. R., et al. (2009). Serum vitamin D, parathyroid hormone levels, and carotid atherosclerosis. *Atherosclerosis* 207, 585–590. doi: 10.1016/j.atherosclerosis.2009.05.030

Riancho, J. A., Zarrabeitia, M. T., De Francisco, A. L., Amado, J. A., Napal, J., Arias, M., et al. (1993). Vitamin D therapy modulates cytokine secretion in patients with renal failure. *Nephron* 65, 364–368. doi: 10.1159/000187514

Richart, T., Li, Y., and Staessen, J. A. (2007). Renal versus extrarenal activation of vitamin D in relation to atherosclerosis, arterial stiffening, and hypertension. *Am. J. Hypertens.* 20, 1007–1015. doi: 10.1016/j.amjhyper.2007.03.017

Riek, A. E., Oh, J., and Bernal-Mizrachi, C. (2013a). 1,25(OH)2 vitamin D suppresses macrophage migration and reverses atherogenic cholesterol metabolism in type 2 diabetic patients. *J. Steroid Biochem. Mol. Biol.* 136, 309–312. doi: 10.1016/j.jsbmb.2012.12.019

Riek, A. E., Oh, J., Darwech, I., Moynihan, C. E., Bruchas, R. R., and Bernal-Mizrachi, C. (2013b). 25(OH) vitamin D suppresses macrophage adhesion and migration by downregulation of ER stress and scavenger receptor A1 in type 2 diabetes. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.10.016. [Epub ahead of print].

Rigby, W. F., Denome, S., and Fanger, M. W. (1987). Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. *J. Clin. Invest.* 79, 1659–1664. doi: 10.1172/JCI113004

Rigby, W. F., Hamilton, B. J., and Waugh, M. G. (1990). 1,25-Dihydroxyvitamin D3 modulates the effects of interleukin 2 independent of IL-2 receptor binding. *Cell. Immunol.* 125, 396–414. doi: 10.1016/0008-8749(90)90094-8

Rigby, W. F., Stacy, T., and Fanger, M. W. (1984). Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol). *J. Clin. Invest.* 74, 1451–1455. doi: 10.1172/JCI111557

Rostkowska-Nadolska, B., Sliupkas-Dyrda, E., Potyka, J., Kusmierz, D., Fraczek, M., Krecicki, T., et al. (2010). Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and interleukin-8 expression in human nasal polyp fibroblast cultures. *Adv. Med. Sci.* 55, 86–92. doi: 10.2478/v10039-010-0012-9

Rothers, J., Wright, A. L., Stern, D. A., Halonen, M., and Camargo, C. A. Jr. (2011). Cord blood 25-hydroxyvitamin D levels are associated with aeroallergen sensitization in children from Tucson, Arizona. *J. Allergy Clin. Immunol.* 128, 1093.e5–1099.e5. doi: 10.1016/j.jaci.2011.07.015

Ryynanen, J., and Carlberg, C. (2013). Primary 1,25-dihydroxyvitamin D3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells. *PLoS ONE* 8:e78170. doi: 10.1371/journal.pone.0078170

Saadi, A., Gao, G., Li, H., Wei, C., Gong, Y., and Liu, Q. (2009). Association study between vitamin D receptor gene polymorphisms and asthma in the Chinese Han population: a case-control study. *BMC Med. Genet.* 10:71. doi: 10.1186/1471-2350-10-71

Sachs, M. C., Brunzell, J. D., Cleary, P. A., Hoofnagle, A. N., Lachin, J. M., Molitch, M. E., et al. (2013). Circulating vitamin D metabolites and subclinical atherosclerosis in type 1 diabetes. *Diabetes Care* 36, 2423–2429. doi: 10.2337/dc12-2020

Saggese, G., Federico, G., Balestri, M., and Toniolo, A. (1989). Calcitriol inhibits the PHA-induced production of IL-2 and IFN-gamma and the proliferation of human peripheral blood leukocytes while enhancing the surface expression of HLA class II molecules. *J. Endocrinol. Invest.* 12, 329–335. doi: 10.1007/BF03349999

Schnatz, P. F., Nudy, M., O'Sullivan, D. M., Jiang, X., Cline, J. M., Kaplan, J. R., et al. (2012a). Coronary artery vitamin D receptor expression and plasma concentrations of 25-hydroxyvitamin D: their association with atherosclerosis. *Menopause* 19, 967–973. doi: 10.1097/gme.0b013e31824cfa8f

Schnatz, P. F., Nudy, M., O'Sullivan, D. M., Jiang, X., Cline, J. M., Kaplan, J. R., et al. (2012b). The quantification of vitamin D receptors in coronary arteries and their association with atherosclerosis. *Maturitas* 73, 143–147. doi: 10.1016/j.maturitas.2012.03.014

Schwalfenberg, G. K. (2011). A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol. Nutr. Food Res.* 55, 96–108. doi: 10.1002/mnfr.201000174

Searing, D. A., Zhang, Y., Murphy, J. R., Hauk, P. J., Goleva, E., and Leung, D. Y. (2010). Decreased serum vitamin D levels in children with asthma are associated with increased corticosteroid use. *J. Allergy Clin. Immunol.* 125, 995–1000. doi: 10.1016/j.jaci.2010.03.008

Selvaraj, P., Vidyarani, M., Alagarasu, K., Prabhu Anand, S., and Narayanan, P. R. (2008). Regulatory role of promoter and 3' UTR variants of vitamin D receptor gene on cytokine response in pulmonary tuberculosis. *J. Clin. Immunol.* 28, 306–313. doi: 10.1007/s10875-007-9152-5

Shanker, J., Maitra, A., Arvind, P., Nair, J., Dash, D., Manchiganti, R., et al. (2011). Role of vitamin D levels and vitamin D receptor polymorphisms in relation to coronary artery disease: the Indian atherosclerosis research study. *Coron. Artery Dis.* 22, 324–332. doi: 10.1097/MCA.0b013e3283472a57

Shapira, Y., Agmon-Levin, N., and Shoenfeld, Y. (2010). Geoepidemiology of autoimmune rheumatic diseases. *Nat. Rev. Rheumatol.* 6, 468–476. doi: 10.1038/nrrheum.2010.86

Sheppard, D. (2006). Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc. Am. Thorac. Soc.* 3, 413–417. doi: 10.1513/pats.200601-008AW

Sochorova, K., Budinsky, V., Rozkova, D., Tobiasova, Z., Dusilova-Sulkova, S., Spisek, R., et al. (2009). Paricalcitol (19-nor-1,25-dihydroxyvitamin D2) and calcitriol (1,25-dihydroxyvitamin D3) exert potent immunomodulatory effects on dendritic cells and inhibit induction of antigen-specific T cells. *Clin. Immunol.* 133, 69–77. doi: 10.1016/j.clim.2009.06.011

Song, G. G., Bae, S. C., and Lee, Y. H. (2012). Association between vitamin D intake and the risk of rheumatoid arthritis: a meta-analysis. *Clin. Rheumatol.* 31, 1733–1739. doi: 10.1007/s10067-012-2080-7

Sorg, B. L., Klan, N., Seuter, S., Dishart, D., Radmark, O., Habenicht, A., et al. (2006). Analysis of the 5-lipoxygenase promoter and characterization of a vitamin D receptor binding site. *Biochim. Biophys. Acta* 1761, 686–697. doi: 10.1016/j.bbalip.2006.04.005

Spach, K. M., and Hayes, C. E. (2005). Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. *J. Immunol.* 175, 4119–4126. doi: 10.4049/jimmunol.175.6.4119

Srviastava, M. D., Deluca, H., and Ambrus, J. L. (1994). Inhibition of IL-6 and IL-8 production in human fibroblast cell lines by 1,25 (OH)2 vitamin D3 and two of its analogs with lower calcemic activity. *Res. Commun. Chem. Pathol. Pharmacol.* 83, 145–150.

Stan, D., Calin, M., Manduteanu, I., Pirvulescu, M., Gan, A. M., Butoi, E. D., et al. (2011). High glucose induces enhanced expression of resistin in human U937 monocyte-like cell line by MAPK- and NF-kB-dependent mechanisms; the modulating effect of insulin. *Cell Tissue Res.* 343, 379–387. doi: 10.1007/s00441-010-1092-3

Stio, M., Martinesi, M., Bruni, S., Treves, C., Mathieu, C., Verstuyf, A., et al. (2007). The Vitamin D analogue TX 527 blocks NF-kappaB activation in peripheral blood mononuclear cells of patients with Crohn's disease. *J. Steroid Biochem. Mol. Biol.* 103, 51–60. doi: 10.1016/j.jsbmb.2006.07.008

Stoffers, K. L., Sorg, B. L., Seuter, S., Rau, O., Radmark, O., and Steinhilber, D. (2010). Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. *J. Mol. Biol.* 395, 884–896. doi: 10.1016/j.jmb.2009.10.022

Stojanovic, O. I., Lazovic, M., Lazovic, M., and Vuceljic, M. (2011). Association between atherosclerosis and osteoporosis, the role of vitamin D. *Arch. Med. Sci.* 7, 179–188. doi: 10.5114/aoms.2011.22066

Subramaniam, N., Leong, G. M., Cock, T. A., Flanagan, J. L., Fong, C., Eisman, J. A., et al. (2001). Cross-talk between 1,25-dihydroxyvitamin D3 and transforming growth factor-beta signaling requires binding of VDR and Smad3 proteins to their cognate DNA recognition elements. *J. Biol. Chem.* 276, 15741–15746. doi: 10.1074/jbc.M011033200

Summerday, N. M., Brown, S. J., Allington, D. R., and Rivey, M. P. (2012). Vitamin D and multiple sclerosis: review of a possible association. *J. Pharm. Pract.* 25, 75–84. doi: 10.1177/0897190011421839

Sun, J., Zhong, W., Gu, Y., Groome, L. J., and Wang, Y. (2013). 1,25(OH)D suppresses COX-2 up-regulation and thromboxane production in placental trophoblast cells in response to hypoxic stimulation. *Placenta* 35, 143–145. doi: 10.1016/j.placenta.2013.12.002

Sun, X., and Zemel, M. B. (2008). Calcitriol and calcium regulate cytokine production and adipocyte-macrophage cross-talk. *J. Nutr. Biochem.* 19, 392–399. doi: 10.1016/j.jnutbio.2007.05.013

Sutherland, E. R., Goleva, E., Jackson, L. P., Stevens, A. D., and Leung, D. Y. (2010). Vitamin D levels, lung function, and steroid response in adult asthma. *Am. J. Respir. Crit. Care Med.* 181, 699–704. doi: 10.1164/rccm.200911-1710OC

Szekely, J. I., and Pataki, A. (2012). Effects of vitamin D on immune disorders with special regard to asthma, COPD and autoimmune diseases: a short review. *Expert Rev. Respir. Med.* 6, 683–704. doi: 10.1586/ers.12.57

Szeto, F. L., Reardon, C. A., Yoon, D., Wang, Y., Wong, K. E., Chen, Y., et al. (2012). Vitamin D receptor signaling inhibits atherosclerosis in mice. *Mol. Endocrinol.* 26, 1091–1101. doi: 10.1210/me.2011-1329

Tabata, T., Shoji, T., Kikunami, K., Matsushita, Y., Inoue, T., Tanaka, S., et al. (1988). *In vivo* effect of 1 alpha-hydroxyvitamin D3 on interleukin-2 production in hemodialysis patients. *Nephron* 50, 295–298. doi: 10.1159/000185191

Taimi, M., Defacque, H., Commes, T., Favero, J., Caron, E., Marti, J., et al. (1993). Effect of retinoic acid and vitamin D on the expression of interleukin-1 beta, tumour necrosis factor-alpha and interleukin-6 in the human monocytic cell line U937. *Immunology* 79, 229–235.

Takahashi, K., Nakayama, Y., Horiuchi, H., Ohta, T., Komoriya, K., Ohmori, H., et al. (2002). Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3. *Immunopharmacol. Immunotoxicol.* 24, 335–347. doi: 10.1081/IPH-120014721

Takeda, M., Yamashita, T., Sasaki, N., Nakajima, K., Kita, T., Shinohara, M., et al. (2010). Oral administration of an active form of vitamin D3 (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. *Arterioscler. Thromb. Vasc. Biol.* 30, 2495–2503. doi: 10.1161/ATVBAHA.110.215459

Takeuchi, A., Reddy, G. S., Kobayashi, T., Okano, T., Park, J., and Sharma, S. (1998). Nuclear factor of activated T cells (NFAT) as a molecular target for 1alpha,25-dihydroxyvitamin D3-mediated effects. *J. Immunol.* 160, 209–218.

Talmor-Barkan, Y., Bernheim, J., Green, J., Benchetrit, S., and Rashid, G. (2011). Calcitriol counteracts endothelial cell pro-inflammatory processes in a chronic kidney disease-like environment. *J. Steroid Biochem. Mol. Biol.* 124, 19–24. doi: 10.1016/j.jsbmb.2011.01.001

Taura, S., Taura, M., Kamio, A., and Kummerow, F. A. (1979). Vitamin D-induced coronary atherosclerosis in normolipemic swine: comparison with human disease. *Tohoku J. Exp. Med.* 129, 9–16. doi: 10.1620/tjem.129.9

Tavakoli, M., Jeddi-Tehrani, M., Salek-Moghaddam, A., Rajaei, S., Mohammadzadeh, A., Sheikhhasani, S., et al. (2011). Effects of 1,25(OH)2 vitamin D3 on cytokine production by endometrial cells of women with recurrent spontaneous abortion. *Fertil. Steril.* 96, 751–757. doi: 10.1016/j.fertnstert.2011.06.075

Thill, M., Becker, S., Fischer, D., Cordes, T., Hornemann, A., Diedrich, K., et al. (2009). Expression of prostaglandin metabolising enzymes COX-2 and 15-PGDH and VDR in human granulosa cells. *Anticancer Res.* 29, 3611–3618.

Thill, M., Fischer, D., Kelling, K., Hoellen, F., Dittmer, C., Hornemann, A., et al. (2010). Expression of vitamin D receptor (VDR), cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in benign and malignant ovarian tissue and 25-hydroxycholecalciferol (25(OH2)D3) and prostaglandin E2 (PGE2) serum level in ovarian cancer patients. *J. Steroid Biochem. Mol. Biol.* 121, 387–390. doi: 10.1016/j.jsbmb.2010.03.049

Thill, M., Hoellen, F., Becker, S., Dittmer, C., Fischer, D., Kummel, S., et al. (2012). Expression of prostaglandin- and vitamin D-metabolising enzymes in benign and malignant breast cells. *Anticancer Res.* 32, 367–372.

Thota, C., Farmer, T., Garfield, R. E., Menon, R., and Al-Hendy, A. (2013). Vitamin D elicits anti-inflammatory response, inhibits contractile-associated proteins, and modulates Toll-like receptors in human myometrial cells. *Reprod. Sci.* 20, 463–475. doi: 10.1177/1933719112459225

Toda, T., Ito, M., Toda, Y., Smith, T., and Kummerow, F. (1985). Angiotoxicity in swine of a moderate excess of dietary vitamin D3. *Food Chem. Toxicol.* 23, 585–592. doi: 10.1016/0278-6915(85)90183-8

Toda, T., Leszczynski, D. E., and Kummerow, F. A. (1983). The role of 25-hydroxy-vitamin D3 in the induction of atherosclerosis in swine and rabbit by hypervitaminosis D. *Acta Pathol. Jpn.* 33, 37–44.

Tolppanen, A. M., Sayers, A., Granell, R., Fraser, W. D., Henderson, J., and Lawlor, D. A. (2013). Prospective association of 25-hydroxyvitamin d3 and d2 with childhood lung function, asthma, wheezing, and flexural dermatitis. *Epidemiology* 24, 310–319. doi: 10.1097/EDE.obo13e318280dd5e

Tomasoni, S., Noris, M., Zappella, S., Gotti, E., Casiraghi, F., Bonazzola, S., et al. (1998). Upregulation of renal and systemic cyclooxygenase-2 in patients with active lupus nephritis. *J. Am. Soc. Nephrol.* 9, 1202–1212.

Tsatsanis, C., Androulidaki, A., Venihaki, M., and Margioris, A. N. (2006). Signalling networks regulating cyclooxygenase-2. *Int. J. Biochem. Cell Biol.* 38, 1654–1661. doi: 10.1016/j.biocel.2006.03.021

Tsoukas, C. D., Provvedini, D. M., and Manolagas, S. C. (1984). 1,25-dihydroxyvitamin D3: a novel immunoregulatory hormone. *Science* 224, 1438–1440. doi: 10.1126/science.6427926

Tsoukas, C. D., Watry, D., Escobar, S. S., Provvedini, D. M., Dinarello, C. A., Hustmyer, F. G., et al. (1989). Inhibition of interleukin-1 production

by 1,25-dihydroxyvitamin D3. *J. Clin. Endocrinol. Metab.* 69, 127–133. doi: 10.1210/jcem-69-1-127

Tuoresmäki, P., Vaisanen, S., Neme, A., Heikkinen, S., and Carlberg, C. (2014). Patterns of genome-wide VDR locations. *PLoS ONE* 9:e96105. doi: 10.1371/journal.pone.0096105

Ueki, N., and Hayman, M. J. (2003). Signal-dependent N-CoR requirement for repression by the Ski oncoprotein. *J. Biol. Chem.* 278, 24858–24864. doi: 10.1074/jbc.M303447200

Urry, Z., Chambers, E. S., Xystrakis, E., Dimeloe, S., Richards, D. F., Gabrysova, L., et al. (2012). The role of 1alpha,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+ and IL-10+ CD4+ T cells. *Eur. J. Immunol.* 42, 2697–2708. doi: 10.1002/eji.201242370

Van Etten, E., Dardenne, O., Gysemans, C., Overbergh, L., and Mathieu, C. (2004). 1,25-Dihydroxyvitamin D3 alters the profile of bone marrow-derived dendritic cells of NOD mice. *Ann. N.Y. Acad. Sci.* 1037, 186–192. doi: 10.1196/annals.1337.030

Van Etten, E., Verlinden, L., Giulietti, A., Ramos-Lopez, E., Branisteanu, D. D., Ferreira, G. B., et al. (2007). The vitamin D receptor gene FokI polymorphism: functional impact on the immune system. *Eur. J. Immunol.* 37, 395–405. doi: 10.1002/eji.200636043

Van Oeffelen, A. A., Bekkers, M. B., Smit, H. A., Kerkhof, M., Koppelman, G. H., Haveman-Nies, A., et al. (2011). Serum micronutrient concentrations and childhood asthma: the PIAMA birth cohort study. *Pediatr. Allergy Immunol.* 22, 784–793. doi: 10.1111/j.1399-3038.2011.01190.x

Verway, M., Bouttier, M., Wang, T. T., Carrier, M., Calderon, M., An, B. S., et al. (2013). Vitamin D induces interleukin-1beta expression: paracrine macrophage epithelial signaling controls *M. tuberculosis* infection. *PLoS Pathog* 9:e1003407. doi: 10.1371/journal.ppat.1003407

Villaggio, B., Soldano, S., and Cutolo, M. (2012). 1,25-dihydroxyvitamin D3 downregulates aromatase expression and inflammatory cytokines in human macrophages. *Clin. Exp. Rheumatol.* 30, 934–938.

Vollmert, C., Illig, T., Altmuller, J., Klugbauer, S., Loesgen, S., Dumitrescu, L., et al. (2004). Single nucleotide polymorphism screening and association analysis–exclusion of integrin beta 7 and vitamin D receptor (chromosome 12q) as candidate genes for asthma. *Clin. Exp. Allergy* 34, 1841–1850. doi: 10.1111/j.1365-2222.2004.02047.x

Wamberg, L., Cullberg, K. B., Rejnmark, L., Richelsen, B., and Pedersen, S. B. (2013). Investigations of the anti-inflammatory effects of vitamin D in adipose tissue: results from an *in vitro* study and a randomized controlled trial. *Horm. Metab. Res.* 45, 456–462. doi: 10.1055/s-0032-1331746

Weng, S., Sprague, J. E., Oh, J., Riek, A. E., Chin, K., Garcia, M., et al. (2013). Vitamin D deficiency induces high blood pressure and accelerates atherosclerosis in mice. *PLoS ONE* 8:e54625. doi: 10.1371/journal.pone.0054625

Willheim, M., Thien, R., Schrattbauer, K., Bajna, E., Holub, M., Gruber, R., et al. (1999). Regulatory effects of 1alpha,25-dihydroxyvitamin D3 on the cytokine production of human peripheral blood lymphocytes. *J. Clin. Endocrinol. Metab.* 84, 3739–3744.

Wingerchuk, D. M., Lesaux, J., Rice, G. P., Kremenchutzky, M., and Ebers, G. C. (2005). A pilot study of oral calcitriol (1,25-dihydroxyvitamin D3) for relapsing-remitting multiple sclerosis. *J. Neurol. Neurosurg. Psychiatr.* 76, 1294–1296. doi: 10.1136/jnnp.2004.056499

Wobke, T. K., Von Knethen, A., Steinhilber, D., and Sorg, B. L. (2013). CD69 is a TGF-beta/1alpha,25-dihydroxyvitamin D3 target gene in monocytes. *PLoS ONE* 8, e64635. doi: 10.1371/journal.pone.0064635

Wu, A. C., Tantisira, K., Li, L., Fuhlbrigge, A. L., Weiss, S. T., and Litonjua, A. (2012). Effect of vitamin D and inhaled corticosteroid treatment on lung function in children. *Am. J. Respir. Crit. Care Med.* 186, 508–513. doi: 10.1164/rccm.201202-0351OC

Xiao, H. Q., Shi, W., Liu, S. X., Zhang, B., Xu, L. X., Liang, X. L., et al. (2009). Podocyte injury is suppressed by 1,25-dihydroxyvitamin D via modulation of transforming growth factor-beta 1/bone morphogenetic protein-7 signalling in puromycin aminonucleoside nephropathy rats. *Clin. Exp. Pharmacol. Physiol.* 36, 682–689. doi: 10.1111/j.1440-1681.2008.05133.x

Yadav, A. K., Banerjee, D., Lal, A., and Jha, V. (2012). Vitamin D deficiency, CD4+CD28null cells and accelerated atherosclerosis in chronic kidney disease. *Nephrology (Carlton)* 17, 575–581. doi: 10.1111/j.1440-1797.2012.01611.x

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., et al. (1999). Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321. doi: 10.1126/science.283.5406.1317

Yang, J., Han, Y., Chen, C., Sun, H., He, D., Guo, J., et al. (2013). EGCG attenuates high glucose-induced endothelial cell inflammation by suppression of PKC and NF-kappaB signaling in human umbilical vein endothelial cells. *Life Sci.* 92, 589–597. doi: 10.1016/j.lfs.2013.01.025

Yoon, J. W., and Jun, H. S. (1999). Cellular and molecular roles of beta cell autoantigens, macrophages and T cells in the pathogenesis of autoimmune diabetes. *Arch. Pharm. Res.* 22, 437–447. doi: 10.1007/BF02979150

Yu, X. P., Bellido, T., and Manolagas, S. C. (1995). Down-regulation of NF-kappa B protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10990–10994. doi: 10.1073/pnas.92.24.10990

Yuan, L., Jiang, R., Yang, Y., Ding, S., and Deng, H. (2012). 1,25-Dihydroxyvitamin D3 inhibits growth of the breast cancer cell line MCF-7 and downregulates cytochrome P4501B1 through the COX-2/PGE2 pathway. *Oncol. Rep.* 28, 2131–2137. doi: 10.3892/or.2012.2031

Zarrabeitia, M. T., Riancho, J. A., Amado, J. A., Olmos, J. M., and Gonzalez-Macias, J. (1992). Effect of calcitriol on the secretion of prostaglandin E2, interleukin 1, and tumor necrosis factor alpha by human monocytes. *Bone* 13, 185–189. doi: 10.1016/8756-3282(92)90010-T

Zemplenyi, T., and Mrhova, O. (1965). Vascular enzyme activity changes accompanying the induction of experimental atherosclerosis. 2. Rats fed excess vitamin D. *J. Atheroscler. Res.* 5, 548–555. doi: 10.1016/S0368-1319(65)80031-X

Zhang, H., Shih, D. Q., and Zhang, X. (2013a). Mechanisms underlying effects of 1,25-Dihydroxyvitamin D3 on the Th17 cells. *Eur. J. Microbiol. Immunol. (Bp).* 3, 237–240. doi: 10.1556/EuJMI.3.2013.4.1

Zhang, J. Z., Maruyama, K., Ono, I., Iwatsuki, K., and Kaneko, F. (1994). Regulatory effects of 1,25-dihydroxyvitamin D3 and a novel vitamin D3 analogue MC903 on secretion of interleukin-1 alpha (IL-1 alpha) and IL-8 by normal human keratinocytes and a human squamous cell carcinoma cell line (HSC-1). *J. Dermatol. Sci.* 7, 24–31. doi: 10.1016/0923-1811(94)90018-3

Zhang, Y., Leung, D. Y., and Goleva, E. (2013b). Vitamin D enhances glucocorticoid action in human monocytes: involvement of granulocyte-macrophage colony-stimulating factor and mediator complex subunit 14. *J. Biol. Chem.* 288, 14544–14553. doi: 10.1074/jbc.M112.427054

Zhang, Y., Leung, D. Y., Richers, B. N., Liu, Y., Remigio, L. K., Riches, D. W., et al. (2012). Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J. Immunol.* 188, 2127–2135. doi: 10.4049/jimmunol.1102412

Zhou, C., Lu, F., Cao, K., Xu, D., Goltzman, D., and Miao, D. (2008). Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice. *Kidney Int.* 74, 170–179. doi: 10.1038/ki.2008.101

Zipitis, C. S., and Akobeng, A. K. (2008). Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis. *Arch. Dis. Child.* 93, 512–517. doi: 10.1136/adc.2007.128579

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The impact of vitamin D in breast cancer: genomics, pathways, metabolism

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Nuclear receptors exert profound effects on mammary gland physiology and have complex roles in the etiology of breast cancer. In addition to receptors for classic steroid hormones such as estrogen and progesterone, the nuclear vitamin D receptor (VDR) interacts with its ligand 1α,25(OH)$_2$D$_3$ to modulate the normal mammary epithelial cell genome and subsequent phenotype. Observational studies suggest that vitamin D deficiency is common in breast cancer patients and that low vitamin D status enhances the risk for disease development or progression. Genomic profiling has characterized many 1α,25(OH)$_2$D$_3$ responsive targets in normal mammary cells and in breast cancers, providing insight into the molecular actions of 1α,25(OH)$_2$D$_3$ and the VDR in regulation of cell cycle, apoptosis, and differentiation. New areas of emphasis include regulation of tumor metabolism and innate immune responses. However, the role of VDR in individual cell types (i.e., epithelial, adipose, fibroblast, endothelial, immune) of normal and tumor tissues remains to be clarified. Furthermore, the mechanisms by which VDR integrates signaling between diverse cell types and controls soluble signals and paracrine pathways in the tissue/tumor microenvironment remain to be defined. Model systems of carcinogenesis have provided evidence that both VDR expression and 1α,25(OH)$_2$D$_3$ actions change with transformation but clinical data regarding vitamin D responsiveness of established tumors is limited and inconclusive. Because breast cancer is heterogeneous, analysis of VDR actions in specific molecular subtypes of the disease may help to clarify the conflicting data. The expanded use of genomic, proteomic and metabolomic approaches on a diverse array of *in vitro* and *in vivo* model systems is clearly warranted to comprehensively understand the network of vitamin D regulated pathways in the context of breast cancer.

Keywords: 1α,25(OH)$_2$D$_3$, vitamin D, calcitriol, VDR, breast cancer, genomics

INTRODUCTION TO BREAST CANCER

In the United States in 2013, breast cancer was estimated to account for 29% of new cancer cases and 14% of cancer deaths in women, making it the most common cancer diagnosed and the second most common cause of cancer mortality in women. While standard tumor pathology focuses on the presence or absence of hormone (estrogen, progesterone) and growth factor (HER2) receptors, it is now clear that breast cancer is an extremely heterogeneous disease. Genomic profiling has identified several molecularly defined sub-types of breast cancer including Luminal A, Luminal B, Basal, HER2, and Claudin-low (Cancer Genome Atlas Network, 2012). The most frequently diagnosed sub-type is Luminal A (51%), followed by Luminal B (29%), Basal (17%), HER2 (12.5%), and Claudin-low (2%). The importance of these molecular subtypes cannot be underestimated as they allow for prediction of therapeutic targets and they display distinct clinical courses (Caan et al., 2014). Women whose tumors fit the Luminal A profile have the best prognosis whereas those whose tumors have Luminal B or Basal profiles have poor prognosis. Although many trials have assessed the impact of nutrients, including vitamin D, on breast cancer risk and progression, few have been designed to stratify results by molecular sub-type. This review will highlight the cumulative data on vitamin D actions in breast cancer while emphasizing the gaps in knowledge regarding its effects on specific molecular subtypes.

OBSERVATIONAL AND INTERVENTION STUDIES ON VITAMIN D AND BREAST CANCER

Population studies on vitamin D in relation to chronic diseases such as breast cancer are complicated by difficulties in accurately assessing dietary sources (confounders include natural foods vs. fortified foods, supplement use, intake of D$_2$ vs. D$_3$, and calcium status) and in estimating the amount of vitamin D$_3$ generated through sunlight exposure (confounders include lifestyle, latitude, pollution, sunscreen, skin pigmentation, and age). Thus, it is not surprising that studies designed to address the impact of vitamin D status on breast cancer have yielded mixed results. While much data is supportive that high vitamin D status as

measured by serum 25-hydroxyvitamin D ($25(OH)D_3$) is associated with decreased risk of breast cancer (Bauer et al., 2013; Bilinski and Boyages, 2013; Wang et al., 2013; Kim et al., 2014), longer disease free survival and reduced mortality (Rose et al., 2013; Maalmi et al., 2014; Mohr et al., 2014), some large studies have failed to support these associations (Kuhn et al., 2013). With respect to endogenous synthesis of vitamin D_3, small scale studies supported the concept that sunlight exposure is associated with reduced risk of breast cancer, however, the associations were dependent on region of residence and skin pigmentation (John et al., 1999, 2007). Larger international studies have consistently demonstrated significant inverse correlations between incident solar radiation and breast cancer rates (Edvardsen et al., 2011; Engel et al., 2011, 2014; Grant, 2013; van der Rhee et al., 2013).

