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VITAMIN D: METABOLISM, MOLECULAR MECHANISM OF ACTION, AND PLEIOTROPIC EFFECTS

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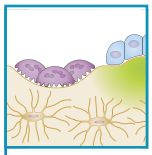


Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev* 96: 365–408, 2016. Published December 16, 2015; doi:10.1152/physrev.00014.2015.—1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is the hormonally active form of vitamin D. The genomic mechanism of 1,25$(OH)_2D_3$ action involves the direct binding of the 1,25$(OH)_2D_3$ activated vitamin D receptor/retinoic X receptor (VDR/RXR) heterodimeric complex to specific DNA sequences. Numerous VDR co-regulatory proteins have been identified, and genome-wide studies have shown that the actions of 1,25$(OH)_2D_3$ involve regulation of gene activity at a range of locations many kilobases from the transcription start site. The structure of the liganded VDR/RXR complex was recently characterized using cryoelectron microscopy, X-ray scattering, and hydrogen deuterium exchange. These recent technological advances will result in a more complete understanding of VDR coactivator interactions, thus facilitating cell and gene specific clinical applications. Although the identification of mechanisms mediating VDR-regulated transcription has been one focus of recent research in the field, other topics of fundamental importance include the identification and functional significance of proteins involved in the metabolism of vitamin D. CYP2R1 has been identified as the most important 25-hydroxylase, and a critical role for CYP24A1 in humans was noted in studies showing that inactivating mutations in CYP24A1 are a probable cause of idiopathic infantile hypercalcemia. In addition, studies using knockout and transgenic mice have provided new insight on the physiological role of vitamin D in classical target tissues as well as evidence of extraskeletal effects of 1,25$(OH)_2D_3$ including inhibition of cancer progression, effects on the cardiovascular system, and immunomodulatory effects in certain autoimmune diseases. Some of the mechanistic findings in mouse models have also been observed in humans. The identification of similar pathways in humans could lead to the development of new therapies to prevent and treat disease.

I. INTRODUCTION

In recent years, vitamin D has received increased attention due to the resurgence of vitamin D deficiency and rickets as a global health issue together with compelling evidence in the laboratory indicating that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the hormonally active form of vitamin D, generates a number of extraskeletal biological responses including inhibition of breast, colon, and prostate cancer cell progression; effects on the cardiovascular system; and protection against a number of autoimmune diseases including multiple sclerosis and inflammatory bowel disease (195). This review summarizes our current understanding of vitamin D and its bioactivation and discusses new developments that have changed our understanding of the mechanism of vitamin D action in classical as well as nonclassical target tissues. This article also evaluates the suggested role of vitamin D in extraskeletal health, provides an overview of 1,25$(OH)_2D_3$ analogs that have been developed, and indicates questions that remain and need to be addressed.

II. VITAMIN D AND ITS BIOACTIVATION

A. Vitamin D and 25$(OH)D_3$

Vitamin D_3 (cholecalciferol), the natural form of vitamin D, is produced in the skin from 7-dehydrocholesterol. Upon irradiation, 7-dehydrocholesterol produces pre-vitamin D_3 which undergoes a temperature-sensitive rearrangement of three double bonds to form vitamin D_3. The synthesis of

vitamin D in the skin is the most important source of vitamin D and depends on the intensity of the ultraviolet irradiation which is dependent on season and latitude. For example, in Boston (42.2°N), no vitamin D is produced from sun-exposed skin from November to February, while in San Juan (18°N) the skin produces vitamin D all year. Melanin and sunscreen markedly diminish the production of vitamin D (194, 483). Vitamin D can also be taken in the diet. However, vitamin D is present in only a few foods (which include fortified dairy products and fish oils). Vitamin D_3 itself is not biologically active. Vitamin D is transported in the blood by vitamin D binding protein (DBP; which binds vitamin D and its metabolites in serum) to the liver. In the liver, vitamin D is hydroxylated at C-25 to produce 25-hydroxyvitamin D_3 [25(OH)D_3]. 25(OH)D_3 is the major circulating form of vitamin D. Its concentration in the serum has served as one of the most reliable biomarkers of vitamin D status (42, 184, 195). The synthesis of 25(OH)D_3 has not been reported to be highly regulated (108). Many cytochrome *P*-450 enzymes (CYPs) including CYP2R1, CYP27A1, and CYP2D25 have been considered as candidates for the enzyme responsible for the conversion of vitamin D to 25(OH)D_3 (523). It has been suggested that CYP2R1, first identified as a microsomal vitamin D 25-hydroxylase by Cheng et al. (80), is the key vitamin D 25-hydroxylase, since patients with a mutation of the CYP2R1 have 25(OH)D_3 deficiency and symptoms of vitamin D-dependent rickets (12, 73, 79, 121, 275). The crystal structure of CYP2R1 in complex with vitamin D_3 has been reported showing that at the active site the 17β-aliphatic side chain of vitamin D is located above the heme plate appropriate for 25-hydroxylation (430). Further strengthening the evidence for the physiological role of CYP2R1 are recent studies using *Cyp2r1* null mutant mice which demonstrate that CYP2R1 is the major enzyme responsible for 25-hydroxylation of vitamin D (524). In the *Cyp2r1* null mice, although 25(OH)D_3 levels are dramatically reduced, synthesis of 25(OH)D_3 is not abolished, suggesting the presence of other vitamin D 25-hydroxylases yet to be identified (524).

25(OH)D_3 is transported by DBP to the kidney and is filtered by the glomerulus. In the kidney megalin, a 600-kDa transmembrane protein, and a member of the low-density lipopoprotein receptor superfamily, acts as a cell surface receptor for DBP resulting in uptake of 25(OH)D in the tubular epithelial cells by endocytic internalization (89). The significance of megalin renal uptake and metabolism of 25(OH)D_3 is demonstrated in studies using *Megalin* knockout mice. Mice lacking *Megalin* lose DBP and 25(OH)D_3 in the urine and have defects of bone metabolism that resemble vitamin D-deficient rickets (336). Cubulin, a second surface receptor for DBP in the proximal tubule, also associates with megalin to internalize complexes of DBP and 25(OH)D_3 (337). In addition, disabled 2 (Dab) 2, a cytoplasmic adaptor protein, also works in conjunction with megalin for the cellular uptake of DBP/25(OH)D_3 by binding to the cytoplasmic tail of megalin enabling the proper routing of the receptor (320, 490).

B. CYP27B1

In the proximal renal tubule, 25(OH)D_3 is hydroxylated at the position of carbon 1 of the A ring, resulting in the formation of 1,25$(OH)_2D_3$, the functional, hormonally active form of vitamin D which is responsible for most, if not all, of the biologic actions of vitamin D. The renal 25(OH)D 1α hydroxylase (mitochondrial CYP27B1), which metabolizes 25(OH)D_3 to 1,25$(OH)_2D_3$, comprises a cytochrome *P*-450, a ferredoxin, and a ferredoxin reductase and is present predominantly in the kidney (proximal straight tubules) and contributes to the circulating concentrations of 1,25$(OH)_2D_3$ (215, 216). Mutations resulting in inactive or deleted CYP27B1 cause vitamin D dependency rickets type 1 (VDDRI) (also known as pseudovitamin D deficiency rickets) despite normal intake of vitamin D, indicating the importance of the CYP27B1 enzyme (234). CYP27B1 has been cloned from rat, mouse, and human (410, 425, 436). The human and rodent CYP27B1 genes comprise nine exons, extending over 5 kbp (234, 318). *Cyp27b1* null mice have provided a mouse model of VDDR type 1. These mice have rickets, undetectable levels of 1,25$(OH)_2D_3$, low serum calcium, and secondary hyperparathyroidism (SHPT) (107, 351). It has been suggested that in healthy animals and humans CYP27B1 is only expressed in kidney and, during pregnancy, in placenta (108). In addition to the kidney, it has been reported that CYP27B1 is present in a number of extrarenal sites. Extrarenal production of CYP27B1 has been convincingly demonstrated in patients with sarcoidosis (4, 31). Macrophages were identified as the source of extrarenal production of 1,25$(OH)_2D_3$ resulting in hypercalcemia and hypercalciuria in these patients. In addition to sarcoidosis, hypercalcemia has also been identified in patients with Crohn's disease (51). It was suggested that activated macrophages of Crohn's granuloma are responsible for the hypercalcemia in Crohn's disease. CYP27B1 produced by macrophages, unlike renal CYP27B1, is not suppressed by elevated 1,25$(OH)_2D_3$ but is upregulated by immune stimuli [interferon-γ and lipopolysaccharide (LPS)]. Regulation by immune stimuli has been reported to involve multiple pathways (including JAK/STAT and NFκB) and to require binding of the C/EBPβ transcription factor to the mouse and human CYP27B1 genes (136, 429). Further evidence of immune derived CYP27B1 is provided by recent studies showing that reconstitution of the hematopoietic cell population from wild-type (WT) mice in *Cyp27b1* knockout mice protects mice from colitis (342). Murine T-cell production of CYP27B1 was demonstrated in the CD8+ but not the CD4+ T cell population (342). Thus it is likely that murine CD8+ T cells as well as other immune cells under activation conditions can produce 1,25$(OH)_2D_3$ to resolve the immune response following antigen specific activation. Cancer cells have also been shown to express CYP27B1 (see sect. V*A*). In addition, CYP27B1 expression has been noted in parathy-

roid gland and in a number of other tissues [see Bikle (42a) for review]. However, whether there is a functional impact of CYP27B1 activity in vivo at sites other than the kidney and placenta under normal physiological conditions remains to be determined.

C. DBP

Although DBP functions as a binding protein for all vitamin D metabolites in the serum [20 times less affinity for $1,25(OH)_2D_3$ than for $25(OH)D_3$], DBP also sequesters actin, can bind fatty acids, and can function as a chemotactic factor with a significant role in neutrophil recruitment (87, 453). In *Dbp* null mice, neutrophil recruitment has been reported to be impaired. It should be noted that although there is a marked decrease in the serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ in *Dbp* null mice (as expected), serum calcium, phosphorus, and PTH are equivalent in *Dbp* null and WT mice (390). However, *Dbp* null mice were more susceptible to develop osteomalacia when given a vitamin D-deficient diet, indicating that DBP may help to maintain stable stores of vitamin D. More recent studies in *Dbp* null mice have shown that these mice are capable of generating tissue levels of $1,25(OH)_2D_3$ comparable to those of WT mice and that induction of vitamin D target genes is similar in WT and *Dbp* null mice (513). Thus the normal serum calcium levels in *Dbp* null mice may be caused by the ability of the vitamin D receptor (VDR) to concentrate $1,25(OH)_2D_3$ in tissues due to its high affinity for $1,25(OH)_2D_3$ resulting in the activation of genes involved in the maintenance of calcium homeostasis. Studies in humans have shown that DBP is highly polymorphic, with three commonly recognized variants (*GC1F, GC1S, GC2*) that are shown to affect protein function. The three common variants with relevance to vitamin D metabolism are determined by two SNPs in *Gc*; rs7041 (aspartic acid switch to glutamic acid at position 432; Gc1f vs. Gc1s) and rs4588 (threonine switch to lysine at position 436; Gc1f vs. Gc2). The resulting variations in DBP amino acid sequence appear to alter the binding affinity of DBP for vitamin D ligands, with Gc1F having the highest affinity for vitamin D metabolites and Gc2 the lowest (23, 54). Genome-wide association studies have shown that the polymorphism rs7041 and rs4588 are associated with circulating $25(OH)D_3$ levels (6, 302, 475); TT carriers for rs7041 (Gc1S) and AA carriers for rs4588 (Gc2) are associated with lower $25(OH)D_3$ levels. The prevalence of these polymorphisms differs between racial groups (97, 132, 219). Black and Asian populations are far more likely to carry the Gc1f form of DBP, while whites more frequently exhibit the Gc1s form of DBP. The Gc2 form is more frequent in people of Asian and European ancestry and rare in the black ethnic groups. An in vitro study showed that addition of DBP of higher affinity genotype (Gc1f/1f) reduced the effect of $25(OH)D_3$ on gene expression in monocytes, compared with lower affinity DBP polymorphic forms (Gc1s or Gc2), indicating that these DBP polymorphisms may influence $25(OH)D_3$ bioavailability (89). More research is needed to fully appreciate the meaning of bioavailable versus total $25(OH)D_3$ as well as $1,25(OH)_2D_3$. It should be noted, however, when determining different vitamin D requirements based on circulating concentrations of DBP, that polyclonal DBP antibodies and not monoclonal antibodies (which discriminate between Gc1f and Gcls and thus could result in an underestimation of DBP concentration) should be used (52, 196).

D. CYP24A1

In the kidney, besides conversion to $1,25(OH)_2D_3$ by CYP27B1, $25(OH)D_3$ can also be converted to $24,25(OH)_2D_3$ by hydroxylation at C-24 by CYP24A1, a mitochondrial inner membrane cytochrome *P*-450 enzyme (214). This enzyme can hydroxylate not only $25(OH)D_3$ but also $1,25(OH)_2D_3$ **(FIGURE 1)**. $1,25(OH)_2D_3$ has been suggested to be the preferred substrate for CYP24A1 (409). CYP24A1 limits the amount of $1,25(OH)_2D_3$ when circulating $1,25(OH)_2D_3$ is elevated by catalyzing the conversion of $1,25(OH)_2D_3$ into 24-hydroxylated products targeted for excretion or by producing $24,25(OH)_2D_3$ thus decreasing the pool of $25(OH)D_3$ available for 1-hydroxylation. CYP24A1 can also catalyze the C23 oxidation pathway resulting in the formation of $1,25(OH)_2D_3$-26, 23 lactone from the substrate $1,25(OH)_2D_3$ (FIGURE 1) and the formation of $25(OH)D_3$-26,23 lactone from $25(OH)D_3$ (214). CYP24A1 is present in all cells containing the VDR. Thus, in addition to regulating circulating concentrations of $1,25(OH)_2D_3$, CYP24A1 may also modulate the levels of $1,25(OH)_2D_3$ within the cell, resulting in an appropriate cellular response. The rat (r) *Cyp24a1* gene spans ~15 kb, is comprised of 12 exons, and is present as a single copy (340). In 2010, the crystal structure of CYP24A1 was reported (21). The crystal structure of CYP24A1 reveals that CYP24A1 has 12 α helices (A-L), and four β-sheet systems (β1-β4), as well as additional helices (A′, B′, G′ on the distal surface and K′ and K″ between β2 and the conserved heme binding motif). The CYP24A1 structure clarifies for the first time the membrane insertion elements and provides new insight on the organization of the CYP24A1 active site. These findings will be important for the design of vitamin D analogs and specific CYP24A1 inhibitors. Studies in *Cyp24a1* null mice provided the first direct in vivo evidence for the role of CYP24A1 in $1,25(OH)_2D_3$ catabolism. About 50% of homozygous mutant mice died before 3 wk of age. *Cyp24a1* null mice that survive post weaning are unable to clear exogenous $1,25(OH)_2D_3$. These animals exhibit an intramembranous bone lesion that is resolved when a double *Cyp24a1/Vdr* null mouse is generated, indicating that elevated $1,25(OH)_2D_3$, acting through VDR, is responsible for the bone defect (424). Although a func-

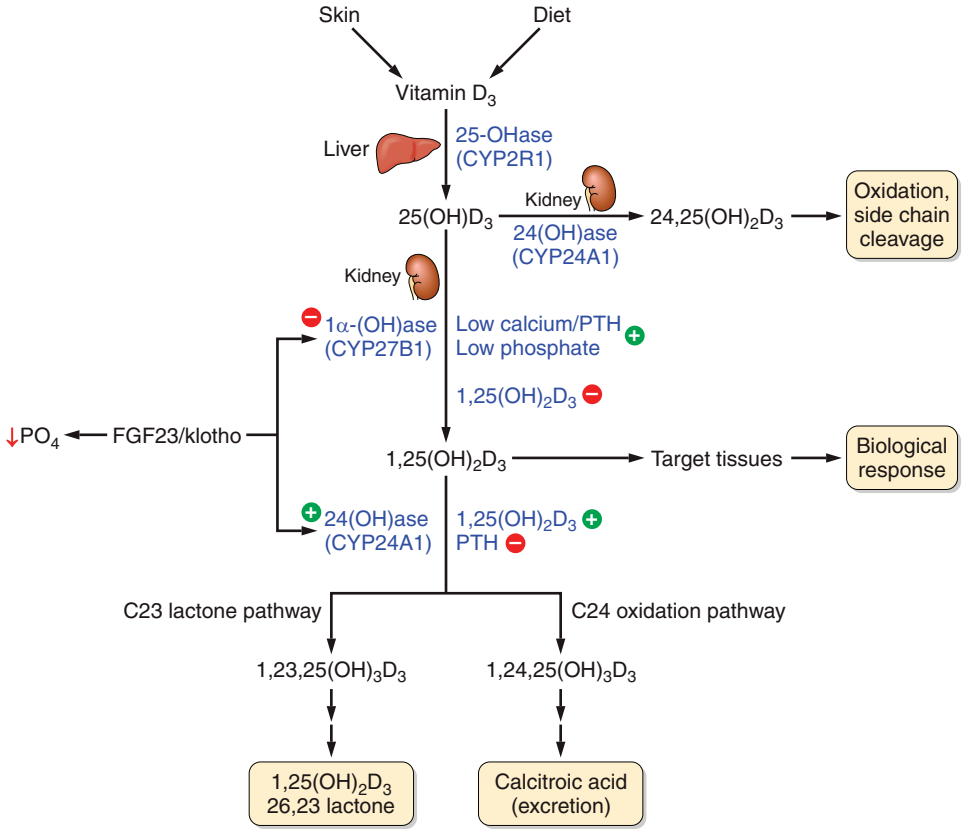


FIGURE 1. The metabolic pathway for vitamin D. CYP2R1 has been identified as a key vitamin D 25-hydroxylase. PTH, FGF23/klotho, and $1,25(OH)_2D_3$ play key roles in the regulation of optimal levels of $1,25(OH)_2D_3$. Only products of $1,25(OH)_2D_3$ are represented for the C23 lactone pathway. See text for 25(OH)D products of the C23 lactone pathway.

tional role for 24 hydroxylated metabolites in bone fracture healing has been suggested (423), a role for 24 hydroxylation other than for elimination of the vitamin D hormone has been a matter of debate (57, 188, 209, 352). Evidence for a critical role of CYP24A1 in humans was noted in recent studies which demonstrated that inactivating mutations in CYP24A1 were a probable cause of idiopathic infantile hypercalcemia (396), thus reinforcing the findings in the *Cyp24a1* null mouse studies and indicating the need for careful administration of vitamin D in infants. Sequence analysis of CYP24A1 in these patients yielded five different mutations [E143del (in frame deletion of E143), E322K, R396W, L409S, and R159Q]. All mutations affect residues of structural importance. In studies in which human CYP24A1 constructs containing the mutants were transfected in cells and compared for catabolism of $1,25(OH)_2D_3$, ablation of CYP24A1 catabolism was noted for all mutations except one mutation, L409S (distal to the active site), which retained a small level of activity. In subsequent reports, CYP24A1 mutations were identified not only in children but also in adult patients (118, 442). These patients were characterized by hypercalcemia, hypercalciuria, and recurrent nephrolithiasis. The findings in adults suggest that CYP24A1 mutations should be considered in diagnosis of long-standing hypercalcemia and hypercalciuria associated with kidney stones, particularly in patients taking vitamin D supplements.

