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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

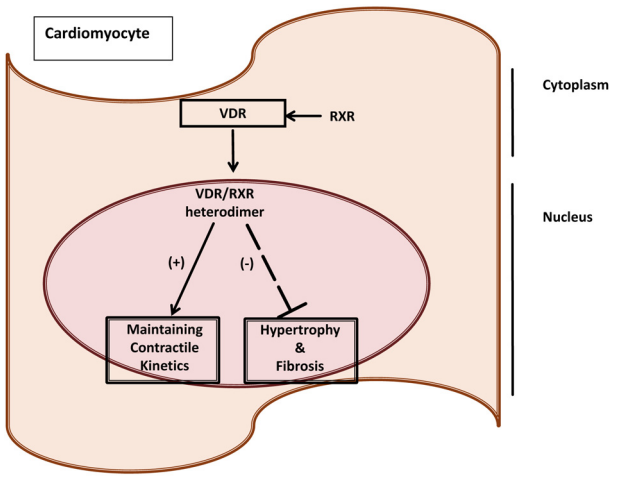

FIGURE 1 | VDR as a marker of cardiac dysfunction induced by cancer cachexia. Vitamin D Receptor (VDR) as a molecular marker of maladaptive responses due to cancer cachexia in the heart. VDR has been linked to having cardioprotective mechanisms by maintaining contractile kinetics, and regulating fibrosis 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.

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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