Data from randomized controlled trials (RCTs) of vitamin D supplementation in relation to breast cancer development have been inconclusive, with only slight benefits of supplementation sometimes observed (Sperati et al., 2013; Redaniel et al., 2014). The large (>30,000 women) Women's Health Initiative (WHI) trial assessed the impact of supplementation with both calcium and vitamin D on multiple health outcomes including cancer. However, the data from this trial was confounded by the low dose of vitamin D (400 IU/day), coadministration of calcium supplements, poor compliance, extensive pre-trial supplement use in the study population and the freedom for trial participants to take additional personal supplements of up to 1000 IU vitamin D per day. Thus it was not surprising that initial data from the WHI trial indicated no significant effects of vitamin D plus calcium supplementation on breast cancer. Subgroup and follow-up analyses of trial participants have yielded mixed results. One report indicated that higher intake of vitamin D was moderately associated with a lower risk of pre- but not post- menopausal breast cancer (Lin et al., 2007). In another sub-group analysis (including only women who were not taking personal calcium or vitamin D supplements at randomization), risk of invasive breast cancers was decreased in women supplemented with calcium and vitamin D (Bolland et al., 2011). The most recent analysis of all WHI diet study participants (assessed 5 years after intervention ended) indicated a reduced incidence of *in situ* breast cancers in the calcium and vitamin D cohort but also suggested that women with the highest vitamin D intakes may have an increased risk of invasive breast cancer (Cauley et al., 2013). Further sub-group analysis based on menopausal status or molecular subtype may shed light on these discrepancies. Regardless, these inconsistent data emphasize the continued need for rigorous, well-designed RCTs to specifically assess the impact of vitamin D supplementation on breast cancer development. Of note, the ongoing VITAL trial of 20,000 men and women taking daily supplements of 2000 IU vitamin D_3 (http://www.vitalstudy.org/) is monitoring breast cancer as one outcome.

As discussed above, genomic studies have demonstrated that breast cancers are heterogeneous and that different subtypes exhibit distinct patterns of disease progression. It is likely that VDR expression or function and thus sensitivity to changes in vitamin D status may be subtype specific, yet this has not rigorously been examined. The limited epidemiologic data that has been stratified by subtype is mixed - one study reported that the relationship between serum 25(OH)D and reduced risk of breast cancer was strongest for high grade, ER negative or triple negative cancers (Yao and Ambrosone, 2013) whereas another found that low serum 25(OH)D was associated with poor prognosis only in women with the luminal subtype of breast cancer (Kim et al., 2011). It should be noted that while vitamin D deficiency is common in all breast cancer patient populations, it is particularly prevalent in those with triple negative/basal-like tumors, the most aggressive form of the disease (Rainville et al., 2009; Peppone et al., 2012; Yao and Ambrosone, 2013). Even without rigorous "proof" of a beneficial effect of supplemental vitamin D on breast cancer, correction of vitamin D deficiency in women at risk for, or living with, breast cancer should be standard practice.

VITAMIN D PATHWAY EXPRESSION IN NORMAL MAMMARY CELLS AND BREAST CANCER PREVENTION

The VDR is present in rat, mouse and human mammary gland and its expression is highest during puberty, pregnancy and lactation (Berger et al., 1987; Zinser et al., 2002). In actively growing murine mammary ducts, VDR expression is high in the ductal epithelium and low in the proliferating terminal end buds. Consistent with this murine data, high content immunohistochemistry of normal human breast epithelium demonstrated that VDR positive cells are exclusively found in the luminal (differentiated) cell layer and do not co-localize with proliferating Ki67 positive cells (Santagata et al., 2014). VDR has also been identified in the stromal compartment including the mammary fibroblasts and adipocytes (Ching et al., 2011; Campos et al., 2013; Knower et al., 2013), highlighting the complexity of $1,25(OH)_2D_3$ signaling in the breast tissue environment.

In addition to VDR, the 25(OH)D activating enzyme CYP27B1 is expressed in murine and human mammary tissue (Zinser and Welsh, 2004a; Townsend et al., 2005; Kemmis et al., 2006; Peng et al., 2009), suggesting that systemic 25(OH)D delivered to the mammary gland can be converted to the biologically active VDR ligand $1,25(OH)_2D_3$. *In vitro* studies confirm that incubation of mammary epithelial cells with physiological (nanomolar) concentrations of 25(OH)D leads to temporal increases in $1,25(OH)_2D_3$ detected in tissue culture media (Kemmis et al., 2006). Although there is still uncertainty regarding how 25(OH)D, which circulates tightly bound to the vitamin D binding protein (DBP), is internalized by non-renal cells, the presence of megalin and cubilin (Rowling et al., 2006) indicates that these accessory proteins could mediate uptake of 25(OH)D-DBP complexes in mammary gland as has been demonstrated for renal cells(Willnow and Nykjaer, 2002). Indeed, *in vitro* studies demonstrate that normal breast epithelial cells and some breast cancer cells internalize 25(OH)D via megalin-mediated endocytosis (Rowling et al., 2006), however, the function of this uptake pathway in intact mammary gland has yet to be confirmed. CYP27B1 is also expressed in mammary adipocytes, which too are capable of converting 25(OH)D to $1,25(OH)_2D_3$ in organoid culture (Ching et al., 2011). Collectively, these studies provide a biological link between vitamin D status [i.e., serum 25(OH)D] and breast cancer risk that is observed at the population level.

The functions of CYP27B1 and VDR in prevention of breast cancer are supported by data from animal models. *In vivo*, both

high dietary vitamin D (Jacobson et al., 1989) and treatment with synthetic VDR agonists (Hussain et al., 2003) inhibit the development of carcinogen induced mammary tumors. Furthermore, VDR ablation enhances the development of hyperplasias and hormone independent mammary tumors after DMBA administration, and VDR haploinsufficiency sensitizes the mammary gland to tumorigenesis driven by the neu oncogene (Zinser et al., 2002; Zinser and Welsh, 2004b). In organ culture models, VDR agonists such as 25(OH)D_3 and 1α,25(OH)$_2D_3$ reduce the incidence of DMBA induced pre-neoplastic lesions (Mehta et al., 1997; Peng et al., 2009) suggesting direct anti-cancer effects of these metabolites on mammary tissue. Collectively, these data support the concept that systemic 25(OH)D delivered to mammary gland is converted to 1,25(OH)$_2D_3$ which activates VDR to protect against carcinogenesis.

Despite these consistent data, the precise mechanisms by which vitamin D inhibits cancer development have yet to be discerned. Both 25(OH)D_3 and 1α,25(OH)$_2D_3$ exert profound effects on non-tumorigenic VDR positive mammary epithelial cells including inhibition of hormone stimulated growth and branching morphogenesis and induction of differentiation biomarkers such as E-cadherin. VDR and CYP27B1 expression in mammary adipocytes also contribute to the anti-cancer effects in the whole tissue, since in response to 25(OH)D adipocytes secrete diffusible signals that inhibit morphogenesis of the adjacent ductal epithelium (Ching et al., 2011). Other potential mechanisms for breast cancer prevention by vitamin D include reduction in DNA damage (possibly via up-regulation of p53 signaling), suppression of oxidative stress and inhibition of angiogenesis, many of which have been demonstrated in tissues or cell types other than mammary gland (Kallay et al., 2002; Peng et al., 2010; Bruce et al., 2011; Hopkins et al., 2011; Krishnan and Feldman, 2011; Bikle, 2012; Dogan et al., 2012; Gordon-Thomson et al., 2012; Ting et al., 2012; Alvarez et al., 2014; Nakai et al., 2014; Sun et al., 2014; Uberti et al., 2014; Zhong et al., 2014). In addition, recent data from our group and others implicate alteration of cellular energy metabolism and innate immune responses in the anti-cancer effects of vitamin D signaling on non-tumorigenic mammary epithelial cells as described below.

Although initially recognized by Otto Warburg more than 50 years ago (Warburg, 1956), attention has recently been re-focused on the role of cellular energy metabolism as a critical component of tumor initiation and progression. It is now recognized that many cancer cells preferentially rely on glycolysis to generate energy and macromolecules that are essential for rapid proliferation, even in the presence of normoxia. The metabolic switch (the "Warburg effect") from oxidative phosphorylation to aerobic glycolysis is triggered in normal cells by activation of oncogenes and/or loss of tumor suppressors which, in part, target glucose and glutamine metabolism. The best characterized oncogenic driver of the metabolic switch, myc, induces a cohort of genes that promote glycolysis and alter glutamine flux. Conversely, tumor suppressors such as p53 normally suppress glycolysis and enhance flux through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. p53 also increases intracellular glutamate which is shunted toward glutathione synthesis, enhancing antioxidant defense against reactive oxygen species. Recent studies support the concept that vitamin D might target energy utilization pathways in non-tumorigenic breast cells to prevent carcinogenesis. The regulation of cellular glucose metabolism by 1,25(OH)$_2D_3$ has been studied (Zheng et al., 2013) in pre-malignant mammary cells (MCF10A cells) compared to their ras transformed malignant derivatives (MCF10A-ras cells). In this model, 4-day 1,25(OH)$_2D_3$ treatment reduced flux of glucose through glycolysis in both MCF10A and MCF10A-ras cells, with a more pronounced effect in the transformed cells. 1,25(OH)$_2D_3$ also reduced the flux of glucose to acetyl-coA and oxaloacetate in both cell lines, suggesting a reduction in TCA cycle activity. The predicted consequences of these 1,25(OH)$_2D_3$ inducedchanges would be limitation in the availability of TCA-derived precursors for macromolecule synthesis, coincident with reduction in proliferation.

In microarray profiling of non-tumorigenic mammary epithelial cells (hTERT-HME1) cells, we identified SLC1A1, which encodes a plasma membrane glutamate transporter, and GLUL, which encodes glutamine synthetase (GS) as novel 1,25(OH)$_2D_3$ targets. We validated that 1,25(OH)$_2D_3$ increases SLC1A1 mRNA (>10-fold) and decreases GLUL mRNA (>4-fold) in these cells, and also demonstrated decreased expression of the cognate proteins GS and SLC1A1 by western blotting. These changes in metabolic gene/protein expression correlated with accumulation of glutathione and changes in respiratory capacity in 1,25(OH)$_2D_3$ treated cells. Furthermore, 1,25(OH)$_2D_3$ pretreatment hindered growth of hTERT-HME1 cells in glutamine-starved media and exogenous glutamine partially rescued 1,25D-mediated growth arrest. These findings are intriguing because: (a) glutamate uptake and glutamate transporters are enhanced during differentiation and deregulated in cancer cells; (b) SLC1A1 null mice exhibit GSH deficiency and high oxidative stress; (c) GS enzymatic activity is necessary for adaption of mammary cells to glutamine depletion; and (d) data compiled from The Human Protein Atlas indicates that SLC1A1 is reduced and GLUL is increased in human breast cancers relative to normal tissue. Thus, we propose that 1,25(OH)$_2D_3$ regulation of SLC1A1 and GLUL synergizes with p53 to alter metabolic flux, prevent the myc-driven metabolic switch and induce quiescence in normal mammary epithelial cells. Collectively, these emerging data demonstrating regulation of amino acid and glucose metabolism (Zheng et al., 2013) in mammary epithelial cells by 1,25(OH)$_2D_3$ provides another mechanism by which vitamin D may act to prevent carcinogenesis in normal breast tissue. These changes may be complemented by alterations in lipid and energy metabolism in adjacent stromal adipocytes by 1,25(OH)$_2D_3$ (Welsh et al., 2011; Narvaez et al., 2013).

Another emerging area in cancer prevention by vitamin D involves suppression of inflammation. We identified CD14, a component of the innate immune system, as the second most highly upregulated gene (second only to CYP24A1) in 1,25(OH)$_2D_3$ treated hTERT-HME1 cells. CD14 is a known VDR target in macrophages and other immune cells, but its regulation by 1,25(OH)$_2D_3$ in mammary cells has not been well studied. In contrast to macrophages where 1,25(OH)$_2D_3$ induces membrane bound CD14, both 1,25(OH)$_2D_3$ and 25(OH)D_3 induce the secretion of large quantities of soluble CD14 (sCD14) from

mammary epithelial cells. As a pattern recognition receptor, sCD14 binds lipopolysaccharide and contributes to protection against mastitis in mammary tissue (Lee et al., 2003; Zheng et al., 2006; Wall et al., 2009). The soluble form of CD14 is also secreted into human milk where it contributes to protection of the neonatal gut from infections (Vidal and Donnet-Hughes, 2008). However, even in the absence of infection or lactation, CD14 and other genes involved in innate immunity are highly induced during regression of the mammary gland after weaning (Stein et al., 2004). The role of CD14 during this period of glandular remodeling may be the recognition and disposal of apoptotic cells (Heidenreich, 1999; Devitt et al., 2004; Tennant et al., 2013). We speculate that vitamin D induction of soluble CD14 in mammary tissue inhibits activation of tissue resident macrophages, suppressing inflammation which is known to drive cancer development and progression(McMahon et al., 2011; Simpson and Brown, 2013). However, it remains to be determined whether vitamin D status regulates any of these proposed functions of CD14 in mammary tissue *in vivo*. One caveat to future experimentation on vitamin D regulation of CD14 is the apparent discordance between the human and murine genomes. $1,25(OH)_2D_3$ does not induce CD14 in the murine mammary epithelial cell line HC11 and CD14 gene expression is not altered in the mammary gland of VDRKO mice (Welsh, unpublished).

VITAMIN D PATHWAY EXPRESSION IN ESTABLISHED BREAST CANCER

Over 30 years ago, the recognition that VDR expression was retained in breast cancers prompted extensive studies to determine whether targeting VDR in tumors would provide therapeutic benefit. VDR expression is retained in the majority of rodent breast tumors, human breast cancers and established breast cancer cell lines (Colston et al., 1986; Buras et al., 1994; Zinser and Welsh, 2004b). In a study of 136 patients with primary breast cancer, it was found that women with VDR negative tumors relapsed significantly earlier than women with VDR positive tumors (Berger et al., 1991). Of note, some data suggests that receptor protein expression declines in highly aggressive tumors (Lopes et al., 2010). We reviewed the frequency of genomic VDR changes in human breast cancers using datasets publically available on The Cancer Genome Atlas (https://tcga-data.nci.nih.gov/tcga/) which annotates mutations, amplifications, deletions and mRNA expression profiles in human tumors (**Table 1**). Analysis of the TCGA invasive breast cancer dataset (Cancer Genome Atlas Network, 2012) of over 450 breast tumors suggests that alterations in the VDR gene are rare in human breast cancer. As shown in **Table 1**, only 5% of human breast tumors exhibited any alteration in VDR sequence or expression. However, when the VDR gene was altered, the most common change was a reduction in mRNA expression (deletions and mutations did not occur). With respect to VDR expression in specific molecular sub-types, the Luminal B subtype had the highest frequency of VDR alterations with 10.5% of tumors displaying reduced VDR mRNA expression compared to 0–3% for Luminal A, Basal, HER2, or Claudin-Low subtypes. These results showing retention of VDR in the majority of human breast tumors are consistent with the data of Santagata et al. (2014) who used a multiplex immunohistochemical approach to map receptor proteins at the single cell level and confirmed that the majority of human breast tumors are VDR positive. Interestingly, this study also demonstrated that breast tumors with the highest expression of VDR, ER, and Androgen Receptor (AR) had the best prognosis. The retention of VDR in tumors may indicate that its function has been somehow abrogated, either by altered function of the VDR despite mutation (i.e., alteration of transcriptional co-regulators), reduced ligand availability (i.e., loss of CYP27B1 and/or gain of CYP24A1), or mutation/deregulation of critical anti-cancer VDR target genes.

Despite these data, *in vitro* studies have demonstrated that specific oncogenes can deregulate VDR expression. For instance, comparison of VDR expression in a series of isogenic,

Table 1 | Frequency of genomic alterations in VDR and CYP24A1 derived from The Cancer Genome Atlas dataset of human breast tumors.

	All tumors ($n = 463$)	Lum A ($n = 235$)	Lum B ($n = 133$)	Basal ($n = 81$)	HER2 ($n = 58$)	CLN Lo ($n = 8$)
VDR						
% Alterations	5.2	3.4	11.3	4.9	3.4	0
Amplifications	0.4	0	0.8	1.2	0	0
Deletions	0	0	0	0	0	0
Mutations	0	0	0	0	0	0
↓ mRNA	3.9	3.0	10.5	3.7	0	0
↑ mRNA	0.9	0.4	0	0	3.4	0
CYP24A1						
% Alterations	9.7	9.8	11.3	7.4	10.3	12.5
Amplifications	5.6	4.7	9.8	1.2	10.3	0
Deletions	0.2	0.2	0	0	0	0
Mutations	0.4	0.2	0.8	0	0	0
↓ mRNA	0	0	0	0	0	0
↑ mRNA	3.5	4.7	0.8	6.2	0	12.5

The data was calculated with the publically available breast invasive carcinoma dataset (Cancer Genome Atlas Network, 2012) at https://tcga-data.nci.nih.gov/tcga/as. Numbers indicate the percentage of the indicated genomic alterations observed in the total number of tumors analyzed within each group (shown in parentheses). LumA, Luminal A; LumB, Luminal B; CLN Lo, Claudin Low.

progressively transformed human mammary epithelial (HME) cell lines indicated that VDR expression and function was reduced more than 70% in HME cells expressing SV40 and/or RAS compared to the non-transformed HME cells from which they were derived (Kemmis and Welsh, 2008). Likewise, SV40 and RAS have been shown to reduce VDR activity in other breast cancer cell model systems (Agadir et al., 1999; Escaleira and Brentani, 1999). Transcriptional repressors linked to the epithelial mesenchymal transition such as SNAIL and SLUG have also been shown to down-regulate VDR (Mittal et al., 2008; Larriba et al., 2010). Thus, it is clear that there are distinct mechanisms targeting both the VDR gene itself and its protein product. Data on receptor expression derived from whole tumors may be somewhat misleading since cancer progression is driven by genetic instability and outgrowth of cells with advantageous mutations, such as activation of oncogenes. These studies indicate that abrogated expression and/or function of VDR may be limited to certain subsets of cells within individual tumors that have sustained specific molecular genetic alterations.

In addition to genetic alterations and effects of oncogenes, VDR abundance is affected by many physiological agents, including $1,25(OH)_2D_3$ itself, estrogens, retinoids and growth factors. Thus, cell sensitivity to $1,25(OH)_2D_3$ may also reflect the activity of other hormone signaling pathways through their impact on VDR expression. In breast cancer, the regulation of VDR expression and activity by estrogens is likely to be clinically significant. ER positive cells tend to express higher levels of VDR than ER negative cells (Buras et al., 1994) and *in vitro* studies have demonstrated that estrogen up-regulates whereas anti-estrogens down regulate VDR in ER positive breast cancer cells (Nolan et al., 1998; Byrne et al., 2000). Further studies are therefore warranted to determine the degree to which estrogen status influences VDR abundance in different $1,25(OH)_2D_3$ target tissues (i.e., breast, bone, uterus), and whether common therapeutic synthetic or natural estrogens act as estrogen agonists or antagonists in regulation of VDR expression. Consistent with this concept, some data suggests that phytoestrogens such as resveratrol and genestein can alter VDR expression and $1,25(OH)_2D_3$ sensitivity in cancer cells *in vitro* (Wietzke and Welsh, 2003; Gilad et al., 2006).

Other tumor-associated changes that can lead to $1,25(OH)_2D_3$ resistance in VDR positive tumors include disruption of VDR transcriptional activation and enhanced catabolism of its ligand. Data from breast, bladder and prostate cancer suggests that alterations in transcriptional co-regulators can abrogate signaling by the $1,25(OH)_2D_3$-VDR complex (Malinen et al., 2008; Abedin et al., 2009). Enhanced mammary cell catabolism of $1,25(OH)_2D_3$ would also be predicted to limit the formation of active VDR complexes. Indeed, amplification of the CYP24A1 gene was reported in human breast tumors (Albertson et al., 2000) and analysis of the datasets from The Cancer Genome Atlas confirms that a subset of human breast cancers (10–13%) exhibit alterations in the CYP24 gene, with the most frequent changes being amplifications and upregulation at the mRNA level (**Table 1**). There is no obvious subtype specificity to CYP24A1 changes, although amplifications were somewhat more frequent in Luminal B and HER2 subtypes whereas increased mRNA levels were more common in Basal and Claudin-low tumors. These data are consistent with analysis of tumor samples which demonstrated higher CYP24A1 protein expression in breast tumors compared to adjacent normal tissue (Townsend et al., 2005; Lopes et al., 2010). Furthermore, splicing variants of CYP24A1 have been reported in breast cancer cell lines (Scheible et al., 2014), suggesting that distinct forms of the enzyme with altered properties may be expressed in tumors. The significance of the CYP24A1 deregulation with respect to overall catabolism of vitamin D metabolites *in situ* has yet to be ascertained.

Given that normal mammary cells utilize $25(OH)D_3$ as substrate for local tissue generation of $1,25(OH)_2D_3$, imbalanced expression of either CYP24A1 or CYP27B1 favoring catabolism could theoretically contribute to escape of developing tumor cells from anti-cancer VDR signaling. In the HME cell model, oncogenic transformation was associated with reduced CYP27B1 expression and activity [as measured by $1,25(OH)_2D_3$ synthesis]. The reductions in CYP27B1 in the oncogene-transformed HME cells were of sufficient magnitude to reduce cellular sensitivity to growth inhibition by $25(OH)D_3$ approximately 100-fold (Kemmis and Welsh, 2008). However, clinical data on CYP27B1 expression in breast cancer is inconsistent (Segersten et al., 2005; Townsend et al., 2005; de Lyra et al., 2006; McCarthy et al., 2009; Lopes et al., 2010) and less than 2% of breast cancers annotated in The Cancer Genome Atlas datasets exhibit genomic alterations in CYP27B1. However, altered splice variants of CYP27B1 have been detected in breast cancer cells (Cordes et al., 2007; Fischer et al., 2007) suggesting the possibility that forms of the CYP27B1 enzyme with altered function could be expressed in breast tumors.

ACTIONS OF VDR AGONISTS ON BREAST CANCER CELLS AND TUMORS

Numerous studies have profiled the cellular and molecular effects of $1,25(OH)_2D_3$ on VDR positive breast cancer cells. Furthermore, a large number of structural analogs of vitamin D developed by pharmaceutical companies and academic researchers have been used to probe the mechanisms of vitamin D mediated growth inhibition. In general, the effects of VDR agonists on breast cancer cells include modulation of key cell cycle regulators to induce G0/G1, induction of differentiation markers, and/or activation of cell death (via apoptosis or autophagy). Notably, studies with cells derived from VDRKO mice has definitely established that the nuclear VDR is required for the anti-proliferative and pro-apoptotic effects of $1,25(OH)_2D_3$ in transformed mammary cells *in vitro* (Zinser et al., 2003; Valrance et al., 2007). In addition to regulation of cell growth and survival, studies in ER negative breast cancer cells, representative of late stage disease, have provided evidence that $1,25(OH)_2D_3$ alters genomic stability, inhibits angiogenesis and reduces invasion and metastasis. For instance, $1,25(OH)_2D_3$ interacts with the 53BP1 protein to eliminate invasive breast cancer cells lacking BRCA1 (Grotsky et al., 2013). $1,25(OH)_2D_3$ and analogs inhibit invasion as measured by the Boyden chamber assay (Flanagan et al., 2003) likely through suppression of extracellular proteases (MMP-9, urokinase-type plasminogen activator, tissue type plasminogen activator), protease inhibitors and adhesion molecules. Comparative studies of breast tumors and normal adjacent breast

tissue in an explant system confirmed that malignant tissue is responsive to $1,25(OH)_2D_3$ but that the magnitude of the response is highly disparate between individual patients (Suetani et al., 2012; Milani et al., 2013). Furthermore, tumor tissue was far less sensitive to $25(OH)D_3$ than $1,25(OH)_2D_3$ (Suetani et al., 2012).

Animal models of established breast cancer have demonstrated that VDR agonists can reduce tumor growth (and in some cases trigger tumor regression) with minimal effects on calcemia [the most common and dangerous side effect of $1,25(OH)_2D_3$ therapy]. In experimental metastasis paradigms, the vitamin D analog EB1089 inhibited secondary tumors, blocked skeletal metastases and improved survival (El Abdaimi et al., 2000; Flanagan et al., 2003). More recently, dietary modifications have been shown to alter breast tumor growth and progression. Increasing dietary vitamin D_3 from 1000 IU/kg diet (rodent standard) to 5000 IU/kg diet significantly reduced growth of established MCF-7 xenografts, with equivalent potency to $1,25(OH)_2D_3$ (Swami et al., 2012). In another study, vitamin D deficiency sufficient to enhance bone turnover promoted the skeletal growth of breast cancer metastases (Ooi et al., 2010). For further details on these and other *in vivo* studies, several recent comprehensive reviews on vitamin D and breast cancer are available (Krishnan and Feldman, 2011; Lopes et al., 2012; Welsh, 2012).

GENOMIC PROFILING OF VDR AGONISTS IN BREAST CANCER MODEL SYSTEMS

Screening for molecular changes induced by $1,25(OH)_2D_3$ or vitamin D analogs in various breast cancer cells has identified scores of VDR regulated genes and proteins, indicating a broad range of downstream targets. Here we will focus on the few comprehensive genomic studies that have allowed for identification of vitamin D responsive pathways in breast cancer (Lee et al., 2006; Campos et al., 2013; Milani et al., 2013; Laporta and Welsh, 2013). In the first study of vitamin D mediated genomic changes in breast cancer, Feldman's groups used early generation arrays to compare gene expression in ER positive MCF-7 cells and ER negative MDA-MB-231 cells treated with 100 nM $1,25(OH)_2D_3$ (Swami et al., 2003). Due to the limited nature of these arrays, which comprised 2000 cancer related genes, direct comparisons with whole genome profiling arrays isn't appropriate. However, comparisons between the two cell lines is of interest. Using a 2-fold cutoff, 62 genes (47 up/15 down) in MCF-7 cells and 20 genes in MDA-MB-231 cells (10 up/10 down) were significantly altered by 24 h treatment with 100 nM $1,25(OH)_2D_3$, with only seven genes commonly altered in both cell lines. The larger number of regulated genes in MCF-7 cells was not surprising as CYP24A1 induction was 10-fold higher in MCF-7 cells (52-fold) than in MDA-MB-231 cells (5.5-fold). Other highly regulated genes in MCF-7 cells treated with $1,25(OH)_2D_3$ for 24 h included RBL2, CTNNA1, RAD23B, NCOA4, BMP5 and IFNG (up) and CEACAM1, CDH6, IL13, IL1R2 and ESR (down). In MDA-MB-231 cells, highly modulated genes at 24 h included CASP4, NF1B, ITGAV, TXNRD1 and TGFB2 (up) and ANGPT1, four MMPs (12, 10, 7, 1) and PRKD1 (down). Thus, in MCF-7 cells, many of the $1,25(OH)_2D_3$ regulated genes were involved in growth factor signaling, cell cycle, apoptosis and immune responses, whereas in MDA-MB-231 cells genes related to disease progression (i.e., invasion and angiogenesis) were altered.

Since the availability of whole genome arrays, four studies (Lee et al., 2006; Campos et al., 2013; Milani et al., 2013; Laporta and Welsh, 2013) on the effects of VDR agonists [three with $1,25(OH)_2D_3$, one with a synthetic VDR agonist] in breast cancer model systems have reported although only one of these datasets (Milani et al., 2013, accession #GSE27220) is publically available on the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). Lee et al. (2006) compared the effects of 1 nM RO3582, a Gemini $1,25(OH)_2D_3$ analog, on pre-malignant (MCF10AT1) and malignant (MCF10CA1a) breast cancer cells and identified distinct gene expression profiles for each cell line. Similar to the comparison of MCF-7 and MDA-MB-231 cells (Swami et al., 2003), more significant changes in gene expression were observed in the less malignant MCF10AT1 cells than in the invasive MCF10CA1a cells (391 vs. 156, respectively, 12 h treatment; 2-fold cutoff). Despite the reduced sensitivity in the more aggressive cells, the overlap in target genes was considerable (about 55% of the genes altered in MCF10AT1 cells were similarly altered in the MCF10CA1a cells); the complete gene lists are available as supplemental data.

Using an approach designed to more accurately represent the tumor microenvironment *in situ*, tumor slices from postmenopausal breast cancer patients with stage I, II, or III breast cancer were cultured with 0.5 or 100 nM $1,25(OH)_2D_3$ for 24 h (Milani et al., 2013). This study identified nine genes that were significantly altered within 24 h of exposure to 0.5 nM $1,25(OH)_2D_3$, a concentration that is physiologically achievable in patients. Of these, CYP24A1 was induced over 7-fold and was validated in another set of tumor samples, clearly indicating activation of VDR signaling. Gene set enrichment analysis (GSEA) indicated a trend toward the enrichment of genes sharing DR3 binding sites, a consensus motif for VDR. Other genes identified in response to 0.5 nM $1,25(OH)_2D_3$ included DPP4, CYP26B1, SPIN3, KCKN3, EFTUD1, TKTL1, and CA2 (up-regulated) and FCGR2C and SAMSN1 (down-regulated). At 100 nM $1,25(OH)_2D_3$, 30 genes (28 up/2 down) were significantly regulated by $1,25(OH)_2D_3$. In addition to those listed above, genes up-regulated by 3-fold or more included IL1RL1, CILP, PI15, TMEM37, and SHE. The two top down-regulated genes (2-fold or more) were P2RY1 and BCOR. Interestingly, CD14 and SLC1A1, two $1,25(OH)_2D_3$ regulated genes we identified in the genomic profiles of normal mammary epithelial cells discussed above, were also induced by $1,25(OH)_2D_3$ in the breast cancer slice model. The significance of this study is that it demonstrated for the first time that $1,25(OH)_2D_3$ could induce genomic changes in intact breast tumor tissue, indicating the functionality of the VDR. Although patient-to-patient variability was considerable, a core set of $1,25(OH)_2D_3$ modulated genes was identified that may represent biomarkers of vitamin D action for future studies.