E. Regulation of Renal CYP27B1 and CYP24A1

CYP27B1 and CYP24A1 are under stringent control. A primary signal mediating the induction of $1,25(OH)_2D_3$ synthesis in the kidney is elevated PTH resulting from hypocalcemia (44, 187, 331, 368). The nuclear orphan receptor 4A2 (NR4A2) [also known as NURR1 (nuclear receptor related 1 protein)] which is induced in the kidney in response to PTH has been shown to be one factor mediating PTH induction of *CYP27B1* transcription (525). $1,25(OH)_2D_3$ in turn suppresses PTH production in the parathyroid gland directly at the level of transcription of the *PTH* gene (111, 239, 279, 387) and indirectly by increasing serum calcium levels and by upregulating the expression and transcription of the calcium sensing receptor (67). $1,25(OH)_2D_3$ regulates its own production by inhibiting CYP27B1 (59) **(FIGURE 1)**. Although negative vitamin D response elements have been identified in the *PTH* gene, further studies are needed to determine genome-wide mechanisms involved in $1,25(OH)_2D_3$-mediated suppression of both *PTH* and *CYP27B1*. When compared with the regulation of CYP27B1, CYP24A1 is reciprocally regulated [stimulated by $1,25(OH)_2D_3$ and inhibited by low calcium and PTH] (44, 187, 368). In addition to calcium, PTH, and $1,25(OH)_2D_3$, the phosphaturic factor fibroblast growth factor 23 (FGF23), which promotes renal phosphate excretion by decreasing

reabsorption in the proximal tubule, is also an important physiological regulator of vitamin D metabolism. FGF23, which belongs to the FGF19 subfamily, is an ~32 kDa protein that is expressed predominantly in osteocytes and osteoblasts and, unlike other FGFs that act in an autocrine/paracrine fashion, acts as an endocrine factor (199, 372). $1,25(OH)_2D_3$ and elevations in serum phosphate independently stimulate the production of FGF23 (269). αKlotho, a 130 kDa transmembrane protein that is highly expressed in the distal tubule of the kidney, acts as an obligate coreceptor for FGF23. αKlotho forms complexes with FGFR1c, FGFR3c, and FGFR4. Klotho is required for FGF23 to activate FGFRs. Together FGF23 and αklotho suppress the expression of CYP27B1 and induce CYP24A1, thereby inhibiting the synthesis and promoting the catabolism of $1,25(OH)_2D_3$ (199) **(FIGURE 1)**. *Fgf23* or *αklotho* deficiency exhibit similar phenotypes including hyperphosphatemia, increased synthesis of $1,25(OH)_2D_3$, ectopic calcification, and premature aging (including atherosclerosis, skin atrophy, and osteoporosis), indicating the cooperation of αklotho and FGF23 in a common signaling pathway (245, 407). Elevated FGF23 is a causative factor of tumor-induced osteomalacia and several hereditary hypophosphatemic disorders including X-linked hypophosphatemic rickets (XLH) and autosomal dominant hypophosphatemic rickets (ADHR) (1, 408, 484, 487). FGF23 is increased in chronic kidney disease (CKD). It has been suggested that increased FGF23, not $1,25(OH)_2D_3$ insufficiency due to loss of functional renal mass, may be the initial event and thus may be an early biomarker for CKD (372). FGF23 would lead to the suppression of $1,25(OH)_2D_3$ and thus to increased PTH observed in CKD.

Calcitonin has also been reported to regulate CYP27B1 in mammalian kidney. In addition to its known role to reduce blood calcium by shrinking osteoclasts under high calcium conditions, calcitonin has been reported to stimulate renal CYP27B1 under normocalcemic conditions (156, 225, 411). Since calcitonin levels as well as $1,25(OH)_2D_3$ levels are elevated during lactation, early studies suggested that calcitonin may have a role to stimulate CYP27B1 resulting in increased plasma $1,25(OH)_2D_3$ and increased intestinal calcium absorption during lactation when the need for calcium is increased (427). Studies have shown an effect of calcitonin on *CYP27B1* transcription (521). In addition to calcitonin, prolactin, which is also elevated during lactation, has been reported to stimulate renal CYP27B1(382). A direct effect of prolactin in cooperation with signal transducer and activator of transcription 5 (STAT5) on renal *CYP27B1* transcription has been observed (8). These findings suggest that prolactin and calcitonin can act as modulators of vitamin D-regulated calcium homeostasis during lactation when there is an increased calcium requirement for the neonate.

F. Aging and Renal Vitamin D Hydroxylases

In aging, as indicated in studies in both animals and humans, there is a decline in the ability of the kidney to synthesize $1,25(OH)_2D_3$ (22, 454). We and others have noted that rat renal CYP24A1 increases with age (22, 295). Thus these findings suggest that the combined effect of a decline in the capacity of the kidney to convert $25(OH)D_3$ to $1,25(OH)_2D_3$ and an increase in the catabolism of $1,25(OH)_2D_3$ by CYP24A1 (and therefore a decline in intestinal calcium absorption) contribute to age-related bone loss.

G. Regulation of Placental CYP27B1

Besides kidney, placenta is also a major site for conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. In the placenta CYP27B1 is expressed in both fetal trophoblasts and maternal decidua (508). Although the placenta was identified as a major site of extrarenal production of CYP27B1 over 30 years ago, the function of $1,25(OH)_2D_3$ in the placenta was unknown. Placental expression of *CYP27B1* mRNA begins early in gestation and has been reported to be highest in the first trimester (508). Recent studies have suggested that synthesis of $1,25(OH)_2D_3$ in the placenta may play an important role in controlling placental responses to infection. Human decidual cells treated with $1,25(OH)_2D_3$ or $25(OH)D_3$ show decreased synthesis of cytokines including tumor necrosis factor, granulocyte-macrophage colony stimulating factor, and interleukin-6 (137). Expression of cathelicidin, an antimicrobial peptide, is also enhanced in response to $1,25(OH)_2D_3$ in trophoblasts and decidual cells, further indicating the importance of $1,25(OH)_2D_3$ as a regulator of immune responses in the placenta (266). When the TLR4 ligand LPS was given in vivo, *Cyp27b1* mRNA was induced in mouse placenta, indicating that placental synthesis of $1,25(OH)_2D_3$ is also sensitive in vivo to immune challenge (267). It has been reported that *CYP24A1* suppression in placenta due to excessive methylation may contribute to increased bioavailability of $1,25(OH)_2D_3$ in human placenta (334). Together these findings suggest the importance of placental CYP27B1 during early feto-placental life as an autocrine/paracrine regulator of both acquired and innate immune responses.

III. THE VITAMIN D RECEPTOR AND GENOMIC MECHANISM OF $1,25(OH)_2D_3$ ACTION

A. Vitamin D Receptor

1. The VDR gene and structural characterization of VDR

The biological actions of $1,25(OH)_2D_3$ are mediated by the VDR. VDR belongs to the steroid receptor family

which includes receptors for retinoic acid, thyroid hormone, sex hormones, and adrenal steroids (287). The VDR gene is evolutionarily conserved among fish, birds, and mammals (183). The human and mouse VDR genes are localized on chromosomes 12 and 15, respectively (510). Both the human and mouse genes are comprised of eight coding exons (510). Two noncoding exons are found in the mouse gene, and at least six noncoding exons are in the human gene. In the human gene there are also at least two promoters. Tissue specific promoter usage has been suggested (100, 511). VDR protein [containing 423 amino acid (mouse VDR) or 427 amino acids (human VDR)] functions as an obligate heterodimer with RXR for activation of vitamin D target genes (183, 364, 510). The two core functional domains of the VDR are the highly conserved NH_2-terminal DNA binding domain (DBD) and the more variable COOH-terminal ligand binding domain (LBD). The DBD is a cysteine-rich zinc finger region. There are two zinc fingers, each of which contains a single zinc atom in a tetrahedral arrangement with four invariant cysteine residues (108, 281, 364). The LBD is comprised of at least 12 α helices [H1-H12; the ligand-dependent activation function (AF2) corresponds to H12] and 3 β sheets (S1-3) (384). $1,25(OH)_2D_3$ binding induces a conformational change that facilitates interaction with RXR and coregulatory complexes required for the transcription of target genes. Although other coactivator interfaces in the LBD of VDR have been identified, repositioning of H12 after $1,25(OH)_2D_3$ binding has been reported to be critical for recruitment of coactivator proteins. The DBD and the LBD are connected through a hinge region. Although the crystal structures of the isolated VDR LBD and the VDR DBD have been reported (384, 403, 404), X-ray crystallographic data of the VDR/RXR complex is currently not available. Recently, the structure of the liganded VDR/RXR DNA complex was characterized using cryoelectron microscopy (345) **(FIGURE 2)**. Findings from this study suggest cooperative and allosteric effects between the LBD and the DBD in VDR-mediated regulation of gene expression. In addition, the structure reveals that the hinge region may stabilize the whole complex, thus facilitating the positioning of the LBD to make the area of H12 accessible for recruitment of coregulators (345). Recent studies using small angle X-ray scattering and hydrogen-deuterium exchange technology also enabled characterization of the VDR/RXR DNA complex and similarly indicated cooperative effects between the VDR DBD and VDR LBD, suggesting mechanisms by which ligands and DNA can act together to fine-tune regulation of gene expression (383, 514). These recent technological advances will allow the visualization of the VDR complexes that have been difficult to crystallize and will result in a more complete understanding of the structural basis for VDR and VDR coactivator action.

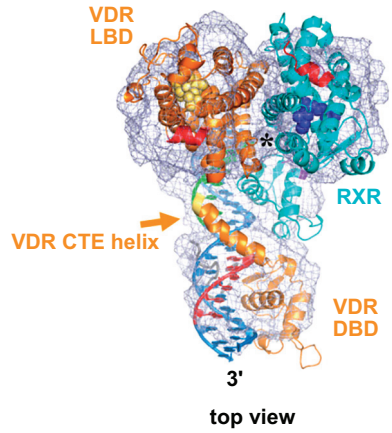


FIGURE 2. Structure of the full human RXR/VDR nuclear receptor heterodimeric complex with its target DNA. The structure of the RXR/VDR complex was determined by single particle cryo-EM and 3D reconstruction. Representation of the cryo-EM map with the fitted crystal structure of the individual RXR and VDR LBDs and DBDs resulting in a molecular model of the full RXR/VDR/DNA complex (top view of the complex). It has been suggested that the carboxy-terminal extension (CTE) of the DBD of VDR extending into the hinge region has a critical role for VDR transcriptional activity. The LBD interface contact comprising helix 4, loop 8/9 of VDR, and helix H7 of RXR is marked with a star. [Adapted from Orlov et al. (335), with permission from John Wiley and Sons.]

2. Hereditary vitamin D-resistant rickets

Hereditary vitamin D-resistant rickets (HVDRR) is a rare autosomal recessive disorder characterized by hypocalcemia, hyperparathyroidism, early-onset rickets, and organ resistance to $1,25(OH)_2D_3$. The resistance to $1,25(OH)_2D_3$ is caused by heterogeneous loss of function mutations in the *VDR*. Affected children may also exhibit alopecia (143). In addition to the findings related to the structure of VDR and the heterodimeric complex, biochemical and genetic analysis of VDR in HVDRR patients has also resulted in important insights related to the functional domains of VDR and the mechanisms involved in VDR signaling. Point mutations in the zinc finger DBD of VDR which caused HVDRR were the first disease-causing mutations identified in the steroid receptor gene superfamily (202). Since that report over 100 cases of HVDRR have been reported, and 45 unique mutations have been identified in VDR as the cause of HVDRR (143). Mutations have also been identified in the LBD that disrupt ligand binding [for example, R274H, a contact point for interaction with the 1α hydroxyl group (15)], that disrupt VDR/RXR interaction [for example, R391S, located in helix 10 (328)] or prevent coactivator recruitment [for example, E420 K located in helix 12 (282)]. The patient with the E420K mutation had rickets but did not have alopecia, indicating that coactivator recruitment by VDR is required to prevent rickets but is not required for hair growth. Treatment of HVDRR patients with intravenous or oral calcium administration has been reported to reverse the mineral and skeletal phenotype of HVDRR (191), suggesting the critical role of VDR/

$1,25(OH)_2D_3$ action on intestinal calcium absorption. Recently, a humanized mouse model of HVDDR without alopecia was developed (254). Transgenic mice expressing the $1,25(OH)_2D_3$-binding-defective hVDR-L233S mutant have no alopecia but still all the characteristics of rickets observed in *Vdr* null mice. Another study describes VDR_{gem} mice, which express a VDR mutant that does not bind $1,25(OH)_2D_3$, also not at supraphysiological doses, but can be selectively activated through the binding of the gemini vitamin D analog (200). These VDR_{gem} mice show more impaired calcium and bone homeostasis compared with *Vdr* null mice. Both interesting models will give in the future new insights in signaling pathways regulated by unliganded or liganded VDR.

B. Genomic Mechanism of $1,25(OH)_2D_3$ Action

1. Diversity of coregulators

The genomic mechanism of $1,25(OH)_2D_3$ action involves the direct binding of $1,25(OH)_2D_3$ activated VDR/RXR to specific DNA sequences [vitamin D response elements (VDREs)] in and around target genes resulting in either activation or repression of transcription. Although significant variability in the sequence of the VDREs has been reported, for activation of transcription, VDREs with high affinity for VDR consist of two direct imperfect repeats of hexanucleotides with a spacer of three nucleotides. The heterodimerization of $1,25(OH)_2D_3$-VDR with RXR leads to high-affinity binding to VDREs. Following the binding of VDR-RXR heterodimer to the VDRE, changes in gene expression are mediated through the ability of the liganded receptor to recruit transcriptional coactivators. The p160 coactivators, steroid receptor activator 1, 2, and 3 (SRC-1, SRC-2, and SRC-3), that have histone acetylase (HAT) activity, are primary coactivators that bind to the AF2 domain of liganded VDR. The SRCs contain LxxL (x = any amino acid) motifs that facilitate binding to VDR and other nuclear receptors. Members of the p160 family recruit proteins as secondary coactivators, such as CBP/p300 (which also have HAT activity), resulting in a multisubunit complex that modifies chromatin and destabilizes histone/DNA interaction (83, 183, 362). In addition to acetylation, methylation also occurs on core histones. Recent studies have shown that methyltransferases may also play a fundamental role in VDR-mediated transcription (84, 402). Liganded VDR also interacts directly or indirectly with basal transcription factors [TFIIB and several TAT binding protein associated factors (TAFs)], resulting in the establishment of a stable preinitiation complex (83). VDR-mediated transcription is facilitated by Mediator, a multi-protein complex (the 205 subunit binds to VDR) which functions through recruitment of RNA polymerase II and promotes formation of the preinitiation complex (124, 505).