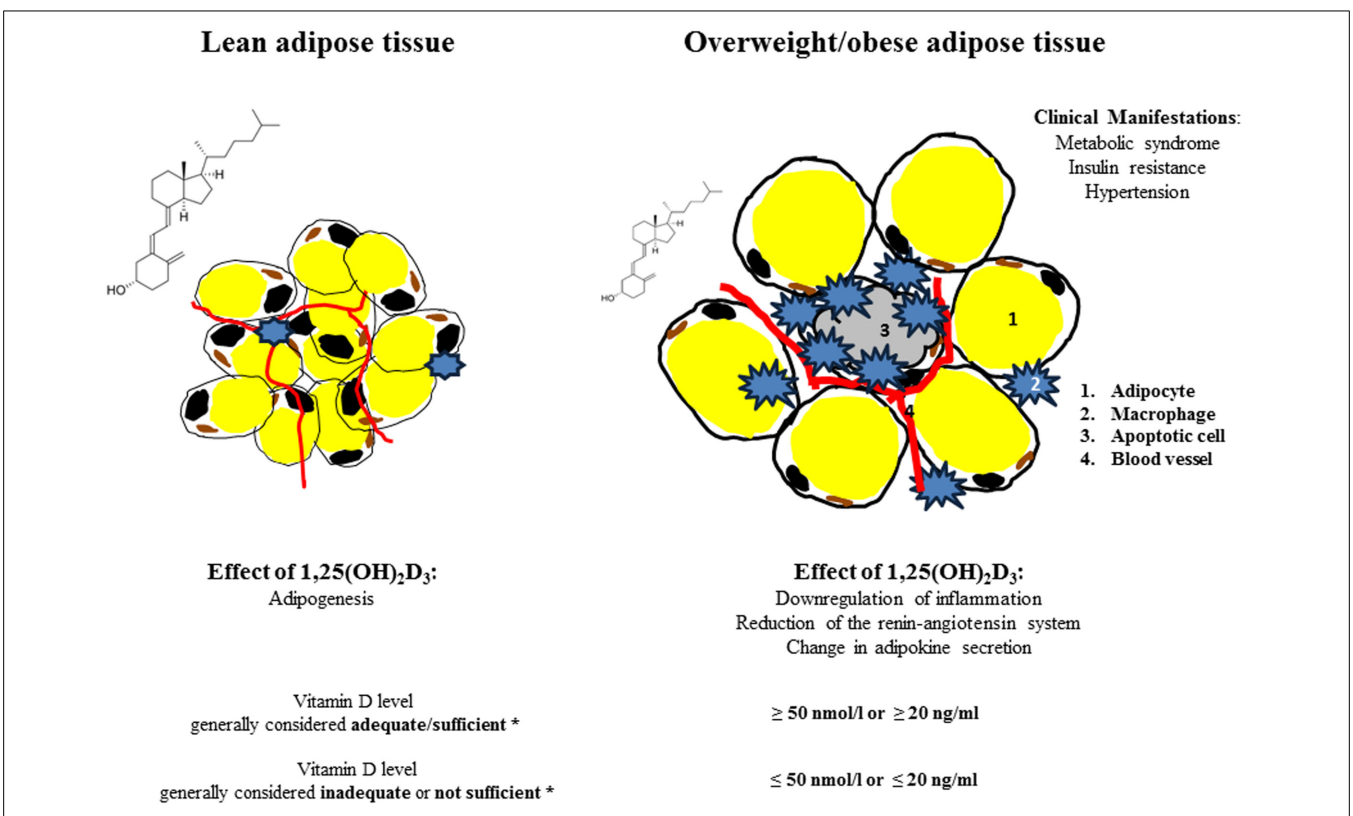

FIGURE 1 | Effect of $1,25(OH)_2D_3$ on adipose tissue/lean and obese state. *Vitamin D levels (25(OH)D) according to the Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2011.

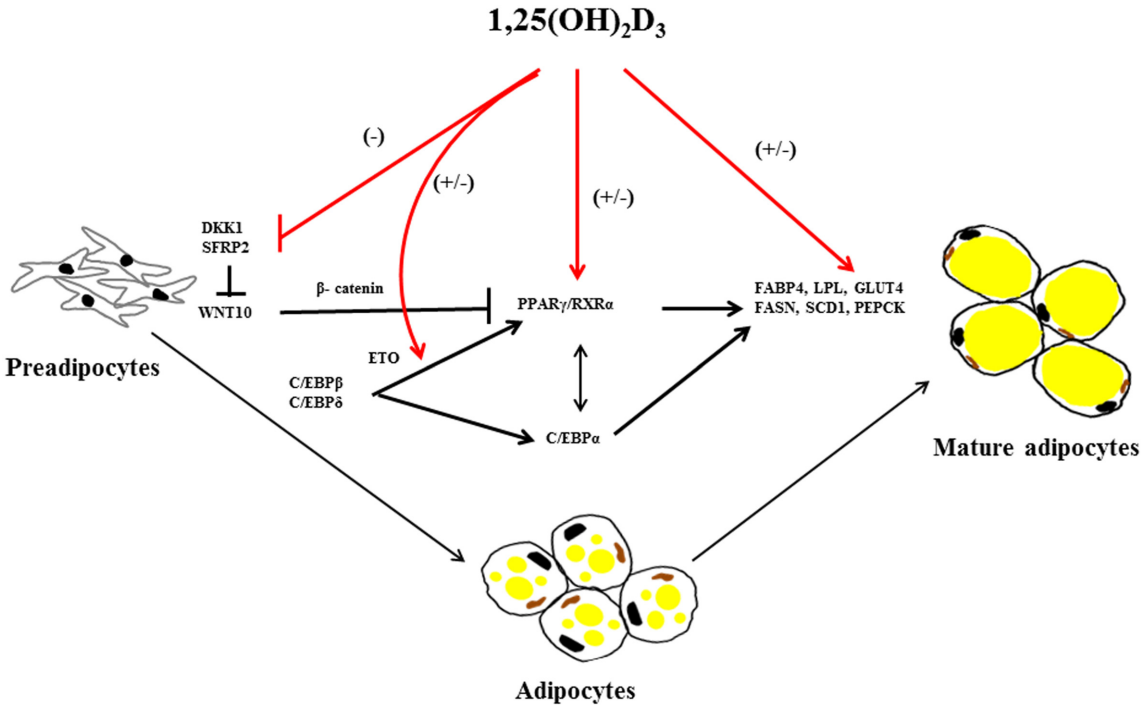

FIGURE 2 | Overview of $1,25(OH)_2D_3$ on regulating factors in adipogenesis. $1,25(OH)_2D_3$ has both stimulating and inhibition effect on adipogenesis. $1,25(OH)_2D_3$ suppress dickkopf1(DKK1) and secreted frizzled-related protein 2 (SFRP2) expression in mouse bone marrow stromal cells (BMSCs) there 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