A fourth genomic study was recently conducted in a mouse mammary tumor model of triple negative breast cancer (Laporta and Welsh, 2013). Cells derived from DMBA induced tumors generated in wildtype (WT) and VDRKO mice were studied after 24 h treatment with 100 nM $1,25(OH)_2D_3$. A unique feature

of this study was the inclusion of VDRKO cells in which the growth inhibitory effects of $1,25(OH)_2D_3$ were restored via stable expression of human VDR (KO^{hVDR} cells). Genomic profiling demonstrated that $1,25(OH)_2D_3$ failed to alter gene expression in VDRKO cells whereas major changes were observed in cells derived from WT mice (WT145 cells) and in KO^{hVDR} cells. With a 2-fold cutoff, 117 transcripts in WT145 cells and 197 transcripts in the KO^{hVDR} clones were significantly altered by $1,25(OH)_2D_3$ with 35 genes found to be commonly regulated in all VDR-positive cell lines (the complete list of genes is included in the manuscript). In addition to *Cyp24a1,* seven genes were validated as $1,25(OH)_2D_3$ responsive and VDR dependent in this system: *Cib2*, *Prelp*, *Enpp1*, *Plau, Hbegf, Postn, and Has2*. The last four of these, whose expression was markedly down regulated by $1,25(OH)_2D_3$, are known to drive breast cancer invasion and metastasis. These data support a model whereby $1,25(OH)_2D_3$ coordinately suppresses multiple proteins that are required for survival of triple-negative/basal-like breast cancer cells.

In summary, while CY24A1 is commonly identified in microarray studies as the most highly upregulated gene in 1,25(OH)D treated cells, other target genes vary greatly depending on the model system. Integration of the existing genomic datasets generated in various mammary cell models with other normal and transformed array profiles and ChIP-Seq studies will assist in identifying common and tissue/cell specific genesets regulated by the $1,25(OH)_2D_3$-VDR complex. In addition, the ENCODE project (http://genome.ucsc.edu/ENCODE/cellTypes.html) includes several breast cancer cell lines (MCF-7, MDA-MB-231, T47-D) which may provide relevant genomic information on nuclear receptor signaling The continued use of complex models such as tumor explants for vitamin D studies is desirable given the expression of VDR in most cell types and the critical interactions between tumor cells and their stromal microenvironment.

CONCLUSIONS

Although meta-analyses of population studies demonstrate an inverse relationship between vitamin D status and breast cancer risk, questions remain regarding mechanisms, tissue specificity, and optimal intakes of vitamin D_3 for potential benefits on cancer. In 2010, the Institute of Medicine recommended an increase in the adult intake of vitamin D_3 (from 200 to 600 IU per day) based on its role in bone health, but concluded that current data is insufficient to support recommendations with respect to cancer prevention. Comprehensive genomic, metabolomic and proteomic profiling approaches combined with mechanistic studies remain highly valuable for identification of relevant biomarkers of tissue vitamin D action that are needed for translational investigations (i.e., supplementation trials).

FUTURE VIEW

Despite the extensive effort to understand the relationship between vitamin D and breast cancer, many issues remain unresolved. Much of the work conducted in cell systems or animal models is consistent, but epidemiological data is somewhat mixed and clinical studies are limited. As discussed above, population studies do support the concept that high serum levels of $1\alpha,25(OH)_2D_3$ and/or its precursor $25(OH)D_3$ are associated with lower risk of initial disease development and may retard progression. However, tissue uptake and cellular metabolism of these metabolites *in vivo* is likely to be highly relevant to cancer biology, yet few studies have successfully measured these parameters. In addition, there is little data on how systemic vitamin D status might interact with other known breast cancer risk factors including genetic (BRCA1, BRCA2, ATM), endocrine (estrogen, progesterone) and environmental (radiation, carcinogens) modulators of breast cancer development. Genomic profiling has characterized many $1\alpha,25(OH)_2D_3$ responsive targets in normal mammary cells and in breast cancers providing valuable insight into the molecular actions of $1\alpha,25(OH)_2D_3$ and the VDR in regulation of cell cycle, apoptosis and differentiation. New areas of emphasis suggested by recent studies include regulation of tumor metabolism and activation of innate immune responses. However, the role of VDR in individual cell types (ie epithelial, adipose, fibroblast, endothelial, immune) of normal and tumor tissues remains to be clarified. Furthermore, there has been limited attention directed at understanding how VDR integrates signaling between these diverse cell types and what soluble signals and paracrine pathways may be regulated by $1\alpha,25(OH)_2D_3$ in the tissue and tumor microenvironment. Finally, the possible interactions of VDR with other nuclear receptors and their ligands (particularly RXR family) in control of mammary cell fate/carcinogenesis will require additional study.

REFERENCES

Abedin, S. A., Thorne, J. L., Battaglia, S., Maguire, O., Hornung, L. B., Doherty, A. P., et al. (2009). Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. *Carcinogenesis* 30, 449–456. doi: 10.1093/carcin/bgp005

Agadir, A., Lazzaro, G., Zheng, Y., Zhang, X. K., and Mehta, R. (1999). Resistance of HBL100 human breast epithelial cells to vitamin D action. *Carcinogenesis* 20, 577–582. doi: 10.1093/carcin/20.4.577

Albertson, D. G., Ylstra, B., Segraves, R., Collins, C., Dairkee, S. H., Kowbel, D., et al. (2000). Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat. Genet.* 25, 144–146. doi: 10.1038/75985

Alvarez, J. A., Chowdhury, R., Jones, D. P., Martin, G. S., Brigham, K. L., Binongo, J. N., et al. (2014). Vitamin D status is independently associated with plasma glutathione and cysteine thiol/disulfide redox status in adults. *Clin. Endocrinol. (Oxf.)*. doi: 10.1111/cen.12449. [Epub ahead of print].

Bauer, S. R., Hankinson, S. E., Bertone-Johnson, E. R., and Ding, E. L. (2013). Plasma vitamin D levels, menopause, and risk of breast cancer: dose-response meta-analysis of prospective studies. *Medicine (Baltimore)* 92, 123–131. doi: 10.1097/MD.0b013e3182943bc2

Berger, U., McClelland, R. A., Wilson, P., Greene, G. L., Haussler, M. R., Pike, J. W., et al. (1991). Immunocytochemical detection of estrogen receptor, progesterone receptor and 1,25-dihydroxyvitamin D_3 receptor in breast cancer and relation to prognosis. *Cancer Res.* 51, 239–244.

Berger, U., Wilson, P., McClelland, R. A., Colston, K., Haussler, M. R., Pike, J. W., et al. (1987). Immunocytochemical detection of 1,25-dihydroxyvitamin D3 receptor in breast cancer. *Cancer Res.* 47, 6793–6799.

Bikle, D. D. (2012). Protective actions of vitamin D in UVB induced skin cancer. *Photochem. Photobiol. Sci.* 11, 1808–1816. doi: 10.1039/c2pp25251a

Bilinski, K., and Boyages, J. (2013). Association between 25-hydroxyvitamin D concentration and breast cancer risk in an Australian population: an observational case-control study. *Breast Cancer Res. Treat.* 137, 599–607. doi: 10.1007/s10549-012-2381-1

Bolland, M. J., Grey, A., Gamble, G. D., and Reid, I. R. (2011). Calcium and vitamin D supplements and health outcomes: a reanalysis of the Women's Health Initiative (WHI) limited-access data set. *Am. J. Clin. Nutr.* 94, 1144–1149. doi: 10.3945/ajcn.111.015032

Bruce, D., Yu, S., Ooi, J. H., and Cantorna, M. T. (2011). Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. *Int. Immunol.* 23, 519–528. doi: 10.1093/intimm/dxr045

Buras, R. R., Schumaker, L. M., Davoodi, F., Brenner, R. V., Shabahang, M., Nauta, R. J., et al. (1994). Vitamin D receptors in breast cancer cells. *Breast Cancer Res. Treat.* 31, 191–202. doi: 10.1007/BF00666153

Byrne, I., Flanagan, L., Tenniswood, M., and Welsh, J. E. (2000). Identification of a hormone responsive promoter immediately upstream of exon 1c in the human vitamin D receptor gene. *Endocrinology* 141, 2829–2836. doi: 10.1210/en.141.8.2829

Caan, B. J., Sweeney, C., Habel, L. A., Kwan, M., Kroenke, C. H., Weltzien, E., et al. (2014). Intrinsic subtypes from the PAM50 gene expression assay in a population-based breast cancer survivor cohort: prognostication of short and long term outcomes. *Cancer Epidemiol. Biomarkers Prev.* 23, 714–724. doi: 10.1158/1055-9965.EPI-13-1017

Campos, L. T., Brentani, H., Roela, R. A., Katayama, M. L., Lima, L., Rolim, C. F., et al. (2013). Differences in transcriptional effects of 1alpha,25 dihydroxyvitamin D3 on fibroblasts associated to breast carcinomas and from paired normal breast tissues. *J. Steroid Biochem. Mol. Biol.* 133, 12–24. doi: 10.1016/j.jsbmb.2012.08.002

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. *Nature* 490, 61–70. doi: 10.1038/nature11412

Cauley, J. A., Chlebowski, R. T., Wactawski-Wende, J., Robbins, J. A., Rodabough, R. J., Chen, Z., et al. (2013). Calcium plus vitamin D supplementation and health outcomes five years after active intervention ended: the Women's Health Initiative. *J. Womens Health (Larchmt)* 22, 915–929. doi: 10.1089/jwh.2013.4270

Ching, S., Kashinkunti, S., Niehaus, M. D., and Zinser, G. M. (2011). Mammary adipocytes bioactivate 25-hydroxyvitamin D(3) and signal via vitamin D(3) receptor, modulating mammary epithelial cell growth. *J. Cell. Biochem.* 112, 3393–3405. doi: 10.1002/jcb.23273

Colston, K., Wilkinson, J. R., and Coombes, R. C. (1986). 1,25-Dihydroxyvitamin D3 binding in estrogen responsive rat breast tumor. *Endocrinology* 119, 397–403. doi: 10.1210/endo-119-1-397

Cordes, T., Diesing, D., Becker, S., Fischer, D., Diedrich, K., and Friedrich, M. (2007). Expression of splice variants of 1alpha-hydroxylase in MCF-7 breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 103, 326–329. doi: 10.1016/j.jsbmb.2006.12.034

de Lyra, E. C., da Silva, I. A., Katayama, M. L., Brentani, M. M., Nonogaki, S., Goes, J. C., et al. (2006). 25(OH)D3 and 1,25$(OH)_2D_3$ serum concentration and breast tissue expression of 1alpha-hydroxylase, 24-hydroxylase and Vitamin D receptor in women with and without breast cancer. *J. Steroid Biochem. Mol. Biol.* 100, 184–192. doi: 10.1016/j.jsbmb.2006.04.009

Devitt, A., Parker, K. G., Ogden, C. A., Oldreive, C., Clay, M. F., Melville, L. A., et al. (2004). Persistence of apoptotic cells without autoimmune disease or inflammation in $CD14^{-/-}$ mice. *J. Cell Biol.* 167, 1161–1170. doi: 10.1083/jcb.200410057

Dogan, M., Cesur, Y., Zehra Dogan, S., Kaba, S., Bulan, K., and Cemek, M. (2012). Oxidant/antioxidant system markers and trace element levels in children with nutritional rickets. *J. Pediatr. Endocrinol. Metab.* 25, 1129–1139. doi: 10.1515/jpem-2012-0153

Edvardsen, K., Veierod, M. B., Brustad, M., Braaten, T., Engelsen, O., and Lund, E. (2011). Vitamin D-effective solar UV radiation, dietary vitamin D and breast cancer risk. *Int. J. Cancer* 128, 1425–1433. doi: 10.1002/ijc.25463

El Abdaimi, K., Dion, N., Papavasiliou, V., Cardinal, P. E., Binderup, L., Goltzman, D., et al. (2000). The vitamin D analogue EB1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. *Cancer Res.* 60, 4412–4418.

Engel, L. S., Satagopan, J., Sima, C. S., Orlow, I., Mujumdar, U., Coble, J., et al. (2014). Sun exposure, vitamin d receptor genetic variants, and risk of breast cancer in the agricultural health study. *Environ. Health Perspect.* 122, 165–171. doi: 10.1289/ehp.1206274

Engel, P., Fagherazzi, G., Mesrine, S., Boutron-Ruault, M. C., and Clavel-Chapelon, F. (2011). Joint effects of dietary vitamin D and sun exposure on breast cancer risk: results from the French E3N cohort. *Cancer Epidemiol. Biomarkers Prev.* 20, 187–198. doi: 10.1158/1055-9965.EPI-10-1039

Escaleira, M. T., and Brentani, M. M. (1999). Vitamin D3 receptor (VDR) expression in HC-11 mammary cells: regulation by growth-modulatory agents, differentiation, and Ha-ras transformation. *Breast Cancer Res. Treat.* 54, 123–133. doi: 10.1023/A:1006198107805

Fischer, D., Seifert, M., Becker, S., Ludders, D., Cordes, T., Reichrath, J., et al. (2007). 25-Hydroxyvitamin D3 1alpha-hydroxylase splice variants in breast cell lines MCF-7 and MCF-10. *Cancer Genomics Proteomics* 4, 295–300.

Flanagan, L., Packman, K., Juba, B., O'Neill, S., Tenniswood, M., and Welsh, J. (2003). Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. *J. Steroid Biochem. Mol. Biol.* 84, 181–192. doi: 10.1016/S0960-0760(03)00028-1

Gilad, L. A., Tirosh, O., and Schwartz, B. (2006). Phytoestrogens regulate transcription and translation of vitamin D receptor in colon cancer cells. *J. Endocrinol.* 191, 387–398. doi: 10.1677/joe.1.06930

Gordon-Thomson, C., Gupta, R., Tongkao-On, W., Ryan, A., Halliday, G. M., and Mason, R. S. (2012). 1alpha,25 dihydroxyvitamin D3 enhances cellular defences against UV-induced oxidative and other forms of DNA damage in skin. *Photochem. Photobiol. Sci.* 11, 1837–1847. doi: 10.1039/c2pp25202c

Grant, W. B. (2013). Update on evidence that support a role of solar ultraviolet-B irradiance in reducing cancer risk. *Anticancer Agents Med. Chem.* 13, 140–146. doi: 10.2174/187152013804487425

Grotsky, D. A., Gonzalez-Suarez, I., Novell, A., Neumann, M. A., Yaddanapudi, S. C., Croke, M., et al. (2013). BRCA1 loss activates cathepsin L-mediated degradation of 53BP1 in breast cancer cells. *J. Cell Biol.* 200, 187–202. doi: 10.1083/jcb.201204053

Heidenreich, S. (1999). Monocyte CD14: a multifunctional receptor engaged in apoptosis from both sides. *J. Leukoc. Biol.* 65, 737–743.

Hopkins, M. H., Owen, J., Ahearn, T., Fedirko, V., Flanders, W. D., Jones, D. P., et al. (2011). Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a randomized, controlled clinical trial. *Cancer Prev. Res. (Phila.)* 4, 1645–1654. doi: 10.1158/1940-6207.CAPR-11-0105

Hussain, E. A., Mehta, R. R., Ray, R., Das Gupta, T. K., and Mehta, R. G. (2003). Efficacy and mechanism of action of 1alpha-hydroxy-24-ethyl-cholecalciferol (1alpha[OH]D5) in breast cancer prevention and therapy. *Recent Results Cancer Res.* 164, 393–411. doi: 10.1007/978-3-642-55580-0_29

Jacobson, E. A., James, K. A., Newmark, H. L., and Carroll, K. K. (1989). Effects of dietary fat, calcium, and vitamin D on growth and mammary tumorigenesis induced by 7,12-dimethylbenz(a)anthracene in female Sprague-Dawley rats. *Cancer Res.* 49, 6300–6303.

John, E. M., Schwartz, G. G., Dreon, D. M., and Koo, J. (1999). Vitamin D and breast cancer risk: the NHANES I Epidemiologic follow-up study, 1971–1975 to 1992. National Health and Nutrition Examination Survey. *Cancer Epidemiol. Biomarkers Prev.* 8, 399–406.

John, E. M., Schwartz, G. G., Koo, J., Wang, W., and Ingles, S. A. (2007). Sun exposure, vitamin D receptor gene polymorphisms, and breast cancer risk in a multiethnic population. *Am. J. Epidemiol.* 166, 1409–1419. doi: 10.1093/aje/kwm259

Kallay, E., Bareis, P., Bajna, E., Kriwanek, S., Bonner, E., Toyokuni, S., et al. (2002). Vitamin D receptor activity and prevention of colonic hyperproliferation and oxidative stress. *Food Chem. Toxicol.* 40, 1191–1196. doi: 10.1016/S0278-6915(02)00030-3

Kemmis, C. M., Salvador, S. M., Smith, K. M., and Welsh, J. (2006). Human mammary epithelial cells express CYP27B1 and are growth inhibited by 25-hydroxyvitamin D-3, the major circulating form of vitamin D-3. *J. Nutr.* 136, 887–892.

Kemmis, C. M., and Welsh, J. (2008). Mammary epithelial cell transformation is associated with deregulation of the vitamin D pathway. *J. Cell. Biochem.* 105, 980–988. doi: 10.1002/jcb.21896

Kim, H. J., Lee, Y. M., Ko, B. S., Lee, J. W., Yu, J. H., Son, B. H., et al. (2011). Vitamin D deficiency is correlated with poor outcomes in patients with luminal-type breast cancer. *Ann. Surg. Oncol.* 18, 1830–1836. doi: 10.1245/s10434-010-1465-6

Kim, Y., Franke, A. A., Shvetsov, Y. B., Wilkens, L. R., Cooney, R. V., Lurie, G., et al. (2014). Plasma 25-hydroxyvitamin D3 is associated with decreased risk of postmenopausal breast cancer in whites: a nested case-control study in the multiethnic cohort study. *BMC Cancer* 14:29. doi: 10.1186/1471-2407-14-29

Knower, K. C., Chand, A. L., Eriksson, N., Takagi, K., Miki, Y., Sasano, H., et al. (2013). Distinct nuclear receptor expression in stroma adjacent to breast tumors. *Breast Cancer Res. Treat.* 142, 211–223. doi: 10.1007/s10549-013-2716-6

Krishnan, A. V., and Feldman, D. (2011). Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. *Annu. Rev. Pharmacol. Toxicol.* 51, 311–336. doi: 10.1146/annurev-pharmtox-010510-100611

Kuhn, T., Kaaks, R., Becker, S., Eomois, P. P., Clavel-Chapelon, F., Kvaskoff, M., et al. (2013). Plasma 25-hydroxyvitamin D and the risk of breast cancer in

the European prospective investigation into cancer and nutrition: a nested case-control study. *Int. J. Cancer* 133, 1689–1700. doi: 10.1002/ijc.28172

Laporta, E., and Welsh, J. (2013). Modeling vitamin D actions in triple-negative/basal-like breast cancer. *J. Steroid Biochem. Mol. Biol.* doi: 10.1016/j.jsbmb.2013.10.022. [Epub ahead of print].

Larriba, M. J., Bonilla, F., and Munoz, A. (2010). The transcription factors Snail1 and Snail2 repress vitamin D receptor during colon cancer progression. *J. Steroid Biochem. Mol. Biol.* 121, 106–109. doi: 10.1016/j.jsbmb.2010.01.014

Lee, H. J., Liu, H., Goodman, C., Ji, Y., Maehr, H., Uskokovic, M., et al. (2006). Gene expression profiling changes induced by a novel Gemini Vitamin D derivative during the progression of breast cancer. *Biochem. Pharmacol.* 72, 332–343. doi: 10.1016/j.bcp.2006.04.030

Lee, J. W., Paape, M. J., Elsasser, T. H., and Zhao, X. (2003). Recombinant soluble CD14 reduces severity of intramammary infection by *Escherichia coli*. *Infect. Immun.* 71, 4034–4039. doi: 10.1128/IAI.71.7.4034-4039.2003

Lin, J., Manson, J. E., Lee, I. M., Cook, N. R., Buring, J. E., and Zhang, S. M. (2007). Intakes of calcium and vitamin D and breast cancer risk in women. *Arch. Intern. Med.* 167, 1050–1059. doi: 10.1001/archinte.167.10.1050

Lopes, N., Paredes, J., Costa, J. L., Ylstra, B., and Schmitt, F. (2012). Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. *Breast Cancer Res.* 14:211. doi: 10.1186/bcr3178

Lopes, N., Sousa, B., Martins, D., Gomes, M., Vieira, D., Veronese, L. A., et al. (2010). Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer* 10:483. doi: 10.1186/1471-2407-10-483

Maalmi, H., Ordonez-Mena, J. M., Schottker, B., and Brenner, H. (2014). Serum 25-hydroxyvitamin D levels and survival in colorectal and breast cancer patients: systematic review and meta-analysis of prospective cohort studies. *Eur. J. Cancer* 50, 1510–1521. doi: 10.1016/j.ejca.2014.02.006

Malinen, M., Saramaki, A., Ropponen, A., Degenhardt, T., Vaisanen, S., and Carlberg, C. (2008). Distinct HDACs regulate the transcriptional response of human cyclin-dependent kinase inhibitor genes to Trichostatin A and 1alpha,25-dihydroxyvitamin D3. *Nucleic Acids Res.* 36, 121–132. doi: 10.1093/nar/gkm913

McCarthy, K., Laban, C., Bustin, S. A., Ogunkolade, W., Khalaf, S., Carpenter, R., et al. (2009). Expression of 25-hydroxyvitamin D-1-alpha-hydroxylase, and vitamin D receptor mRNA in normal and malignant breast tissue. *Anticancer Res.* 29, 155–157.

McMahon, L., Schwartz, K., Yilmaz, O., Brown, E., Ryan, L. K., and Diamond, G. (2011). Vitamin D-mediated induction of innate immunity in gingival epithelial cells. *Infect. Immun.* 79, 2250–2256. doi: 10.1128/IAI.00099-11

Mehta, R. G., Moriarty, R. M., Mehta, R. R., Penmasta, R., Lazzaro, G., Constantinou, A., et al. (1997). Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1alpha-hydroxyvitamin D5. *J. Natl. Cancer Inst.* 89, 212–218. doi: 10.1093/jnci/89.3.212

Milani, C., Katayama, M. L., de Lyra, E. C., Welsh, J., Campos, L. T., Brentani, M. M., et al. (2013). Transcriptional effects of 1,25 dihydroxyvitamin D(3) physiological and supra-physiological concentrations in breast cancer organotypic culture. *BMC Cancer* 13:119. doi: 10.1186/1471-2407-13-119

Mittal, M. K., Myers, J. N., Misra, S., Bailey, C. K., and Chaudhuri, G. (2008). *In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. *Biochem. Biophys. Res. Commun.* 372, 30–34. doi: 10.1016/j.bbrc.2008.04.187

Mohr, S. B., Gorham, E. D., Kim, J., Hofflich, H., and Garland, C. F. (2014). Meta-analysis of Vitamin D sufficiency for improving survival of patients with breast cancer. *Anticancer Res.* 34, 1163–1166.

Nakai, K., Fujii, H., Kono, K., Goto, S., Kitazawa, R., Kitazawa, S., et al. (2014). Vitamin D activates the nrf2-keap1 antioxidant pathway and ameliorates nephropathy in diabetic rats. *Am. J. Hypertens.* 27, 586–595. doi: 10.1093/ajh/hpt160

Narvaez, C. J., Simmons, K. M., Brunton, J., Salinero, A., Chittur, S. V., and Welsh, J. E. (2013). Induction of STEAP4 correlates with 1,25-dihydroxyvitamin D3 stimulation of adipogenesis in mesenchymal progenitor cells derived from human adipose tissue. *J. Cell. Physiol.* 228, 2024–2036. doi: 10.1002/jcp.24371

Nolan, E., Donepudi, M., VanWeelden, K., Flanagan, L., and Welsh, J. E. (1998). Dissociation of vitamin D3 and anti-estrogen mediated growth regulation in MCF-7 breast cancer cells. *Mol. Cell. Biochem.* 188, 13–20. doi: 10.1007/978-1-4615-5763-0_2

Ooi, L. L., Zhou, H., Kalak, R., Zheng, Y., Conigrave, A. D., Seibel, M. J., et al. (2010). Vitamin D deficiency promotes human breast cancer growth in a murine model of bone metastasis. *Cancer Res.* 70, 1835–1844. doi: 10.1158/0008-5472.CAN-09-3194

Peng, X., Hawthorne, M., Vaishnav, A., St-Arnaud, R., and Mehta, R. G. (2009). 25-Hydroxyvitamin D3 is a natural chemopreventive agent against carcinogen induced precancerous lesions in mouse mammary gland organ culture. *Breast Cancer Res. Treat.* 113, 31–41. doi: 10.1007/s10549-008-9900-0

Peng, X., Vaishnav, A., Murillo, G., Alimirah, F., Torres, K. E., and Mehta, R. G. (2010). Protection against cellular stress by 25-hydroxyvitamin D3 in breast epithelial cells. *J. Cell. Biochem.* 110, 1324–1333. doi: 10.1002/jcb.22646

Peppone, L. J., Rickles, A. S., Janelsins, M. C., Insalaco, M. R., and Skinner, K. A. (2012). The association between breast cancer prognostic indicators and serum 25-OH vitamin D levels. *Ann. Surg. Oncol.* 19, 2590–2599. doi: 10.1245/s10434-012-2297-3

Rainville, C., Khan, Y., and Tisman, G. (2009). Triple negative breast cancer patients presenting with low serum vitamin D levels: a case series. *Cases J.* 2:8390. doi: 10.4076/1757-1626-2-8390

Redaniel, M. T., Gardner, M. P., Martin, R. M., and Jeffreys, M. (2014). The association of vitamin D supplementation with the risk of cancer in postmenopausal women. *Cancer Causes Control* 25, 267–271. doi: 10.1007/s10552-013-0328-4

Rose, A. A., Elser, C., Ennis, M., and Goodwin, P. J. (2013). Blood levels of vitamin D and early stage breast cancer prognosis: a systematic review and meta-analysis. *Breast Cancer Res. Treat.* 141, 331–339. doi: 10.1007/s10549-013-2713-9

Rowling, M. J., Kemmis, C. M., Taffany, D. A., and Welsh, J. (2006). Megalin-mediated endocytosis of vitamin D binding protein correlates with 25-hydroxycholecalciferol actions in human mammary cells. *J. Nutr.* 136, 2754–2759.