A number of other transcription factors have been reported to affect the transcriptional activity of VDR. Ras activated Ets transcription factor has been reported to have a critical role in induction of *Cyp24a1* (126). VDR-induced *Cyp24a1* and rat osteocalcin (*Bglap)* transcription are repressed by YY1, a multifunctional transcription factor (178, 375). There is increasing evidence that specific CAAT enhance binding protein (C/EBP) family members may be key mediators of $1,25(OH)_2D_3$ action. C/EBPβ is induced by $1,25(OH)_2D_3$ in kidney and osteoblastic cells and cooperates with $1,25(OH)_2D_3$ and VDR in enhancing *Cyp24a1* and *Bglap* transcription (113, 180). In the regulation of *Bglap* transcription, cooperation between C/EBPβ and VDR and between Runx2 and C/EBPβ has been reported (180). C/EBPs and Runx2 have also been reported to regulate *VDR* transcription (511). C/EBPα and VDR cooperate in the transcriptional regulation of the human antimicrobial peptide cathelicidin in lung epithelial cells and Runx2 and VDR cooperate in the transcriptional regulation of mouse osteopontin in osteoblastic cells (114, 405). C/EBPβ, Runx2, and VDR all contribute to the control of *Mmp*13 (matrix metalloproteinase 13) gene transcription (308).

The SWI/SNF complexes, that remodel chromatin using the energy of ATP hydrolysis, also contribute to transcriptional activation by VDR. C/EBPβ recruits the SWI/SNF complex to promote $1,25(OH)_2D_3$ induction of *Cyp24a1* and *Bglap* transcription (402, 468). An interplay between the SWI/SNF complex, C/EBPβ, and protein arginine methyltransferase 5 in epigenetic modification of VDR-mediated *Cyp24a1* transcription has also recently been shown, suggesting that these are key factors involved in the regulation of $1,25(OH)_2D_3$ catabolism and therefore in the maintenance of calcium homeostasis (402). It has been suggested that cell and gene specific functions of steroid receptors may be mediated through differential recruitment of coregulatory proteins (coactivators and their associated proteins and corepressors and their associated proteins) (426). Since VDR coregulatory proteins are master regulators of $1,25(OH)_2D_3$ action, further studies identifying VDR coactivators and corepressors as well as epigenetic regulation of VDR function will yield significant new insight into the complex mechanisms by which $1,25(OH)_2D_3$ acts to direct its multiple biological activities.

2. Genome-wide studies

The complexity of the molecular mechanisms involved in $1,25(OH)_2D_3$ action is not only indicated by the diversity of coregulators and their activities but also through genome-wide studies which have shown that the actions of $1,25(OH)_2D_3$, similar to other steroids, involve regulation of gene activity at a range of locations many kilobases upstream as well as downstream of the transcription start site (TSS) and within introns and intergenic

regions. VDR binding to these sites is largely but not exclusively dependent on activation by $1,25(OH)_2D_3$. Global networks regulated by VDR are beginning to be addressed in osteoblastic, intestinal carcinoma, immune, and hepatic stellate cells (72, 181, 361, 374). This review will focus on $1,25(OH)_2D_3$-regulated genes involved in the regulation of calcium homeostasis. Recent ChIP-chip and ChIP-seq approaches have provided new insight into the mechanisms of regulation of $1,25(OH)_2D_3$ targets in bone cells including VDR, RANKL which induces osteoclast differentiation from hematopoetic precursors, LRP5 (low-density lipoprotein receptor related protein 5) which facilitates β-catenin activation and is known to play a role in bone formation, and CYP24A1. With regard to regulation of VDR, $1,25(OH)_2D_3$ has been shown to autoregulate VDR in bone cells not in intestine (253, 493). ChIP-seq analysis of MC3T3 osteoblastic cells revealed the presence of $1,25(OH)_2D_3$-induced VDR and RXR at two intronic sites (+19 and +29) downstream and one intergenic site (−6 kb) upstream of the TSS of the mouse *Vdr* gene. These regulatory sites are conserved in the human and mouse VDR genes (510, 511). Genome-wide analysis of $1,25(OH)_2D_3$ regulation of RANKL resulted in the identification of five distal VDREs upstream of the mouse *Rankl* (*Tnfsfl 1*) gene promoter (at −16, −22, −60, −69, and −75 kb). The most distal of the five enhancers was found to be the dominant mediator of $1,25(OH)_2D_3$ activity in the mouse *Rankl* gene (233). Five enhancers were also identified in the human *RANKL* gene (between −20 and −96). However, unlike the mouse *Rankl* gene, the most proximal element at −20 kb was the dominant mediator of $1,25(OH)_2D_3$ activity (326). The regulatory region responsive to $1,25(OH)_2D_3$ in the mouse *Lrp5* gene is at +19 kb (153). Although the induction by $1,25(OH)_2D_3$ of RANKL as well as LRP5 suggests that $1,25(OH)_2D_3$ action in bone cells can be anabolic as well as catabolic, it should be noted that $1,25(OH)_2D_3$ was unable to induce VDR binding in the human *LRP5* gene (153). With regard to CYP24A1, ChIP-chip and ChIP-seq studies confirmed the regulatory region previously defined, which is located proximal to the transcription start site of the mouse *Cyp24a1* gene (at −160 and at −265), and in addition a novel intergenic region was identified at +35 and +37 kb (309). Occupancy of C/EBPβ was found to be enhanced in response to $1,25(OH)_2D_3$ at −345 nt (confirming previous data obtained using chromatin immunoprecipitation) (113, 363). In intestine, transient potential vanilloid type 6 (TRPV6) is an epithelial calcium channel regulated at the transcriptional level by $1,25(OH)_2D_3$. The human *TRPV6* gene was found to contain multiple VDR/RXR binding sites (at −1.2, −2.1, −3.5, −4.3, and −5.5 kb) (310). The elements at −2.1 and −4.3 kb were found to be $1,25(OH)_2D_3$ responsive. Collectively, these genome-wide studies have provided a new perspective on mechanisms involved in the regulation of gene expression by $1,25(OH)_2D_3$ and suggest a chromatin looping mechanism whereby the regulatory regions can be brought into close proximity with the gene's promoter via protein-protein interaction.

IV. CLASSICAL ROLE OF VITAMIN D

A. Intestine

1. Overall process of vitamin D-mediated intestinal calcium absorption

The principal action of $1,25(OH)_2D_3$ and the VDR is intestinal calcium absorption. This conclusion is based on the observation that mineral and skeletal phenotypes of HVDRR patients are reversed when these patients are treated with intravenous or high oral calcium (191). In addition, when *Vdr* null mice (which represent an animal model of HVDRR) are fed a rescue diet high in calcium and lactose, rickets and osteomalacia are prevented (18, 258, 292), further indicating that impaired bone mineralization as a consequence of defective VDR signaling results from impaired intestinal calcium absorption. Although the studies in HVDRR patients and in *Vdr* null mice establish the importance of the intestine in $1,25(OH)_2D_3$-mediated regulation of calcium and bone homeostasis, the mechanisms involved in vitamin D regulation of intestinal calcium absorption have remained incomplete. The facilitated diffusion model is the most studied mechanism of vitamin D-regulated calcium absorption. In this model, transcellular calcium transport is a saturable process comprised of three $1,25(OH)_2D_3$ regulated steps: *1*) entry of calcium through the apical membrane calcium channel TRPV6, *2*) binding to the calcium binding protein calbindin-D_{9k}, and *3*) extrusion of calcium across the basolateral membrane by PMCA1b. TRPV6 and calbindin-D_{9k} have been evaluated as the major intestinal targets of $1,25(OH)_2D_3$. They are colocalized in the intestine (similar to VDR they are expressed in all segments of the small and large intestine) and their expression is strongly correlated to transcellular calcium absorption efficiency (85, 380, 420, 459). However, studies in *Trpv6* and calbindin-D_{9k} (*S100g*) KO mice show that $1,25(OH)_2D_3$-mediated calcium transport is similar to WT in the absence of TRPV6 or calbindin, suggesting compensation by other calcium channels and other calcium binding proteins yet to be identified (9, 37). Although bone mass is comparable in the WT and *Trpv6* KO mice under conditions of normal dietary calcium, when dietary calcium is low, excessive bone turnover and impaired mineralization have been observed in the *Trpv6* KO mice, suggesting a role for TRPV6 under low dietary calcium conditions (262) (see model **FIGURE 3**). Transgenic mice overexpressing *Trpv6* in the intestine develop hypercalciuria, hypercalcemia, and soft tissue calcification (102). Thus, although there may be

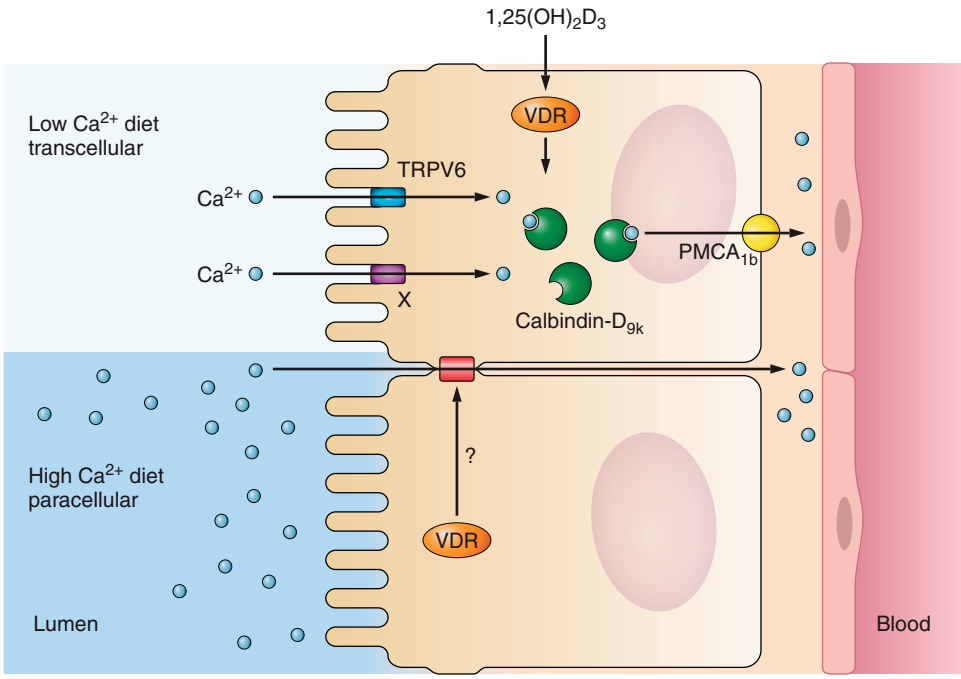


FIGURE 3. Effects of $1,25(OH)_2D_3$ in the intestine. An important function of $1,25(OH)_2D_3$ is the stimulation of transcellular intestinal calcium transport by increasing the expression of the apical membrane calcium channel TRPV6 and calcium binding protein calbindin-D_{9k}. The extrusion of calcium is across the basolateral membrane by PMCA1b. This process is especially enhanced when dietary calcium intake is low. Mouse genetic studies however suggest that other calcium transporters (X) are likely involved. When calcium intake is high, the paracellular calcium transport prevails, but studies suggest that this pathway may also be regulated by $1,25(OH)_2D_3$.

compensation in the KO mouse, these findings indicate a direct role for TRPV6 in intestinal calcium absorption. Unlike the single KO mice in which active intestinal calcium absorption in response to $1,25(OH)_2D_3$ is similar to WT, the ability of the intestine to absorb calcium in response to $1,25(OH)_2D_3$ is reduced by 60% in the *Calbindin-D_{9k}/Trpv6* double KO mice, suggesting that TRPV6 and calbindin act together to affect calcium absorption (37). It is possible that calbindin may act to modulate TRPV6-mediated calcium influx. Calbindin may also act to buffer calcium preventing toxic levels from accumulating in intestinal cells. In the cytosol, calcium may be bound to other calcium binding proteins besides calbindin. In addition, intracellular organelles could also sequester calcium in the intestinal cell. Although the mechanism involved has been a matter of debate, it has been suggested that $1,25(OH)_2D_3$ can also stimulate active phosphate absorption in the intestine (489).

2. Vitamin D and the distal intestine

Most of what is known about the mechanisms involved in $1,25(OH)_2D_3$ regulation of intestinal calcium absorption comes from studies that utilize the duodenum. The capacity to absorb calcium is most rapid in the duodenum. However, only 8-10% of calcium absorption takes place in the duodenum (481). Although little is known about $1,25(OH)_2D_3$ action in other parts of the intestine, the importance of regions other than the proximal small intestine has been suggested. Vitamin D and $1,25(OH)_2D_3$-regulated calcium transport has been reported in ileum, cecum, and colon as well as duodenum (140, 141, 250). The highest expression levels of TRPV6 are in the distal intestine (517). Studies from rats and humans show that total calcium absorption is significantly higher when the colon is preserved after extensive small bowel resection (203, 491). In addition, we recently showed that transgenic expression of *VDR* specifically in ileum, cecum, and colon of *Vdr* null mice is sufficient to prevent the abnormal calcium homeostasis phenotype of *Vdr* KO mice (86) **(FIGURE 4)**. Together these findings indicate that the distal segments of the intestine, in addition to the duodenum, play an important role in intestinal calcium absorption and proper bone mineralization.

3. Paracellular calcium transport

In addition to transcellular calcium transport, calcium is absorbed by the paracellular path that occurs between epithelial cells. The vitamin D dependency of this nonsaturable component of calcium absorption has been a subject of debate and is much less defined than vitamin D-mediated transcellular calcium transport. Early studies using cultured chick intestine as well as in vivo studies in rats provided evidence that $1,25(OH)_2D_3$ enhanced paracellular permeability (101, 223). More recent studies have shown the paracellular associated proteins including claudin-2 and claudin-12 (transmembrane components of tight junctions), cadherin-17 (a cell adhesion protein), and aquaporin 8 (a tight junction channel) can be regulated by $1,25(OH)_2D_3$ in the intestine, suggesting that vitamin D can regulate calcium absorption by the paracellular as well as the transcellular pathway (155, 246) **(FIGURE 3)**. Further studies are needed, however, to determine the role of these intercellular adhesion molecules in intestinal physiology and the significance of the regulation by $1,25(OH)_2D_3$ in intestinal calcium absorption.

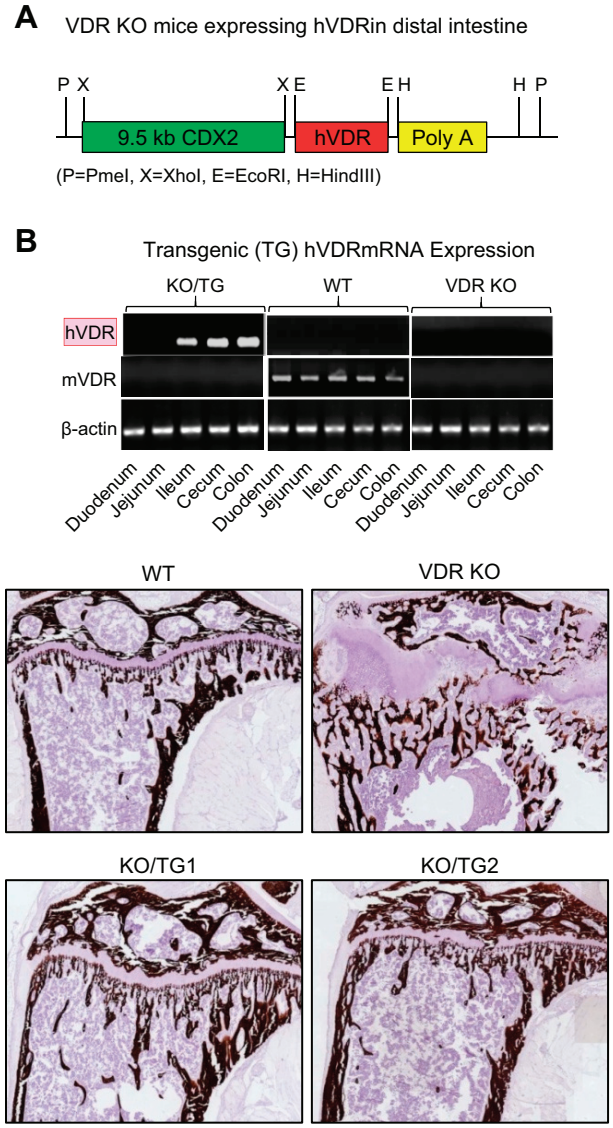


FIGURE 4. Transgenic (TG) expression of VDR specifically in ileum, cecum, and colon of VDR null mice prevents the abnormal calcium phenotype of VDR null mice. *A*: the full-length hVDR cDNA was introduced into the multiple cloning cassette under control of the 9.5-kb CDX2 promoter region (9.5 kb CDX2 from E. Fearon). Mice expressing VDR exclusively in the ileum, cecum, and colon were generated by breeding VDR null mice with TG mice expressing hVDR under the control of 9.5-kb CDX2. *B*, *top panel*: expression of hVDR was restricted to ileum, cecum, and colon. Mouse (m) VDR was present in WT but not in TG mice or VDR null (KO) mice. Levels of VDR in the distal intestine in TG mice were equivalent or 1.5 upregulated compared with WT. *Bottom panel*: Van Kossa staining of histolocial sections of tibia showing that the expression of hVDR in the distal intestine (KO/TG1 and KO/TG2) rescues the bone defects associated with systemic VDR deficiency. Serum PTH and serum calcium are normalized in KO/TG1 and KO/TG2 mice (not shown). [From Dhawan et al. (112).]