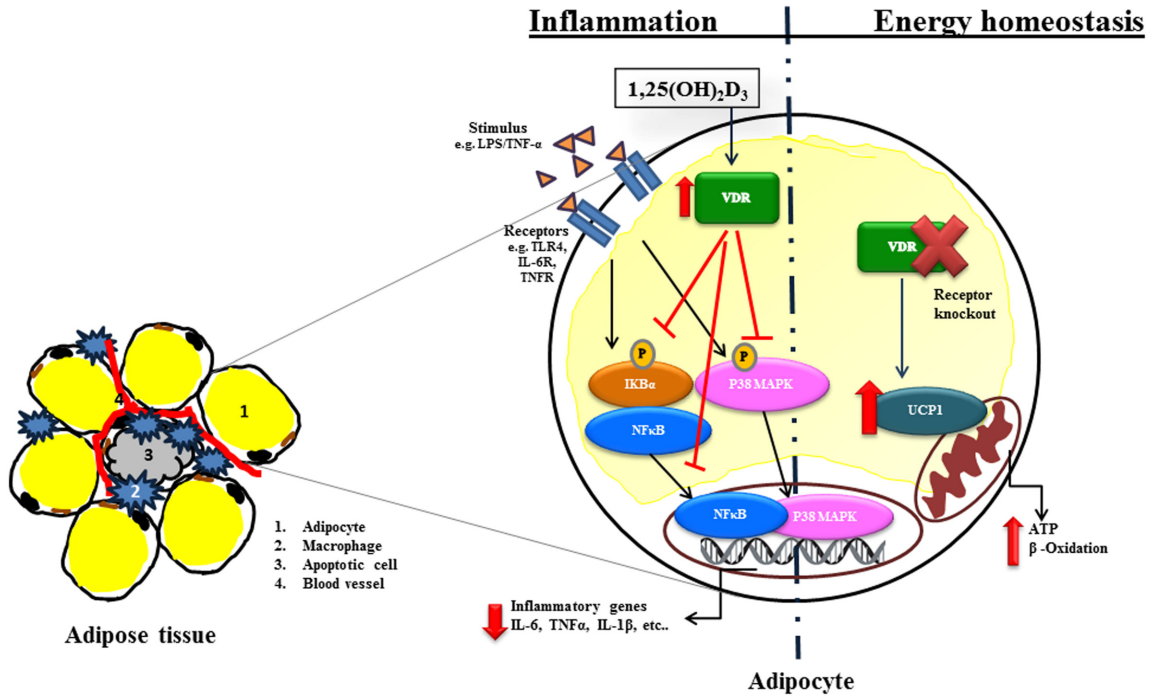

FIGURE 3 | Molecular actions of $1,25(OH)_2D_3$ in inflammation and energy homeostasis in adipocytes. Stimulation via e.g., lipopolysaccharide (LPS), TNF-α via specific receptors e.g., Toll like receptor (TLR), IL-6 receptors (IL-6R) activate Nuclear factor kappa-B (NFκB) or p38 mitogen-activated protein 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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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

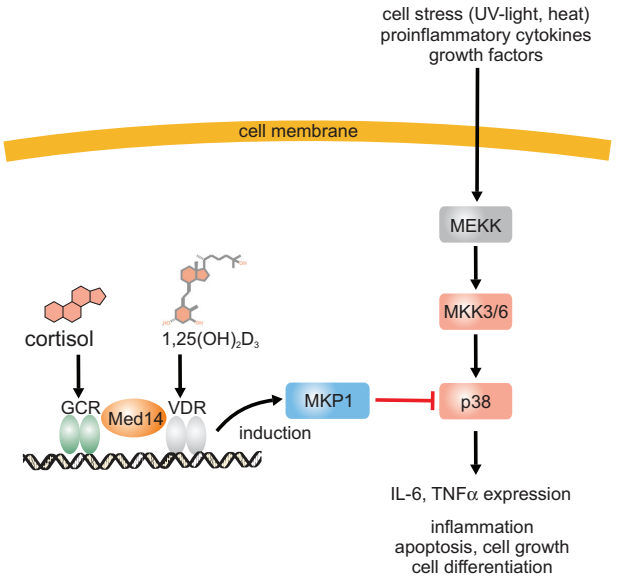

FIGURE 1 | Inhibition of the p38 MAP kinase pathway by 1α,25(OH)$_2$D$_3$ and a mechanism for the synergistic anti-inflammatory effects of 1α,25(OH)$_2$D$_3$ and glucocorticoids. Proinflammatory stimuli lead to p38 MAP kinase phosphorylation and activation which subsequently induces expression of many 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