Santagata, S., Thakkar, A., Ergonul, A., Wang, B., Woo, T., Hu, R., et al. (2014). Taxonomy of breast cancer based on normal cell phenotype predicts outcome. *J. Clin. Invest.* 124, 859–870. doi: 10.1172/JCI70941

Scheible, C., Thill, M., Baum, S., Solomayer, E., and Friedrich, M. (2014). Implication of CYP24A1 splicing in breast cancer. *Anticancer Agents Med. Chem.* 14, 109–114. doi: 10.2174/18715206113139990311

Segersten, U., Holm, P. K., Bjorklund, P., Hessman, O., Nordgren, H., Binderup, L., et al. (2005). 25-Hydroxyvitamin D3 1alpha-hydroxylase expression in breast cancer and use of non-1alpha-hydroxylated vitamin D analogue. *Breast Cancer Res.* 7, R980–R986. doi: 10.1186/bcr1332

Simpson, E. R., and Brown, K. A. (2013). Minireview: obesity and breast cancer: a tale of inflammation and dysregulated metabolism. *Mol. Endocrinol.* 27, 715–725. doi: 10.1210/me.2013-1011

Sperati, F., Vici, P., Maugeri-Sacca, M., Stranges, S., Santesso, N., Mariani, L., et al. (2013). Vitamin D supplementation and breast cancer prevention: a systematic review and meta-analysis of randomized clinical trials. *PLoS ONE* 8:e69269. doi: 10.1371/journal.pone.0069269

Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., et al. (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res.* 6, R75–R91. doi: 10.1186/bcr753

Suetani, R. J., Ho, K., Jindal, S., Manavis, J., Neilsen, P. M., Pishas, K. I., et al. (2012). A comparison of vitamin D activity in paired non-malignant and malignant human breast tissues. *Mol. Cell. Endocrinol.* 362, 202–210. doi: 10.1016/j.mce.2012.06.022

Sun, J., Zhong, W., Gu, Y., Groome, L. J., and Wang, Y. (2014). $1,25(OH)_2D_3$ suppresses COX-2 up-regulation and thromboxane production in placental trophoblast cells in response to hypoxic stimulation. *Placenta* 35, 143–145. doi: 10.1016/j.placenta.2013.12.002

Swami, S., Krishnan, A. V., Wang, J. Y., Jensen, K., Horst, R., Albertelli, M. A., et al. (2012). Dietary vitamin D(3) and 1,25-dihydroxyvitamin D(3) (calcitriol) exhibit equivalent anticancer activity in mouse xenograft models of breast and prostate cancer. *Endocrinology* 153, 2576–2587. doi: 10.1210/en.2011-1600

Swami, S., Raghavachari, N., Muller, U. R., Bao, Y. P., and Feldman, D. (2003). Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. *Breast Cancer Res. Treat.* 80, 49–62. doi: 10.1023/A:1024487118457

Tennant, I., Pound, J. D., Marr, L. A., Willems, J. J., Petrova, S., Ford, C. A., et al. (2013). Innate recognition of apoptotic cells: novel apoptotic cell-associated

molecular patterns revealed by crossreactivity of anti-LPS antibodies. *Cell Death Differ.* 20, 698–708. doi: 10.1038/cdd.2012.165

Ting, H. J., Yasmin-Karim, S., Yan, S. J., Hsu, J. W., Lin, T. H., Zeng, W., et al. (2012). A positive feedback signaling loop between ATM and the vitamin D receptor is critical for cancer chemoprevention by vitamin D. *Cancer Res.* 72, 958–968. doi: 10.1158/0008-5472.CAN-11-0042

Townsend, K., Banwell, C. M., Guy, M., Colston, K. W., Mansi, J. L., Stewart, P. M., et al. (2005). Autocrine metabolism of vitamin D in normal and malignant breast tissue. *Clin. Cancer Res.* 11, 3579–3586. doi: 10.1158/1078-0432.CCR-04-2359

Uberti, F., Lattuada, D., Morsanuto, V., Nava, U., Bolis, G., Vacca, G., et al. (2014). Vitamin D protects Human Endothelial Cells from oxidative stress through the autophagic and survival pathways. *J. Clin. Endocrinol. Metab.* 99, 1367–1374. doi: 10.1210/jc.2013-2103

Valrance, M. E., Brunet, A. H., and Welsh, J. (2007). Vitamin D receptor-dependent inhibition of mammary tumor growth by EB1089 and ultraviolet radiation *in vivo*. *Endocrinology* 148, 4887–4894. doi: 10.1210/en.2007-0267

van der Rhee, H., Coebergh, J. W., and de Vries, E. (2013). Is prevention of cancer by sun exposure more than just the effect of vitamin D? A systematic review of epidemiological studies. *Eur. J. Cancer* 49, 1422–1436. doi: 10.1016/j.ejca.2012.11.001

Vidal, K., and Donnet-Hughes, A. (2008). CD14: a soluble pattern recognition receptor in milk. *Adv. Exp. Med. Biol.* 606, 195–216. doi: 10.1007/978-0-387-74087-4_7

Wall, R., Powell, A., Sohn, E., Foster-Frey, J., Bannerman, D., and Paape, M. (2009). Enhanced host immune recognition of mastitis causing *Escherichia coli* in CD-14 transgenic mice. *Anim. Biotechnol.* 20, 1–14. doi: 10.1080/10495390802594206

Wang, D., Velez de-la-Paz, O. I., Zhai, J. X., and Liu, D. W. (2013). Serum 25-hydroxyvitamin D and breast cancer risk: a meta-analysis of prospective studies. *Tumour Biol.* 34, 3509–3517. doi: 10.1007/s13277-013-0929-2

Warburg, O. (1956). On the origin of cancer cells. *Science* 123, 309–314. doi: 10.1126/science.123.3191.309

Welsh, J. (2012). Cellular and molecular effects of vitamin D on carcinogenesis. *Arch. Biochem. Biophys.* 523, 107–114. doi: 10.1016/j.abb.2011.10.019

Welsh, J., Zinser, L. N., Mianecki-Morton, L., Martin, J., Waltz, S. E., James, H., et al. (2011). Age-related changes in the epithelial and stromal compartments of the mammary gland in normocalcemic mice lacking the vitamin D3 receptor. *PLoS ONE* 6:e16479. doi: 10.1371/journal.pone.0016479

Wietzke, J. A., and Welsh, J. (2003). Phytoestrogen regulation of a Vitamin D3 receptor promoter and 1,25-dihydroxyvitamin D3 actions in human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 84, 149–157. doi: 10.1016/S0960-0760(03)00024-4

Willnow, T. E., and Nykjaer, A. (2002). Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D3. *Curr. Opin. Lipidol.* 13, 255–260. doi: 10.1097/00041433-200206000-00004

Yao, S., and Ambrosone, C. B. (2013). Associations between vitamin D deficiency and risk of aggressive breast cancer in African-American women. *J. Steroid Biochem. Mol. Biol.* 136, 337–341. doi: 10.1016/j.jsbmb.2012.09.010

Zheng, J., Watson, A. D., and Kerr, D. E. (2006). Genome-wide expression analysis of lipopolysaccharide-induced mastitis in a mouse model. *Infect. Immun.* 74, 1907–1915. doi: 10.1128/IAI.74.3.1907-1915.2006

Zheng, W., Tayyari, F., Gowda, G. A., Raftery, D., McLamore, E. S., Shi, J., et al. (2013). 1,25-dihydroxyvitamin D regulation of glucose metabolism in Harvey-ras transformed MCF10A human breast epithelial cells. *J. Steroid Biochem. Mol. Biol.* 138, 81–89. doi: 10.1016/j.jsbmb.2013.03.012

Zhong, W., Gu, B., Gu, Y., Groome, L. J., Sun, J., and Wang, Y. (2014). Activation of vitamin D receptor promotes VEGF and CuZn-SOD expression in endothelial cells. *J. Steroid Biochem. Mol. Biol.* 140, 56–62. doi: 10.1016/j.jsbmb.2013.11.017

Zinser, G., Packman, K., and Welsh, J. (2002). Vitamin D(3) receptor ablation alters mammary gland morphogenesis. *Development* 129, 3067–3076.

Zinser, G. M., McEleney, K., and Welsh, J. (2003). Characterization of mammary tumor cell lines from wild type and vitamin D(3) receptor knockout mice. *Mol. Cell. Endocrinol.* 200, 67–80. doi: 10.1016/S0303-7207(02)00416-1

Zinser, G. M., and Welsh, J. (2004a). Accelerated mammary gland development during pregnancy and delayed postlactational involution in vitamin D3 receptor null mice. *Mol. Endocrinol.* 18, 2208–2223. doi: 10.1210/me.2003-0469

Zinser, G. M., and Welsh, J. (2004b). Vitamin D receptor status alters mammary gland morphology and tumorigenesis in MMTV-neu mice. *Carcinogenesis* 25, 2361–2372. doi: 10.1093/carcin/bgh271

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Vitamin D, intermediary metabolism and prostate cancer tumor progression

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Epidemiological data have demonstrated an inverse association between serum vitamin D_3 levels, cancer incidence and related mortality. However, the effects of vitamin D on prostate cancer biology and its utility for prevention of prostate cancer progression are not as well-defined. The data are often conflicting: some reports suggest that vitamin D_3 induces apoptosis in androgen dependent prostate cancer cell lines, while others suggest that vitamin D_3 only induces cell cycle arrest. Recent molecular studies have identified an extensive synergistic crosstalk between the vitamin D- and androgen-mediated mRNA and miRNA expression, adding an additional layer of post-transcriptional regulation to the known VDR- and AR-regulated gene activation. The Warburg effect, the inefficient metabolic pathway that converts glucose to lactate for rapid energy generation, is a phenomenon common to many different types of cancer. This process supports cell proliferation and promotes cancer progression via alteration of glucose, glutamine and lipid metabolism. Prostate cancer is a notable exception to this general process since the metabolic switch that occurs early during malignancy is the reverse of the Warburg effect. This "anti-Warburg effect" is due to the unique biology of normal prostate cells that harbor a truncated TCA cycle that is required to produce and secret citrate. In prostate cancer cells, the TCA cycle activity is restored and citrate oxidation is used to produce energy for cancer cell proliferation. $1,25(OH)_2D_3$ and androgen together modulates the TCA cycle via transcriptional regulation of zinc transporters, suggesting that $1,25(OH)_2D_3$ and androgen maintain normal prostate metabolism by blocking citrate oxidation. These data demonstrate the importance of androgens in the anti-proliferative effect of vitamin D in prostate cancer and highlight the importance of understanding the crosstalk between these two signaling pathways.

Keywords: vitamin D, androgen, prostate, warburg, miRNA, mRNA

OVERVIEW ON PROSTATE CANCER BIOLOGY

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in males in North America (Altekruse et al., 2010). This disease is usually considered to be an androgen dependent cancer, since the normal prostate is clearly dependent on androgens for its structure and function. Paradoxically, the age-dependent incidence and associated mortality of prostate cancer between 50 and 60 years of age increase after serum testosterone levels start to decline significantly, particularly after age of 65 (**Figure 1A**) (Siegel et al., 2014). Prostate adenocarcinomas are slow growing tumors that are characterized by low mitotic index and a long natural history (McNeal, 1968). The progression from normal prostate to prostatic intraepithelial neoplasia (PIN), and eventually to localized adenocarcinoma takes place over several decades (**Figure 1B**). Autopsy studies have shown that prostatic adenocarcinoma and the pre-malignant PIN are evident in men in their early and mid-thirties. The development of advanced, locally invasive prostate cancer and metastatic disease is a relatively late process for which there are limited treatments, and hormone ablation therapy used at this late stage applies selective stress that probably is responsible for the development of castration-resistant prostate cancer (CRPC).

VITAMIN D AND PROSTATE

There are many epidemiological studies that suggest high serum vitamin D levels, usually measured as serum 25(OH)-vitamin D_3 ($25(OH)D_3$) may be important in preventing various cancers, including breast, ovarian and colon cancer (Thorne and Campbell, 2008; Giovannucci, 2009). The risk of developing and dying of these cancers appears to be inversely correlated with sun exposure, and/or vitamin D status, suggesting that vitamin D has chemopreventive properties (Garland et al., 2009). Some studies have also suggested that vitamin D may play a role in prostate cancer prevention (Tseng et al., 2004; Schwartz and Skinner, 2007), but the data are less conclusive than in other cancers and several recent meta-analyses have found weak or no associations between $25(OH)D_3$ levels and prostate tumor incidence or progression (Yin et al., 2009; van der et al., 2009; Barnett et al., 2010; Park et al., 2010; Holt et al., 2013). However, a recent study of men diagnosed with prostate cancer showed that 72% of men with recurrent disease and 68% with clinically localized disease were insufficient or deficient in serum $25(OH)D_3$ levels, less than 20 ng/mL (desirable levels >40 ng/mL) (Trump et al., 2010). These data suggest that the majority of men with prostate cancer have low circulating androgen and low $25(OH)D_3$ levels

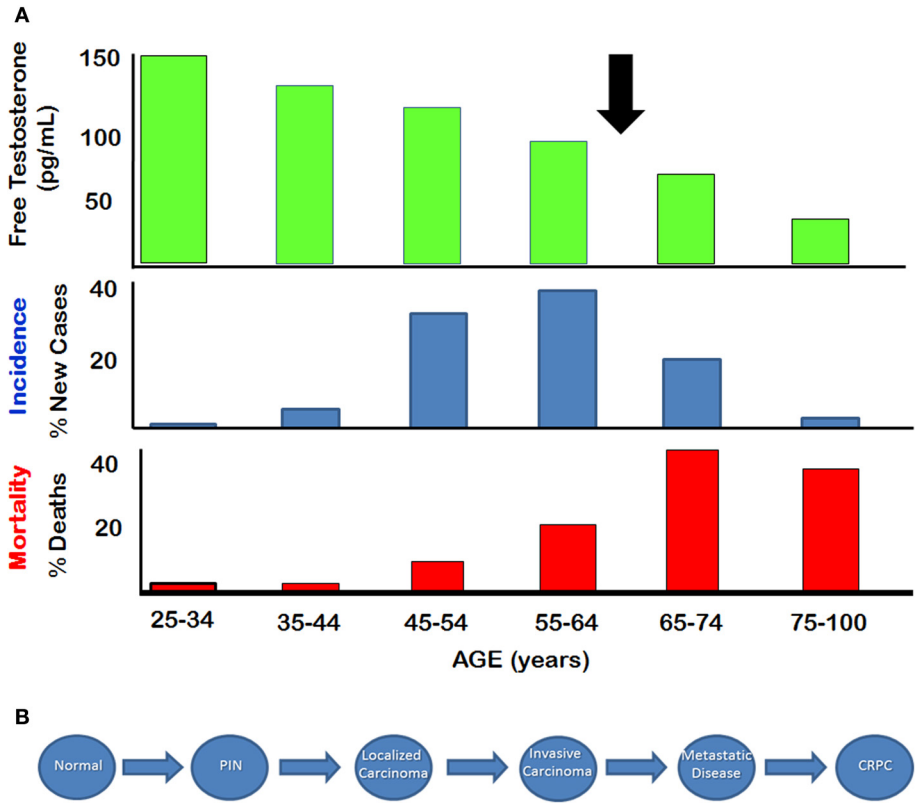


FIGURE 1 | Natural History of Prostate Cancer (A) Relationship between serum free testosterone and incidence and mortality of prostate cancer. Arrow indicates approximate age at which free testosterone declines below 50% of the level seen in young adults. **(B)** Stepwise depiction of progression from normal disease to metastatic disease. Castration resistant prostate cancer (CRPC) appears to emerge after hormone therapy.(Figure adapted from SEER database and from Framington Heart Study).

at the time of diagnosis. Based on many *in vitro* studies (Miller, 1998; Blutt et al., 2000; Peehl et al., 2003), preclinical and clinical studies (Deeb et al., 2007), it has been suggested that vitamin D can be used either as chemopreventative or as therapeutic agent for prostate cancer. Despite extensive research, the importance of vitamin D as a chemopreventive agent for prostate cancer is still a matter of considerable controversy (van der et al., 2009; Park et al., 2010), and the results from therapeutic intervention using 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D_3 or its less calcemic analogs, have been generally disappointing (Vijayakumar et al., 2005; Beer and Myrthue, 2006; Wagner et al., 2013). In low-risk prostate cancer patients who enrolled in active surveillance, high dose of vitamin D_3 supplementation decreases Gleason score or the number of positive cores in more than 50% of patient population (Marshall et al., 2012), whereas $1,25(OH)_2D_3$ supplementation at adjuvant settings have provided mixed results in CRPC or recurrent diseases (Flaig et al., 2006; Chan et al., 2008; Srinivas and Feldman, 2009; Chadha et al., 2010; Scher et al., 2011; Shamseddine et al., 2013).

Various reports suggest that the action of vitamin D in prostate cancer cells is androgen dependent (Esquenet et al., 1995; Murthy et al., 2005; Weigel, 2007; Mordan-McCombs et al., 2010). In Sprague–Dawley rats, $1,25(OH)_2D_3$ administration decreases prostatic size in intact males, but not castrated groups (Leman et al., 2003). Longitudinal studies have demonstrated a positive correlation between $25(OH)D_3$ levels and the production of androgen (Wehr et al., 2010; Pilz et al., 2011; Nimptsch et al., 2012), which has been further validated *in vitro* (Lundqvist et al., 2011). However, vitamin D also induces *CYP3A4* and *CYP3A5* expression, enzymes that metabolize and inactivate testosterone and androstanediol in prostate cells, suggesting that vitamin D signaling may play a role in limiting androgen levels in the prostate (Maguire et al., 2012). Previous *in vitro* studies have shown that $1,25(OH)_2D_3$ also induces moderate increases in *AR*, *PSA*, and *TMPRSS2* transcript levels (Hsieh et al., 1996; Zhao et al., 1999; Krishnan et al., 2004; Washington and Weigel, 2010), however this finding does not translate into clinical setting where $1,25(OH)_2D_3$ appears to decrease the PSA velocity (Krishnan et al., 2003). Based on these findings, serum vitamin D levels appear to have a significant impact on androgen-mediated signaling and the crosstalk between androgen and vitamin D probably plays an important role in prostate cancer biology. While there have been many studies examining the effects of androgens or $1,25(OH)_2D_3$ individually on gene expression in prostate cancer cells, there have been very few studies that explored the crosstalk between the two signaling pathways and the biological consequences of this crosstalk.

GENOMIC OVERLAY OF VDR AND AR SIGNALING

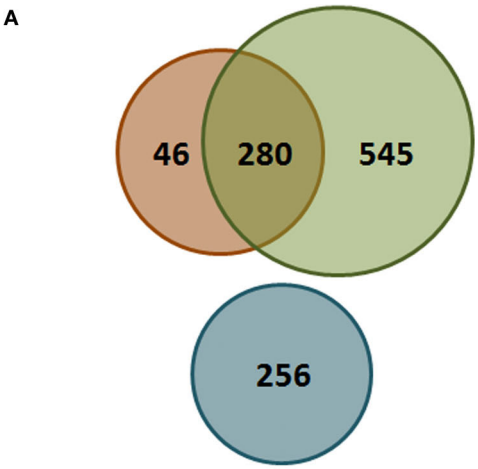
The crosstalk between VDR- and AR-mediated gene expression was first demonstrated in LNCaP cells (Qiao and Tuohimaa, 2004). Induction of *FACL3* (long-chain fatty-acid CoA ligase 3) is dependent on both vitamin D and androgen levels, and treatment with bicalutamide inhibits $1,25(OH)_2D_3$-induced *FACL3* expression. This coordinated effect on gene expression has recently been validated by a comprehensive microarray study using the same *in vitro* model (Wang et al., 2011). $1,25(OH)_2D_3$ and androgen share many common targets and coordinately modulate these transcript levels in the same direction (**Figures 2A,B**). More importantly, the combination of the two hormones regulates additional genes, including both mRNAs and miRNAs that have not been previously identified. The significance of this additional layer of transcriptional control is best illustrated by bioinformatic analysis which demonstrates the coordinated effect of $1,25(OH)_2D_3$ and androgen on cellular processes, including cell homeostasis, proliferation, differentiation and metabolism, all of which have significant impact on prostate tumorigenesis (**Figure 2C**). Most of these processes are more significantly regulated by $1,25(OH)_2D_3$ and androgen together than by either hormone alone, demonstrating the interaction between the two signaling pathways. Several genes identified from the expression microarray analysis are validated VDR and AR targets, contains functional VDRE (within 10kb upstream and 5 kb downstream of the structural gene) and ARE sites, and some genes exhibit additive induction after testosterone and $1,25(OH)_2D_3$ stimulation. Both androgen and $1,25(OH)_2D_3$ induces *PSA* mRNA levels while addition of testosterone blunts the early vitamin D dependent induction of *Cyp24A1*, the main enzyme involved in the catabolism of $1,25(OH)_2D_3$. This suggests that the half-life of $1,25(OH)_2D_3$ is extended in the presence of exogenous androgen.

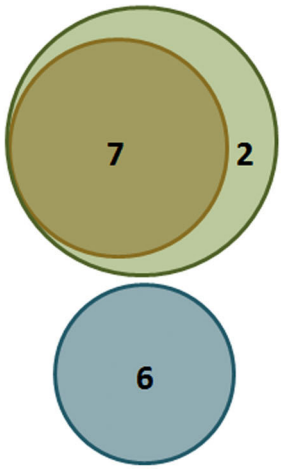
More than 50% of the responsive genes found from microarray data lack functional response elements in their promoters when comparing to existing genome-wide screens for VDREs and androgen responsive database (Wang et al., 2005; Jiang et al., 2009), raising issues regarding the regulation of these genes, particularly genes that are only expressed if both hormones are present.

The anti-neoplastic effect of vitamin D has been linked to its regulation of miRNA levels. This include the repression of *miR-181ab* expression (Wang et al., 2009) and the induction of *miR-100*, *miR-125b*, and *miR-22* levels by $1,25(OH)_2D_3$ (Alvarez-Diaz et al., 2012; Giangreco et al., 2013). Dysregulated miR-106b expression, which is required for the $1,25(OH)_2D_3$-induced feed-forward loop regulating *p21* expression in non-malignant RWPE-1 cells, has also been implicated in prostate cancer biology (Poliseno et al., 2010; Thorne et al., 2011). Microarray analysis that interrogates the differential miRNA expression in LNCaP cells after treatment with $1,25(OH)_2D_3$ and testosterone, either alone or in combination suggests that VDR plays a critical role in miRNA regulation (Wang et al., 2011) and further highlights the important interactions between VDR- and AR-mediated miRNA expression. These include the additive induction of *miR-22*, *miR-29ab*, *miR-134*, *miR-371-5p*, *miR-663*, and *miR-1207-5p* and the synergistic down-regulation of the oncogenic *miR-17/92* cluster by testosterone and $1,25(OH)_2D_3$. Both *miR-22* and members of the *miR-29* family are candidate tumor suppressors (Alvarez-Diaz et al., 2012; Szczyrba et al., 2013; Wu et al., 2013) and their induction is consistent with the anti-proliferative effect of vitamin D in prostate cancer. In comparison, elevated *miR-371-5p* and *miR-663* expression have been correlated with cancer progression and *miR-663* expression positively associates with the Gleason score used to stage prostate cancer (Zhou et al., 2012; Liu et al., 2013; Jiao et al., 2014). In contrast, the *miR-17/92* cluster is known to play an oncogenic role and its expression has been linked to more advanced prostate cancer (He et al., 2005; Volinia et al., 2006; Sylvestre et al., 2007; Yu et al., 2008; Diosdado et al., 2009; Trompeter et al., 2011; Yang et al., 2013). In addition, this cluster is a well-validated target for c-Myc, which itself is a direct target of VDR (Simpson et al., 1987; O'Donnell et al., 2005), and a recent report has proposed a regulatory role for the *miR-17/92* cluster on PPARα levels, linking *miR-17/92* to energy metabolism in prostate cancer cells (Wang et al., 2013). This concurrent analysis of VDR- and AR-mediated mRNA and miRNA expression reveals an extensive and complex transcription network that interconnects c-Myc, PPARα and other transcription factor-mediated signaling, which is only active when both androgen and vitamin D are present. A recent comprehensive analysis of 24 nuclear receptors and 14 transcription factors (TFs) in the MCF-7 breast cancer cell line has demonstrated a similar finding and has identified genomic regions enriched with nuclear receptors and TFs binding sites, which generates extensive regulatory networks that may modulate target gene expression (Kittler et al., 2013). Such functional interactions between nuclear receptors and TFs, including the antagonistic interaction between RARs and AR and PPARδ (Rivera-Gonzalez et al., 2012; Kittler et al., 2013), and the agonistic interaction between VDR and AR (Wang et al., 2011) provide valuable information that can be used to improve cancer prevention and therapy. The functional interactions between AR and VDR, as well as other nuclear receptors and TFs may also be important for disease management, especially now that nutritional intervention has become more widely accepted as an effective approach to prevent cancer progression. These experimental data suggest that $1,25(OH)_2D_3$, and androgens as well as other hormones and growth factors trigger at least three mechanisms to modulate global gene expression. These include AR- and VDR-mediated gene transactivation; miRNA-mediated mRNA degradation and translational repression; and transcription factor-mediated feed-forward signaling. These mechanisms do not appear to be mutually exclusive and act together to regulate many vitamin D- and androgen-mediated cellular processes that have significant implication in prostate carcinogenesis.

INTERMEDIATE METABOLISM: THE WARBURG EFFECT

A number of studies have suggested that vitamin D has a novel role in regulating energy metabolism. The vitamin D receptor knockout (VDRKO) and the *Cyp27b1* knockout (Cyp27b1KO) mice exhibit elevated energy expenditure with subsequent loss of body fat over time (Narvaez et al., 2009; Wong et al., 2011). In human adipocytes, $1,25(OH)_2D_3$ inhibits uncoupling protein-1 expression and alters Ca^{2+} homeostasis, suggesting a regulatory role of vitamin D in thermogenesis and provides





C

Biological Process	Testosterone		$1,25(OH)_2D_3$		TD	
	Median Change	p-value	Median Change	p-value	Median Change	p-value
Mitosis	-1.54	3.51E-10	-1.98	3.63E-07	-3.63	1.27E-15
Microtubule-based movement	-1.62	2.54E-05	-2.11	1.17E-04	-4.19	5.78E-07
Chromosome segregation	-1.61	1.11E-03	-1.99	2.13E-03	-3.44	1.12E-04
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	-1.47	5.90E-03	n/a	n/a	-3.63	2.10E-03
DNA repair	n/a	n/a	-1.77	1.08E-02	-2.55	2.24E-03
Chromosome organization	n/a	n/a	n/a	n/a	-3.74	1.04E-03
Elevation of cytosolic calcium ion concentration	n/a	n/a	2.02	1.76E-02	3.43	2.95E-02
Angiogenesis	n/a	n/a	n/a	n/a	3	1.99E-02
DNA recombination	n/a	n/a	-2.02	4.30E-02	-3.42	1.41E-02
Phosphoinositide-mediated signaling	n/a	n/a	-1.73	4.97E-02	-2.4	1.60E-02

FIGURE 2 | The effect of T and $1,25(OH)_2D_3$ on mRNA and miRNA expression in LNCaP cells. (A) Venn diagram analysis of the gene expression microarray data [Green: $1,25(OH)_2D_3$-moduated, Orange: androgen-modulated, Blue: synergistically modulated genes by androgen and $1,25(OH)_2D_3$]. **(B)** Venn diagram analysis of the miRNA microarray data [Green: $1,25(OH)_2D_3$-modulated, Orange: androgen-modulated, Blue: synergistically modulated genes by androgen and $1,25(OH)_2D_3$]. **(C)** Gene set enrichment analysis of representative gene sets identified as significantly enriched after $1,25(OH)_2D_3$ treatment in the presence or absence of androgen in LNCaP cells. False discovery rate <5%.

rationale for the observed lean phenotype in VDRKO and Cyp27b1KO mice (Xue et al., 1998; Shi et al., 2001, 2002). Similarly, both $25(OH)D_3$ and $1,25(OH)_2D_3$ promotes lipogenesis in primary human preadipocytes, adipocyte and adipose-derived mesenchymal progenitor cells, which is associated with increased expression of differentiation markers *C/EBPα* and *PPARγ* (Nimitphong et al., 2012; Narvaez et al., 2013). However, this effect may be cell type and lineage specific since $1,25(OH)_2D_3$ inhibits lipid accumulation in mouse 3T3-L1 preadipocytes and prevents high fat diet-induced fatty liver syndrome in Sprague–Dawley male rats (Rayalam et al., 2008; Yin et al., 2012).

In T47D breast cancer cells, $1,25(OH)_2D_3$ induces lipid synthesis, which has been associated with its effect on cell differentiation and reduced cell growth (Lazzaro et al., 2000). This lipogenic effect of $1,25(OH)_2D_3$ is recapitulated in LNCaP cells and is enhanced in the presence of androgen (Esquenet et al., 1997; Wang et al., 2013), highlighting the coordinated effect of AR and VDR signaling. Increase in PPARα expression and its associated lipogenic gene signature, including the elevation of fatty acid synthase (*FASN*) expression, accounts for vitamin D- and androgen-induced lipid production. However, this occurs without significant changes in nuclear sterol regulatory element-binding protein (SREBP-1) levels. Nuclear activation of

SREBP-1 has been implicated in *de novo* lipogenesis in more aggressive cancers, including prostate cancer (Menendez and Lupu, 2007; Huang et al., 2012). A recent comprehensive parallel analysis of various genomic studies using prostate cancer cell lines has uncovered a critical regulatory role of AR in the energy metabolic network, with lipid synthesis being the predominate AR-regulated process. These data suggest that altered AR signaling and its effects on the downstream targets of calcium/calmodulin-dependent protein kinase kinase 2, beta (*CAMKK2*), which regulates the activity of a key energy sensor AMP-activated protein kinase (AMPK), promotes the metabolic switch that provides the energy for prostate cancer growth and progression (Massie et al., 2011). These data suggest a divergent role of lipid production in prostate tumors: SREBP-1 dependent up-regulation of fatty acids production for phospholipid and membrane synthesis and signaling molecules that are essential for tumor progression (Currie et al., 2013; Soga, 2013); or SREBP-1-independent elevation of neutral and inactive lipid accumulation which restricts energy expenditure and limits tumor growth.

In addition to the modulation of lipid metabolism by vitamin D and androgen, qPCR analysis has suggested a regulatory role of these two hormones on the TCA cycle in prostate cancer cells. In most normal cells, the TCA cycle is utilized to generate energy for normal cellular functions. This process is relatively slow and ATP production does not meet the demand for highly proliferative cancer cells. As a result, cancer cells often disengage mitochondrial oxidative phosphorylation from glycolysis for rapid ATP production by employing the fermentation process, a process referred as the Warburg effect (Warburg et al., 1927; Warburg, 1956; Soga, 2013). Prostate cancer cells are a notable exception, and the metabolic switch that occurs is more appropriately regarded as an "anti-Warburg" effect. The prostate gland normally secrets high levels of citrate into the seminal fluid, a function that is supported by a truncated TCA cycle activity. The prostate has the highest levels of intracellular zinc of any tissue in the body. This high level of zinc inactivates m-aconitase 2 activity, the enzyme that converts citrate to isocitrate in the mitochondria. In prostate cancer cells, zinc transporters are down-regulated, which leads to lower intracellular zinc levels. This restores m-aconitase 2 function and the conversion of citrate to isocitrate for ATP production via the TCA cycle (Costello and Franklin, 1991a,b). This is supported by both clinical and *in vitro* data, demonstrating a minimal reliance of prostate cancer cells on glycolysis for proliferation especially during the early phases of tumor progression. This precludes the usage of fluorine-18-labeled 2-deoxy-2-fluoro-$_D$-glucose (FDG-PET) for prostate cancer detection and diagnosis (Hofer et al., 1999; Jadvar, 2011). In comparison, androgen stimulates glucose usage to facilitate citrate accumulation in normal prostate epithelial cells (Harkonen, 1981; Harkonen et al., 1982) and this androgenic effect is maintained in androgen responsive prostate cancer cells, although in these cells, elevated citrate is funneled for the production of lipid (Moon et al., 2011).

In LNCaP cells, 1,25$(OH)_2D_3$ and androgen together down-regulate mitochondrial thiamine pyrophosphate (TPP) carrier (*SLC25A19*) and up-regulates two zinc transporters, (*SLC39A1* and *SLC39A11*) (supplemental data to Wang et al., 2011). Low expression of *SLC39A1* in adenocarcinomatous glands and PIN foci has been documented and linked to depleted zinc levels (Franklin et al., 2005). In comparison, *SLC39A11* is less well-characterized, but studies have shown that it is abundantly expressed in murine testes and digestive system, and is associated with zinc import (Yu et al., 2013). This suggests that vitamin D and androgen cooperate to reset zinc levels, inhibiting m-aconitase activity in prostate cancer cells. In comparison, down-regulation of the TPP carrier, *SLC25A19* (Lindhurst et al., 2006; Kang and Samuels, 2008) affects mitochondrial coenzyme TPP levels, leading to decreased activities of pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (OGDH) activities. In comparison, *in vivo* studies have shown that administration of testosterone up-regulates the expression and activities of PDH and mitochondrial aspartate aminotransferase to increase the substrate pools for citrate synthesis, acetyl-CoA and oxaloacetate (Costello and Franklin, 1993; Qian et al., 1993). This suggests that vitamin D and androgen supplementation facilitate the reversion of the metabolic switch that occurs during prostate carcinogenesis by preventing citrate oxidation, partially restoring the normal prostatic function and shunting citrate into the cytoplasm for secretion and lipid synthesis (**Figure 3**). This is supported by the observation that LNCaP cells retain the sensitivity to androgen-induced citrate production and accumulation (Franklin et al., 1995). This suggests that vitamin D facilitates and maintains this differentiated phenotype, rendering prostate cancer cells less aggressive. This also suggests that maintaining or restoring adequate levels of androgen, accompanied by vitamin D supplement will significantly delay prostate cancer progression in aging men.