Thus $1,25(OH)_2D_3$-mediated intestinal calcium absorption is more complex than has been suggested by the three-step model. Future studies defining the multiple mechanisms by which $1,25(OH)_2D_3$ acts in both proximal and distal segments of the intestine are needed to identify new therapeutic approaches to sustain calcium balance.

B. Kidney

1. Overall process of renal calcium reabsorption: role of $1,25(OH)_2D_3$

Most of the calcium that is filtered through the glomerulus will be reabsorbed in both the proximal and distal tubule resulting in only 1% to 2% of filtered calcium appearing in the urine. Approximately 65% of the filtered calcium is passively reabsorbed at the proximal tubules in a $1,25(OH)_2D_3$-independent way. In the distal tubules, calcium absorption is regulated by $1,25(OH)_2D_3$ and PTH. Calcium reabsorption in the proximal tubule is passive and follows a sodium gradient, whereas calcium reabsorption in the distal tubule involves an active transcellular mechanism and resembles intestinal calcium absorption (see sect. IV*A1*). The model consists of calcium entry through TRPV5, transfer of calcium in the cytoplasm by binding to calbindin-D_{9k} and calbindin-D_{28k}, and calcium extrusion by the sodium/calcium exchanger (NCX1) and plasma membrane calcium pump 1b. Inactivation of *Trpv5* results in hypercalciuria, but normocalcemia is maintained in these mice by compensatory increase of intestinal calcium absorption stimulated by high serum $1,25(OH)_2D_3$ levels (193). These findings suggest that calcium uptake by TRPV5 is a rate-limiting step in renal calcium reabsorption. *Calbindin-D_{28k}* deletion has no effect on urinary calcium excretion, but its role is mostly compensated by calbindin-D_{9k} (520). Ablation of *Calbindin-D_{28k}* in *Trpv5* null mice did not worsen the *Trpv5* null phenotype (165). Active renal calcium reabsorption is regulated by PTH and $1,25(OH)_2D_3$, which both increase calcium reabsorption. Indeed, *Cyp27b1* null mice show decreased expression of TRPV5, calbindin-D_{9k}, calbindin-D_{28k}, and NCX1 mRNAs, and this reduced expression was rescued by $1,25(OH)_2D_3$ treatment (192). However, in the different *Vdr* null strains, only calbindin-D_{9k} mRNA was consistently decreased (133, 460, 506). Nevertheless, *Vdr* null mice display reduced renal calcium reabsorption, as shown by the inappropriately high urinary calcium levels given the hypocalcemia (133, 259). Besides PTH and $1,25(OH)_2D_3$, αKlotho and FGF23 can also regulate TRPV5 expression. Two pathways are proposed: the first model states that αKlotho hydrolyzes extracellular residues of TRPV5 and thereby ensures that TRPV5 is entrapped in the apical plasma membrane (74); the second model, proposed by a recent study, suggests that FGF23 signaling through the FGFR1-αKlotho complex at the basolateral membrane regulates intracellular TRPV5 trafficking and TRPV5 abundance at the apical membrane (20). In accordance with these findings, *αKlotho* and *Fgf23* null mice exhibit hypercalciuria (14, 20) (**FIGURE 5,** *bottom panel*).

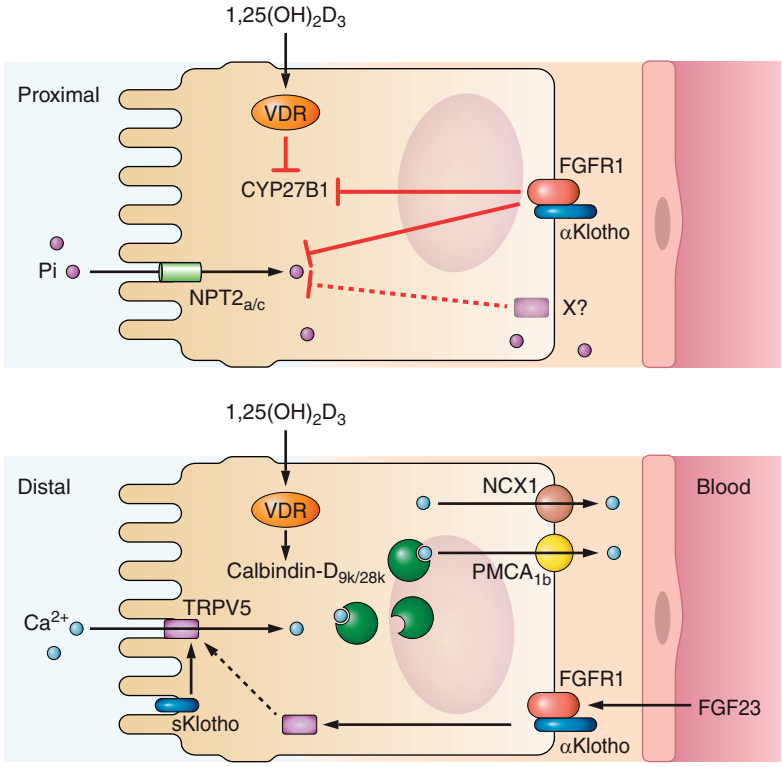


FIGURE 5. Renal VDR actions. In the proximal tubule cells, CYP27B1 expression is suppressed by $1,25(OH)_2D_3$ and FGF23. FGF23 also stimulates phosphate excretion by decreasing the expression of the phosphate transporters NPT2a/c in the apical membrane. FGF23 may signal by binding to the few FGFR1-Klotho complexes in the proximal tubules or by inducing a paracrine factor (factor X) in the distal tubules where abundant FGFR1-Klotho complexes are present. Renal calcium reabsorption in the distal tubule is stimulated by $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ increases the expression of calbindin-D_{9k} and calbindin-D_{28k} and to a lesser extent of TRPV5. The extrusion of calcium at the basolateral side is mediated by PMCA1b and NCX1. Two models are proposed on how Klotho and FGF23 regulate TRPV5 expression: *1*) secreted Klotho (sKlotho) is considered to hydrolyze sugar residues from the glycan chains on TRPV5 resulting in better entrapment of TRPV5 in the apical membrane; and *2*) FGF23 binds to the basolateral FGFR1-Klotho complex, which stimulates intracellular transport of TRPV5 to the plasma membrane.

2. Regulation of $1,25(OH)_2D_3$ synthesis and phosphate absorption

The proximal tubules of the kidney are also the major site of $1,25(OH)_2D_3$ synthesis (see sect. II*B*; CYP27B1) and of phosphate absorption. CYP27B1 expression is upregulated by PTH but downregulated by FGF23 and $1,25(OH)_2D_3$ (see sect. II*E*: regulation of renal CYP27B1 and CYP24A1) (**FIGURE 5,** *top panel*). Approximately 80% of filtered phosphate is reabsorbed from urine under normal dietary phosphate intake and most of it occurs within the proximal tubule (469a). Phosphate transport across the proximal tubule epithelium is mediated by sodium-phosphate cotransporters NPT2a and NPT2c and the energy derived from the transport of sodium down its gradient is used to transport phosphate into the cell (49a). This phosphate reabsorption in the proximal tubules is regulated by several factors including FGF23, PTH, and $1,25(OH)_2D_3$. PTH and FGF23 promote renal phosphate loss by decreasing the abundance of the sodium-phosphate cotransporter (NPT2a/2c) at the apical membrane: PTH stimulates the internalization and lysosomal degradation of these transporters (27), whereas FGF23 decreases their expression (507) (**FIGURE 5,** *top panel*). How FGF23 signaling regulates NPT2a and NPT2c expression is not fully clarified since αKlotho is predominantly expressed in the distal tubules, whereas the effects of FGF23 on phosphate absorption are mainly observed in the proximal tubules (257). Possibly, the small number of FGFR1-αKlotho complexes found in the proximal tubules is sufficient for signaling. On the other hand, recent findings suggest that a paracrine factor is released from the distal tubules that acts on adjacent proximal tubules (139). Beside PTH and FGF23, $1,25(OH)_2D_3$ may regulate phosphate homeostasis by increasing FGF23 expression in osteocytes (see sect. II*D*) and αKlotho expression in the distal tubule (150), factors that both stimulate renal phosphate loss.

Thus $1,25(OH)_2D_3$ action regulates renal calcium reabsorption and phosphate loss, but the molecular mechanism is still incompletely characterized.

C. Bone

1. Link between bone metabolism and calcium homeostasis

Calcium and bone homeostasis are highly intertwined, as calcium is a major constituent of the bone and provides strength to the skeleton, but the bone is also the largest store of calcium in the body. The bone structural integrity thus relies on sufficient calcium supply from the serum and therefore indirectly from intestinal calcium absorption and renal calcium reabsorption, but on the other hand, calcium can be removed from the bone to preserve normal serum calcium levels in case of a negative calcium balance. In the adult, bone is continuously remodeled, and bone resorption by osteoclasts is in balance with bone formation by osteoblasts to maintain bone mass. During growth, bone lengthening is highly dependent on the coordinated growth and differentiation of chondrocytes. Studies of humans and mice lacking VDR or CYP27B1 systemically have evidenced that the

bone characteristics of rickets and osteomalacia are rescued when sufficient calcium absorption is ensured by dietary (18, 107, 258, 350, 351, 446) or genetic (499) means, indicating an indirect role of VDR signaling for bone homeostasis by regulating intestinal calcium absorption and controlling phosphate homeostasis. Indeed, the low serum phosphate levels in *Vdr* null mice decrease apoptosis of hypertrophic chondrocytes resulting in widening and expansion of the epiphyseal growth plate (123, 389). In addition, the mineral supply for bone matrix mineralization is decreased because of the hypocalcemia and hypophosphatemia, leading to osteomalacia. Nevertheless, VDR has specific actions in osteogenic cells, and we will first discuss the contribution of VDR signaling to mineral homeostasis, next its role for bone metabolism, and finally the effects of vitamin D supplementation as part of the strategies to treat osteoporosis.

2. The paracrine role of VDR signaling in osteogenic cells for mineral homeostasis

Osteoblast VDR signaling participates in calcium metabolism primarily during a negative calcium balance. Indeed, in the situation that dietary calcium acquisition is lower than bodily calcium use and renal calcium loss, calcium is mobilized from the bone to preserve normal serum calcium levels. This skeletal response was especially evidenced in a mouse model lacking *Vdr* expression specifically in the intestine, resulting in markedly reduced intestinal calcium absorption (263). In this condition, serum levels of PTH and $1,25(OH)_2D_3$ increase, which lead to a marked depletion of calcium from the bone to maintain normal serum calcium levels. The effect on the bone consisted of increased bone resorption accompanied by impaired bone mineralization **(FIGURE 6)**.

VDR signaling enhances bone resorption mostly indirectly by acting on osteoblasts rather than on osteoclasts. Indeed, osteoblast VDR signaling exerts direct transcriptional control on the expression of RANKL, an important osteoclastogenic factor (233) (see sect. III*B2*). RANKL binds to its cognate receptor RANK in osteoclast precursors and increases osteoclast formation and action (431) **(FIGURE 6)**. This action can be blocked by the naturally occurring soluble decoy receptor of RANKL, termed osteoprotegerin (OPG). In vitro co-culture experiments have shown that osteoblast VDR signaling is necessary for $1,25(OH)_2D_3$-induced osteoclast formation, whereas VDR activity in osteoclasts is not (435). The increase in bone resorption during a negative calcium balance is necessary to maintain normocalcemia, as evidenced by a reduction in serum calcium levels when bone resorption is pharmacologically blocked in the intestinal-specific *Vdr* null mice (263).

Besides stimulating bone resorption during a negative calcium balance, $1,25(OH)_2D_3$ also inhibits bone matrix mineralization and thereby contributes to preserving normal serum calcium levels (263). These mineralization defects are characterized by abundant unmineralized bone matrix and reduced mineral content of the mineralized bone. Mechanistically, $1,25(OH)_2D_3$ suppresses mineralization by increasing the pyrophosphate (PP_i) levels and *Osteopontin* expression, both potent mineralization inhibitors. VDR signaling in osteoblasts regulates PP_i levels by controlling the expression of several genes: increasing the expression of ectonucleotide pyrophosphatase phosphodiesterase (*Enpp*)1 and Ennp3, which generate PP_i from trinucleotides, and increasing the levels of progressive ankylosis (*Ank*), which mediates the transport of PP_i (169, 263, 307). VDR signaling in osteogenic cells not only influences mineral homeostasis by regulating local processes of bone mineralization and remodeling, but also controls calcium and phosphate homeostasis in an endocrine manner by stimulating FGF23 expression as has been discussed in section II*E*.

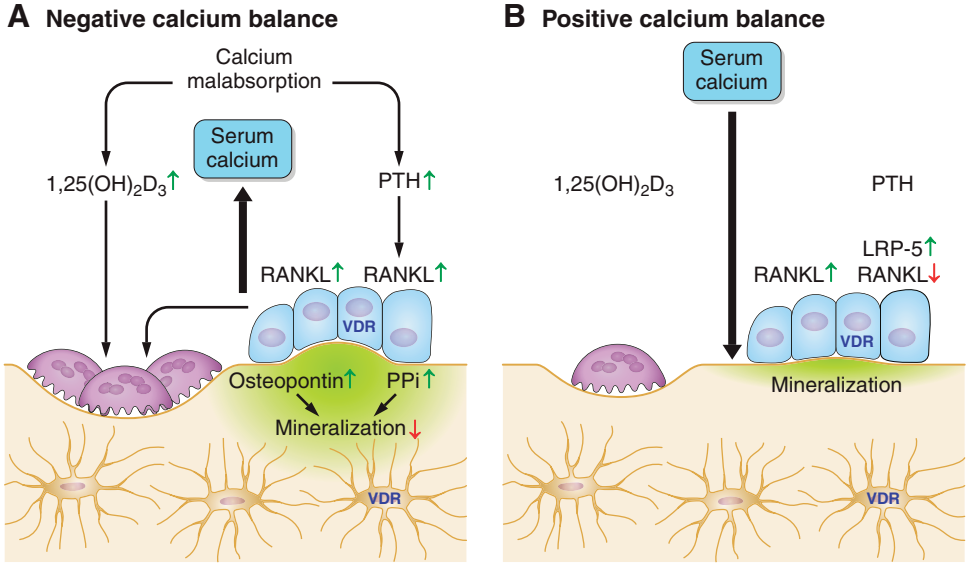


FIGURE 6. Skeletal effects of $1,25(OH)_2D_3$ signaling. During a negative calcium balance, when VDR action in the intestine is impaired or dietary calcium intake is low, intestinal calcium absorption is decreased. Normal serum calcium levels can however be maintained by increased $1,25(OH)_2D_3$ and PTH levels, which will increase bone resorption and reduce bone matrix mineralization. During a normal or positive calcium balance, normal serum $1,25(OH)_2D_3$ levels promote intestinal calcium absorption. This pathway will deliver sufficient calcium for adequate bone matrix mineralization. VDR signaling in osteoprogenitors increases RANKL expression and stimulates osteoclastogenesis, whereas VDR action in mature osteoblasts has anticatabolic actions, by decreasing RANKL expression, and anabolic effects by stimulating LRP-5 signaling.

3. Role of osteoblastic/osteocytic VDR signaling in bone homeostasis

As discussed, VDR signaling in osteogenic cells during a negative calcium balance is mainly directed to preserve serum calcium levels, and the increased bone resorption and impaired bone mineralization occur at the expenses of skeletal integrity. The role of VDR signaling in bone cells during a positive calcium balance is still not fully elucidated, but the specific effects likely depend on the osteoblast differentiation stage **(FIGURE 6)**. VDR signaling in osteoprogenitors and osteoblasts has a positive effect on osteoclast formation and bone resorption and thus negatively regulates bone mass, as shown by *Vdr* inactivation in mice using the collagen type I promoter which resulted in increased bone mass (501). On the other hand, VDR activity in more mature osteoblasts has anabolic and anticatabolic activity and increases bone mass, as evidenced by *Vdr* overexpression using the osteocalcin promotor (29, 157). The anti-resorptive effect is mediated by decreased RANKL/OPG ratio, whereas the anabolic effect may rely on increased LRP-5 expression. The mouse *Lrp-5* gene is regulated by VDR signaling. LRP-5 functions as a co-receptor in wingless (Wnt) signaling (153), a pathway known to mediate anabolic effects in osteoblasts. Finally, VDR signaling in osteocytes is redundant for bone metabolism, as *Vdr* inactivation in mature osteoblasts and osteocytes [using the dentin matrix protein 1 (Dmp1) promoter] has no effect on bone mass, formation, resorption, or mineralization (263). Since all these osteogenic differentiation stages coexist, the physiological relevance of the differential, and even opposing, effects of VDR signaling in osteogenic cells are still not fully defined and need further investigation.