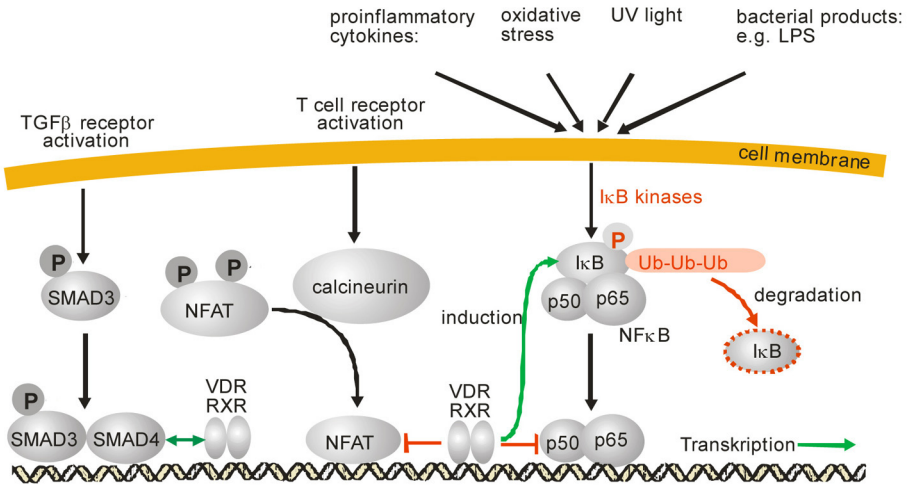

FIGURE 2 | SMAD, NFAT and NFκB signaling and modulation of these signaling pathways by 1α,25(OH)$_2$D$_3$, respective VDR/RXR. IκB phosphorylation after various cell stress signals leads to its ubiquitinylation and subsequent proteosomal degradation. After IκB degradation, NFκB is released and translocates 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).

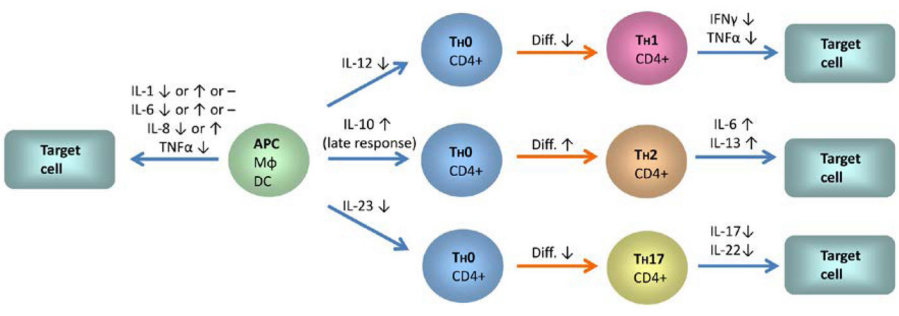

FIGURE 3 | The influence of $1\alpha,25(OH)_2D_3$ on the expression of interleukins, TNFα and IFNγ in monocytes, dendritic cells, and different T-cell subsets. Blue arrows indicate IL signaling between the different cell types and red arrows indicate differentiation processes. IL-12 and IL-23 expression 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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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.

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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