To further highlight the impact of vitamin D and androgen on resetting cancer cell metabolism, 1,25$(OH)_2D_3$ and androgen also down-regulate c-Myc levels, whose many functions include metabolic reprogramming to drive tumor progression, including the induction of glycolysis and glutaminolysis (Shim et al., 1998; Wise et al., 2008; Soga, 2013; Zirath et al., 2013). While there is good evidence suggesting a positive correlation between serum glutamate levels and more aggressive prostate cancer (Koochekpour et al., 2012), the dependence of prostate cancer on glutaminolysis for energy generation and progression is not well-studied. Nevertheless, it is reasonable to suggest that in response to vitamin D and androgen stimulation, prostate cancer cells reverse or block the metabolic switch that occurs early in the course of the disease and further blocks c-Myc-mediated metabolic reprogramming, which may occur independently of the initial metabolic switch.

CONCLUSION

Recent studies have shown a complex relationship between vitamin D_3- and androgen-mediated signaling in the normal prostate and prostate cancer through their coordinated effect on mRNA and miRNA transcription, cell proliferation and cancer metabolism. These data suggest that the effect of vitamin D_3 on global gene expression is dependent on the activity of androgen and their combined effect on miRNA transcription and other TFs. Phenotypically, the two hormones maintain normal prostatic metabolism to prevent de-differentiation of prostate cancer

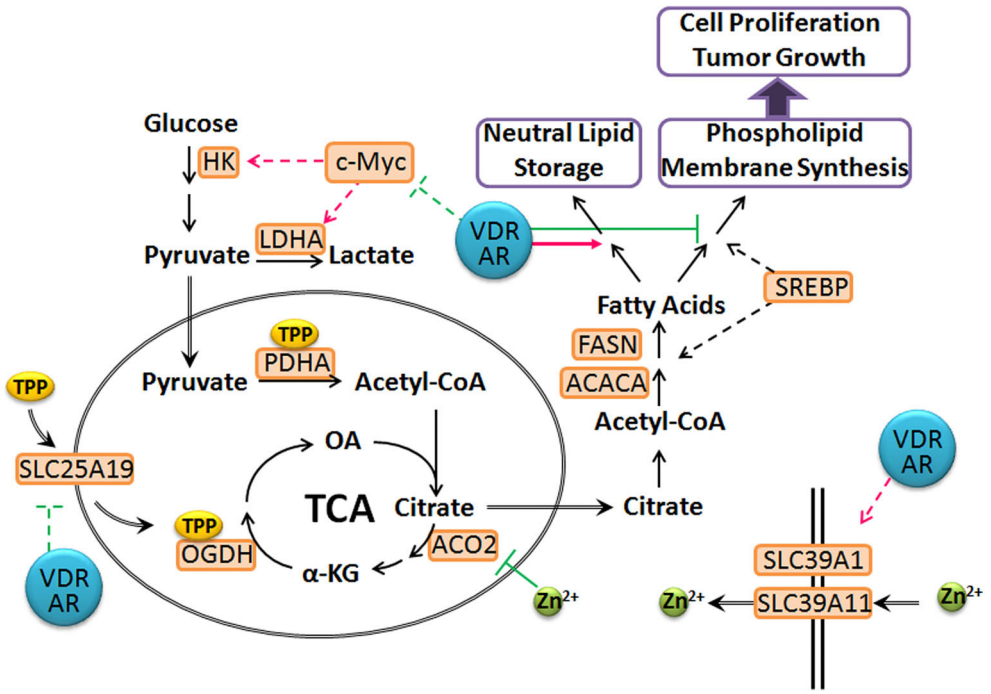


FIGURE 3 | Proposed model of T- and 1,25(OH)$_2$D$_3$-mediated prostate cancer metabolism. The VDR and AR axes modulate the expression of SLC25A19 and SLC39A11, leading to elevated intracellular zinc levels and deplete mitochondrial TPP pool, resulting in a truncated TCA cycle. Citrate is shunted into lipid synthesis and storage instead of phospholipid and membrane synthesis, which prevents cancer cell proliferation. In addition, VDR and AR repress c-Myc levels and associated metabolic reprogramming to maintain prostate cancer cells in a more differentiated state. (dashed line: transcriptional regulation; green: inhibition; magenta: stimulation).

cells into more aggressive phenotype. These newly emerging data provide a explanation for the discrepancies observed from epidemiological and experimental studies of vitamin D$_3$ in prostate cancer since these studies do not take the synergistic interactions between the two pathways into account. These data also suggest that maintenance of adequate levels of vitamin D$_3$ and androgen will slow or halt prostate cancer progression especially for patients diagnosed with early stage, locally confined disease. Case-control clinical studies will be needed to fully evaluate the risk and benefit of combining these two hormones in prostate cancer patients.

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REFERENCES

Altekruse, S. F., Huang, L., Cucinelli, J. E., McNeel, T. S., Wells, K. M., and Oliver, M. N. (2010). Spatial patterns of localized-stage prostate cancer incidence among white and black men in the southeastern United States, 1999-2001. *Cancer Epidemiol. Biomarkers Prev.* 19, 1460–1467. doi: 10.1158/1055-9965.EPI-09-1310

Alvarez-Diaz, S., Valle, N., Ferrer-Mayorga, G., Lombardia, L., Herrera, M., Dominguez, O., et al. (2012). MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells. *Hum. Mol. Genet.* 21, 2157–2165. doi: 10.1093/hmg/dds031

Barnett, C. M., Nielson, C. M., Shannon, J., Chan, J. M., Shikany, J. M., Bauer, D. C., et al. (2010). Serum 25-OH vitamin D levels and risk of developing prostate cancer in older men. *Cancer Causes Control* 21, 1297–1303. doi: 10.1007/s10552-010-9557-y

Beer, T. M., and Myrthue, A. (2006). Calcitriol in the treatment of prostate cancer. *Anticancer Res.* 26, 2647–2651.

Blutt, S. E., Polek, T. C., Stewart, L. V., Kattan, M. W., and Weigel, N. L. (2000). A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice. *Cancer Res.* 60, 779–782.

Chadha, M. K., Tian, L., Mashtare, T., Payne, V., Silliman, C., Levine, E., et al. (2010). Phase 2 trial of weekly intravenous 1,25 dihydroxy cholecalciferol (calcitriol) in combination with dexamethasone for castration-resistant prostate cancer. *Cancer* 116, 2132–2139. doi: 10.1002/cncr.24973

Chan, J. S., Beer, T. M., Quinn, D. I., Pinski, J. K., Garzotto, M., Sokoloff, M., et al. (2008). A phase II study of high-dose calcitriol combined with mitoxantrone and prednisone for androgen-independent prostate cancer. *BJU Int.* 102, 1601–1606. doi: 10.1111/j.1464-410X.2008.08017.x

Costello, L. C., and Franklin, R. B. (1991a). Concepts of citrate production and secretion by prostate. 1. metabolic relationships. *Prostate* 18, 25–46. doi: 10.1002/pros.2990180104

Costello, L. C., and Franklin, R. B. (1991b). Concepts of citrate production and secretion by prostate: 2. hormonal relationships in normal and neoplastic prostate. *Prostate* 19, 181–205. doi: 10.1002/pros.2990190302

Costello, L. C., and Franklin, R. B. (1993). Testosterone regulates pyruvate dehydrogenase activity of prostate mitochondria. *Horm. Metab. Res.* 25, 268–270. doi: 10.1055/s-2007-1002094

Currie, E., Schulze, A., Zechner, R., Walther, T. C., and Farese, R. V. Jr. (2013). Cellular fatty acid metabolism and cancer. *Cell Metab.* 18, 153–161. doi: 10.1016/j.cmet.2013.05.017

Deeb, K. K., Trump, D. L., and Johnson, C. S. (2007). Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat. Rev. Cancer* 7, 684–700. doi: 10.1038/nrc2196

Diosdado, B., van de Wiel, M. A., Terhaar Sive Droste, J. S., Mongera, S., Postma, C., Meijerink, W. J., et al. (2009). MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. *Br. J. Cancer* 101, 707–714. doi: 10.1038/sj.bjc.6605037

Esquenet, M., Swinnen, J. V., Heyns, W., and Verhoeven, G. (1995). Triiodothyronine modulates growth, secretory function and androgen receptor concentration in the prostatic carcinoma cell line LNCaP. *Mol. Cell. Endocrinol.* 109, 105–111. doi: 10.1016/0303-7207(95)03490-X

Esquenet, M., Swinnen, J. V., Van Veldhoven, P. P., Denef, C., Heyns, W., and Verhoeven, G. (1997). Retinoids stimulate lipid synthesis and accumulation in LNCaP prostatic adenocarcinoma cells. *Mol. Cell. Endocrinol.* 136, 37–46. doi: 10.1016/S0303-7207(97)00210-4

Flaig, T. W., Barqawi, A., Miller, G., Kane, M., Zeng, C., Crawford, E. D., et al. (2006). A phase II trial of dexamethasone, vitamin D, and carboplatin in patients with hormone-refractory prostate cancer. *Cancer* 107, 266–274. doi: 10.1002/cncr.21982

Franklin, R. B., Feng, P., Milon, B., Desouki, M. M., Singh, K. K., Kajdacsy-Balla, A., et al. (2005). hZIP1 zinc uptake transporter down regulation and zinc depletion in prostate cancer. *Mol. Cancer* 4, 32. doi: 10.1186/1476-4598-4-32

Franklin, R. B., Juang, H. H., Zou, J., and Costello, L. C. (1995). Regulation of citrate metabolism by androgen in the LNCaP human prostate carcinoma cell line. *Endocrine* 3, 603–607. doi: 10.1007/BF02953026

Garland, C. F., Gorham, E. D., Mohr, S. B., and Garland, F. C. (2009). Vitamin D for cancer prevention: global perspective. *Ann. Epidemiol.* 19, 468–483. doi: 10.1016/j.annepidem.2009.03.021

Giangreco, A. A., Vaishnav, A., Wagner, D., Finelli, A., Fleshner, N., Van der, K. T., et al. (2013). Tumor suppressor microRNAs, miR-100 and -125b, are regulated by 1,25-dihydroxyvitamin D in primary prostate cells and in patient tissue. *Cancer Prev. Res. (Phila)* 6, 483–494. doi: 10.1158/1940-6207.CAPR-12-0253

Giovannucci, E. (2009). Expanding roles of vitamin, D. *J. Clin. Endocrinol. Metab.* 94, 418–420. doi: 10.1210/jc.2008-2695

Harkonen, P. (1981). Androgenic control of glycolysis, the pentose cycle and pyruvate dehydrogenase in the rat ventral prostate. *J. Steroid Biochem.* 14, 1075–1084. doi: 10.1016/0022-4731(81)90219-3

Harkonen, P. L., Kostian, M. L., and Santti, R. S. (1982). Indirect androgenic control of citrate accumulation in rat ventral prostate. *Arch. Androl.* 8, 107–116. doi: 10.3109/01485018208987026

He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., et al. (2005). A microRNA polycistron as a potential human oncogene. *Nature* 435, 828–833. doi: 10.1038/nature03552

Hofer, C., Laubenbacher, C., Block, T., Breul, J., Hartung, R., and Schwaiger, M. (1999). Fluorine-18-fluorodeoxyglucose positron emission tomography is useless for the detection of local recurrence after radical prostatectomy. *Eur. Urol.* 36, 31–35. doi: 10.1159/000019923

Holt, S. K., Kolb, S., Fu, R., Horst, R., Feng, Z., and Stanford, J. L. (2013). Circulating levels of 25-hydroxyvitamin D and prostate cancer prognosis. *Cancer Epidemiol.* 37, 666–670. doi: 10.1016/j.canep.2013.07.005

Hsieh, T. Y., Ng, C. Y., Mallouh, C., Tazaki, H., and Wu, J. M. (1996). Regulation of growth, PSA/PAP and androgen receptor expression by 1 alpha,25-dihydroxyvitamin D3 in the androgen-dependent LNCaP cells. *Biochem. Biophys. Res. Commun.* 223, 141–146. doi: 10.1006/bbrc.1996.0859

Huang, W. C., Li, X., Liu, J., Lin, J., and Chung, L. W. (2012). Activation of androgen receptor, lipogenesis, and oxidative stress converged by SREBP-1 is responsible for regulating growth and progression of prostate cancer cells. *Mol. Cancer Res.* 10, 133–142. doi: 10.1158/1541-7786.MCR-11-0206

Jadvar, H. (2011). Prostate cancer: PET with 18F-FDG, 18F- or 11C-acetate, and 18F- or 11C-choline. *J. Nucl. Med.* 52, 81–89. doi: 10.2967/jnumed.110.077941

Jiang, M., Ma, Y., Chen, C., Fu, X., Yang, S., Li, X., et al. (2009). Androgen-responsive gene database: integrated knowledge on androgen-responsive genes. *Mol. Endocrinol.* 23, 1927–1933. doi: 10.1210/me.2009-0103

Jiao, L., Deng, Z., Xu, C., Yu, Y., Li, Y., Yang, C., et al. (2014). MicroRNA-663 induces castration-resistant prostate cancer transformation and predicts clinical recurrence. *J. Cell. Physiol.* 229, 834–844. doi: 10.1002/jcp.24510

Kang, J., and Samuels, D. C. (2008). The evidence that the DNC (SLC25A19) is not the mitochondrial deoxyribonucleotide carrier. *Mitochondrion* 8, 103–108. doi: 10.1016/j.mito.2008.01.001

Kittler, R., Zhou, J., Hua, S., Ma, L., Liu, Y., Pendleton, E., et al. (2013). A comprehensive nuclear receptor network for breast cancer cells. *Cell Rep.* 3, 538–551. doi: 10.1016/j.celrep.2013.01.004

Koochekpour, S., Majumdar, S., Azabdaftari, G., Attwood, K., Scioneaux, R., Subramani, D., et al. (2012). Serum glutamate levels correlate with gleason score and glutamate blockade decreases proliferation, migration, and invasion and induces apoptosis in prostate cancer cells. *Clin. Cancer Res.* 18, 5888–5901. doi: 10.1158/1078-0432.CCR-12-1308

Krishnan, A. V., Peehl, D. M., and Feldman, D. (2003). Inhibition of prostate cancer growth by vitamin D: regulation of target gene expression. *J. Cell. Biochem.* 88, 363–371. doi: 10.1002/jcb.10334

Krishnan, A. V., Shinghal, R., Raghavachari, N., Brooks, J. D., Peehl, D. M., and Feldman, D. (2004). Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. *Prostate* 59, 243–251. doi: 10.1002/pros.20006

Lazzaro, G., Agadir, A., Qing, W., Poria, M., Mehta, R. R., Moriarty, R. M., et al. (2000). Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors. *Eur. J. Cancer* 36, 780–786. doi: 10.1016/S0959-8049(00)00016-2

Leman, E. S., Arlotti, J. A., Dhir, R., and Getzenberg, R. H. (2003). Vitamin D and androgen regulation of prostatic growth. *J. Cell. Biochem.* 90, 138–147. doi: 10.1002/jcb.10605

Lindhurst, M. J., Fiermonte, G., Song, S., Struys, E., De Leonardis, F., Schwartzberg, P. L., et al. (2006). Knockout of Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15927–15932. doi: 10.1073/pnas.0607661103

Liu, R. Y., Diao, C. F., Zhang, Y., Wu, N., Wan, H. Y., Nong, X. Y., et al. (2013). miR-371-5p down-regulates pre mRNA processing factor 4 homolog B (PRPF4B) and facilitates the G1/S transition in human hepatocellular carcinoma cells. *Cancer Lett.* 335, 351–360. doi: 10.1016/j.canlet.2013.02.045

Lundqvist, J., Norlin, M., and Wikvall, K. (2011). 1alpha,25-Dihydroxyvitamin D3 exerts tissue-specific effects on estrogen and androgen metabolism. *Biochim. Biophys. Acta* 1811, 263–270. doi: 10.1016/j.bbalip.2011.01.004

Maguire, O., Pollock, C., Martin, P., Owen, A., Smyth, T., Doherty, D., et al. (2012). Regulation of CYP3A4 and CYP3A5 expression and modulation of "intracrine" metabolism of androgens in prostate cells by liganded vitamin D receptor. *Mol. Cell. Endocrinol.* 364, 54–64. doi: 10.1016/j.mce.2012.08.007

Marshall, D. T., Savage, S. J., Garrett-Mayer, E., Keane, T. E., Hollis, B. W., Horst, R. L., et al. (2012). Vitamin D3 supplementation at 4000 international units per day for one year results in a decrease of positive cores at repeat biopsy in subjects with low-risk prostate cancer under active surveillance. *J. Clin. Endocrinol. Metab.* 97, 2315–2324. doi: 10.1210/jc.2012-1451

Massie, C. E., Lynch, A., Ramos-Montoya, A., Boren, J., Stark, R., Fazli, L., et al. (2011). The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. *EMBO J.* 30, 2719–2733. doi: 10.1038/emboj.2011.158

McNeal, J. E. (1968). Regional morphology and pathology of the prostate. *Am. J. Clin. Pathol.* 49, 347–357.

Menendez, J. A., and Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* 7, 763–777. doi: 10.1038/nrc2222

Miller, G. J. (1998). Vitamin D and prostate cancer: biologic interactions and clinical potentials. *Cancer Metastasis Rev.* 17, 353–360. doi: 10.1023/A:1006102124548

Moon, J. S., Jin, W. J., Kwak, J. H., Kim, H. J., Yun, M. J., Kim, J. W., et al. (2011). Androgen stimulates glycolysis for de novo lipid synthesis by increasing the activities of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 in prostate cancer cells. *Biochem. J.* 433, 225–233. doi: 10.1042/BJ20101104

Mordan-McCombs, S., Brown, T., Wang, W. L., Gaupel, A. C., Welsh, J., and Tenniswood, M. (2010). Tumor progression in the LPB-Tag transgenic model of prostate cancer is altered by vitamin D receptor and serum testosterone status. *J. Steroid Biochem. Mol. Biol.* 121, 368–371. doi: 10.1016/j.jsbmb.2010.03.062

Murthy, S., Agoulnik, I. U., and Weigel, N. L. (2005). Androgen receptor signaling and vitamin D receptor action in prostate cancer cells. *Prostate* 64, 362–372. doi: 10.1002/pros.20251

Narvaez, C. J., Matthews, D., Broun, E., Chan, M., and Welsh, J. (2009). Lean phenotype and resistance to diet-induced obesity in vitamin D receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue. *Endocrinology* 150, 651–661. doi: 10.1210/en.2008-1118

Narvaez, C. J., Simmons, K. M., Brunton, J., Salinero, A., Chittur, S. V., and Welsh, J. E. (2013). Induction of STEAP4 correlates with 1,25-dihydroxyvitamin D3 stimulation of adipogenesis in mesenchymal progenitor cells derived from human adipose tissue. *J. Cell. Physiol.* 228, 2024–2036. doi: 10.1002/jcp.24371

Nimitphong, H., Holick, M. F., Fried, S. K., and Lee, M. J. (2012). 25-hydroxyvitamin D(3) and 1,25-dihydroxyvitamin D(3) promote the differentiation of human subcutaneous preadipocytes. *PLoS ONE* 7:e52171. doi: 10.1371/journal.pone.0052171

Nimptsch, K., Platz, E. A., Willett, W. C., and Giovannucci, E. (2012). Association between plasma 25-OH vitamin D and testosterone levels in men. *Clin. Endocrinol. (Oxf.)* 77, 106–112. doi: 10.1111/j.1365-2265.2012.04332.x

O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435, 839–843. doi: 10.1038/nature03677

Park, S. Y., Cooney, R. V., Wilkens, L. R., Murphy, S. P., Henderson, B. E., and Kolonel, L. N. (2010). Plasma 25-hydroxyvitamin D and prostate cancer risk: the multiethnic cohort. *Eur. J. Cancer* 46, 932–936. doi: 10.1016/j.ejca.2009.12.030

Peehl, D. M., Krishnan, A. V., and Feldman, D. (2003). Pathways mediating the growth-inhibitory actions of vitamin D in prostate cancer. *J. Nutr.* 133, 2461S–2469S.

Pilz, S., Frisch, S., Koertke, H., Kuhn, J., Dreier, J., Obermayer-Pietsch, B., et al. (2011). Effect of vitamin D supplementation on testosterone levels in men. *Horm. Metab. Res.* 43, 223–225. doi: 10.1055/s-0030-1269854

Poliseno, L., Salmena, L., Riccardi, L., Fornari, A., Song, M. S., Hobbs, R. M., et al. (2010). Identification of the miR-106b∼25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. *Sci. Signal.* 3, ra29. doi: 10.1126/scisignal.200059

Qian, K., Franklin, R. B., and Costello, L. C. (1993). Testosterone regulates mitochondrial aspartate aminotransferase gene expression and mRNA stability in prostate. *J. Steroid Biochem. Mol. Biol.* 44, 13–19. doi: 10.1016/0960-0760(93)90146-N

Qiao, S., and Tuohimaa, P. (2004). The role of long-chain fatty-acid-CoA ligase 3 in vitamin D3 and androgen control of prostate cancer LNCaP cell growth. *Biochem. Biophys. Res. Commun.* 319, 358–368. doi: 10.1016/j.bbrc.2004.05.014

Rayalam, S., Della-Fera, M. A., Ambati, S., Yang, J. Y., Park, H. J., and Baile, C. A. (2008). Enhanced effects of 1,25(OH)(2)D(3) plus genistein on adipogenesis and apoptosis in 3T3-L1 adipocytes. *Obesity (Silver Spring)* 16, 539–546. doi: 10.1038/oby.2007.90

van der, R. H., Coebergh, J. W., and de Vries, E. (2009). Sunlight, vitamin D and the prevention of cancer: a systematic review of epidemiological studies. *Eur. J. Cancer Prev.* 18, 458–475. doi: 10.1097/CEJ.0b013e32832f9bb1

Rivera-Gonzalez, G. C., Droop, A. P., Rippon, H. J., Tiemann, K., Pellacani, D., Georgopoulos, L. J., et al. (2012). Retinoic acid and androgen receptors combine to achieve tissue specific control of human prostatic transglutaminase expression: a novel regulatory network with broader significance. *Nucleic Acids Res.* 40, 4825–4840. doi: 10.1093/nar/gks143

Scher, H. I., Jia, X., Chi, K., de Wit, R., Berry, W. R., Albers, P., et al. (2011). Randomized, open-label phase III trial of docetaxel plus high-dose calcitriol versus docetaxel plus prednisone for patients with castration-resistant prostate cancer. *J. Clin. Oncol.* 29, 2191–2198. doi: 10.1200/JCO.2010.32.8815

Schwartz, G. G., and Skinner, H. G. (2007). Vitamin D status and cancer: new insights. *Curr. Opin. Clin. Nutr. Metab. Care* 10, 6–11. doi: 10.1097/MCO.0b013e328011aa60

Shamseddine, A., Farhat, F. S., Elias, E., Khauli, R. B., Saleh, A., and Bulbul, M. A. (2013). High-dose calcitriol, docetaxel and zoledronic acid in patients with castration-resistant prostate cancer: a phase II study. *Urol. Int.* 90, 56–61. doi: 10.1159/000343780

Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2001). 1alpha,25-Dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. *FASEB J.* 15, 2751–2753. doi: 10.1096/fj.01-0584fje

Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B. (2002). 1alpha,25-dihydroxyvitamin D3 inhibits uncoupling protein 2 expression in human adipocytes. *FASEB J.*16, 1808–1810. doi: 10.1096/fj.02-0255fje

Shim, H., Chun, Y. S., Lewis, B. C., and Dang, C. V. (1998). A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1511–1516. doi: 10.1073/pnas.95.4.1511

Siegel, R., Ma, J., Zou, Z., and Jemal, A. (2014). Cancer statistics, 2014. *CA Cancer J. Clin.* 64, 9–29. doi: 10.3322/caac.21208

Simpson, R. U., Hsu, T., Begley, D. A., Mitchell, B. S., and Alizadeh, B. N. (1987). Transcriptional regulation of the c-myc protooncogene by 1,25-dihydroxyvitamin D3 in HL-60 promyelocytic leukemia cells. *J. Biol. Chem.* 262, 4104–4108.

Soga, T. (2013). Cancer metabolism: key players in metabolic reprogramming. *Cancer Sci.* 104, 275–281. doi: 10.1111/cas.12085

Srinivas, S., and Feldman, D. (2009). A phase II trial of calcitriol and naproxen in recurrent prostate cancer. *Anticancer Res.* 29, 3605–3610.

Sylvestre, Y., De, G. V., Querido, E., Mukhopadhyay, U. K., Bourdeau, V., Major, F., et al. (2007). An E2F/miR-20a autoregulatory feedback loop. *J. Biol. Chem.* 282, 2135–2143. doi: 10.1074/jbc.M608939200

Szczyrba, J., Nolte, E., Hart, M., Doll, C., Wach, S., Taubert, H., et al. (2013). Identification of ZNF217, hnRNP-K, VEGF-A and IPO7 as targets for microRNAs that are downregulated in prostate carcinoma. *Int. J. Cancer* 132, 775–784. doi: 10.1002/ijc.27731

Thorne, J., and Campbell, M. J. (2008). The vitamin D receptor in cancer. *Proc. Nutr. Soc.* 67, 115–127. doi: 10.1017/S0029665108006964

Thorne, J. L., Maguire, O., Doig, C. L., Battaglia, S., Fehr, L., Sucheston, L. E., et al. (2011). Epigenetic control of a VDR-governed feed-forward loop that regulates p21(waf1/cip1) expression and function in non-malignant prostate cells. *Nucleic Acids Res.* 39, 2045–2056. doi: 10.1093/nar/gkq875

Trompeter, H. I., Abbad, H., Iwaniuk, K. M., Hafner, M., Renwick, N., Tuschl, T., et al. (2011). MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. *PLoS ONE* 6:e16138. doi: 10.1371/journal.pone.0016138

Trump, D. L., Deeb, K. K., and Johnson, C. S. (2010). Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy. *Cancer J.* 16, 1–9. doi: 10.1097/PPO.0b013e3181c51ee6

Tseng, M., Breslow, R. A., DeVellis, R. F., and Ziegler, R. G. (2004). Dietary patterns and prostate cancer risk in the national health and nutrition examination survey epidemiological follow-up study cohort. *Cancer Epidemiol. Biomarkers Prev.* 13, 71–77. doi: 10.1158/1055-9965.EPI-03-0076

Vijayakumar, S., Mehta, R. R., Boerner, P. S., Packianathan, S., and Mehta, R. G. (2005). Clinical trials involving vitamin D analogs in prostate cancer. *Cancer J.* 11, 362–373. doi: 10.1097/00130404-200509000-00002

Volinia, S., Calin, G. A., Liu, C. G., Ambs, S., Cimmino, A., Petrocca, F., et al. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2257–2261. doi: 10.1073/pnas.0510565103

Wagner, D., Trudel, D., Van der, K. T., Nonn, L., Giangreco, A. A., Li, D., et al. (2013). Randomized clinical trial of vitamin D3 doses on prostatic vitamin D metabolite levels and ki67 labeling in prostate cancer patients. *J. Clin. Endocrinol. Metab.* 98, 1498–1507. doi: 10.1210/jc.2012-4019

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., et al. (2005). Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol. Endocrinol.* 19, 2685–2695. doi: 10.1210/me.2005-0106

Wang, W. L., Chatterjee, N., Chittur, S. V., Welsh, J., and Tenniswood, M. P. (2011). Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Mol. Cancer* 10, 58. doi: 10.1186/1476-4598-10-58

Wang, W. L., Welsh, J., and Tenniswood, M. (2013). 1,25-Dihydroxyvitamin D3 modulates lipid metabolism in prostate cancer cells through miRNA mediated regulation of PPARA. *J. Steroid Biochem. Mol. Biol.* 136, 247–251. doi: 10.1016/j.jsbmb.2012.09.033

Wang, X., Gocek, E., Liu, C. G., and Studzinski, G. P. (2009). MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D3. *Cell Cycle* 8, 736–741. doi: 10.4161/cc.8.5.7870

Warburg, O. (1956). [Origin of cancer cells]. *Oncologia* 9, 75–83. doi: 10.1159/000223920

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* 8, 519–530. doi: 10.1085/jgp.8.6.519

Washington, M. N., and Weigel, N. L. (2010). 1{alpha},25-Dihydroxyvitamin D3 inhibits growth of VCaP prostate cancer cells despite inducing the growth-promoting TMPRSS2:ERG gene fusion. *Endocrinology* 151, 1409–1417. doi: 10.1210/en.2009-0991

Wehr, E., Pilz, S., Boehm, B. O., Marz, W., and Obermayer-Pietsch, B. (2010). Association of vitamin D status with serum androgen levels in men. *Clin. Endocrinol. (Oxf.)* 73, 243–248. doi: 10.1111/j.1365-2265.2009.03777.x

Weigel, N. L. (2007). Interactions between vitamin D and androgen receptor signaling in prostate cancer cells. *Nutr. Rev.* 65, S116–S117. doi: 10.1301/nr.2007.aug.S116-S117

Wise, D. R., DeBerardinis, R. J., Mancuso, A., Sayed, N., Zhang, X. Y., Pfeiffer, H. K., et al. (2008). Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc. Natl. Acad. Sci. U.S.A* 105, 18782–18787. doi: 10.1073/pnas.0810199105

Wong, K. E., Kong, J., Zhang, W., Szeto, F. L., Ye, H., Deb, D. K., et al. (2011). Targeted expression of human vitamin D receptor in adipocytes decreases energy expenditure and induces obesity in mice. *J. Biol. Chem.* 286, 33804–33810. doi: 10.1074/jbc.M111.257568

Wu, Z., Huang, X., Huang, X., Zou, Q., and Guo, Y. (2013). The inhibitory role of Mir-29 in growth of breast cancer cells. *J. Exp. Clin. Cancer Res.* 32, 98. doi: 10.1186/1756-9966-32-98

Xue, B., Moustaid, N., Wilkison, W. O., and Zemel, M. B. (1998). The agouti gene product inhibits lipolysis in human adipocytes via a Ca2+-dependent mechanism. *FASEB J.* 12, 1391–1396.