4. VDR signaling in chondrocytes

Not only osteoblasts and osteocytes express the vitamin D machinery, but also growth plate chondroyctes express the VDR (478). Mouse genetic studies using *Vdr* inactivation in the chondrocytes (collagen 2 promoter) have indicated that VDR signaling in these cells is especially important during bone growth, when chondrocytes are abundantly present (293). In young mice, VDR activity in chondrocytes regulates RANKL expression and hereby trabecular bone remodeling. In addition, it indirectly contributes to FGF23 production in osteocytes and thereby vitamin D homeostasis. These effects diminish in adult mice, when VDR signaling in osteoblasts and osteocytes become more important as these cell types are then the major source of RANKL as well as FGF23 (322, 373, 496).

5. Human studies

Severe vitamin D deficiency in children, due to lack of exposure of sunlight and low vitamin D intake, is still endemic in several areas of the world (166, 369). In these children, serum 25OHD levels are usually below 10 ng/ml, which is considered as the threshold for 25OHD to control intestinal calcium absorption (55, 325). Below 10 ng/ml 25OHD there is a deficit in substrate leading to lower serum1,25$(OH)_2$D levels and a decrease in intestinal calcium absorption (325). Extensive clinical experience and some randomized control trials indicate that daily intake of 400 IU vitamin D_3 is sufficient to prevent this type of rickets in children (55, 264). In adults, clinical vitamin D-related osteomalacia is usually found in individuals with low sun exposure or in patients with impaired intestinal vitamin D absorption as part of intestinal fat malabsorption, like after bariatric surgery or with inflammatory bowel disease (395).

A negative calcium balance is often found in ageing individuals and is explained by low dietary calcium intake, vitamin D deficiency, and a decrease in VDR levels in the intestine (127) leading to reduced intestinal calcium absorption. As a consequence, SHPT develops with increased bone resorption and decreased bone mineralization (45, 241, 370). Numerous clinical trials and meta-analyses have therefore been performed to investigate the effect of vitamin D supplementation with or without calcium on fracture incidence. In general, the effect of vitamin D alone compared with placebo had no effect on fracture risk, whereas meta-analyses on the combination of vitamin D and calcium were inconclusive showing a 12–26% reduction in fracture risk in some meta-analyses, but no effect in others (264). Several factors may explain these inconsistencies. The dose of vitamin D has to be adequate (>400 IU) to reduce fracture risk, and dosage has been different between several clinical trials (46). In addition, baseline values of serum 25$(OH)D_3$ may be different and vitamin D supplementation is mainly effective in individuals with documented or at high risk of vitamin D deficiency and low calcium intake (90, 99). Finally, compliance and persistence with calcium and vitamin D are essential, but compliance with the supplements is often low in healthy and community-dwelling individuals (439). In general, a vitamin D supplement of 600–800 IU per day in combination with calcium may reduce the incidence of nonvertebral fractures by ~10–20% in old, vitamin D-deficient population (386).

Thus vitamin D actions control bone metabolism mainly indirectly by regulating mineral homeostasis, but the exact role of the VDR in osteogenic cells for bone homeostasis during a normal calcium balance requires further investigation. Several ongoing large-scale clinical trials will help to define the dosage of vitamin D supplement that is best for skeletal health.

V. PLEIOTROPIC ACTIONS OF VITAMIN D

Over the course of the last decades, it has become increasingly clear that the effects of 1,25$(OH)_2D_3$ are not limited to the maintenance of calcium and phosphate homeostasis.

Indeed, $1,25(OH)_2D_3$ regulates multiple cellular processes with effects on normal and malignant cell growth and differentiation (including differentiation of keratinocytes; see Ref. 43 for review), on the innate and adaptive immune function, on cardiovascular function, and on the complex interplay with other hormones. In this review we focus on effect on cancer, the cardiovascular system (including results of randomized controlled trials), and the immune system.

A. Cancer

1. VDR expression and vitamin D metabolism in cancer cells

Already more than three decades ago, Colston et al. (95) demonstrated that doubling times of melanoma cells increase after treatment with $1,25(OH)_2D_3$. Abe et al. (3) reported shortly thereafter that HL60 leukemia cells differentiate towards the macrophage lineage upon incubation with $1,25(OH)_2D_3$. Ever since, numerous studies have shown that $1,25(OH)_2D_3$ and its analogs slow down cancer cell growth by arresting cells in the G_0/G_1 phase of the cell cycle, by inducing their differentiation or by the induction of apoptotic cell death. Furthermore, $1,25(OH)_2D_3$ influences angiogenesis, alters cell adhesion and migration, and reduces the invasiveness of cancer cells. Interestingly, most cancer cells do not only express VDR, but also CYP27B1 and CYP24A1, which allows the cells to locally regulate $1,25(OH)_2D_3$ metabolism. Although locally produced $1,25(OH)_2D_3$ concentrations are considered not to contribute to calcium homeostasis, they may have significant implications for cancer cell progression (190, 433).

A) VDR. The presence of the VDR in tumor cells is a prerequisite for the antineoplastic effects of $1,25(OH)_2D_3$. In most tumors, VDR expression is retained and, according to Narvaez et al. (323), alterations in the *VDR* gene are only seen in 5% of cancers in The Cancer Genome atlas. Several studies suggested that enhanced tumor VDR expression levels are correlated with a better prognosis and prolonged overall survival (119, 186). This was recently confirmed by Santagata et al. (391) who used large-scale immunohistochemistical stainings to develop a phylogenetic classification scheme. Their data showed that tumors that expressed next to the estrogen receptor (ER) and the androgen receptor (AR) also the VDR had the better prognosis and they suggested that ER/AR/VDR expression was correlated with the differentiation grade. Moreover, these observations were more pronounced at the protein level than at mRNA level (391). Interestingly, more than 900 allelic variants have been described at the *VDR* locus (39), and numerous studies have investigated the association of SNPs in *VDR* (e.g., *ApaI* [rs7975232], *BsmI* [rs1544410], *FokI* [rs10735810], *TaqI* [rs731236]) and cancer risk with inconclusive results (179, 238, 270, 516).

B) CYP27B1. Expression of CYP27B1 has been extensively studied in cancer cell lines as well as in primary tumors. In cancer cell lines, enhanced as well as reduced expression of *CYP27B1* is reported (488). Interestingly, upon oncogenic transformation of a mammary epithelial cell line *CYP27B1* expression levels decrease significantly which results in a reduced cellular sensitivity to $25(OH)D_3$ and $1,25(OH)_2D_3$ (227). Recently, Narvaez et al. (323) reported that in The Cancer Genome Atlas datasets <2% of breast cancers exhibit genomic alterations (including amplifications, deletions, mutations, and changes in mRNA) (323). Nonetheless, multiple studies reported that in human cancer biopsies *CYP27B1* mRNA expression (32, 47) and CYP27B1 protein levels (93, 272, 299) tend to be higher in well-differentiated tumors, whereas they are lower in more malignant and poorly differentiated tumors. In addition, in lung cancer, high *CYP27B1* is associated with better overall survival (237). Multiple single nucleotide polymorphisms (SNP) exist in the gene that encodes for *CYP27B1*. Although conflicting results are reported on the associations of SNPs in the *CYP27B1* gene and cancer risk, these SNPs may alter $1,25(OH)_2D_3$ production as these SNPs may lead to a reduced enzymatic activity (207). Although associations have been reported, it is unclear at this time whether cancer cell *CYP27B1* has a role in affecting disease progression.

C) CYP24A1. Not only the enzyme responsible for the production of $1,25(OH)_2D_3$, but also the catabolic enzyme of $1,25(OH)_2D_3$, CYP24A1, is expressed in cancer cells. *CYP24A1* expression has been reported to be enhanced in the more malignant and metastatic tumors. It has been suggested that increased CYP24A1 expression is associated with increased resistance to $1,25(OH)_2D_3$ action (154). Increased *CYP24A1* levels may result from gene amplification as was demonstrated in breast tumors, where the chromosomal region 20q13.2, which contains the *CYP24A1* gene, was found to be amplified (13). Also in colorectal cancer, increased *CYP24A1* gene copy number is shown, whereas no differences in *CYP24A1* promoter methylation are seen (189). In agreement with these findings, 10–13% of human breast cancers in the dataset from The Cancer Genome Atlas show altered *CYP24A1* expression, most often due to gene amplification and characterized by enhanced mRNA levels (323). Of note, high CYP24A1 expression significantly correlates with poor survival in lung cancer cohorts (50). In addition, in the context of p53 loss, suppression of *CYP24A1* caused by inhibition of the miR-17~92 cluster was toxic in non-small cell lung cancer (50). Genetic variants in *CYP2R1*, *7-DHCR* but also in *CYP24A1* (rs6013897) are significantly correlated with vitamin D status as was reported in two recent genome-wide association studies (6, 475). Of interest, many SNPs in the *CYP24A1* gene are characterized by a reduced enzymatic activity, suggesting that vitamin D catabolism may be influenced by genetic factors (207). Multiple recent studies have investi-

gated whether polymorphisms in the *CYP24A1* gene could be associated with cancer risk. However, until now, no consensus exists on the association between common variants in the *CYP24A1* gene and cancer risk (316, 379, 394). This might not be surprising as the described SNPs only explain a small amount of the variation in $25(OH)D_3$ levels, and therefore the contribution of the specific SNPs to the predicted cancer risk may be too weak to be identified in genome-wide association studies (142).

2. In vitro antineoplastic effects of $1,25(OH)_2D_3$

A) ANTIPROLIFERATIVE EFFECTS. One of the earliest and best-described effects of $1,25(OH)_2D_3$ includes its growth-inhibitory and prodifferentiation effects. Indeed, differentiation along the macrophage lineage of HL60 cells is accompanied by a reduction in cell proliferation (3). However, regulation of differentiation and cell proliferation are not always coupled and seem to be cell-type dependent (38). In most cancer cells expressing a functional VDR, incubation with $1,25(OH)_2D_3$ leads to an accumulation of cells in the G_0/G_1 phase of the cell cycle (211). Downregulation of the abundance of cyclins and cyclin-dependent kinases (CDKs) and/or upregulation of different CDK inhibitors, such as p21 and p27, by $1,25(OH)_2D_3$ results in a reduced CDK activity, the formation of an intact retinoblastoma (Rb)-E2F complex, a decrease in E2F and E2F-target genes, and subsequent growth inhibition (355, 441, 469, 477) **(FIGURE 7)**. However, when Rb is downregulated in prostate cells, $1,25(OH)_2D_3$ is still able to retard the growth of these cells, suggesting that redundant growth inhibitory pathways compensate for the loss of Rb (479). In analogy, *Rb*-deficient murine embryonal fibroblasts (MEFs) remained sensitive to the growth-inhibitory effect of $1,25(OH)_2D_3$, whereas the antiproliferative effect of $1,25(OH)_2D_3$ is lost in MEFs in which the pocket proteins p107 and p130 were both deleted (467). In addition, induction of C/EBPα by $1,25(OH)_2D_3$ and enhancement of VDR transcription by C/EBPα has been suggested as one mechanism involved in $1,25(OH)_2D_3$-mediated inhibition of proliferation of breast cancer (115). In human colon carcinoma, $1,25(OH)_2D_3$ antagonizes the Wnt/β-catenin signaling, which finally results in a reduced cell proliferation. Treatment with $1,25(OH)_2D_3$ leads to a diminished interaction between β-catenin and T-cell factor (TCF) in favor of an enhanced interaction between VDR and β-catenin. Moreover, $1,25(OH)_2D_3$ enhances E-cadherin expression, leading to the nuclear export of β-catenin, and induces the expression of Dickkopf (DKK) 1, an extracellular Wnt inhibitor. As a consequence, the transcription of TCF-target decreases, among which that of c-myc, a key regulator of cell cycle progression (246a). Very recently, Chang et al. (75) reported that $1,25(OH)_2D_3$ induces the expression of the microRNA miR-145 in a dose- and VDR-dependent manner. Interestingly, inhibition of miR-145 abrogates the $1,25(OH)_2D_3$-induced downregulation of E2F3 and reverses the growth-inhibitory effect of $1,25(OH)_2D_3$ (75). In addition, $1,25(OH)_2D_3$ also interferes with other growth regulatory pathways initiated by transforming growth factor (TGF)-β (76), epidermal growth factor (36), insulin-like growth factor (58), platelet-derived growth factor (324), and fibroblast growth factor 2 (385). Moreover, it intervenes in other mitogenic signaling pathways (e.g., ERK/mitogen-activated protein kinase pathway and *c-myc*) (344, 480). In BRCA1-positive breast cancer cells, liganded VDR associates with BRCA1, and this complex occupies VDREs in the p21 promoter to enhance promoter acetylation and p21 expression, revealing a novel aspect of BRCA unrelated to DNA repair (360).

Recent work demonstrated that VDR acts as transcriptional regulator in pancreatic stellate cells and primes them to differentiate towards a more quiescent phenotype (117). These findings are potentially very interesting because there is increasing evidence that activated pancreatic stellate cells are characterized by a pathological matrix secretion, which results in a physical barrier for chemotherapy. Moreover, they produce mitogenic factors that may promote pancreatic cancer cell proliferation, survival, and migration. Interestingly, when the vitamin D analog Calcipotriol is administered in mice with pancreatitis, less fibrosis and inflammation is observed. In addition, extensive stromal remodeling is induced, whereas tumor-supportive signaling is reduced. As a consequence, the efficacy of a co-administered chemotherapeutic agent is enhanced, and in parallel, survival increases. Previous work from the same research group on hepatic stellate cells reveals that VDR promotes quiescence of hepatic stellate cells by temporally inhibiting TGF-β1/mothers against decapentaplegic homolog 3 (SMAD3) signaling via genomic competition (406). Recent research has pointed out that $1,25(OH)_2D_3$ is able to target the cancer stem cell population. Cancer stem cells have the capacity to continuously self-renew and retain multilineage differentiation potential. Therefore, these cells constitute a relevant target for chemoprevention and chemotherapy. Indeed, upon growth arrest and differentiation, these cells will lose their self-renewal competence and their capacity to initiate tumorigenesis. Maund et al. (300) demonstrated that upon incubation with $1,25(OH)_2D_3$ normal adult prostate progenitor/stem cells undergo cell-cycle arrest, senescence, and differentiation. In breast cancer research, several studies have been performed on mammosphere cell cultures to enrich for mammary progenitor cells and putative breast cancer stem cells. In mammosphere cultures of SKBR3, MCF7, and $HMLE^{H\text{-}RAS}$ breast cancer cell lines, VDR expression is downregulated, and little growth inhibition is seen upon treatment with $1,25(OH)_2D_3$. Upon overexpression of VDR, the ability to form mammospheres is reduced and cell differentiation increased (358). On the other hand, in MCF10DCIS mammosphere cultures, $1,25(OH)_2D_3$ and its analogs decrease mammosphere forming efficiency and repress markers that are associated with stem cells and pluripotency such as CD44, CD49f, c-Notch1, and OCT4 (416, 417, 471). In pancreatic cancer, $1,25(OH)_2D_3$ also suppresses cancer cell stemness through inhibition of FOXM1 signaling (261).