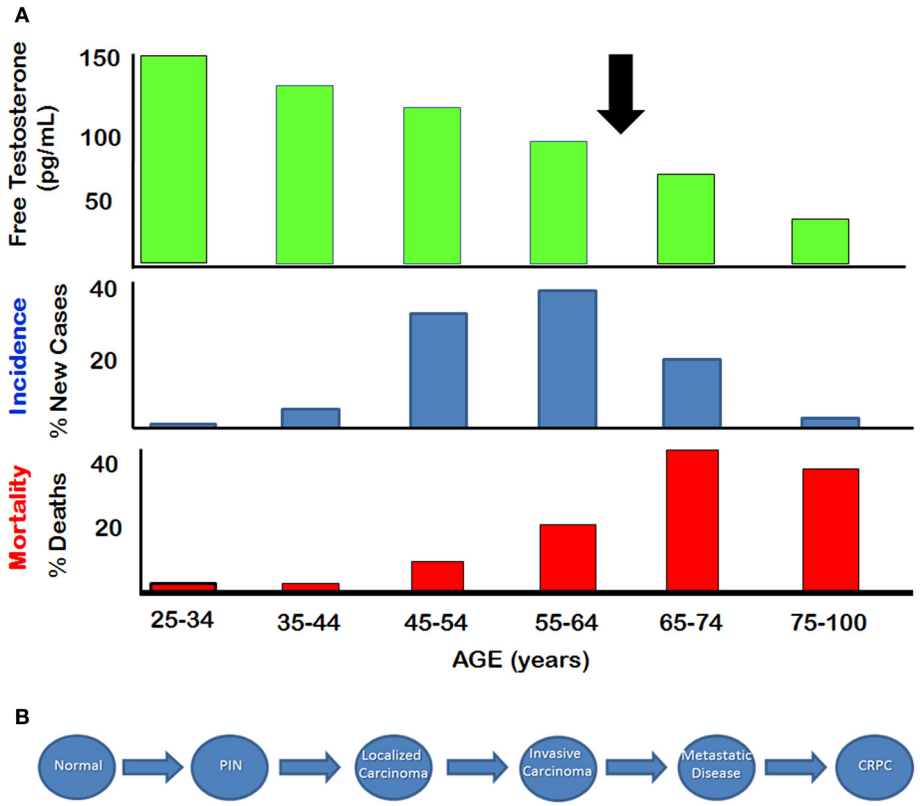


FIGURE 1 | Natural History of Prostate Cancer (A) Relationship between serum free testosterone and incidence and mortality of prostate cancer. Arrow indicates approximate age at which free testosterone declines below 50% of the level seen in young adults. **(B)** Stepwise depiction of progression from 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

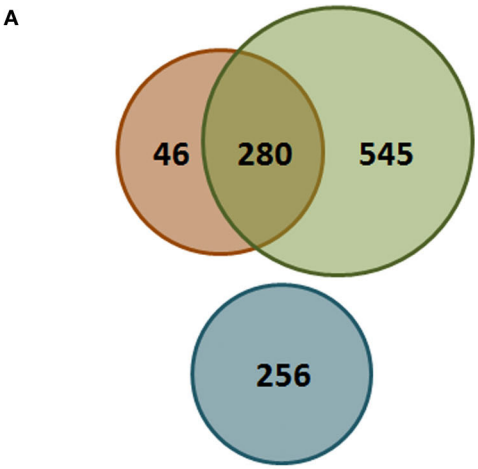
The crosstalk between VDR- and AR-mediated gene expression was first demonstrated in LNCaP cells (Qiao and Tuohimaa, 2004). Induction of *FACL3* (long-chain fatty-acid CoA ligase 3) is dependent on both vitamin D and androgen levels, and treatment with bicalutamide inhibits $1,25(OH)_2D_3$-induced *FACL3* 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.

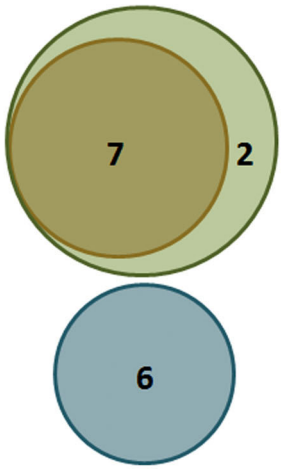
More than 50% of the responsive genes found from microarray data lack functional response elements in their promoters when comparing to existing genome-wide screens for VDREs and androgen responsive database (Wang et al., 2005; Jiang et al., 2009), raising issues regarding the regulation of these genes, 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





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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