Yang, X., Du, W. W., Li, H., Liu, F., Khorshidi, A., Rutnam, Z. J., et al. (2013). Both mature miR-17-5p and passenger strand miR-17-3p target TIMP3 and induce prostate tumor growth and invasion. *Nucleic Acids Res.* 41, 9688–9704. doi: 10.1093/nar/gkt680

Yin, L., Raum, E., Haug, U., Arndt, V., and Brenner, H. (2009). Meta-analysis of longitudinal studies: serum vitamin D and prostate cancer risk. *Cancer Epidemiol.* 33, 435–445. doi: 10.1016/j.canep.2009.10.014

Yin, Y., Yu, Z., Xia, M., Luo, X., Lu, X., and Ling, W. (2012). Vitamin D attenuates high fat diet-induced hepatic steatosis in rats by modulating lipid metabolism. *Eur. J. Clin. Invest.* 42, 1189–1196. doi: 10.1111/j.1365-2362.2012.02706.x

Yu, Y., Wu, A., Zhang, Z., Yan, G., Zhang, F., Zhang, L., et al. (2013). Characterization of the GufA subfamily member SLC39A11/Zip11 as a zinc transporter. *J. Nutr. Biochem.* 24, 1697–1708. doi: 10.1016/j.jnutbio.2013.02.010

Yu, Z., Wang, C., Wang, M., Li, Z., Casimiro, M. C., Liu, M., et al. (2008). A cyclin D1/microRNA 17/20 regulatory feedback loop in control of breast cancer cell proliferation. *J. Cell Biol.* 182, 509–517. doi: 10.1083/jcb.200801079

Zhao, X. Y., Ly, L. H., Peehl, D. M., and Feldman, D. (1999). Induction of androgen receptor by 1alpha,25-dihydroxyvitamin D3 and 9-cis retinoic acid in LNCaP human prostate cancer cells. *Endocrinology* 140, 1205–1212. doi: 10.1210/endo.140.3.6561

Zhou, A. D., Diao, L. T., Xu, H., Xiao, Z. D., Li, J. H., Zhou, H., et al. (2012). beta-Catenin/LEF1 transactivates the microRNA-371-373 cluster that modulates the Wnt/beta-catenin-signaling pathway. *Oncogene* 31, 2968–2978. doi: 10.1038/onc.2011.461

Zirath, H., Frenzel, A., Oliynyk, G., Segerstrom, L., Westermark, U. K., Larsson, K., et al. (2013). MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10258–10263. doi: 10.1073/pnas.12224 04110

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The future of vitamin D analogs

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The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3, is a major regulator of bone and calcium homeostasis. In addition, this hormone also inhibits the proliferation and stimulates the differentiation of normal as well as malignant cells. Supraphysiological doses of 1,25-dihydroxyvitamin D_3 are required to reduce cancer cell proliferation. However, these doses will lead *in vivo* to calcemic side effects such as hypercalcemia and hypercalciuria. During the last 25 years, many structural analogs of 1,25-dihydroxyvitamin D_3 have been synthesized by the introduction of chemical modifications in the A-ring, central CD-ring region or side chain of 1,25-dihydroxyvitamin D_3 in the hope to find molecules with a clear dissociation between the beneficial antiproliferative effects and adverse calcemic side effects. One example of such an analog with a good dissociation ratio is calcipotriol (Daivonex®), which is clinically used to treat the hyperproliferative skin disease psoriasis. Other vitamin D analogs were clinically approved for the treatment of osteoporosis or secondary hyperparathyroidism. No vitamin D analog is currently used in the clinic for the treatment of cancer although several analogs have been shown to be potent drugs in animal models of cancer. Transcriptomics studies as well as *in vitro* cell biological experiments unraveled basic mechanisms involved in the antineoplastic effects of vitamin D and its analogs. 1,25-dihydroxyvitamin D_3 and analogs act in a cell type- and tissue-specific manner. Moreover, a blockade in the transition of the G0/1 toward S phase of the cell cycle, induction of apoptosis, inhibition of migration and invasion of tumor cells together with effects on angiogenesis and inflammation have been implicated in the pleiotropic effects of 1,25-dihydroxyvitamin D_3 and its analogs. In this review we will give an overview of the action of vitamin D analogs in tumor cells and look forward how these compounds could be introduced in the clinical practice.

Keywords: vitamin D, analogs, pleiotropic effects, cancer

INTRODUCTION

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$; **1**] (**Table 1**), is mostly known for its effects on bone, calcium, and phosphate homeostasis. Next to these classical effects, $1,25(OH)_2D_3$ also exerts so-called non-classical effects on various tissues which express the vitamin D receptor (VDR) as well as the enzymes that are responsible for activating the hydroxylations of vitamin D_3, which is essential for the formation of $1,25(OH)_2D_3$. Thus, most tissues have the ability to convert vitamin D_3 into its active form, $1,25(OH)_2D_3$, which in turn will bind the VDR in order to positively or negatively influence target genes via binding of the $1,25(OH)_2D_3$/VDR complex to vitamin D receptor elements (VDRE). Non-classical properties of $1,25(OH)_2D_3$ include prodifferentiating and antiproliferative effects on normal and cancer cells (Colston et al., 1981; Jensen et al., 2001) as well as immunomodulatory effects. However, in order to obtain these non-classical effects, $1,25(OH)_2D_3$ doses of the nanomolar range are necessary, while physiological $1,25(OH)_2D_3$ serum concentrations are in the picomolar range. Since supraphysiological doses of $1,25(OH)_2D_3$ result in hypercalcemia, $1,25(OH)_2D_3$ analogs were developed to minimize the calcemic side effects while preserving or augmenting the beneficial effects of $1,25(OH)_2D_3$. Both industry and academic institutions have synthesized a vast amount of vitamin D analogs. Some of these analogs have tissue-specific effects with low calcemic side effects and can be given at higher doses compared to the mother compound.

CLINICALLY APPROVED VITAMIN D ANALOGS

Given the huge amount of vitamin D analogs that have been synthesized during the years, it is nearly impossible to give an overview of them all. In the first part we will discuss vitamin D analogs that are clinically approved (**Table 2**). For several conditions such as secondary hyperparathyroidism, psoriasis and osteoporosis, vitamin D analogs are frequently used as a treatment option. Paricalcitol [**2**] and doxercalciferol [**3**] are vitamin D_2 analogs approved for therapeutic use of secondary hyperparathyroidism. In Japan falecalcitriol [**4**] and maxacalcitol [**5**] are also used to treat this disease. Secondary hyperparathyroidism is characterized by elevated parathyroid hormone (PTH) levels in response to hypocalcemia and is often caused by chronic kidney disease. Above-mentioned vitamin D analogs suppress PTH, as does $1,25(OH)_2D_3$, but without inducing severe hypercalcemia. Clinical studies with chronic kidney disease patients show that different analogs induce a stronger PTH suppression compared to placebo treatment (Hamdy et al., 1995; Coburn et al., 2004; Coyne et al., 2006). Also, end-stage renal disease patients treated with these analogs often have a better survival (Teng et al., 2003;

Table 1 | Overview of vitamin D analogs.

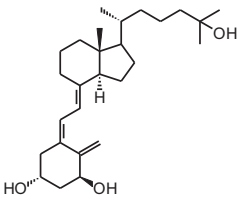
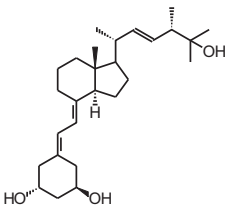
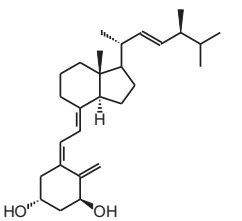
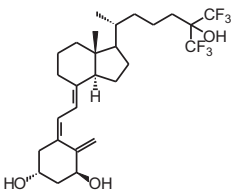
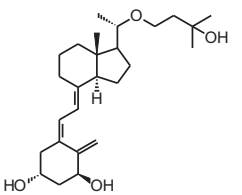
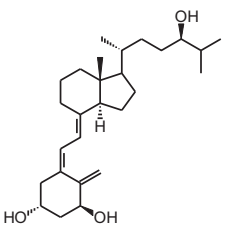
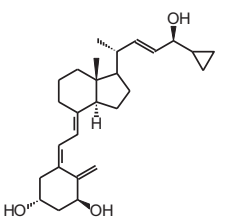
Identification number	Name	Structure
[1]	1α,25(OH)$_2$D$_3$	
[2]	Paricalcitol (19-nor-1α,25(OH)$_2$D$_2$)	
[3]	Doxercalciferol (1α(OH)D$_2$)	
[4]	Falecalcitriol (26,27 F6-1α,25(OH)$_2$D$_3$)	
[5]	Maxacalcitol (22oxa-1α,25(OH)$_2$D$_3$)	
[6]	Tacalcitol (1α,24(R)(OH)$_2$D$_3$)	
[7]	Calcipotriol (22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$)	

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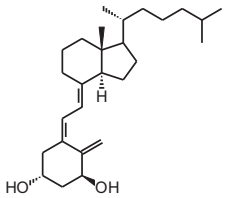
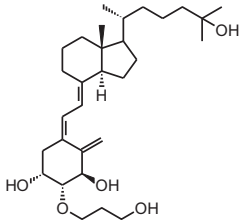
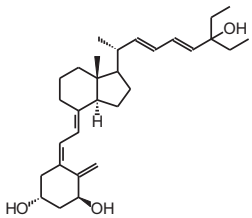
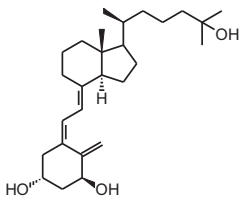
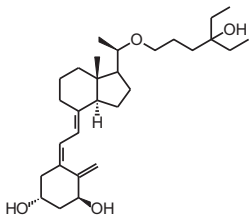
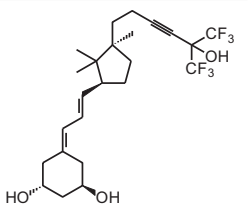
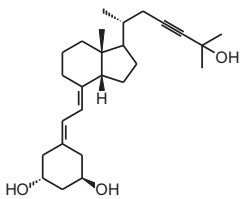
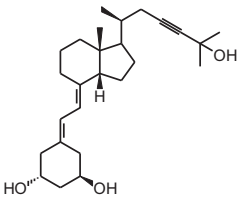
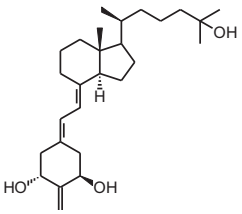
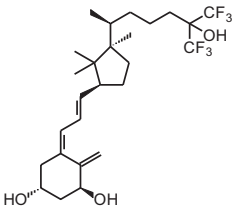
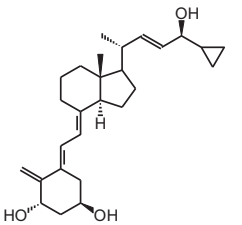
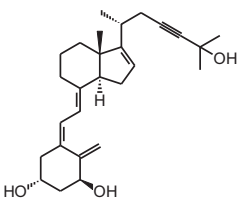
Table 1 | Continued

Identification number	Name	Structure
[8]	Alfacalcidol (1α(OH)D$_3$)	
[9]	Eldecalcidol (2β-(3-hydroxypropoxy)-1α,25(OH)$_2$D$_3$)	
[10]	Seocalcitol (22,24-diene-24,26,27-trishomo-1α,25(OH)$_2$D$_3$)	
[11]	20-epi-1α,25(OH)$_2$D$_3$	
[12]	Lexicalcitol (20-epi-22-oxa-24,26,27-trishomo-1α,25(OH)$_2$D$_3$)	
[13]	CD578 (17-methyl-19-nor-21-nor-23-yne-26,27-F6-1α,25(OH)$_2$D$_3$)	
[14]	Inecalcitol (19-nor-14-epi-23-yne-1α,25(OH)$_2$D$_3$)	

(Continued)

Table 1 | Continued

Identification number	Name	Structure
[15]	TX527 (19-nor-14,20-bisepi-23-yne-1α,25(OH)$_2$D$_3$)	
[16]	2MD (2-methylene-19-nor-(20S)-1α,25(OH)$_2$D$_3$)	
[17]	WY1112 (Seco-C-9,11-bisnor-17-methyl-20-epi-26,27-F6-1α,25(OH)$_2$D$_3$)	
[18]	PRI-2205 ((5E,7E)-22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$)	
[19]	ILX23-7553 (16-ene-23-yne-1α,25(OH)$_2$D$_3$)	

Tentori et al., 2006; Shinaberger et al., 2008). However, few studies with chronic kidney disease and end-stage renal disease patients directly compare the effects of 1,25(OH)$_2$D$_3$ with its analogs.

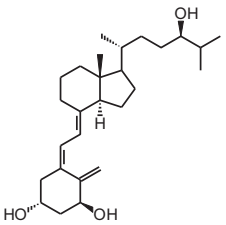
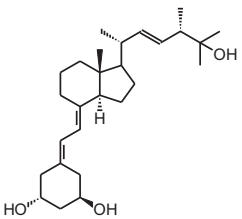
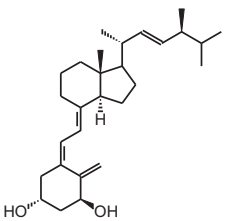
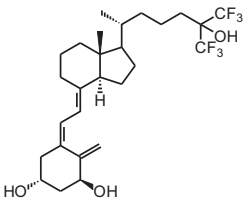
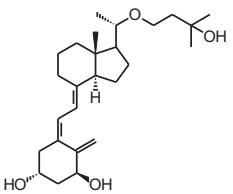
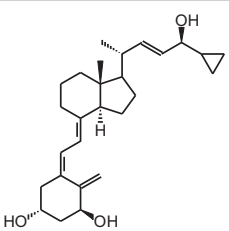
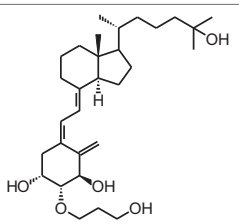
Psoriasis, a hyperproliferative condition of the skin, is also treated with vitamin D analogs. Tacalcitol [**6**], calcipotriol [**7**] and the recently approved maxacalcitol [**5**] are used either as monotherapy or in combination with topical steroids such as betamethasone dipropionate to treat psoriasis. The analogs exert prodifferentiating and antiproliferative effects on keratinocytes and also possess important anti-inflammatory properties. Furthermore, alfacalcidol ([1α(OH)D$_3$; **8**], actually a pre-metabolite of 1,25(OH)$_2$D$_3$) and eldecalcitol (ED-71) [**9**] are used in Japan in the treatment of osteoporosis. The recently approved eldecalcitol [**9**] is more effective than 1,25(OH)$_2$D$_3$ and alfacalcidol [**8**] in increasing bone mineral density and mechanical strength in ovariectomized rats (Uchiyama et al., 2002). Various studies in mouse models as well as in patients show that treatment with eldecalcitol [**9**] leads to higher lumbar and hip bone mineral density and a lower incidence of new vertebral fractures (Ito et al., 2011; Matsumoto et al., 2011; Harada et al., 2012; Hagino et al., 2013), making eldecalcitol [**9**] a very promising new analog for the treatment of osteoporosis.

GENOME- AND TRANSCRIPTOME-WIDE EFFECTS OF VITAMIN D ANALOGS

The exact mechanism of action of vitamin D analogs still has to be deciphered. The reason why specific analogs have superagonistic actions on specific tissues remains unknown, however several studies have tried to elucidate the mechanisms behind these tissue-specific effects. The catabolism of vitamin D analogs is one of the mechanisms that have an effect on their potency. Modifications of the side chain of 1,25(OH)$_2$D$_3$ are known to slow down its catabolism by CYP24A1 (Jones, 1997). Seocalcitol (EB1089) [**10**] and 20-epi-1,25(OH)$_2$D$_3$ [**11**] are degraded slower compared to 1,25(OH)$_2$D$_3$ leading to a longer exposure of these analogs to the tissues (Hansen and Maenpaa, 1997; Kissmeyer et al., 1997; Shankar et al., 1997; Zella et al., 2009). The metabolites formed after catabolism of lexicalcitol [**12**] are more active than the ones formed after 1,25(OH)$_2$D$_3$ is catabolized (Dilworth et al., 1997) and this analog is also more effective in slowing down the degradation rate of the VDR (van den Bemd et al., 1996). Moreover, since some cell types prefer specific catabolism pathways and enzymes above others, the degradation process may also contribute to the tissue-specific activity of vitamin D analogs. The affinity for the vitamin D binding protein (DBP) also plays a role in the activity of vitamin D analogs. Maxacalcitol [**5**] for example has a 500 times lower affinity for DBP and is thus cleared faster from the circulation than 1,25(OH)$_2$D$_3$ (Okano et al., 1989a). This analog has a short effect on bone and intestine, tissues responsible for calcium homeostasis, and a longer effect on PTH levels, making this analog ideal for the treatment of secondary hyperparathyroidism. However, it is still unknown why the duration of the effects is different in these tissues. Eldecalcitol [**9**] on the other hand has a higher DBP affinity compared to the mother compound, leading to longer sustained plasma levels and is thus more suitable for the treatment of osteoporosis (Okano et al., 1989b).

Another mechanism that contributes to the superagonistic effects of vitamin D analogs is their interaction with the VDR, co-activators and VDREs. 20-epi-1,25(OH)$_2$D$_3$, a C-20 epimer of 1,25(OH)$_2$D$_3$ [**11**], promotes heterodimerization between VDR and retinoid X receptor (RXR) (Liu et al., 2001). 20-epi-1,25(OH)$_2$D$_3$ [**11**] and other analogs like maxacalcitol [**5**], CD578 [**13**], inecalcitol [**14**], and TX527 [**15**] require lower concentrations to recruit specific coactivators to the VDR/RXR/VDRE complex (Liu et al., 2000; Eelen et al., 2005, 2008; Schwinn and DeLuca, 2007). Approximately 10- fold lower doses of inecalcitol [**14**] and TX527 [**15**] are needed, compared to 1,25(OH)$_2$D$_3$, to acquire the same amount of co-activator interactions (Eelen et al.,

Table 2 | Overview of clinically approved vitamin D analogs.

Name	Structure	Indication	Brand name
Tacalcitol (1α,24(R)$(OH)_2D_3$)		Psoriasis	Curatoderm® (Almirall Hermal), Bonalfa® (ISDIN, Teijin Pharma),...
Paricalcitol (19-nor-1α,25$(OH)_2D_2$)		Secondary hyperparathyroidism	Zemplar® (Abbott)
Doxercalciferol (1α$(OH)D_2$)		Secondary hyperparathyroidism	Hectorol® (Genzyme corp)
Falecalcitriol (26,27 F6-1α,25$(OH)_2D_3$)		Secondary hyperparathyroidism (Japan only)	Fulstan® (Dainippon Sumitomo) and Hornel® (Taisho Yakuhin)
Maxacalcitol (22oxa-1α,25$(OH)_2D_3$)		Secondary hyperparathyroidism and psoriasis (Japan only)	Oxarol® (Chugai Pharmaceutical)
Calcipotriol (22-ene-26,27-dehydro-1α,25$(OH)_2D_3$)		Psoriasis	Daivonex®, Dovonex® (LEO Pharma), Sorilux® (Stiefel)
Eldecalcitol (2β-(3-hydroxypropoxy)-1α,25$(OH)_2D_3$)		Osteoporosis (Japan only)	Edirol® (Chugai Pharmaceutical)

2005). Vitamin D analogs might also be able to induce tissue-specific effects by favoring binding to specific *VDRE* motifs in target gene promoters. Analogs with a 20-methyl group as well as seocalcitol [**10**] bound to a VDR/RXR complex preferably interact with the IP9 type of VDRE (Danielsson et al., 1996; Quack and Carlberg, 1999).

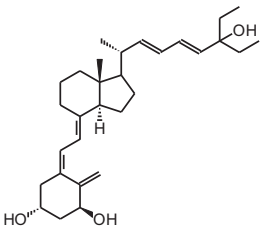
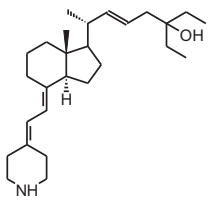
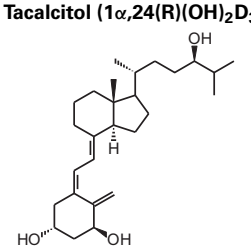
On the genome level, studies using chromatin immunoprecipitation (ChIP) and micro-array techniques have investigated $1,25(OH)_2D_3$-regulated genes in different cell lines. One ChIP study compared the binding sites of the VDR in intestinal tissue after $1,25(OH)_2D_3$ or 20-epi-$1,25(OH)_2D_3$ [**11**] treatment. This study shows that both $1,25(OH)_2D_3$ and 20-epi-$1,25(OH)_2D_3$ [**11**] induce VDR binding to *CYP24A1* and *TRPV6* loci in the intestine, but the analog elicits a prolonged VDR binding to these genes leading to its superagonistic characteristics such as hypercalcemia *in vivo* (Zella et al., 2009). Other ChIP studies have tried to investigate the molecular mechanisms of some analogs in different tissues. In osteoblast cell models 2MD [**16**] bound to the VDR is able to bind VDREs at lower concentrations compared to $1,25(OH)_2D_3$ (Yamamoto et al., 2003). Seocalcitol [**10**], on the other hand, mediates the dissociation of Williams syndrome transcription factor of the aromatase promoter leading to inhibition of aromatase expression and activity in breast cancer cells which is one of the main therapeutic strategies in breast cancer patients (Lundqvist et al., 2013). In a recent paper binding sites of VDR and mothers against decapentaplegic homolog 3 (SMAD3) were investigated in hepatic stellar cells. These transforming growth factor β1 (TGFβ1)-activated cells play an important role in liver fibrosis. In this study it is shown that VDR and SMAD3 can at least transiently co-occupy the same genomic sites and function as enhancers of pro-fibrotic gene expression. However, when calcipotriol [**7**] is added, the TGFβ1-induced recruitment of SMAD3 is compromised and binding of VDR to these genomic sites is enhanced 10-fold meaning that liganded VDR antagonizes SMAD residency on chromatin and thereby suppresses pro-fibrotic gene expression (Ding et al., 2013). This genomic feedback circuit is a previously unknown mechanism of calcipotriol [**7**].

Micro-array studies in various cancer cell lines such as leukemia, prostate, breast, colorectal, and ovarian cancer show that a variety of gene clusters are influenced by $1,25(OH)_2D_3$ and its analogs (reviewed in Kriebitzsch et al., 2009). Cell growth, apoptosis, cellular adhesion and extracellular matrix composition, oxidative stress, immune function, intra- and intercellular signaling and steroid/lipid metabolism are frequently modulated processes in cells by $1,25(OH)_2D_3$ and its analogs. However, when different cell lines are compared, few $1,25(OH)_2D_3$/analog-regulated genes overlap, which suggests that $1,25(OH)_2D_3$ and its analogs behave in a cell type- and tissue-specific way. Also studies using human T-cells (Baeke et al., 2011), rat ventricular heart tissue (Bae et al., 2011), and bone marrow-derived mouse dendritic cells (Griffin et al., 2004) have researched the impact of $1,25(OH)_2D_3$ analogs on gene expression. In these studies genes important for cell growth, cell death and cell signaling are regulated, but also a large set of genes implicated in the migration of T-cells and dendritic cells are influenced. TX527 [**15**] imprints human T-cells with a migratory signature and targets them to sites of inflammation (Baeke et al., 2011). Paricalcitol [**2**] treatment of rats with cardiac hypertrophy prevents the progression of cardiac hypertrophy and the development into chronic heart failure. The genomic changes associated with cardiac hypertrophy in the ventricular heart tissue of these rats are, in part, reversed by paricalcitol [**2**] administration (Bae et al., 2011). Furthermore, other studies investigated if $1,25(OH)_2D_3$ analogs are able to bind and regulate different genes compared to $1,25(OH)_2D_3$. All conducted studies conclude that $1,25(OH)_2D_3$ and its analogs induce or repress the same set of genes. Seocalcitol [**10**] induces a less malignant phenotype in SCC25 squamous cell carcinoma cells and modulates expression of genes important in cell cycle progression, cell adhesion, extracellular matrix composition, intra- and intercellular signaling, G-protein coupled function, redox balance, and steroid metabolism. In these cells, seocalcitol [**10**] regulates the same genes compared to $1,25(OH)_2D_3$, however gene regulation by $1,25(OH)_2D_3$ is more transient (Lin et al., 2002). Also WY1112, a seco-9,11-*bis*nor-17-methyl analog lacking the C-ring and with a 21-epi side chain which is fluorinated on C26 and C27 [**17**], was investigated in MCF-7 breast cancer cells. Despite the 400-fold stronger antiproliferative capacity of WY1112 [**17**], the same genes are upregulated after $1,25(OH)_2D_3$ or WY1112 [**17**] treatment. However, the induction ability is much higher for the analog (Vanoirbeek et al., 2009). When treating human coronary artery smooth muscle cells with equal amounts of $1,25(OH)_2D_3$ or paricalcitol [**2**] the same genes are regulated (Wu-Wong et al., 2007; Shalhoub et al., 2010). In conclusion, differences in action and capacity of vitamin D analogs are more due to their specific sensitivities to metabolism and their specific interaction with the VDR, co-activators and VDREs than from different gene regulations. However, to our knowledge no studies have yet looked into the potential differences elicited by analogs compared to $1,25(OH)_2D_3$ in the fields of proteomics and epigenetics, which could help to understand the molecular mechanism of $1,25(OH)_2D_3$ and its analogs on different cell and tissue types.

EFFECTS OF VITAMIN D ANALOGS IN CANCER

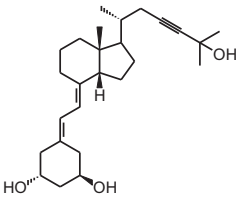
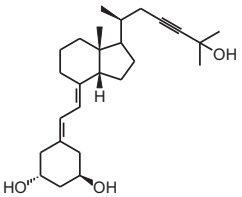
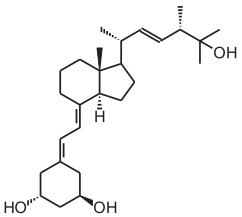
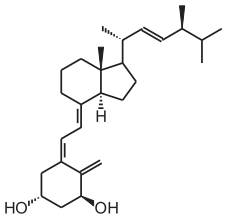
The use of $1,25(OH)_2D_3$ for the treatment of cancer gained interest since many tissues express vitamin D metabolizing enzymes as well as the VDR and because $1,25(OH)_2D_3$ has potent antiproliferative and prodifferentiating effects on normal and malignant cell lines. Several analogs evaluated *in vitro* show stronger antiproliferative and prodifferentiating effects compared to the mother compound in different cancer cell lines. These compounds are further evaluated in rodent models to assess their anti-cancer activity and safety *in vivo*. All *in vivo* studies using rodent cancer models that were published between 2007 and 2013 are summarized in **Table 3**. For studies preceding 2007, the reader is referred to other reviews (Eelen et al., 2007). In most studies the growth of the tumor is inhibited without inducing severe hypercalcemia when appropriate doses of vitamin D analogs are used (Abe et al., 1991; Kawa et al., 1996, 2005; Akhter et al., 1997, Blutt et al., 2000; Prudencio et al., 2001; Grostern et al., 2002; Flanagan et al., 2003; Albert et al., 2004a; Wietrzyk et al., 2004; Zhang et al., 2005; Fichera et al., 2007; van Ginkel et al., 2007; Ghous et al., 2008; Lee et al., 2008; Schwartz et al., 2008; Gonzalez-Pardo et al., 2010; Seubwai et al., 2010; Berkovich et al., 2013;

Table 3 | *In vivo* studies in rodent cancer models treated with vitamin D analogs (intraperitoneal i.p.; subcutaneous s.c.) published between 2007 and 2013.