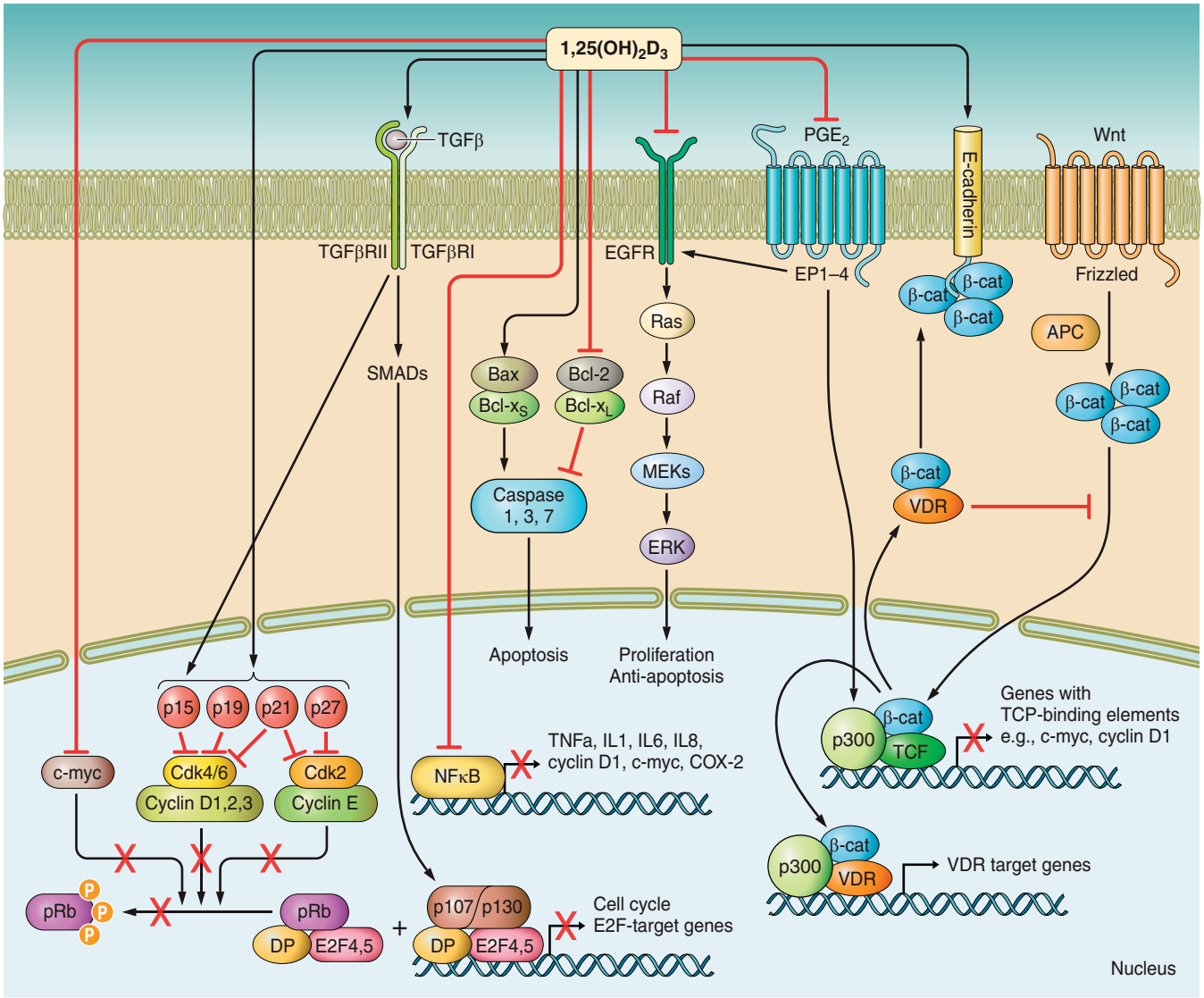


FIGURE 7. $1,25(OH)_2D_3$-induced signaling pathways involved in the regulation of cell proliferation, apoptosis, and inflammation in cancer. $1,25(OH)_2D_3$ hampers the transition from the G_1 to the S phase of the cell cycle either directly, through upregulation of different cyclin-dependent kinase inhibitors, or indirectly through the induction of other growth factors (e.g., TGF-β, EGF). In addition, $1,25(OH)_2D_3$ induces apoptosis through activation of the intrinsic apoptotic pathway or by interference with other signaling pathways such as TNF-α, EGF, β-catenin, and prostaglandins. $1,25(OH)_2D_3$ has also an immunosuppressive activity, as indicated by the repression of NFκB-mediated gene transcription, which results in a suppressed production of inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α.

B) EFFECTS ON APOPTOSIS. $1,25(OH)_2D_3$ induces apoptosis in a wide variety of cancer cells, and although the underlying mechanisms seem to be cell type-specific, several studies indicated that the nuclear VDR is required for the proapoptotic effects of $1,25(OH)_2D_3$ (171, 526). Many studies have pointed to an activation of the intrinsic apoptotic pathway by $1,25(OH)_2D_3$ as illustrated by suppression of the antiapoptotic B-cell lymphoma 2 (Bcl2) proteins and B-cell lymphoma-extra large (Bcl-xl) and activation of the proapoptotic protein Bax **(FIGURE 7)**. This subsequently leads to the release of cytochrome *c* from the mitochondria and the activation of downstream caspases such as caspase 3 and 9 (171, 470, 515). In addition, $1,25(OH)_2D_3$ may induce apoptosis by interfering with other signaling pathways such as tumor-necrosis factor (TNF)-α (168, 303). Interestingly, the induction of apoptosis by the vitamin D analog EB1089 in MCF-7 cells is suggested to occur through a pathway that involves Beclin 1-dependent autophagy (197).

In contrast, in acute myeloid leukemia (AML), treatment with $1,25(OH)_2D_3$ results in differentiation of these cells which is accompanied by an enhanced cell survival. Changes in the anti-apoptotic protein Mcl-1 and in the Bcl2/Bad ratio contribute to these prosurvival effects (477).

Recently, the increased expression of miR-32 in AML cell lines after incubation with $1,25(OH)_2D_3$ has been implicated in the antiapoptotic effect of $1,25(OH)_2D_3$ through suppression of the proapoptotic protein Bim (167).

C) EFFECTS ON MIGRATION AND INVASION. Cancer invasion into the surrounding tissue is an important hallmark of cancer and is regulated by signaling pathways that regulate the cytoskeleton, induce the turnover of cell matrix, and control cell-cell junctions and cell adhesion. Interestingly, $1,25(OH)_2D_3$ impacts each of these different processes. Indeed, in squamous cell carcinoma, $1,25(OH)_2D_3$ leads to an altered cell morphology and actin organization (276). Moreover, $1,25(OH)_2D_3$ inhibits expression of the cytoskeletal protein vimentin, which results in a decreased cell motility (449). Of note, in MCF-7 breast cancer cells, PDZ-LIM domain-containing protein 2 (PDLIM2), an adaptor molecule that links different components of the cytoskeleton, is identified as a direct target gene of $1,25(OH)_2D_3$. Furthermore, the induction of PDLIM2 by $1,25(OH)_2D_3$ mediates the proadhesion, anti-migration, and anti-invasion effects of $1,25(OH)_2D_3$ (466). Different proteases such as the matrix metalloproteinases (MMPs), the plasminogen activators (PAs), and the cathepsins (CPs) are involved in tumor invasion as they are able to degrade the extracellular matrix (30). $1,25(OH)_2D_3$ not only reduces the expression and secretion of metalloproteinase (MMP) 2 and 9, but also decreases cathepsin K activity, increases tissue inhibitor of MMP1 (TIMP1) and regulates different components of the plasminogen activator system (30, 204, 235). In addition, in multiple cell types treatment with $1,25(OH)_2D_3$ leads to an altered expression of adhesion molecules. As such, the cell surface adhesion molecules $\alpha 6$ integrin and $\beta 4$ integrin (432) as well as the intracellular adhesion molecule 1 (428) are decreased upon treatment with $1,25(OH)_2D_3$. Expression of E-cadherin, a tumor suppressor that is inversely correlated to metastasis, is induced by $1,25(OH)_2D_3$ in different cell types, and this induction may lead to suppression of cellular motility (66, 347).

D) EFFECTS ON INFLAMMATION IN CANCER. Numerous studies demonstrated that $1,25(OH)_2D_3$ has an immunosuppressive activity by stimulation of the innate immune system and by suppression of the adaptive immune system (457). Because chronic inflammation is regarded as a risk factor for the development of cancer, the suppression of inflammation by $1,25(OH)_2D_3$ may contribute to its antineoplastic activity. In cancer, the synthesis of prostaglandins, small-molecule derivatives of arachidonic acid which play a key role in the generation of the inflammatory response, is targeted by $1,25(OH)_2D_3$. Indeed, $1,25(OH)_2D_3$ leads to a decrease in cyclooxygenase-2 and an increase in 15-hydroxyprostaglandin dehydrogenase resulting in a reduced prostaglandin synthesis (319, 444). In addition, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, a major mediator of the immune response, is inhibited in various cancers by $1,25(OH)_2D_3$ by acting on different members of this pathway (304, 455) **(FIGURE 7)**. A suppressed production of inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, IL-17, and TNF-α is consistently demonstrated in in vitro and in vivo cancer models (5, 162, 240, 330) **(FIGURE 7)**. In a preclinical SMAD3 knockout model of colitis and colon cancer, Meeker et al. (304) showed that a high vitamin D diet decreases NF-κB activation in colonic epithelial cells, which is reflected by decreased inflammatory cell infiltrates and reduced expression of proinflammatory cytokines during initiation of carcinogenesis (304). In line with this, a strong inverse association is found between 25(OH)D status and the risk of colorectal cancer with high-level lymphocytic reactions which suggests a role for vitamin D in cancer immunoprevention through modulation of the host-tumor interaction (419). A recent whole genome profiling on cells derived from normal mammary tissue and breast cancer reveals the regulation of a set of immune genes by $1,25(OH)_2D_3$. Among these genes, soluble CD14b, which may contribute to the protection against breast cancer, seems to be consistently induced by $1,25(OH)_2D_3$ (412). Induction of CD14 by $1,25(OH)_2D_3$ is confirmed by a microarray study on fresh tumor slices exposed to $1,25(OH)_2D_3$ (311).

E) EFFECTS OF $1,25(OH)_2D_3$ ON CELL METABOLISM. Recent research has focused on the role of $1,25(OH)_2D_3$ on cellular energy metabolism in the context of cancer. In contrast to quiescent cells, which rely mainly on oxidative phosphorylation for their energy demands, most cancer cells rely on aerobic glycolysis to provide energy and biosynthetic intermediates for the production of nucleotides, amino acids, and fatty acids to sustain their rapid proliferation (464). Therefore, alterations in energy metabolism by $1,25(OH)_2D_3$ may contribute to its antineoplastic activity. In untransformed human breast epithelial cells MCF10A as well as in *ras*-transformed MCF10A cells, treatment with $1,25(OH)_2D_3$ results in reduced glycolysis. In both cell types flux of glucose to acetyl-coA and flux to oxaloacetate is decreased, indicative of a reduced tricarboxylic acid (TCA) cycle activity. However, further research is required to decipher how the $1,25(OH)_2D_3$-induced metabolic reprogramming contributes to its chemopreventive capacity (518). Consiglio et al. (96) genetically silenced *VDR* expression in HaCaT keratinocytes, which are insensitive to the antiproliferative effects of $1,25(OH)_2D_3$. In these cells, *VDR* silencing results in a clear growth reduction and a strong increase in mitochondrial membrane potential that leads to oxidation of metabolic intermediates that can no longer be used in biosynthetic pathways. Intriguingly, these results are indicative of a role of the VDR as an enhancer of cell proliferation (96). Microarray profiling of nontumorigenic mammary epithelial cells after treatment with $1,25(OH)_2D_3$ revealed the regulation of a whole set of metabolic genes such as *SLC1A1* and *GLUL* by $1,25(OH)_2D_3$ (412). *SLCA1* encodes a plasma membrane glutamate transporter that is induced by $1,25(OH)_2D_3$, whereas *GLUL*, which encodes glutamine synthetase, is repressed by $1,25(OH)_2D_3$. These

changes are accompanied by an accumulation of glutathione and changes in respiratory capacity, suggesting that $1,25(OH)_2D_3$ leads to a metabolic switch that might induce quiescence in mammary epithelial cells (323).

F) REGULATION OF MICRORNAS BY $1,25(OH)_2D_3$. MicroRNAs (miRNAs) are endogenous (~22 nucleotides long) single-strand RNA molecules that target mRNAs for cleavage or translational repression and hence fulfill important regulatory roles in a wide variety of biological processes (34). miRNAs usually target sequences at the 3' end of a gene, which position facilitates the control of mRNA stability. The regulation of miRNA expression by $1,25(OH)_2D_3$ is an emerging field of interest and has been shown to be part of the signaling cascades responsible for the effects of $1,25(OH)_2D_3$ on cell proliferation, differentiation, apoptosis, and gene regulation (**TABLE 1**). Sing et al. (414) undertook miRNA microarray profiling in different prostate cell lines ranging from nonmalignant to highly malignant cell types and showed that miRNA expression profiles are highly cell-specific. In addition, they demonstrated in nonmalignant RWPE-1 cells that miRNAs are significantly coregulated with mRNA regulation (414). Such integration studies will be important to improve miRNA target predictions and to unravel the complex signaling pathways induced by $1,25(OH)_2D_3$.

3. In vivo anticancer effects of $1,25(OH)_2D_3$ in animals models

A) VITAMIN D OR VDR-DEFICIENT MOUSE MODELS. Different lines of evidence indicate that an impaired vitamin D signaling, either due to absence of ligand or *Vdr* ablation, is correlated with decreased tumor growth.

I) Vitamin D deficiency. Several studies reported that decreased circulating serum levels of 25(OH)D in mice on a vitamin D-deficient diet, are associated with an increased tumor growth in mice that were inoculated with cancer cells (343, 376, 440). In parallel, a Western diet, low in calcium and vitamin D but high in fat, promotes colonic tumor formation (502).

II) Vdr deficiency. Several studies in $Vdr^{-/-}$ mice pointed out that the absence of the VDR leads to enhanced proliferation and higher susceptibility to carcinogenesis, both in genetic models as upon exposure to carcinogenic products (526). Interestingly, two different research groups investigated the effect of genetic inactivation of *Vdr* in $Apc^{min/+}$ mice. Both groups reported that the number of small intestinal and colonic tumors is not different between $Vdr^{+/+}Apc^{min/+}$ and $Vdr^{-/-}Apc^{min/+}$ mice, which suggests that not tumor formation but rather tumor growth is inhibited by $1,25(OH)_2D_3$. However, the size of the tumors is bigger in the absence of a functional *Vdr* (247, 519). Tumors from $Vdr^{-/-}Apc^{min/+}$ mice express higher levels of β-catenin/TCF target genes, indicating that upon *Vdr* deficiency $1,25(OH)_2D_3$ is no longer able to relocate β-catenin from the nucleus to the plasma membrane and to suppress transcription of β-catenin/TCF target genes (247).

B) CHEMOPREVENTIVE OR THERAPEUTIC EFFECTS OF VITAMIN D, $1,25(OH)_2D_3$, OR ITS ANALOGS. *I) Vitamin D supplementation*. In preclinical cancer models, it was demonstrated that elevating serum 25(OH)D levels is capable to reduce tumor growth. Indeed, when rodents on a Western diet were supplemented with sufficient amounts of calcium and vitamin D_3, colonic tumor formation was repressed (327). Supplementing rodent chow with 5,000 IU vitamin D_3/kg is as potent as treatment with $1,25(OH)_2D_3$ in inhibiting tumor growth in mouse xenograft models of prostate and breast cancer (433). A recent study in intestine-specific *Apc* mutant mice demonstrated that vitamin D_3 supplementation (1,500 IU vitamin D_3/kg diet) but also UV-B irradiation are able to repress outgrowth and malignant progression of primary intestinal tumors (377).

II) Therapeutic effects of $1,25(OH)_2D_3$ or its analogs. In most rodent models for various cancer types, including but not limited to breast, colon, and prostate cancer, the use of $1,25(OH)_2D_3$ has been proven to reduce tumor growth and metastasis. However, the adverse calcemic effects of $1,25(OH)_2D_3$ hamper its clinical applicability, and different analogs of $1,25(OH)_2D_3$ showed clear antitumor activity without inducing hypercalcemia (354). Several studies reported that treatment with $1,25(OH)_2D_3$ or its analogs ameliorates the response to chemotherapy (277, 312). Recently, it also became clear that $1,25(OH)_2D_3$ could be used as a cryosensitizing agent (392). The avenue of combined vitamin D therapy is interesting since lower doses of chemo- or cryotherapy could be given resulting in fewer side effects without losing the efficacy of the treatment.