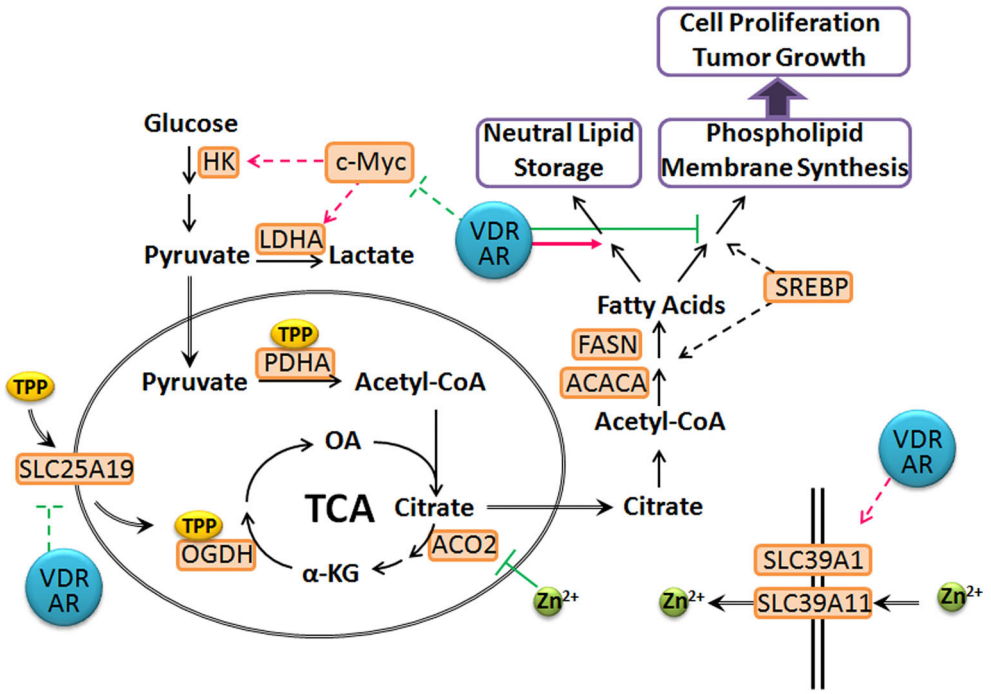

FIGURE 3 | Proposed model of T- and 1,25(OH)$_2$D$_3$-mediated prostate cancer metabolism. The VDR and AR axes modulate the expression of SLC25A19 and SLC39A11, leading to elevated intracellular zinc levels and deplete mitochondrial TPP pool, resulting in a truncated TCA cycle. Citrate is shunted into 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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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.

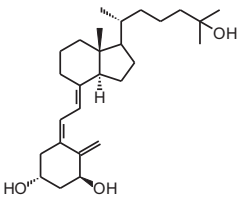
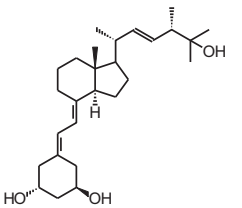
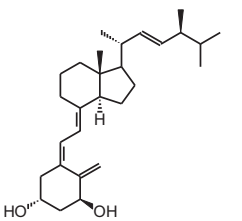
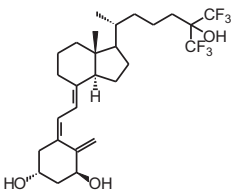
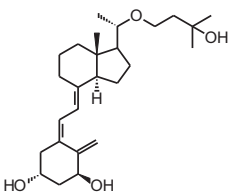
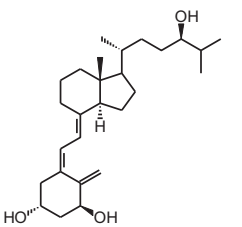
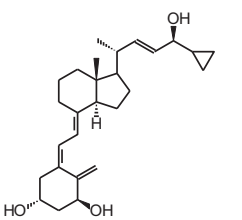
Identification number	Name	Structure
[1]	1α,25(OH)$_2$D$_3$	
[2]	Paricalcitol (19-nor-1α,25(OH)$_2$D$_2$)	
[3]	Doxercalciferol (1α(OH)D$_2$)	
[4]	Falecalcitriol (26,27 F6-1α,25(OH)$_2$D$_3$)	
[5]	Maxacalcitol (22oxa-1α,25(OH)$_2$D$_3$)	
[6]	Tacalcitol (1α,24(R)(OH)$_2$D$_3$)	
[7]	Calcipotriol (22-ene-26,27-dehydro-1α,25(OH)$_2$D$_3$)	

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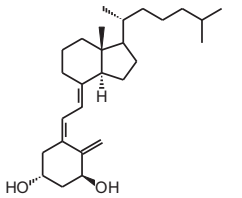
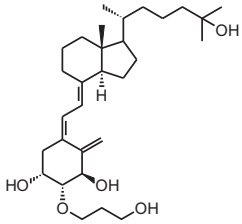
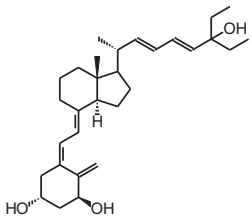
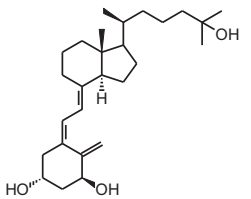
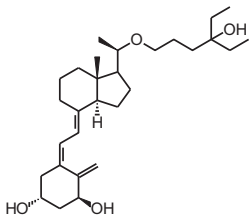
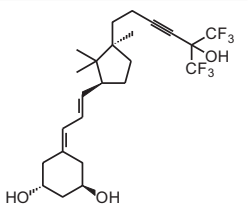
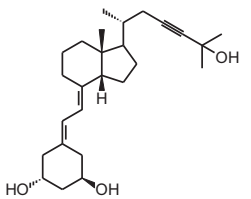
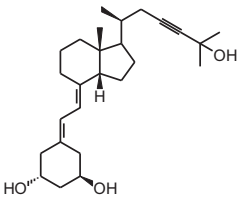
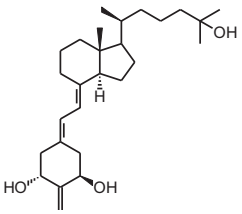
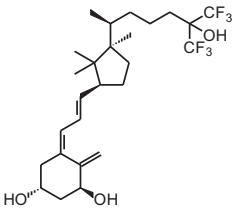
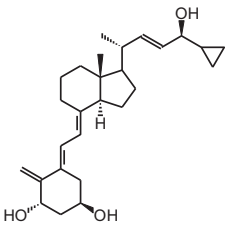
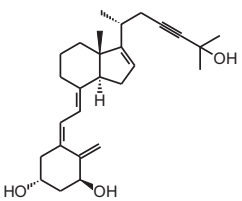
Table 1 | Continued