Cancer type	Dosage vitamin D analog	Duration of treatment	Outcome	References
Seocalcitol (22,24-diene-24,26,27-trishomo-1α,25(OH)$_2$D$_3$) 				
Chemically-induced breast cancer	7 μg/kg/week	Approximately 80 days	Decreased tumor burden and volume	Liska et al., 2012
Chemically-induced breast cancer	Oral, 7 μg/kg/week	116 or 156 days	Prolonged latency of mammary gland tumors	Macejova et al., 2011
Prostate cancer xenograft	i.p., 0.5 μg/kg every other day	45 days	Reversal of growth stimulatory effects of PTHrP	Bhatia et al., 2009
Hepatocellular carcinoma xenograft	Oral and i.p., 0.02/0.1/0.5 μg/kg/d	Approximately 21 days	Inhibition of tumor growth	Ghous et al., 2008
Inoculation with mice breast cancer cells	i.p., 20 ng 3×/week	6 weeks	Inhibition of tumor growth, no inhibition of tumor angiogenesis	Valrance et al., 2007
HY-11 (2-amino-3-deoxy-19-nor-22-ene-26-dihomo-27-dihomo-25(OH)D$_3$) 				
Inoculation with mice leukemia cells	i.p., 10^{-5} M/d	26 days	50% increase in survival	Yoon et al., 2008
Tacalcitol (1α,24(R)(OH)$_2$D$_3$) 				
Inoculation with mice colorectal cancer cells	Different concentrations s.c. (3 or 5×/week) or oral (3×/week) in combination with different concentrations of 5-fluorouracil	Variable duration	1 μg/kg/d optimal dose + prolongation of life span of mice (synergistic effect when combined with chemotherapy)	Milczarek et al., 2013a
Inoculation with mice or human colorectal cancer cells	s.c., Different concentrations in combination with different concentraties of irinotecan or oxaliplatin	Variable duration	Under certain experimental conditions vitamin D analogs and chemotherapy can work synergistically	Milczarek et al., 2013b

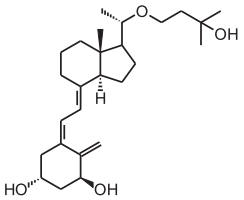
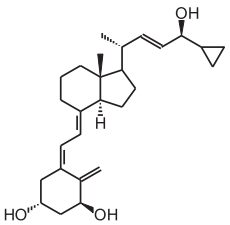
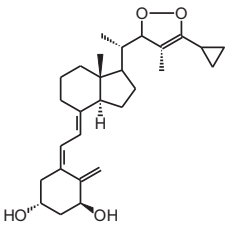
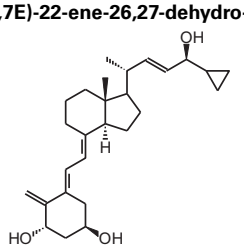
(Continued)

Table 3 | Continued

Cancer type	Dosage vitamin D analog	Duration of treatment	Outcome	References
		Inecalcitol (19-nor-14-epi-23-yne-1α,25(OH)$_2$D$_3$) 		
Squamous cell carcinoma xenograft	i.p., 80/160/320 μg/mouse/d	3 days	Inhibition of tumor growth, increased apoptosis, decreased proliferation	Ma et al., 2013
Prostate cancer xenograft	i.p., 1300 μg/kg 3×/week	42 days	Delay of tumor growth, 50% decrease in tumor weight and decreased tumor vascularity	Okamoto et al., 2012
		TX527 (19-nor-14,20-bisepi-23-yne-1α,25(OH)$_2$D$_3$) 		
Kaposi sarcoma xenograft	i.p., 10 μg/kg/d	4 days	Decreased tumor progression	Gonzalez-Pardo et al., 2010
		Paricalcitol (19-nor-1α,25(OH)$_2$D$_2$) 		
Gastric cancer xenograft	s.c., 100 ng/d 3×/week	4 weeks	Lower tumor volume, reduced growth of intraperitoneal metastasis	Park et al., 2012
Pancreatic cancer xenograft	s.c., 2.5 μg/kg 3×/week	Variable duration	Inhibition of tumor growth	Schwartz et al., 2008
		Doxercalciferol (1α(OH)D$_2$) 		
Neuroblastoma xenograft	Oral, 0.15/0.3 μg/d	5 weeks	Lower tumor volume	van Ginkel et al., 2007

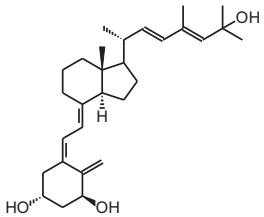
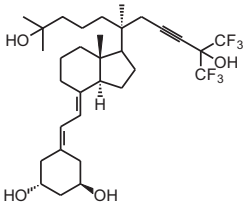
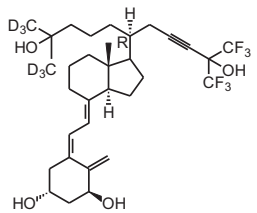
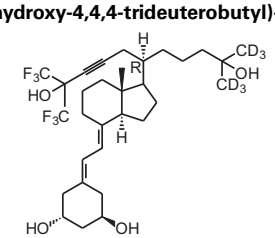
(Continued)

Table 3 | Continued

Cancer type	Dosage vitamin D analog	Duration of treatment	Outcome	References
		Maxacalcitol (22oxa-1α,25(OH)$_2$D$_3$) 		
Cholangiocarcinoma xenograft	i.p., 15 μg/kg/d	17 days	Inhibition of tumor growth	Seubwai et al., 2010
		Calcipotriol (22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$) 		
UV-induced non-melanoma skin cancer	Topical application in combination with diclofenac and difluoromethylornithine	17 weeks	Decrease in number and area of tumors when combined with diclofenac	Pommergaard et al., 2013
		BGP-13 (1R, 3S, 5Z)-5-((8E)-2-((3R)-3-((2R, 3S)-3-(5-cyclopropyl-3H-1,2-dioxol-3-yl)-2-ethyl-3-methylcyclohexylidene) ethylidene)-4-methylenecyclohexane-1,3-diol) 		
Colorectal cancer xenograft	i.p., 2 μg/kg every 2 days	8 days	Inhibition of tumor growth	Berkovich et al., 2013
		PRI-2205 ((5E,7E)-22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$) 		
Breast and lung cancer xenograft	s.c., 10 μg/kg 2 or 3×/week + cytostatics	18–21 days	Combination of analogs with low doses of cytostatics is not effective	Wietrzyk et al., 2007

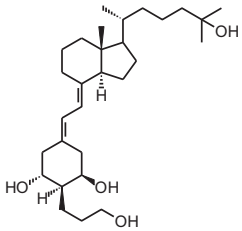
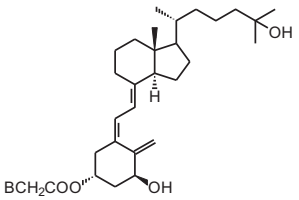
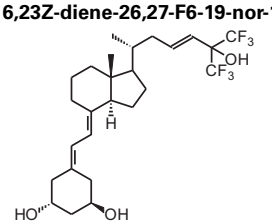
(Continued)

Table 3 | Continued

Cancer type	Dosage vitamin D analog	Duration of treatment	Outcome	References
PRI-1906 ((24E)-(1S)-24-dehydro-24a-homo-1α,25(OH)$_2$D$_3$) 				
Inoculation with mice breast cancer cells	s.c., 0.1 or 1 μg/kg/d	9 or 11 days	No effects	Wietrzyk et al., 2008
BXL-01-0126 (20R-(4-hydroxy-4-methylpentyl)-23-yne-26,27-hexafluoro-19-nor-1α,25(OH)$_2$D$_3$) 				
Acute myeloid leukemia xenograft	i.p., 0.0625 μg	1 injection	Cathelicidin antimicrobial peptide present in systemic circulation	Okamoto et al., 2014
BXL0124 (20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-1α,25(OH)$_2$D$_3$) 				
Breast cancer xenograft	i.p., 0.1 μg/kg or oral 0.03/0.1 μg/kg 6 days/week	5 weeks	Suppressed tumor growth	So et al., 2011
Transgenic mice with breast cancer (ErbB2/Her-2/neu overexpressing tumors)	i.p., 0.3 μg/kg 3×/week	Approximately 38 weeks	Inhibition of tumor growth and regulation of ErbB2/AKT/ERK pathway	Lee et al., 2010
Gemini0097 (20R-21(3-trideuteromethyl-3-hydroxy-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-1α,25(OH)$_2$D$_3$) 				
ER-negative breast cancer xenograft	i.p., 0.1 μg/kg/d	9 weeks	Suppressed tumor growth	Lee et al., 2008
Chemically-induced breast cancer (ER positive)	i.p., 0.03/0.1/0.3 μg/kg 5days/week	9 weeks	Inhibition of tumor burden	

(Continued)

Table 3 | Continued

Cancer type	Dosage vitamin D analog	Duration of treatment	Outcome	References
	MART-10 (19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$) 			
Pancreatic cancer xenograft	i.p., 0.3 μg/kg 2×/week	3 weeks	Inhibition of tumor growth	Chiang et al., 2013
	1α,25(OH)$_2$D$_3$-3-bromoacetate 			
Kidney cancer xenograft	i.p., 0.75 μg/kg every third day	80 days	Reduced tumor size and increased apoptosis	Lambert et al., 2010
	Ro26-2198 (16,23Z-diene-26,27-F6-19-nor-1α,25(OH)$_2$D$_3$) 			
Chemically-induced colorectal cancer	0.01 μg/kg/d via mini-osmotic pump	28 days	Inhibition of dysplasia progression and inhibition of proliferation and pro-inflammatory signals	Fichera et al., 2007

For studies preceding 2007 the reader is referred to other reviews (Eelen et al., 2007).

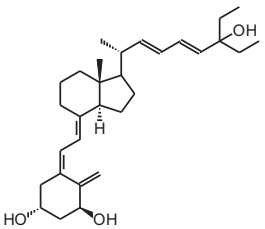
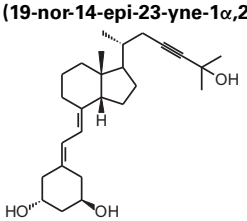
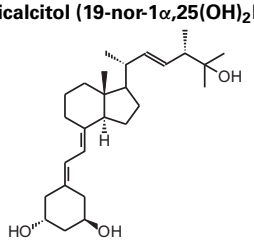
Chiang et al., 2013). However, in some models the analog dose that is effective in inhibiting tumor growth also causes hypercalcemia and lower survival of the treated animals (Albert et al., 2004b). Not only tumor proliferation is modulated by vitamin D analogs, also apoptosis, angiogenesis, migration of tumor cells, etc. are affected by some analogs. In xenograft studies where apoptosis in the tumor was investigated after vitamin D analog treatment, apoptosis or the necrotic field in the tumor is augmented (James et al., 1998; VanWeelden et al., 1998; Hara et al., 2001; Vegesna et al., 2003; Lambert et al., 2010; Park et al., 2012). Inecalcitol (Hybrigenics, France) [**14**] treatment of mice with squamous cell carcinoma xenografts increases apoptosis in the tumors and this increase is higher for the analog compared to 1,25(OH)$_2$D$_3$, while the capacity of the analog to inhibit proliferation is equal compared to the mother compound (Ma et al., 2013). Most studies agree that vitamin D analogs also have an effect on tumor metastasis. Seocalcitol [**10**] reverses the growth-stimulatory effects of parathyroid hormone-related protein (PTHrP), which plays a major role in prostate cancer progression and metastasis, in a xenograft mouse model of prostate cancer. The same study shows that seocalcitol [**10**] also inhibits migration and invasion of these prostate cancer cells *in vitro* (Bhatia et al., 2009). This analog also reduces the number and surface area of bone metastasis originating from intracardially injected breast cancer cells (El Abdaimi et al., 2000). Vitamin D analogs are thus able to reduce the number and growth of metastasis originating from various types of cancer cells (Sato et al., 1984; Lokeshwar et al., 1999; Nakagawa et al., 2005; Park et al., 2012). However, in a study using mice with chemically induced breast cancer, the invasion capacity of the tumor after seocalcitol [**10**] treatment is not affected (Liska et al., 2012). The effect of vitamin D analogs on angiogenesis has also been studied *in vivo*,

but here the results are more conflicting. Some studies show no effect of vitamin D analogs on angiogenesis (Oades et al., 2002; Valrance et al., 2007), while others find decreased angiogenesis of xenograft tumors. Intraperitoneal injections of inecalcitol [**14**] decrease the vascularity of xenografted prostate cancer cells (Okamoto et al., 2012) and oral treatment of colorectal tumors in rats with alfacalcidol [**8**] also inhibits tumor angiogenesis (Iseki et al., 1999). All *in vivo* studies conclude that vitamin D analogs inhibit tumor growth but vitamin D and its analogs often do not influence tumor number. Seocalcitol [**10**] was given as chemoprevention in a transgenic mouse model for androgen-independent prostate cancer. Tumor growth is inhibited, however, there is no prevention in the development of tumors (Perez-Stable et al., 2002).

Since vitamin D and its analogs do not possess cytostatic properties, many *in vivo* studies have focused on vitamin D analog cancer treatment combined with radiotherapy and/or chemotherapy. When seocalcitol [**10**] is combined with radiotherapy in a xenograft model for breast cancer, the anti-cancer effects are more effective compared to monotherapy (Sundaram et al., 2003). Another analog, tacalcitol [**6**], has been investigated in colorectal cancer xenograft in combination with different standard chemotherapies. Different concentrations as well as administration routes of tacalcitol [**6**] or PRI-2205 (an analog of calcipotriol) [**18**] were used in combination with different concentrations of 5-fluorouracil (5-FU). Using specific analog doses and chemotherapy schedules, a synergistic effect on the prolongation of the life span of the mice is achieved (Milczarek et al., 2013a). Also the combination with irinotecan or oxaliplatin was investigated. In this study the mice also show a longer life span and a stronger tumor growth inhibition compared to monotherapy when certain doses of tacalcitol [**6**] and chemotherapy were used. However, some combinations were more toxic than the monotherapies (Milczarek et al., 2013b). Other studies report better effects when combining calcipotriol [**7**] and diclofenac in a non-melanoma skin cancer model (Pommergaard et al., 2013). However, the combination of vitamin D analogs with chemotherapy does not always result in additive or synergistic effects. Combining maxacalcitol [**5**] and 5-FU did not enhance anti-tumor effects in a chemically induced breast cancer model (Iino et al., 1992). Another study investigated calcipotriol [**7**] and its derivatives in breast and lung cancer *in vivo* models and concluded that these analogs and low dose cytostatics are not effective in the used models (Wietrzyk et al., 2007). Also tacalcitol [**6**] in combination with cyclophosphamide does not lead to a significant difference in tumor growth inhibition compared to the vehicle treatment (Wietrzyk et al., 2008).

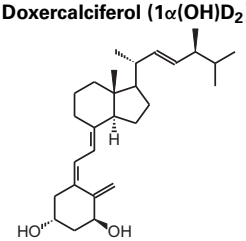
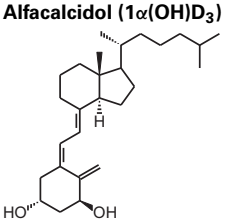
In view of the promising results that certain vitamin D analogs show against cancer *in vitro* and *in vivo* animal models, some analogs have been tested in cancer patients (**Table 4**). Seocalcitol [**10**] is an analog that has been extensively studied *in vitro* and *in vivo* in different cancer models, however in clinical trials the results are rather disappointing. Patients with advanced breast or colon cancer were treated with different doses of seocalcitol [**10**] (most patients tolerate 7 μg/d) but none of them showed a complete or partial response (Gulliford et al., 1998). Also oral seocalcitol [**10**] treatment in patients with inoperable pancreatic cancer exhibited no objective anti-tumor activity (Evans et al., 2002). Two out of 33 patients with inoperable hepatocellular carcinoma showed a complete response after oral seocalcitol [**10**] treatment, however the majority of the patients presented stable or progressive disease (Dalhoff et al., 2003). Inecalcitol [**14**] is in an early stage II of its clinical trial in chronic lymphocytic leukemia. Fifteen patients received 2 mg/d orally and one patient had a 90% decrease in blood lymphocyte count after 10 months of treatment, in 8 other patients blood lymphocyte count stopped growing when the treatment started (Hybrigenics, 2014). Intravenous administration of paricalcitol [**2**], an analog that is approved for secondary hyperparathyroidism, also displayed no objective responses in patients with androgen-independent prostate cancer. However, elevated serum PTH levels, which are common for advanced prostate cancers, are reduced by the analog (Schwartz et al., 2005). Doxercalciferol [**3**], also used in the treatment against secondary hyperparathyroidism, was investigated in androgen-independent prostate cancer patients. A phase I study administered oral doxercalciferol [**3**] between 5 and 15 μg/d, which was well tolerated by the patients (Liu et al., 2002). In the following phase II study, patients were treated with 12.5 μg/d for a minimum of 8 weeks and 30% of these patients experienced stable disease for over 6 months (Liu et al., 2003). Oral treatment of non-Hodgkin's lymphoma patients with 1 μg/d alfacalcidol [**8**], a pre-metabolite of $1,25(OH)_2D_3$, resulted in a low overall response. Out of 34 treated patients, only 4 had a complete response and 4 others showed a partial response to the treatment (Raina et al., 1991). Calcipotriol [**7**] is often used to treat skin psoriasis and has thus been investigated in patients with locally advanced or cutaneous metastases from breast cancer. In both studies the analog was applied topically at a dose of 100 μg/d. One study reported no response after 3 months of treatment (O'Brien et al., 1993), while in the other study 3 patients showed a 50% reduction in the diameter of treated lesions after 6 weeks (Bower et al., 1991). A more recently developed analog, ILX23-7553 [**19**], was investigated in 16 patients with advanced solid tumors but no objective response was seen (Jain et al., 2011). Similar to the *in vivo* studies, clinical trials have also combined vitamin D analogs with standard radiotherapy or chemotherapy. Metastatic breast cancer patients were given oral paricalcitol [**2**] doses between 2 and 7 μg/d in combination with taxane-based chemotherapy and this regimen was well tolerated by the patients (Lawrence et al., 2013). Oral inecalcitol [**14**] was given to patients with hormone-refractory prostate cancer in combination with docetaxel for maximum 18 weeks. This study had a response rate of 85% based on a PSA decline of at least 30% within 3 months of treatment (Hybrigenics, 2014). In a small study with acute non-lymphoid leukemia patients the combination of alfacalcidol [**8**] and chemotherapy resulted in 17% of the patients with a complete response and 45% with a partial response (Petrini et al., 1991). The same analog was combined with standard treatment of surgery, radiotherapy, and/or chemotherapy in glioblastoma and anaplastic astrocytomas. Here, 0.04 μg/kg/d alfacalcidol [**8**] was administered resulting in 27% of the patients with progressive regression of the lesion and complete clinical remission (Trouillas et al., 2001). In metastatic renal cell carcinoma patients, oral treatment of 1 μg alfacalcidol/d [**8**]

Table 4 | Clinical trials with vitamin D analog supplementation.

Cancer type	Sample size	Dosage vitamin D analog	Duration of treatment	Outcome	References
Seocalcitol (22,24-diene-24,26,27-trishomo-1α,25(OH)$_2$D$_3$) 					
Inoperable hepatocellular carcinoma	33	Oral individual dosage, most patients tolerate 10 μg/d	Up to 1 year	2 patients with complete response; 12 with stable disease; 19 with progressive disease	Dalhoff et al., 2003 (uncontrolled trial)
Inoperable pancreatic cancer	36	Oral individual dosage, most patients tolerate 10–15 μg/d	Minimum 8 weeks	No objective anti-tumor activity	Evans et al., 2002 (uncontrolled trial)
Advanced breast cancer and colorectal cancer	36	Individual dosage (solution), most patients tolerate 7 μg/d	From 5 days up to 1 year	No complete or partial responses	Gulliford et al., 1998 (uncontrolled trial)
Inecalcitol (19-nor-14-epi-23-yne-1α,25(OH)$_2$D$_3$) 					
Hormone-refractory prostate cancer	54	Oral individual dosage, maximum tolerated dose is 4 mg/d + docetaxel (chemotherapy)	Maximum 18 weeks	85% response rate based on a PSA decline of at least 30% within 3 months	Hybrigenics, 2014 (uncontrolled trial)
Chronic lymphocytic leukemia	15	Oral 2 mg/d	Not found	1 patient had a 90% decrease in blood lymphocyte count after 10 months of treatment; in 8 patients blood lymphocyte count stopped increasing when treatment was started; 6 patients showed no response	Hybrigenics, 2014 (uncontrolled trial)
Paricalcitol (19-nor-1α,25(OH)$_2$D$_2$) 					
Metastatic breast cancer	24	Oral individual dosage, 2–7 μg/d + taxane-based chemotherapy	8 weeks	Well tolerated regimen	Lawrence et al., 2013 (uncontrolled)

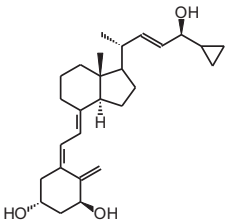
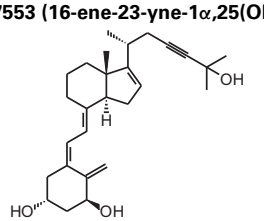
(Continued)

Table 4 | Continued

Cancer type	Sample size	Dosage vitamin D analog	Duration of treatment	Outcome	References
Androgen-independent prostate cancer	18	i.v., Individual dosage, 3×/week 5–25 μg	Up to 12 weeks	No objective response, reduced serum PTH levels	Schwartz et al., 2005 (uncontrolled)
		Doxercalciferol (1α(OH)D_2) 			
Localized prostate cancer and high grade prostatic intraepithelial neoplasia	31	Oral, 10 μg/d	4 weeks	No beneficial effects in serum and tissue markers	Gee et al., 2013 (placebo-controlled)
Metastatic androgen-independent prostate cancer	70	Oral, 10 μg/d + docetaxel	4 weeks	No enhanced PSA response rate or survival	Attia et al., 2008 (placebo-controlled)
Advanced androgen-independent prostate cancer	26	Oral, 12.5 μg/d	Minimum 8 weeks	30% experienced stable disease for over 6 months	Liu et al., 2003 (uncontrolled)
Advanced androgen-independent prostate cancer	25	Oral individual dosage, 5–15 μg/d	Minimum 8 weeks	Well tolerated, maximal tolerated dose was not reached	Liu et al., 2002 (uncontrolled)
		Alfacalcidol (1α(OH)D_3) 			
Metastatic renal cell carcinoma	16	Oral, 1 μg/d + interferon-α (3×/week)	Minimum 3 months	25% had partial response	Obara et al., 2008 (uncontrolled)
Glioblastoma and anaplastic astrocytomas	11	0.04 μg/kg/d + surgery/chemotherapy/ radiotherapy	Not found	27% showed progressive regression of the lesion and had a complete clinical remission	Trouillas et al., 2001 (uncontrolled)
Myelodysplastic syndromes	30	Oral, 4–6 μg/d	Median 17 months	Prolongation of leukemic transformation-free survival	Motomura et al., 1991 (placebo-controlled)
Acute non-lymphoid leukemia	11	Analog + chemotherapy	Not found	17% complete remission, 45% partial remission	Petrini et al., 1991 (uncontrolled)
Progressive low-grade non-Hodgkin's lymphoma	34	Oral, 1 μg/d	Minimum 8 weeks	4 patients has a complete response, 4 other patients had a partial response	Raina et al., 1991 (uncontrolled)

(Continued)

Table 4 | Continued

Cancer type	Sample size	Dosage vitamin D analog	Duration of treatment	Outcome	References
		Calcipotriol (22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$) 			
Locally advanced or cutaneous metastatic breast cancer	19	Topical 100 μg/d	6 weeks	3 patients showed 50% reduction in diameter of treated lesions	Bower et al., 1991 (uncontrolled)
Locally advanced or cutaneous metastatic breast cancer	15	Topical 100 μg/d	3 months	No response	O'Brien et al., 1993 (uncontrolled)
		ILX23-7553 (16-ene-23-yne-1α,25(OH)$_2$D$_3$) 			
Advanced solid tumors	16	Oral individual dosage, 1.7–37.3 μg/m^2/d for 3 consecutive days, repeated in 7-day cycle	Minimum 3 weeks	No objective response	Jain et al., 2011 (uncontrolled)

was combined with a 3 weekly administration of interferon-α for minimal 3 months. In these patients 25% had a partial response to the combination therapy (Obara et al., 2008). Randomized, placebo-controlled studies have been conducted with oral doxercalciferol [**3**] or alfacalcidol [**8**]. One study administered 10 μg/d doxercalciferol [**3**] or placebo during 4 weeks to patients with localized prostate cancer or high-grade prostatic intraepithelial neoplasia. However, no beneficial effects in serum or tissue markers were seen (Gee et al., 2013). Another study used the same dose in metastatic androgen-independent prostate cancer patients but combined the treatment with docetaxel. Also here, no enhanced PSA response rate or survival rate was seen after 4 weeks of treatment (Attia et al., 2008). Oral alfacalcidol [**8**] or placebo was given to patients with myelodysplastic syndromes. In the patients treated with the analog, a prolongation of leukemic transformation-free survival was seen compared to the placebo group (Motomura et al., 1991). Despite the promising *in vitro* and *in vivo* results of vitamin D analogs in cancer models, clinical trials have failed to proof the effects of vitamin D analogs in cancer patients. Vitamin D and its analogs lack cytotoxic activity, so using these analogs in combination with standard therapies such as radio- and chemotherapy is probably more effective than using the analogs as monotherapy. Next to the combination of analogs with standard cancer therapies, it is also possible that these analogs need to be given for a longer period of time or that treatment with analogs has to be started earlier, for example in early stages of disease or even as chemoprevention.

CONCLUSIONS AND PERSPECTIVES

Vitamin D and its analogs exhibit strong antiproliferative and prodifferentiating effects on different normal and malignant cell types. Several vitamin D analogs have been approved for treating psoriasis, osteoporosis, and secondary hyperparathyroidism and are often used as first or second-line treatment option. Despite promising *in vitro* as well as *in vivo* results in various cancer models, vitamin D analog treatment in clinical trials with cancer patients failed to prove efficacy in most trials. Different combinations of analogs and standard cancer therapies should be further explored as well as the correct duration and timing of administration. To unravel the exact working mechanisms of vitamin D analogs more research studies should compare the effects of vitamin D analogs in different cell types to the mother compound.

Furthermore, differences between $1,25(OH)_2D_3$ and its analogs are probably more due to their differences in metabolism and coactivator recruitment than from different genetic regulations. However, some fields such as epigenetics and proteomics remain largely unexplored in comparing the potentially distinctive effects of $1,25(OH)_2D_3$ and its analogs. Since all current genomic and transcriptomic studies focus on established human cell lines, micro-array, and ChIP techniques comparing the effects of $1,25(OH)_2D_3$ and its analogs on human primary tumor tissues should be investigated in the future.

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REFERENCES

Abe, J., Nakano, T., Nishii, Y., Matsumoto, T., Ogata, E., and Ikeda, K. (1991). A novel vitamin D3 analog, 22-oxa-1,25-dihydroxyvitamin D3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* 129, 832–837. doi: 10.1210/endo-129-2-832

Akhter, J., Chen, X., Bowrey, P., Bolton, E. J., and Morris, D. L. (1997). Vitamin D3 analog, EB1089, inhibits growth of subcutaneous xenografts of the human colon cancer cell line, LoVo, in a nude mouse model. *Dis. Colon Rectum* 40, 317–321. doi: 10.1007/BF02050422

Albert, D. M., Kumar, A., Strugnell, S. A., Darjatmoko, S. R., Lokken, J. M., Lindstrom, M. J., et al. (2004a). Effectiveness of 1alpha-hydroxyvitamin D2 in inhibiting tumor growth in a murine transgenic pigmented ocular tumor model. *Arch. Ophthalmol.* 122, 1365–1369. doi: 10.1001/archopht.122.9.1365

Albert, D. M., Kumar, A., Strugnell, S. A., Darjatmoko, S. R., Lokken, J. M., Lindstrom, M. J., et al. (2004b). Effectiveness of vitamin D analogues in treating large tumors and during prolonged use in murine retinoblastoma models. *Arch. Ophthalmol.* 122, 1357–1362. doi: 10.1001/archopht.122.9.1357

Attia, S., Eickhoff, J., Wilding, G., McNeel, D., Blank, J., Ahuja, H., et al. (2008). Randomized, double-blinded phase II evaluation of docetaxel with or without doxercalciferol in patients with metastatic, androgen-independent prostate cancer. *Clin. Cancer Res.* 14, 2437–2443. doi: 10.1158/1078-0432.CCR-07-4274

Bae, S., Yalamarti, B., Ke, Q., Choudhury, S., Yu, H., Karumanchi, S. A., et al. (2011). Preventing progression of cardiac hypertrophy and development of heart failure by paricalcitol therapy in rats. *Cardiovasc. Res.* 91, 632–639. doi: 10.1093/cvr/cvr133

Baeke, F., Korf, H., Overbergh, L., Verstuyf, A., Thorrez, L., Van Lommel, L., et al. (2011). The vitamin D analog, TX527, promotes a human CD4+CD25highCD127low regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation. *J. Immunol.* 186, 132–142. doi: 10.4049/jimmunol.1000695

Berkovich, L., Sintov, A. C., and Ben-Shabat, S. (2013). Inhibition of cancer growth and induction of apoptosis by BGP-13 and BGP-15, new calcipotriene-derived vitamin D3 analogs, *in-vitro* and *in-vivo* studies. *Invest. New Drugs* 31, 247–255. doi: 10.1007/s10637-012-9839-1

Bhatia, V., Saini, M. K., Shen, X., Bi, L. X., Qiu, S., Weigel, N. L., et al. (2009). EB1089 inhibits the parathyroid hormone-related protein-enhanced bone metastasis and xenograft growth of human prostate cancer cells. *Mol. Cancer Ther.* 8, 1787–1798. doi: 10.1158/1535-7163.MCT-09-0064

Blutt, S. E., Polek, T. C., Stewart, L. V., Kattan, M. W., and Weigel, N. L. (2000). A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice. *Cancer Res.* 60, 779–782.