4. Human studies

A) OBSERVATIONAL STUDIES ON VITAMIN D AND CANCER. Since the initial observation of an inverse association between sunlight exposure and colorectal cancer mortality (158), multiple studies have investigated the association between vitamin D and cancer risk. As a person's vitamin D status depends both on sunlight exposure and vitamin D intake, studies on the association between circulating 25(OH)D levels may be more informative. Indeed, synthesis of 25(OH)D is not under the strict control of calciotrophic hormones, and 25(OH)D is a stable molecule with a half-life of 3 wk. In this review, we have focused on recent meta-analyses of prospective studies where 25(OH)D in serum was measured at the initiation of the study, thus before disease onset. This is in contrast to postdiagnostic measurements, which may be prone to inverse causality. Lower postdiagnostic $25(OH)D_3$ levels may not be the cause but rather the consequence of the disease due to disease-related factors such as lower sun exposure and physi-

Table 1. *Overview of miRNAs that are involved in antineoplastic effects of 1,25(OH)$_2$D$_3$*

microRNA	Cell Type	Regulation by 1,25(OH)$_2$D$_3$	Target of microRNA	Biological Effect	Reference Nos.
miR-22	Colon cancer, SW480-ADH, and HCT116 cell lines	Increased expression		Implicated in antiproliferative and antimigratory effect of 1,25(OH)$_2$D$_3$	17
miR-27b mmu-miR-298	Colon adenocarcinoma LS-180 and pancreatic PANC1 cell lines	Not investigated	VDR	Overexpression of miR-27b and mmu-miR-298 results in reduced VDR protein levels	349
miR-27b	Melanoma SK-Mel5, SK-Mel28, and IGR cell lines	Reduced expression in conjunction with 5-azacytidine	VDR	Reduced miR-27b expression correlates with increased VDR mRNA	135
miR-32	Human myeloid leukemia HL60 and U937 cells	Increased expression	BIM	Implicated in apoptotic cell death after 1,25(OH)$_2$D$_3$ treatment	167
miR-98	Prostate cancer LNCaP cells	Increased expression	CCNJ	Implicated in the antiproliferative effect of 1,25(OH)$_2$D$_3$	445
miR-100 miR-125b	Prostate cancer RWPE-2 cell line	Increased expression	E2F3, PLK1	Implicated in decreased proliferation and migration after 1,25(OH)$_2$D$_3$ treatment	163
miR-125b	Human breast MCF7 cell line	Not investigated	VDR, CYP24A1	Mature miR-125 levels negatively correspond to decreased VDR protein Overexpression of miR-125b leads to reduced antiproliferative effect of 1,25(OH)$_2$D$_3$ CYP24 protein levels in cancer tissues are inversely associated with their miR-125b levels	315
miR-125b	Melanoma MeWo and SK-Mel28 cell lines	Not investigated	VDR	VDR protein levels are inversely associated with miR125b levels	135
miR-145	Gastric SGC-7901 and AGS cell line	Increased expression	E2F3	Implicated in the antiproliferative effect of 1,25(OH)$_2$D$_3$	75
miR-181a miR-181b	Human myeloid leukemia HL60 and U937 cells	Reduced expression	p27^{KIP1}	Regulation of miR-181a is implicated in the differentiation inducing effect of 1,25(OH)$_2$D$_3$	476
miR-302c miR-520c	Hematological tumor Kasumi-1 and breast cancer MDA-MB-231 cell line	Reduced expression	MICA/B and ULBP2 (NKG2D ligands)	Implicated in the immuno-attack of NK cells against malignant cells	313
miR-498	Various ovarian, endometrium, and breast cancer cell lines	Increased expression	TERT	Implicated in decreased cell proliferation after 1,25(OH)$_2$D$_3$ treatment	224
miR-627	Colorectal HT29 cell line	Increased expression	JMJD1A	Increased methylation of histone H3K9, Suppressed expression of proliferative factors	346

cal activity, lower food intake, or systemic effects of the cancer itself (26, 175, 176, 182, 244).

Two independent meta-analyses of prospective studies on the association between serum 25(OH)D and colorectal cancer risk identified a 30–40% decreased relative risk in patients with high circulating 25(OH)D levels, compared with those with the lowest serum concentrations (252, 278). In a meta-analysis on prediagnostic 25(OH)D and breast cancer risk, Bauer et al. (35) included nine prospective studies. Their analysis suggests no relationship between circulating 25(OH)D levels and breast cancer risk, whereas in postmenopausal women, a stepwise inverse correlation is observed in a narrow range of 25(OH)D concentrations

between 27 and 35 ng/ml. Above the 35 ng/ml threshold, the association flattens with no additional benefit of higher serum 25(OH)D concentrations (35). Another recent meta-analysis on 14 prospective studies identified an overall significant inverse association between serum 25(OH)D levels and breast cancer risk. Whereas there is a protective effect of 25(OH)D levels in Americans, no statistically significant associations are observed in European studies, suggesting that ethnic differences, living environment, and dietary habits may influence the association (472). In analogy with the meta-analysis of Bauer et al. (35), an inversely statistically significant association is observed in postmenopausal women, but not in premenopausal women (472). For other cancers, such as for prostate, non-melanoma skin cancer, and bladder cancer, meta-analyses did not reveal a significant association between circulating 25(OH)D and relative cancer risk (65, 317, 498). Several recent meta-analyses investigated the impact of circulating 25(OH)D levels (at or near the time of diagnosis cancer) on patient outcome. Li et al. (256) analyzed 25 studies and found a significant inverse association between 25(OH)D levels and overall survival of colorectal and breast cancer patients and patients with lymphoma. The authors concluded that a 4 ng/ml increment in circulating 25(OH)D levels confers a hazard ratio of 0.96 for overall survival of the cancer patients (256). Two additional meta-analyses concentrated on the relationship between prediagnostic circulating 25(OH)D concentrations and all-cause and cancer-related mortality. Schottker et al. (398) performed a meta-analysis from eight prospective studies and found that lower 25(OH)D levels are inversely associated with all-cause mortality, but with respect to cancer-related mortality, an association is only observed in subjects with a history of cancer. Chowdhury et al. (81) performed a meta-analysis of primary prevention observational studies and randomized controlled trials. Analysis of the observational studies (73 cohort studies) indicates an inverse association of circulating 25(OH)D with risks of death due to cardiovascular disease, cancer, and other causes. In conclusion, the inverse association between 25(OH)D levels and cancer risk is most consistent for colorectal cancer, while for other cancers contradicting results have been described. However, several recent meta-analyses support the inverse association between circulating 25(OH)D concentrations and (cancer-related) mortality, suggesting that the effects of vitamin D may be stronger for mortality than for cancer risk.

b) randomized controlled trial of vitamin d supplements and cancer risk. More direct evidence to unequivocally assess the association between vitamin D and cancer risk is to be expected from randomized controlled trials (RCTs) with vitamin D supplements. Yet the existing RCTs, in which the effects of vitamin D are studied, have limited power, and their primary endpoint was not cancer incidence or mortality (48, 229). Therefore, awaiting results from ongoing RCTs, meta-analyses of RCTs on vitamin D (vitamin D_2, vitamin D_3, or calcitriol) supplementation and cancer incidence or mortality may be more informative than the results derived from individual trials. Interestingly, most of these meta-analyses agree on the finding that there is no association with vitamin D supplementation and cancer incidence but a trend for an inverse association between vitamin D supplementation and overall mortality (48, 49, 90, 229). The most elaborate meta-analyses were performed by Bjelakovic et al. who investigated the effects of vitamin D supplementation on mortality (48) and on prevention of cancer in adults (49). In their study, data from 18 RCTs with 50,623 participants were analyzed. Most of the trials included mainly elderly community-dwelling women. Vitamin D supplementation was given for a weighted mean of 6 yr. Most trials studied vitamin D_3 supplementation, one trial D_2 supplementation, and three trials investigated supplementation with calcitriol. The authors concluded that there is no evidence for an association between vitamin D supplementation (in whatever form) and cancer incidence. No substantial differences in the effect of vitamin D and cancer are found in a subgroup analysis of trials including participants with vitamin D levels below 20 ng/ml at entry compared with trials in which participants had levels above 20 ng/ml at entry. However, there is a trend towards a protective effect of vitamin D_3 supplementation on cancer mortality and of vitamin D supplementation on overall mortality. The latter conclusions have to be taken with caution as sequential trial analysis reveals that this finding could be due to random errors (48, 49). The findings of this study are in agreement with those of the systematic review on evidence for the use of multivitamins or single nutrients for the United States Preventive Services Task Force. Indeed, in this study, no evidence is found for benefit from vitamin D supplementation for the prevention and cancer and cardiovascular disease (151). Keum and Giovannucci (229) also performed a recent analysis on vitamin D supplementation and cancer incidence and mortality, but they limited their meta-analysis to RCTs of 2–7 yr of duration, involving moderate doses of supplemental vitamin D (400-1,100 IU per day), and for reasonable numbers for total cancer incidence and mortality ($n = 4$). Also this analysis failed to provide evidence for an effect of vitamin D supplementation on total cancer incidence. However, the authors propose a significant benefit from vitamin D supplementation on cancer mortality (229). There are some considerations that have to be taken into account when interpreting the results of these meta-analyses. As outlined above, only a few trials had cancer occurrence as a primary end-point. Furthermore, the duration of supplementation and follow-up is rather short in some studies, certainly when taking into account that carcinogenesis is a long process (48). Meanwhile, other RCTs are ongoing among which the large VITAL trial in the United States, that assesses the effect of vitamin D supplementation (2,000 IU/day), whether or not in combination with omega-3 fatty acids (1 g/day), on the risk of developing cancer as a primary end-point in more

than 20,000 participants (285). All participants are enrolled and the trial is anticipated to be finished in June 2016. In Europe, a similar but smaller RCT (DO-Health, same compounds, same dosages), is running; however, in this trial cancer is not included as a primary end-point. Analyses of these trials may provide information on the dosage, the form, and the duration of vitamin D supplementation that may or may not result in beneficial extraskeletal effects.

5. Conclusion

There is ample evidence for the in vitro antineoplastic activities of the active form of vitamin D_3 as numerous studies have reported beneficial effects of $1,25(OH)_2D_3$ on reducing the malignant behavior of cancer cells. However, upon analysis of the preclinical in vivo models of cancer, most studies seem to agree that vitamin D signaling mainly affects cancer progression rather than carcinogenesis itself. In line with this, most up to date meta-analyses of observational studies and randomized clinical trials argue for a positive effect of vitamin D status on (cancer-related) mortality but not on cancer incidence.

B. Cardiovascular System

1. Vitamin D and cardiovascular disease: associations

Interest in the role of vitamin D in cardiovascular disease (CVD) came from animal studies, but also from epidemiological studies reporting the increase in cardiovascular events in winter and at increasing distance from the equator (147, 321, 400). In general, increasing observational evidence supports an association between low $25(OH)D_3$ levels and cardiovascular disease, although not all observational studies find significant associations, and these relationships may be prone to confounding factors. In addition, RCTs on vitamin D supplementation show inconsistent effects in relation to cardiovascular events. We will review major findings with relation to cardiovascular disease in general and to hypertension in particular, but we will start with discussing potential mechanisms revealed by animal studies.

2. Mechanisms: in vitro data and mouse data

Data from systemic and tissue-specific *Vdr* null mice provide insight into the role of vitamin D signaling in the cardiovascular system. Indeed, *Vdr* or *Cyp27B1* null mice have increased levels of renin and consequently of angiotensin II, resulting in hypertension and cardiac hypertrophy (260, 522). The renin-angiotensin system is an important endocrine system controlling vascular tone, peripheral vascular resistance, and volume homeostasis. However, serum PTH levels were still increased in normocalcemic *Vdr* null mice, hindering interpretation of the data as PTH has been shown to increase serum renin levels (62). On the other hand, aged normocalcemic *Vdr* null mice fed with a rescue diet develop endothelial dysfunction, increased arterial stiffness, increased aortic impedance, and impaired systolic and diastolic function. Mechanistically, the lack of *Vdr* signaling results in chronically lower bioavailability of the vasodilator nitric oxide (NO) due to reduced expression of NO synthesizing enzyme, and these effects are independent of changes in the renin-angiotensin system (19). In accordance herewith, deletion of the *Vdr* specifically in endothelial cells results in endothelial dysfunction evidenced by impaired blood vessel relaxation, an effect that was associated with reduced endothelial NO synthase expression (329). Moreover, mice with cardiomyocyte-selective deletion of *Vdr* also develop cardiac hypertrophy, independent of changes in the renin-angiotensin system and thus indicating a direct in vivo antihypertrophic effect of $1,25(OH)_2D_3$ (78). Finally, *Vdr* null mice display a prothrombotic state that was associated with a decrease in antithrombin and thrombomodulin (7). The therapeutic potential of $1,25(OH)_2D_3$ was tested in rat models that develop chronic hypertension and cardiac hypertrophy and progress to congestive heart failure. Treatment with $1,25(OH)_2D_3$ reduced cardiac hypertrophy especially in rats fed a high-salt diet (283). Together, these findings indicate that VDR signaling has effects on several aspects of the cardiovascular system and may promote endothelial and cardiac function, although the functional significance in normal physiology requires further investigation.

3. Human studies

a) Vitamin D levels and endpoints of cardiovascular disease. *I) Observational studies*. Several prospective observational studies investigated $25(OH)D_3$ levels and the risk of CVD using as endpoints myocardial infarction (MI), combined CVD, stroke, and CV mortality. Most studies observed that low $25(OH)D_3$ levels were associated with a high risk of CVD, although this relationship was not always significant. The Framingham Offspring Study followed 1,739 white participants free of CVD at baseline. Over an average follow-up of 5 years, the risk of cardiovascular events was 1.62 times higher in those with $25(OH)D_3$ levels <15 ng/ml versus the remainder (95% CI = 1.11–2.36; $P = 0.01$). Noteworthy, the significant increased risk of CVD with vitamin D deficiency was noted in hypertensive subjects but not in those without hypertension (474). The Health Professionals Follow-up Study with a nested case-control study design evaluated more than 18,000 men and observed that the incidence of acute myocardial infarction was 2.42 times higher in men with $25(OH)D_3$ levels <15 ng/ml compared with those with levels above 30 ng/ml (95% CI = 1.53–3.84) (164). An 8-year follow-up cohort study of more than 3,000 patients undergoing coronary angiography noted that subjects with low $25(OH)D_3$ levels (<8 ng /ml) had significantly higher CV mortality compared with patients with higher levels (>28 ng/ml) (120). On the other hand,

the NHANES III study, which included data from more than 13,300 participants followed for 8.7 years, showed only a trend toward an increased risk in the lowest (<17.8 ng/ml) compared with the highest 25(OH)D_3 levels (305). A 10-year follow-up study of 755 patients noted no significant association of serum 25(OH)D_3 levels with MI incidence, but an association with stroke was observed (288). In a prospective cohort study, as subset of the MrOS study, 813 men were followed for 4.4 years. No significant association was found between 25(OH)D_3 deficiency (<15 ng/ml) and CVD incidence (coronary heart disease and cerebrovascular attack) compared with vitamin D sufficiency (> 30 ng/ml) (HR = 1.34; 95% CI = 0.65–2.77) (306). A recent meta-analysis of prospective observational studies revealed an inverse relationship between levels of 25(OH)D_3 and risk of CVD, including coronary heart disease, stroke, and total CVD mortality (473). These increased risks for coronary heart disease and ischemic stroke or cerebrovascular disease for individuals with the lowest 25(OH)D_3 levels were also observed in other meta-analyses (60, 61, 82).

Several studies evaluated not only changes in risk with low serum 25(OH)D_3 levels, but also the contribution of higher levels. Most studies suggest that risk does not continue to decrease with levels >30 ng/ml as shown by the Framingham Osteoporosis Study and the NHANES study (228, 305, 474). Some studies even suggested a possible U-shaped relation, with a slight increase in CVD risk at high 25(OH)D_3 levels (>60 ng/ml) (125, 305, 474). The IOM report concluded from the observational data that evidence is provided for an association between low 25(OH)D_3 levels and greater risk of CVD but that evidence is limited to support the view that higher levels of 25(OH)D_3 are linked with a further decrease of risk (Institute of Medicine 2011).

II) Limitations of observational studies. Observational data are potentially subject to residual confounding. Many disease states will reduce activity and thus sun exposure, leading to reverse causality. In addition, several risk factors for CVD are known to lead to low 25(OH)D_3 levels, including age, obesity, smoking, and physical inactivity (291). It is therefore important to investigate the independent effect of vitamin D from these other risk factors.

III) Randomized control trials. The number of RCTs investigating only vitamin D versus placebo with respect to CVD is limited, as often vitamin D supplementation is combined with calcium. In addition, no large RCTs have been published that were designed specifically to test the effect of vitamin D supplementation on cardiovascular events. Indeed, most randomized vitamin D therapy trials to date were designed to investigate its protective skeletal effects, and because of the older age group, many subjects had established cardiovascular disease or risk factors.

One RCT used 100,000 IU four times per year, and they found a nonsignificant trend toward a reduction in CV deaths (RR = 0.84; 95% CI = 0.055–1.10) (452). Another trial added vitamin D to ongoing calcium supplementation and observed a decrease in ischemic heart disease event rates (371). In the large WHI trial, 36,000 women received vitamin D and calcium supplements and were followed for 7 years. No significant effect was reported on MI, coronary heart disease death, or stroke (198). Of note, a low dose of vitamin D (400 IU) was used in this large WHI trial, which may account for the lack of relationship. A recent meta-analysis reported on the few eligible studies and found no significant effect of vitamin D on MI or stroke (131). Together, the evidence from RCTs is currently insufficient to define a relationship between vitamin D and decrease in CV events and to support recommending vitamin D supplementation for lowering CVD risk (131).

b) VITAMIN D AND HYPERTENSION. *I) Observational studies*. The most convincing evidence for the involvement of vitamin D metabolism in CVD is obtained in studies on hypertension. Several cross-sectional studies reported an inverse association between levels of 25(OH)D_3 and the risk of hypertension (218, 291, 401). Also numerous prospective studies have shown an inverse relationship between 25(OH)D_3 levels and blood pressure (BP). A recent meta-analysis reported that 25(OH)D_3 levels were inversely associated with hypertension and with a significant dose-response effect: every 16 ng/ml increase in serum 25(OH)D_3 was associated with a 16% decreased risk of hypertension (64). Another recent large meta-analysis included more than 283,000 participants with a mean follow-up of 9 years (242) and reported a significant inverse association of 25(OH)D_3 levels and risk of incident hypertension (RR = 0.70; 95% CI = 0.57–0.86) when comparing the highest with the lowest tertile of baseline 25(OH)D_3 levels.