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.

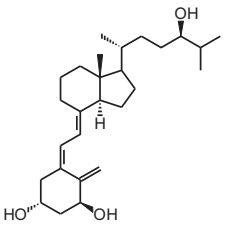
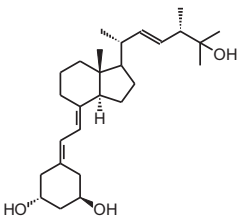
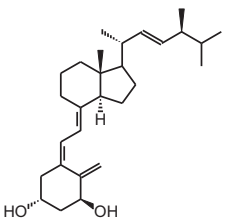
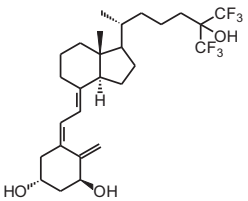
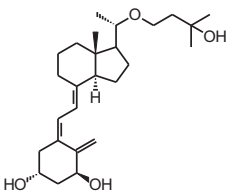
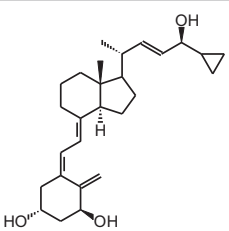
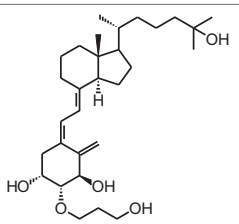
Psoriasis, a hyperproliferative condition of the skin, is also treated with vitamin D analogs. Tacalcitol [**6**], calcipotriol [**7**] and the recently approved maxacalcitol [**5**] are used either as monotherapy or in combination with topical steroids such as betamethasone dipropionate to treat psoriasis. 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).

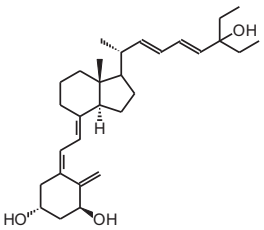
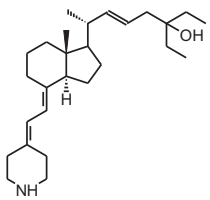
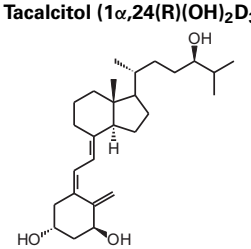
On the genome level, studies using chromatin immunoprecipitation (ChIP) and micro-array techniques have investigated $1,25(OH)_2D_3$-regulated genes in different cell lines. One ChIP study compared the binding sites of the VDR in intestinal tissue after $1,25(OH)_2D_3$ or 20-epi-$1,25(OH)_2D_3$ [**11**] 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

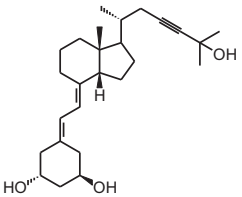
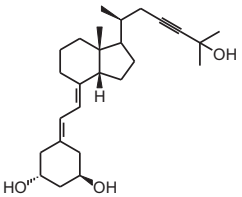
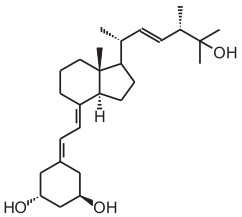
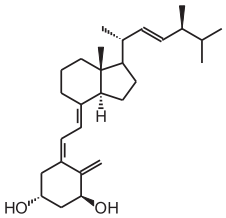
The use of $1,25(OH)_2D_3$ for the treatment of cancer gained interest since many tissues express vitamin D metabolizing enzymes as well as the VDR and because $1,25(OH)_2D_3$ has potent antiproliferative and prodifferentiating effects on normal and malignant cell lines. Several analogs evaluated *in 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