Bower, M., Colston, K. W., Stein, R. C., Hedley, A., Gazet, J. C., Ford, H. T., et al. (1991). Topical calcipotriol treatment in advanced breast cancer. *Lancet* 337, 701–702. doi: 10.1016/0140-6736(91)90280-3

Chiang, K. C., Yeh, C. N., Hsu, J. T., Yeh, T. S., Jan, Y. Y., Wu, C. T., et al. (2013). Evaluation of the potential therapeutic role of a new generation of vitamin D analog, MART-10, in human pancreatic cancer cells *in vitro* and *in vivo*. *Cell Cycle* 12, 1316–1325. doi: 10.4161/cc.24445

Coburn, J. W., Maung, H. M., Elangovan, L., Germain, M. J., Lindberg, J. S., Sprague, S. M., et al. (2004). Doxercalciferol safely suppresses PTH levels in patients with secondary hyperparathyroidism associated with chronic kidney disease stages 3 and 4. *Am. J. Kidney Dis.* 43, 877–890. doi: 10.1053/j.ajkd.2004.01.012

Colston, K., Colston, M. J., and Feldman, D. (1981). 1,25-dihydroxyvitamin D3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology* 108, 1083–1086. doi: 10.1210/endo-108-3-1083

Coyne, D., Acharya, M., Qiu, P., Abboud, H., Batlle, D., Rosansky, S., et al. (2006). Paricalcitol capsule for the treatment of secondary hyperparathyroidism in stages 3 and 4 CKD. *Am. J. Kidney Dis.* 47, 263–276. doi: 10.1053/j.ajkd.2005.10.007

Dalhoff, K., Dancey, J., Astrup, L., Skovsgaard, T., Hamberg, K. J., Lofts, F. J., et al. (2003). A phase II study of the vitamin D analogue Seocalcitol in patients with inoperable hepatocellular carcinoma. *Br. J. Cancer* 89, 252–257. doi: 10.1038/sj.bjc.6601104

Danielsson, C., Nayeri, S., Wiesinger, H., Thieroff-Ekerdt, R., and Carlberg, C. (1996). Potent gene regulatory and antiproliferative activities of 20-methyl analogues of 1,25 dihydroxyvitamin D3. *J. Cell. Biochem.* 63, 199–206. doi: 10.1002/(SICI)1097-4644(19961101)63:2<199::AID-JCB7>3.0.CO;2-T

Dilworth, F. J., Williams, G. R., Kissmeyer, A. M., Nielsen, J. L., Binderup, E., Calverley, M. J., et al. (1997). The vitamin D analog, KH1060, is rapidly degraded both *in vivo* and *in vitro* via several pathways: principal metabolites generated retain significant biological activity. *Endocrinology* 138, 5485–5496. doi: 10.1210/en.138.12.5485

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Eelen, G., Gysemans, C., Verlinden, L., Vanoirbeek, E., De Clercq, P., Van Haver, D., et al. (2007). Mechanism and potential of the growth-inhibitory actions of vitamin D and ana-logs. *Curr. Med. Chem.* 14, 1893–1910. doi: 10.2174/092986707781058823

Eelen, G., Valle, N., Sato, Y., Rochel, N., Verlinden, L., De Clercq, P., et al. (2008). Superagonistic fluorinated vitamin D3 analogs stabilize helix 12 of the vitamin D receptor. *Chem. Biol.* 15, 1029–1034. doi: 10.1016/j.chembiol.2008.08.008

Eelen, G., Verlinden, L., Rochel, N., Claessens, F., De Clercq, P., Vandewalle, M., et al. (2005). Superagonistic action of 14-epi-analogs of 1,25-dihydroxyvitamin D explained by vitamin D receptor-coactivator interaction. *Mol. Pharmacol.* 67, 1566–1573. doi: 10.1124/mol.104.008730

El Abdaimi, K., Dion, N., Papavasiliou, V., Cardinal, P. E., Binderup, L., Goltzman, D., et al. (2000). The vitamin D analogue EB 1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. *Cancer Res.* 60, 4412–4418.

Evans, T. R., Colston, K. W., Lofts, F. J., Cunningham, D., Anthoney, D. A., Gogas, H., et al. (2002). A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. *Br. J. Cancer* 86, 680–685. doi: 10.1038/sj.bjc.6600162

Fichera, A., Little, N., Dougherty, U., Mustafi, R., Cerda, S., Li, Y. C., et al. (2007). A vitamin D analogue inhibits colonic carcinogenesis in the AOM/DSS model. *J. Surg. Res.* 142, 239–245. doi: 10.1016/j.jss.2007.02.038

Flanagan, L., Packman, K., Juba, B., O'Neill, S., Tenniswood, M., and Welsh, J. (2003). Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. *J. Steroid Biochem. Mol. Biol.* 84, 181–192. doi: 10.1016/S0960-0760(03)00028-1

Gee, J., Bailey, H., Kim, K., Kolesar, J., Havighurst, T., Tutsch, K. D., et al. (2013). Phase II open label, multi-center clinical trial of modulation of intermediate endpoint biomarkers by 1alpha-hydroxyvitamin D2 in patients with clinically localized prostate cancer and high grade pin. *Prostate* 73, 970–978. doi: 10.1002/pros.22644

Ghous, Z., Akhter, J., Pourgholami, M. H., and Morris, D. L. (2008). Inhibition of hepatocellular cancer by EB1089: *in vitro* and *in vive* study. *Anticancer Res.* 28, 3757–3761.

Gonzalez-Pardo, V., Martin, D., Gutkind, J. S., Verstuyf, A., Bouillon, R., de Boland, A. R., et al. (2010). 1 Alpha,25-dihydroxyvitamin D3 and its TX527 analog inhibit the growth of endothelial cells transformed by Kaposi sarcoma-associated herpes virus G protein-coupled receptor *in vitro* and *in vivo*. *Endocrinology* 151, 23–31. doi: 10.1210/en.2009-0650

Griffin, M. D., Xing, N., and Kumar, R. (2004). Gene expression profiles in dendritic cells conditioned by 1alpha,25-dihydroxyvitamin D3 analog.

J. Steroid Biochem. Mol. Biol. 89–90, 443–448. doi: 10.1016/j.jsbmb.2004.03.039

Grostern, R. J., Bryar, P. J., Zimbric, M. L., Darjatmoko, S. R., Lissauer, B. J., Lindstrom, M. J., et al. (2002). Toxicity and dose-response studies of 1alpha-hydroxyvitamin D2 in a retinoblastoma xenograft model. *Arch. Ophthalmol.* 120, 607–612. doi: 10.1001/archopht.120.5.607

Gulliford, T., English, J., Colston, K. W., Menday, P., Moller, S., and Coombes, R. C. (1998). A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer. *Br. J. Cancer* 78, 6–13. doi: 10.1038/bjc.1998.434

Hagino, H., Takano, T., Fukunaga, M., Shiraki, M., Nakamura, T., and Matsumoto, T. (2013). Eldecalcitol reduces the risk of severe vertebral fractures and improves the health-related quality of life in patients with osteoporosis. *J. Bone Miner. Metab.* 31, 183–189. doi: 10.1007/s00774-012-0397-6

Hamdy, N. A., Kanis, J. A., Beneton, M. N., Brown, C. B., Juttmann, J. R., Jordans, J. G., et al. (1995). Effect of alfacalcidol on natural course of renal bone disease in mild to moderate renal failure. *BMJ* 310, 358–363. doi: 10.1136/bmj.310.6976.358

Hansen, C. M., and Maenpaa, P. H. (1997). EB 1089, a novel vitamin D analog with strong antiproliferative and differentiation-inducing effects on target cells. *Biochem. Pharmacol.* 54, 1173–1179. doi: 10.1016/S0006-2952(97)00181-0

Hara, K., Kusuzaki, K., Takeshita, H., Kuzuhara, A., Tsuji, Y., Ashihara, T., et al. (2001). Oral administration of 1 alpha hydroxyvitamin D3 inhibits tumor growth and metastasis of a murine osteosarcoma model. *Anticancer Res.* 21, 321–324.

Harada, S., Mizoguchi, T., Kobayashi, Y., Nakamichi, Y., Takeda, S., Sakai, S., et al. (2012). Daily administration of eldecalcitol (ED-71), an active vitamin D analog, increases bone mineral density by suppressing RANKL expression in mouse trabecular bone. *J. Bone Miner. Res.* 27, 461–473. doi: 10.1002/jbmr.555

Hybrigenics. (2014). *Inecalcitol. [Internet] Paris, France: Hybrigenics* [cited 2014 29/01]. Available online at: http://www.hybrigenics.com/contents/pharma-1/inecalcitol-1

Iino, Y., Yoshida, M., Sugamata, N., Maemura, M., Ohwada, S., Yokoe, T., et al. (1992). 1 alpha-hydroxyvitamin D3, hypercalcemia, and growth suppression of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumors. *Breast Cancer Res. Treat.* 22, 133–140. doi: 10.1007/BF01833343

Iseki, K., Tatsuta, M., Uehara, H., Iishi, H., Yano, H., Sakai, N., et al. (1999). Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 of colon carcinogenesis induced by azoxymethane in Wistar rats. *Int. J. Cancer* 81, 730–733. doi: 10.1002/(SICI)1097-0215(19990531)81:5<730::AID-IJC11>3.0.CO;2-Q

Ito, M., Nakamura, T., Fukunaga, M., Shiraki, M., and Matsumoto, T. (2011). Effect of eldecalcitol, an active vitamin D analog, on hip structure and biomechanical properties: 3D assessment by clinical CT. *Bone* 49, 328–334. doi: 10.1016/j.bone.2011.05.002

Jain, R. K., Trump, D. L., Egorin, M. J., Fernandez, M., Johnson, C. S., and Ramanathan, R. K. (2011). A phase I study of the vitamin D3 analogue ILX23-7553 administered orally to patients with advanced solid tumors. *Invest. New Drugs* 29, 1420–1425. doi: 10.1007/s10637-010-9492-5

James, S. Y., Mercer, E., Brady, M., Binderup, L., and Colston, K. W. (1998). EB1089, a synthetic analogue of vitamin D, induces apoptosis in breast cancer cells *in vivo* and *in vitro*. *Br. J. Pharmacol.* 125, 953–962. doi: 10.1038/sj.bjp.0702103

Jensen, S. S., Madsen, M. W., Lukas, J., Binderup, L., and Bartek, J. (2001). Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol. Endocrinol.* 15, 1370–1380. doi: 10.1210/mend.15.8.0673

Jones, G. (1997). "Analog metabolism," in *Vitamin D*. 1st Edn., ed D. Feldman (San Diego, CA: Academic Press), 973–994.

Kawa, S., Yoshizawa, K., Nikaido, T., and Kiyosawa, K. (2005). Inhibitory effect of 22-oxa-1,25-dihydroxyvitamin D3, maxacalcitol, on the proliferation of pancreatic cancer cell lines. *J. Steroid Biochem. Mol. Biol.* 97, 173–177. doi: 10.1016/j.jsbmb.2005.06.021

Kawa, S., Yoshizawa, K., Tokoo, M., Imai, H., Oguchi, H., Kiyosawa, K., et al. (1996). Inhibitory effect of 22-oxa-1,25-dihydroxyvitamin D3 on the proliferation of pancreatic cancer cell lines. *Gastroenterology* 110, 1605–1613. doi: 10.1053/gast.1996.v110.pm8613068

Kissmeyer, A. M., Binderup, E., Binderup, L., Mork Hansen, C., Andersen, N. R., Makin, H. L., et al. (1997). Metabolism of the vitamin D analog EB 1089: identification of *in vivo* and *in vitro* liver metabolites and their biological activities. *Biochem. Pharmacol.* 53, 1087–1097. doi: 10.1016/S0006-2952(96)00816-7

Kriebitzsch, C., Verlinden, L., Eelen, G., Tan, B. K., Van Camp, M., Bouillon, R., et al. (2009). The impact of 1,25(OH)2D3 and its structural analogs on gene expression in cancer cells–a microarray approach. *Anticancer Res.* 29, 3471–3483.

Lambert, J. R., Eddy, V. J., Young, C. D., Persons, K. S., Sarkar, S., Kelly, J. A., et al. (2010). A vitamin D receptor-alkylating derivative of 1alpha,25-dihydroxyvitamin D3 inhibits growth of human kidney cancer cells and suppresses tumor growth. *Cancer Prev. Res. (Phila.)* 3, 1596–1607. doi: 10.1158/1940-6207.CAPR-10-0122

Lawrence, J. A., Akman, S. A., Melin, S. A., Case, L. D., and Schwartz, G. G. (2013). Oral paricalcitol (19-nor-1,25-dihydroxyvitamin D2) in women receiving chemotherapy for metastatic breast cancer: a feasibility trial. *Cancer Biol. Ther.* 14, 476–480. doi: 10.4161/cbt.24350

Lee, H. J., Paul, S., Atalla, N., Thomas, P. E., Lin, X., Yang, I., et al. (2008). Gemini vitamin D analogues inhibit estrogen receptor-positive and estrogen receptor-negative mammary tumorigenesis without hypercalcemic toxicity. *Cancer Prev. Res. (Phila.)* 1, 476–484. doi: 10.1158/1940-6207.CAPR-08-0084

Lee, H. J., So, J. Y., DeCastro, A., Smolarek, A., Paul, S., Maehr, H., et al. (2010). Gemini vitamin D analog suppresses ErbB2-positive mammary tumor growth via inhibition of ErbB2/AKT/ERK signaling. *J. Steroid Biochem. Mol. Biol.* 121, 408–412. doi: 10.1016/j.jsbmb.2010.03.053

Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., et al. (2002). Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. *Mol. Endocrinol.* 16, 1243–1256. doi: 10.1210/mend.16.6.0874

Liska, J., Macejova, D., Ondkova, S., and Brtko, J. (2012). Morphology of 1-methyl-1-nitrosourea induced rat mammary tumours after treatment with precursor of phytanic acid or its combination with vitamin D analogue. *Endocr. Regul.* 46, 21–26. doi: 10.4149/endo_2012_021

Liu, G., Oettel, K., Ripple, G., Staab, M. J., Horvath, D., Alberti, D., et al. (2002). Phase I trial of 1alpha-hydroxyvitamin d(2) in patients with hormone refractory prostate cancer. *Clin. Cancer Res.* 8, 2820–2827.

Liu, G., Wilding, G., Staab, M. J., Horvath, D., Miller, K., Dresen, A., et al. (2003). Phase II study of 1alpha-hydroxyvitamin D(2) in the treatment of advanced androgen-independent prostate cancer. *Clin. Cancer Res.* 9, 4077–4083.

Liu, Y. Y., Nguyen, C., Ali Gardezi, S. A., Schnirer, I., and Peleg, S. (2001). Differential regulation of heterodimerization by 1alpha,25-dihydroxyvitamin D(3) and its 20-epi analog. *Steroids* 66, 203–212. doi: 10.1016/S0039-128X(00)00151-3

Liu, Y. Y., Nguyen, C., and Peleg, S. (2000). Regulation of ligand-induced heterodimerization and coactivator interaction by the activation function-2 domain of the vitamin D receptor. *Mol. Endocrinol.* 14, 1776–1787. doi: 10.1210/mend.14.11.0560

Lokeshwar, B. L., Schwartz, G. G., Selzer, M. G., Burnstein, K. L., Zhuang, S. H., Block, N. L., et al. (1999). Inhibition of prostate cancer metastasis *in vivo*: a comparison of 1,25-dihydroxyvitamin D (calcitriol) and EB1089. *Cancer Epidemiol. Biomarkers Prev.* 8, 241–248.

Lundqvist, J., Hansen, S. K., and Lykkesfeldt, A. E. (2013). Vitamin D analog EB1089 inhibits aromatase expression by dissociation of comodulator WSTF from the CYP19A1 promoter-a new regulatory pathway for aromatase. *Biochim. Biophys. Acta* 1833, 40–47. doi: 10.1016/j.bbamcr.2012.10.012

Ma, Y., Yu, W. D., Hidalgo, A. A., Luo, W., Delansorne, R., Johnson, C. S., et al. (2013). Inecalcitol, an analog of 1,25D3, displays enhanced antitumor activity through the induction of apoptosis in a squamous cell carcinoma model system. *Cell Cycle* 12, 743–752. doi: 10.4161/cc.23846

Macejova, D., Ondkova, S., Jakubikova, L., Mlynarcikova, A., Scsukova, S., Liska, J., et al. (2011). MNU-induced mammary gland carcinogenesis: chemopreventive and therapeutic effects of vitamin D and Seocalcitol on selected regulatory vitamin D receptor pathways. *Toxicol. Lett.* 207, 60–72. doi: 10.1016/j.toxlet.2011.07.029

Matsumoto, T., Ito, M., Hayashi, Y., Hirota, T., Tanigawara, Y., Sone, T., et al. (2011). A new active vitamin D3 analog, eldecalcitol, prevents the risk of osteoporotic fractures–a randomized, active comparator, double-blind study. *Bone* 49, 605–612. doi: 10.1016/j.bone.2011.07.011

Milczarek, M., Psurski, M., Kutner, A., and Wietrzyk, J. (2013a). Vitamin D analogs enhance the anticancer activity of 5-fluorouracil in an *in vivo* mouse colon cancer model. *BMC Cancer* 13:294. doi: 10.1186/1471-2407-13-294

Milczarek, M., Rosinska, S., Psurski, M., Maciejewska, M., Kutner, A., and Wietrzyk, J. (2013b). Combined colonic cancer treatment with vitamin D analogs and irinotecan or oxaliplatin. *Anticancer Res.* 33, 433–444.

Motomura, S., Kanamori, H., Maruta, A., Kodama, F., and Ohkubo, T. (1991). The effect of 1-hydroxyvitamin D3 for prolongation of leukemic transformation-free survival in myelodysplastic syndromes. *Am. J. Hematol.* 38, 67–68. doi: 10.1002/ajh.2830380112

Nakagawa, K., Sasaki, Y., Kato, S., Kubodera, N., and Okano, T. (2005). 22-Oxa-1alpha,25-dihydroxyvitamin D3 inhibits metastasis and angiogenesis in lung cancer. *Carcinogenesis* 26, 1044–1054. doi: 10.1093/carcin/bgi049

Oades, G. M., Dredge, K., Kirby, R. S., and Colston, K. W. (2002). Vitamin D receptor-dependent antitumour effects of 1,25-dihydroxyvitamin D3 and two synthetic analogues in three *in vivo* models of prostate cancer. *BJU Int.* 90, 607–616. doi: 10.1046/j.1464-410X.2002.02964.x

Obara, W., Mizutani, Y., Oyama, C., Akaza, H., Ishii, N., Kohri, K., et al. (2008). Prospective study of combined treatment with interferon-alpha and active vitamin D3 for Japanese patients with metastatic renal cell carcinoma. *Int. J. Urol.* 15, 794–799. doi: 10.1111/j.1442-2042.2008.02086.x

O'Brien, M. E., Talbot, D., Maclennan, K., and Smith, I. E. (1993). Inefficacy of calcipotriol in skin metastases from breast cancer. *Lancet* 342, 994. doi: 10.1016/0140-6736(93)92042-R

Okamoto, R., Delansorne, R., Wakimoto, N., Doan, N. B., Akagi, T., Shen, M., et al. (2012). Inecalcitol, an analog of 1alpha,25(OH)(2) D(3), induces growth arrest of androgen-dependent prostate cancer cells. *Int. J. Cancer* 130, 2464–2473. doi: 10.1002/ijc.26279

Okamoto, R., Gery, S., Kuwayama, Y., Borregaard, N., Ho, Q., Alvarez, R., et al. (2014). Novel Gemini vitamin D3 analogs: large structure/function analysis and ability to induce antimicrobial peptide. *Int. J. Cancer* 134, 207–217. doi: 10.1002/ijc.28328

Okano, T., Tsugawa, N., Masuda, S., Takeuchi, A., Kobayashi, T., and Nishii, Y. (1989a). Protein-binding properties of 22-oxa-1 alpha,25-dihydroxyvitamin D3, a synthetic analogue of 1 alpha,25-dihydroxyvitamin D3. *J. Nutr. Sci. Vitaminol. (Tokyo)* 35, 529–533. doi: 10.3177/jnsv.35.529

Okano, T., Tsugawa, N., Masuda, S., Takeuchi, A., Kobayashi, T., Takita, Y., et al. (1989b). Regulatory activities of 2 beta-(3-hydroxypropoxy)-1 alpha, 25-dihydroxyvitamin D3, a novel synthetic vitamin D3 derivative, on calcium metabolism. *Biochem. Biophys. Res. Commun.* 163, 1444–1449. doi: 10.1016/0006-291X(89)91140-6

Park, M. R., Lee, J. H., Park, M. S., Hwang, J. E., Shim, H. J., Cho, S. H., et al. (2012). Suppressive effect of 19-nor-1alpha-25-dihydroxyvitamin D2 on gastric cancer cells and peritoneal metastasis model. *J. Korean Med. Sci.* 27, 1037–1043. doi: 10.3346/jkms.2012.27.9.1037

Perez-Stable, C. M., Schwartz, G. G., Farinas, A., Finegold, M., Binderup, L., Howard, G. A., et al. (2002). The G gamma / T-15 transgenic mouse model of androgen-independent prostate cancer: target cells of carcinogenesis and the effect of the vitamin D analogue EB 1089. *Cancer Epidemiol. Biomarkers Prev.* 11, 555–563.

Petrini, M., Caracciolo, F., Corini, M., Valentini, P., Sabbatini, A. R., and Grassi, B. (1991). Low-dose ARA-C and 1(OH) D3 administration in acute non lymphoid leukemia: pilot study. *Haematologica* 76, 200–203.

Pommergaard, H. C., Burcharth, J., Rosenberg, J., and Raskov, H. (2013). Combination chemoprevention with diclofenac, calcipotriol and difluoromethylornithine inhibits development of non-melanoma skin cancer in mice. *Anticancer Res.* 33, 3033–3039.

Prudencio, J., Akutsu, N., Benlimame, N., Wang, T., Bastien, Y., Lin, R., et al. (2001). Action of low calcemic 1alpha,25-dihydroxyvitamin D3 analogue EB1089 in head and neck squamous cell carcinoma. *J. Natl. Cancer Inst.* 93, 745–753. doi: 10.1093/jnci/93.10.745

Quack, M., and Carlberg, C. (1999). Selective recognition of vitamin D receptor conformations mediates promoter selectivity of vitamin D analogs. *Mol. Pharmacol.* 55, 1077–1087. doi: 10.1124/mol.55.6.1077

Raina, V., Cunningham, D., Gilchrist, N., and Soukop, M. (1991). Alfacalcidol is a nontoxic, effective treatment of follicular small-cleaved cell lymphoma. *Br. J. Cancer* 63, 463–465. doi: 10.1038/bjc.1991.108

Sato, T., Takusagawa, K., Asoo, N., and Konno, K. (1984). Effect of 1 alpha-hydroxyvitamin D3 on metastasis of rat ascites hepatoma K-231. *Br. J. Cancer* 50, 123–125. doi: 10.1038/bjc.1984.148

Schwartz, G. G., Eads, D., Naczki, C., Northrup, S., Chen, T., and Koumenis, C. (2008). 19-nor-1 alpha,25-dihydroxyvitamin D2 (paricalcitol) inhibits the proliferation of human pancreatic cancer cells *in vitro* and *in vivo*. *Cancer Biol. Ther.* 7, 430–436. doi: 10.4161/cbt.7.3.5418

Schwartz, G. G., Hall, M. C., Stindt, D., Patton, S., Lovato, J., and Torti, F. M. (2005). Phase I/II study of 19-nor-1alpha-25-dihydroxyvitamin D2 (paricalcitol) in advanced, androgen-insensitive prostate cancer. *Clin. Cancer Res.* 11(24 Pt 1), 8680–8685. doi: 10.1158/1078-0432.CCR-05-1237

Schwinn, M. K., and DeLuca, H. F. (2007). Differential recruitment of coactivators to the vitamin D receptor transcriptional complex by 1alpha,25-dihydroxyvitamin D3 analogs. *Arch. Biochem. Biophys.* 465, 443–451. doi: 10.1016/j.abb.2007.06.015

Seubwai, W., Wongkham, C., Puapairoj, A., Okada, S., and Wongkham, S. (2010). 22-oxa-1,25-dihydroxyvitamin D3 efficiently inhibits tumor growth in inoculated mice and primary histoculture of cholangiocarcinoma. *Cancer* 116, 5535–5543. doi: 10.1002/cncr.25478

Shalhoub, V., Shatzen, E. M., Ward, S. C., Young, J. I., Boedigheimer, M., Twehues, L., et al. (2010). Chondro/osteoblastic and cardiovascular gene modulation in human artery smooth muscle cells that calcify in the presence of phosphate and calcitriol or paricalcitol. *J. Cell. Biochem.* 111, 911–921. doi: 10.1002/jcb.22779

Shankar, V. N., Dilworth, F. J., Makin, H. L., Schroeder, N. J., Trafford, D. J., Kissmeyer, A. M., et al. (1997). Metabolism of the vitamin D analog EB1089 by cultured human cells: redirection of hydroxylation site to distal carbons of the side-chain. *Biochem. Pharmacol.* 53, 783–793. doi: 10.1016/S0006-2952(96)00815-5

Shinaberger, C. S., Kopple, J. D., Kovesdy, C. P., McAllister, C. J., van Wyck, D., Greenland, S., et al. (2008). Ratio of paricalcitol dosage to serum parathyroid hormone level and survival in maintenance hemodialysis patients. *Clin. J. Am. Soc. Nephrol.* 3, 1769–1776. doi: 10.2215/CJN.01760408

So, J. Y., Lee, H. J., Smolarek, A. K., Paul, S., Wang, C. X., Maehr, H., et al. (2011). A novel Gemini vitamin D analog represses the expression of a stem cell marker CD44 in breast cancer. *Mol. Pharmacol.* 79, 360–367. doi: 10.1124/mol.110.068403

Sundaram, S., Sea, A., Feldman, S., Strawbridge, R., Hoopes, P. J., Demidenko, E., et al. (2003). The combination of a potent vitamin D3 analog, EB 1089, with ionizing radiation reduces tumor growth and induces apoptosis of MCF-7 breast tumor xenografts in nude mice. *Clin. Cancer Res.* 9, 2350–2356.

Teng, M., Wolf, M., Lowrie, E., Ofsthun, N., Lazarus, J. M., and Thadhani, R. (2003). Survival of patients undergoing hemodialysis with paricalcitol or calcitriol therapy. *N. Engl. J. Med.* 349, 446–456. doi: 10.1056/NEJMoa022536

Tentori, F., Hunt, W. C., Stidley, C. A., Rohrscheib, M. R., Bedrick, E. J., Meyer, K. B., et al. (2006). Mortality risk among hemodialysis patients receiving different vitamin D analogs. *Kidney Int.* 70, 1858–1865. doi: 10.1038/sj.ki.5001868

Trouillas, P., Honnorat, J., Bret, P., Jouvet, A., and Gerard, J. P. (2001). Redifferentiation therapy in brain tumors: long-lasting complete regression of glioblastomas and an anaplastic astrocytoma under long term 1-alpha-hydroxycholecalciferol. *J. Neurooncol.* 51, 57–66. doi: 10.1023/A:1006437003352

Uchiyama, Y., Higuchi, Y., Takeda, S., Masaki, T., Shira-Ishi, A., Sato, K., et al. (2002). ED-71, a vitamin D analog, is a more potent inhibitor of bone resorption than alfacalcidol in an estrogen-deficient rat model of osteoporosis. *Bone* 30, 582–588. doi: 10.1016/S8756-3282(02)00682-8

Valrance, M. E., Brunet, A. H., and Welsh, J. (2007). Vitamin D receptor-dependent inhibition of mammary tumor growth by EB1089 and ultraviolet radiation *in vivo*. *Endocrinology* 148, 4887–4894. doi: 10.1210/en.2007-0267

van den Bemd, G. C., Pols, H. A., Birkenhager, J. C., and van Leeuwen, J. P. (1996). Conformational change and enhanced stabilization of the vitamin D receptor by the 1,25-dihydroxyvitamin D3 analog KH1060. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10685–10690. doi: 10.1073/pnas.93.20.10685

van Ginkel, P. R., Yang, W., Marcet, M. M., Chow, C. C., Kulkarni, A. D., Darjatmoko, S., et al. (2007). 1 alpha-Hydroxyvitamin D2 inhibits growth of human neuroblastoma. *J. Neurooncol.* 85, 255–262. doi: 10.1007/s11060-007-9418-z

Vanoirbeek, E., Eelen, G., Verlinden, L., Marchal, K., Engelen, K., De Moor, B., et al. (2009). Microarray analysis of MCF-7 breast cancer cells treated with 1,25-dihydroxyvitamin D3 or a 17-methyl-D-ring analog. *Anticancer Res.* 29, 3585–3590.

VanWeelden, K., Flanagan, L., Binderup, L., Tenniswood, M., and Welsh, J. (1998). Apoptotic regression of MCF-7 xenografts in nude mice treated

with the vitamin D3 analog, EB1089. *Endocrinology* 139, 2102–2110. doi: 10.1210/en.139.4.2102

Vegesna, V., O'Kelly, J., Said, J., Uskokovic, M., Binderup, L., and Koeffle, H. P. (2003). Ability of potent vitamin D3 analogs to inhibit growth of prostate cancer cells *in vivo*. *Anticancer Res.* 23, 283–289.

Wietrzyk, J., Nevozhay, D., Filip, B., Milczarek, M., and Kutner, A. (2007). The antitumor effect of lowered doses of cytostatics combined with new analogs of vitamin D in mice. *Anticancer Res.* 27, 3387–3398.

Wietrzyk, J., Nevozhay, D., Milczarek, M., Filip, B., and Kutner, A. (2008). Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cyclophosphamide in a mouse mammary cancer model. *Cancer Chemother. Pharmacol.* 62, 787–797. doi: 10.1007/s00280-007-0666-6

Wietrzyk, J., Pelczynska, M., Madej, J., Dzimira, S., Kusnierczyk, H., Kutner, A., et al. (2004). Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D3 (PRI-2191). *Steroids* 69, 629–635. doi: 10.1016/j.steroids.2004.05.015

Wu-Wong, J. R., Nakane, M., Ma, J., Ruan, X., and Kroeger, P. E. (2007). VDR-mediated gene expression patterns in resting human coronary artery smooth muscle cells. *J. Cell. Biochem.* 100, 1395–1405. doi: 10.1002/jcb.21133

Yamamoto, H., Shevde, N. K., Warrier, A., Plum, L. A., DeLuca, H. F., and Pike, J. W. (2003). 2-Methylene-19-nor-(20S)-1,25-dihydroxyvitamin D3 potently stimulates gene-specific DNA binding of the vitamin D receptor in osteoblasts. *J. Biol. Chem.* 278, 31756–31765. doi: 10.1074/jbc.M304737200

Yoon, J. S., Kim, J. Y., Park, H. K., Kim, E. S., Ahn, K. S., Yoon, S. S., et al. (2008). Antileukemic effect of a synthetic vitamin D3 analog, HY-11, with low potential to cause hypercalcemia. *Int. J. Oncol.* 32, 387–396. doi: 10.3892/ijo.32.2.387

Zella, L. A., Meyer, M. B., Nerenz, R. D., and Pike, J. W. (2009). The enhanced hypercalcemic response to 20-epi-1,25-dihydroxyvitamin D3 results from a selective and prolonged induction of intestinal calcium-regulating genes. *Endocrinology* 150, 3448–3456. doi: 10.1210/en.2009-0113

Zhang, X., Jiang, F., Li, P., Li, C., Ma, Q., Nicosia, S. V., et al. (2005). Growth suppression of ovarian cancer xenografts in nude mice by vitamin D analogue EB1089. *Clin. Cancer Res.* 11, 323–328.

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