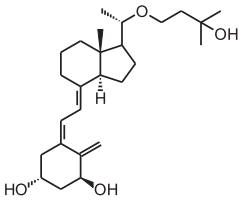
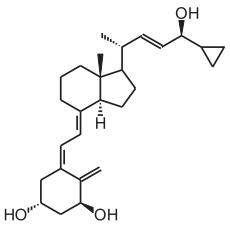
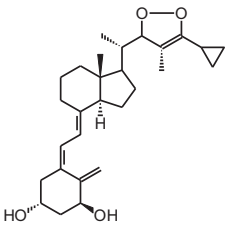
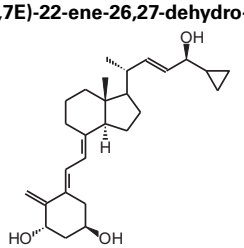
(Continued)

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

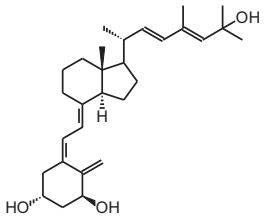
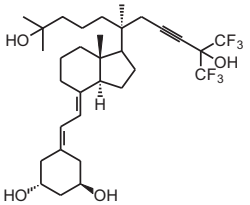
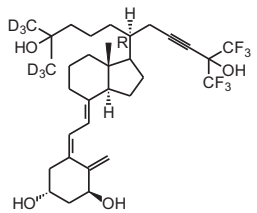
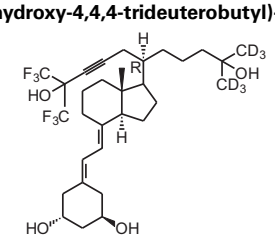
(Continued)

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

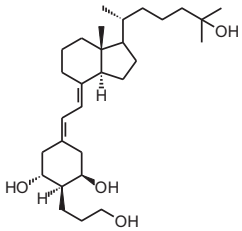
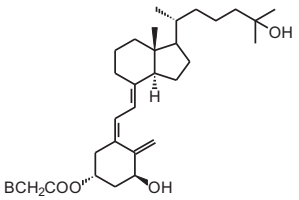
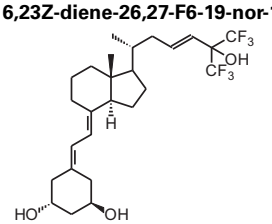
(Continued)

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).

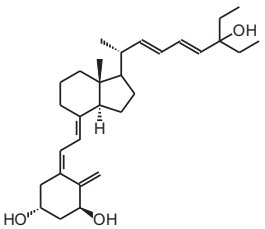
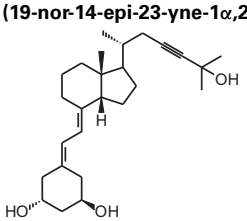
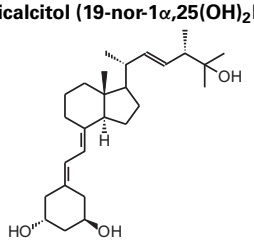
Chiang et al., 2013). However, in some models the analog dose that is effective in inhibiting tumor growth also causes hypercalcemia and lower survival of the treated animals (Albert et al., 2004b). Not only tumor proliferation is modulated by vitamin D analogs, also apoptosis, angiogenesis, migration 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).

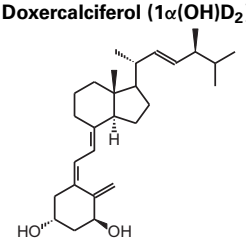
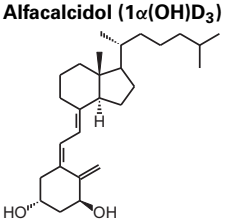
In view of the promising results that certain vitamin D analogs show against cancer *in vitro* and *in vivo* animal models, some analogs have been tested in cancer patients (**Table 4**). Seocalcitol [**10**] is an analog that has been extensively studied *in vitro* and *in vivo* in different cancer 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)

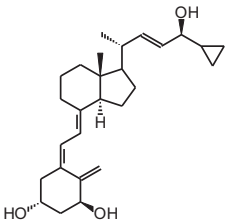
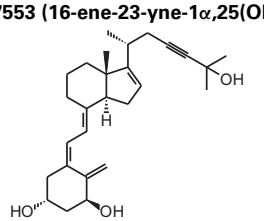
(Continued)

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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