II) Randomized control trials. RCTs of vitamin D supplementation offer the highest clinical evidence for establishing whether vitamin D deficiency is causally related to high BP. Results of RCTs have been conflicting and remain inconclusive, with some studies, but not others, suggesting a positive effect of vitamin D supplementation (148, 248, 286, 359, 367, 492). A meta-analysis from 2009 demonstrated a modest but significant decrease in BP with vitamin D treatment especially in patients with elevated mean BP at baseline (492), whereas another meta-analysis did not find a significant effect of vitamin D supplementation on BP (495). More recently, a meta-analysis reported that vitamin D supplementation resulted in a nonsignificant reduction in systolic and diastolic BP (243). A significant decrease in diastolic BP was however observed in participants with preexisting cardiometabolic disease. A recent meta-analysis on a large number of studies found no evidence of vitamin D supplementation on BP reduction (40). Also, two recent RCT in hypertensive patients with low 25(OH)D_3 levels did

not find a significant effect of vitamin D supplementation on BP (24, 365). Together, these findings do not support the use of vitamin D as treatment for hypertension.

c) MECHANISTIC INSIGHT FROM HUMAN STUDIES. Some of the mechanistic findings in mouse models were also observed in humans. Several studies reported an inverse association between $25(OH)D_3$ and parameters of endothelial dysfunction (11, 206, 434) and arterial stiffness (161). Vitamin D deficiency may increase CVD risk by activating an inflammatory cascade, which results in endothelial dysfunction and increased arterial stiffness, both of which contribute to high BP and are risk markers for CVD (160). A small RCT reported that vitamin D supplementation improved arterial stiffness (122). In other observational studies, renin activity and hypertension have been found to be inversely associated with $25(OH)D_3$ levels (64, 149, 366, 450). Another possible mechanism for this association of low vitamin D and high BP is that vitamin D deficiency leads to high PTH levels that are known to be linked with myocardial hypertrophy and higher BP levels (415).

4. Conclusions and future directions

Observational evidence supports an association between low $25(OH)D_3$ levels and both cardiovascular risk factors and cardiovascular events. However, the major problem with epidemiological studies is that 25(OH)D status may just be a surrogate for sociodemographic risk factors and poor metabolic health, thereby confounding any observational associations with CVD. The low $25(OH)D_3$ levels may also be a result of cardiovascular disorders rather than the cause of the disease, as sunlight exposure is the major source of vitamin D. In addition, existing trial data show inconsistent effects on cardiovascular risk factors, and the current evidence therefore does not support routine supplementation with vitamin D to reduce cardiovascular risk at the population level.

The inconsistent results reported by clinical trials have been attributed to several reasons. These include limited sample sizes to detect incremental differences in BP, heterogeneity in study populations, short follow-up periods, and the fact that the majority of trials reported results from post hoc subgroup analyses. Useful insights are therefore expected from the ongoing VITamin D and OmegA-3 TriaL (VITAL), with over 20,000 healthy participants randomized to daily dietary supplements of vitamin D_3 or omega-3 fatty acids during 5 years (285).

C. Immune System

1. Mechanism of $1,25(OH)_2D_3$ action in the immune system

Vitamin D as an immune system regulator was suggested in early studies with the discovery of the presence of VDR in activated T cells (41). More recent studies have shown that $1,25(OH)_2D_3$ regulates both adaptive and innate immunity but in opposite directions. $1,25(OH)_2D_3$ inhibits the adaptive immune response and promotes the innate immune response [for reviews, see Chun et al. (88), Cantorna (68), and Baeke et al. (28)]. The immunosuppressive effect of $1,25(OH)_2D_3$ is correlated with a decrease in inflammatory cytokines including IL-2 and interferon (IFN)-γ (255). The suppressive effect of $1,25(OH)_2D_3$ on IL-2 transcription involves blockage of the formation of the NFAT/AP-1 complex by VDR/RXR and association of VDR/RXR with the NFAT element in the IL-2 promoter as well as sequestration of Runx1 by VDR (16, 217). It has been suggested that the repressive effect of $1,25(OH)_2D_3$ on IFN-γ transcription is due to direct binding of VDR/RXR to a silencer region in the hIFN-γ promoter (92). $1,25(OH)_2D_3$ also stimulates the production of IL-4 by Th2 cells (280). $1,25(OH)_2D_3$ has also been reported to result in an enhancement of T regulatory (T_{reg}) cells (a subset of $CD4^+$T cells important for inhibition of inflammation) (456, 458, 462, 463) and to induce Foxp3 (a lineage specific transcription factor involved in the development and function of T_{reg} cells) (210, 217). IL-17 and IL-9 secreting T cells (Th17 and Th9 cells; distinct from Th1, Th2, and T_{reg} subsets) have also been shown to be $1,25(OH)_2D_3$ targets. IL-17 and IL-9, which have been implicated in many autoimmune diseases, are repressed by $1,25(OH)_2D_3$ (217, 357, 457). The absence of IL-10 signaling completely prevented the inhibitory effect of $1,25(OH)_2D_3$ on Th9 cells but had no effect on $1,25(OH)_2D_3$-mediated inhibition of Th17 cells (348). The mechanism of repression of IL-17 by $1,25(OH)_2D_3$ involves blockage of NFAT binding to the IL-17 promoter, sequestration of Runx1 by VDR, and induction of Foxp3 (which associates with and inhibits NFAT and Runx1 function) (217). Moreover, Palmer et al. (348) demonstrated that $1,25(OH)_2D_3$ diminishes Th17 development partially through inhibition of the transcription factor RORγt, both in the presence and absence of IL-23 signaling. The stimulatory effects of $1,25(OH)_2D_3$ on IL-4 and IL-10 and perhaps other cytokines may be indirect, and the immune response to $1,25(OH)_2D_3$ may be dependent on the interaction of multiple cell types and activation states. In the immune cascade, one of the main targets of $1,25(OH)_2D_3$ is the dendritic cells. Exposure of in vitro differentiating DCs to $1,25(OH)_2D_3$ interferes with their maturation locking the cells in a semi-mature state. The altered dendritic cells have a reduced expression of class II MHC, costimulatory molecules (CD40, CD80, CD86) and a changed IL12/IL10 ratio (145, 174, 356). The mechanism of the repression of IL-12 by $1,25(OH)_2D_3$ has been reported to involve binding of VDR/RXR to the NF-kappaB site in the IL-12p40 promoter (104). The dendritic cells are able to alter the behavior of T lymphocytes, inducing T cell anergy and increasing apoptosis levels while shifting T cell cytokine responses from a pro-inflammatory, with T-helper(Th)1 and Th17, to a more tolerogenic one, with Th2 and T regulatory cells

(145). Moreover $1,25(OH)_2D_3$-treated mouse NOD dendritic cells exhibit an intact functional migratory capacity and successfully dampen proliferation of activated T cells in vivo (144). Recent data demonstrate that $1,25(OH)_2D_3$ affects the phenotype and behavior of dendritic cells through its early and transcriptionally mediated reprogramming of metabolic pathways, namely, the increase of glycolysis and oxidative phosphorylation at the same time (146). We are only beginning to understand the factors involved in the regulation of the immune system by vitamin D. Although global networks regulated by VDR are beginning to be addressed in immune cells, further studies related to genome-wide, proteome, and metabolic analyses are needed to define multiple roles of vitamin D in immune function.

2. In vivo studies in mouse models of autoimmunity

The physiological significance of these effects of $1,25(OH)_2D_3$ on the immune system has been suggested by in vivo studies in mouse models of autoimmunity. $1,25(OH)_2D_3$ can protect against a number of experimental autoimmune diseases including inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE; mouse model for multiple sclerosis, MS) (110). Dietary calcium has been reported to be required for the suppressive effect of $1,25(OH)_2D_3$ on IBD and EAE (70, 110). $1,25(OH)_2D_3$ has also been reported to reverse as well as to prevent paralysis of EAE mice (69, 217). The protective effect of $1,25(OH)_2D_3$ in EAE is associated with inhibition of IL-12 and IL-17 and requires IL-10 signaling (217, 298, 421). Not only $1,25(OH)_2D_3$ but also high-dose dietary vitamin D was found to attenuate EAE. The combination of high-dose dietary vitamin D (20 IU/g diet) and IFN-β was more effective than either high-dose dietary vitamin D alone or IFN-β alone in diminishing paralysis in mice with ongoing EAE **(FIGURE 8)**. These findings provide a rationale for trials combining vitamin D and IFN-β in MS patients. With regard to IBD, VDR KO mice show more severe IBD which is associated with overproduced IFN-γ and IL-17 cells (68). Recently several studies demonstrated the importance of the VDR expression specifically in intestinal epithelial cells in IBD (232, 269a, 494). Treatment with $1,25(OH)_2D_3$ of experimentally induced colitis results in inhibition of Th1 and Th17 cells, induction of T_{reg} cells, and reduced inflammation (71, 106). Protection against autoimmune diabetes in nonobese diabetic (NOD) mice by $1,25(OH)_2D_3$ has also been reported. Induction of Treg cells and decreased numbers of effector T cells have been suggested as the basis for this protection by $1,25(OH)_2D_3$ (173, 294, 437). Although these findings are suggestive of a protective effect of $1,25(OH)_2D_3$ against the pathogenesis of autoimmune inflammation, whether vitamin D supplementation or treatment with analogs of $1,25(OH)_2D_3$ is beneficial clinically in the treatment of autoimmune diseases is not known. Adequately powered, RCTs are needed to demonstrate the suggested benefit of vitamin D.

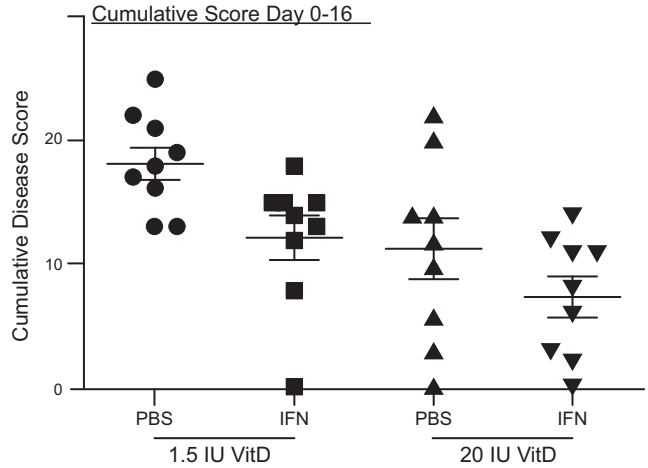


FIGURE 8. Cooperative effects of dietary vitamin D and IFN-β treatments in attenuation of EAE. On *day 0*, EAE was induced by immunization with myelin oligodendrocyte glycoprotein (MOG) p35-55 in C57BL/6 mice and scored daily for degree of paralysis. On *day 5*, the diet was changed to 20 IU vitamin D/g diet or left at 1.5 IU vitamin D/g diet. On *day 7*, treatment with IFN-β (a first line treatment for multiple sclerosis) was initiated and continued every other day through *day 16*. The combination of high-dose vitamin D and IFN-β was more effective than high dietary vitamin D alone or IFN-β alone in diminishing paralysis in EAE (R. Axtell, L. Steinman, and S. Christakos, unpublished data).

3. Effects of vitamin D on innate immunity

With regard to effects on innate immunity, in vitro studies have shown that $1,25(OH)_2D_3$ induces the antimicrobial peptide cathelicidin in both myeloid and epithelial cells with the subsequent killing of bacteria (170, 268, 486, 504). Despite significant in vitro evidence showing a negative effect of $1,25(OH)_2D_3$ on bacterial growth, relatively few studies have examined the effect of $1,25(OH)_2D_3$ in vivo on host resistance against bacteria. The studies in experimental animal models of infection have not supported a consistent beneficial or adverse effect of vitamin D. Vitamin D-deficient mice are more susceptible to *Mycobacterium bovis* infection than WT mice due to an effect on NO production (482). Using *Vdr* KO mice, it was shown that VDR is not required for the clearance of *L. monocytogenes* following either primary or secondary infection (63). $1,25(OH)_2D_3$ treatment was reported to impair host defense against colitis induced by *Citobacter rodentium* (388). $1,25(OH)_2D_3$ treatment of infected mice resulted in increased pathogen burdens, exaggerated tissue pathology, and significantly reduced numbers of Th17 cells. Although IL-17 plays a pathological role in inflammatory diseases, this cytokine also plays a protective role against infection. IL-17 can enhance host defense by induction of antimicrobial peptides and by induction of chemokines to recruit neutrophils (222, 230, 503) It was suggested that $1,25(OH)_2D_3$ by suppressing Th17 T cell responses in vivo resulted in impaired host defense against *Citrobacter rodentium* (388). Thus $1,25(OH)_2D_3$ treatment can be a double-edged sword. Fur-

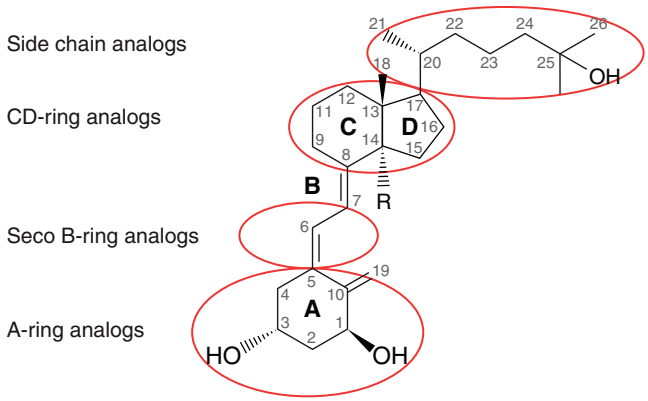


FIGURE 9. Chemical structure of $1,25(OH)_2D_3$ and possible analog modifications.

ther in vivo data are needed to support a relationship between vitamin D and host resistance to infection.

VI. VITAMIN D ANALOGS

A. Analog Development: Introduction

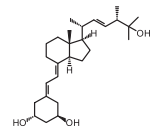
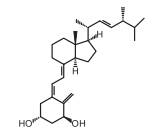
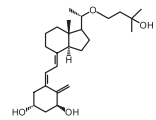
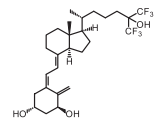
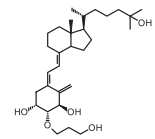
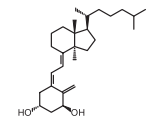
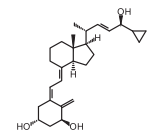
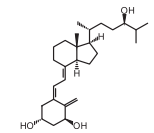
Since the discovery that supraphysiological doses of $1,25(OH)_2D_3$ (calcitriol) can stimulate the differentiation of promeylocytic leukemia cells towards mature macrophages (3) and can inhibit the proliferation of melanoma cells (95), academic and nonacademic institutions started to develop analogs of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ is a very flexible molecule that can be structurally modified to yield analogs with altered biological properties. Chemical modifications **(FIGURE 9)** were introduced in the A-ring, seco-B ring, central CD-region, or side chain of $1,25(OH)_2D_3$ with the objective to minimize the calcemic side effects and keep or increase the antiproliferative or prodifferentiating effects of $1,25(OH)_2D_3$ (53). Until the end of the 20st century, this new field of vitamin D analog research was based on a "trial and error" approach. Once the biological profile of some analogs was characterized, it became possible to predict the biological implications of some, but not all, chemical modifications ("educated guess"). Several modifications such as 19-nor, 16-ene, 23-yne, and 20-epi were shown to diminish the calcemic effects and/or enhance the antipoliferative effects of the mother compound. In 2000, the group of Moras and Rochel (384) was able to crystallize the VDR (see sect. III*A1*), and this VDR crystal model led to the rational design of a second generation of vitamin D analogs (carbon-2 substituted analogs; gemini analogs). In this overview we will first focus on analogs that are used for disorders linked to the classical effects of $1,25(OH)_2D_3$ to regulate calcium and bone homeostasis. A second group of analogs are characterized by a dissociation of the calcemic effects and the nonclassical effects of $1,25(OH)_2D_3$ on proliferation and differentiation. These analogs have the eligible profile for the treatment of hyperproliferative disorders such as psoriasis and cancer. Next we discuss the possible mechanism of action of these compounds.

B. Analogs Related to the Classical Function of $1,25(OH)_2D_3$

1. Kidney disease

Therapy with $1,25(OH)_2D_3$ in patients with CKD can lead to an increase in calcium and phosphorus absorption with hypercalcemia and hyperphosphatemia as a result (103). The existence of vitamin D analogs, which selectively decrease PTH levels without a major rise of serum calcium levels, opened new perspectives for the commercialization of these compounds for chronic renal failure (**FIGURE 10**). Paricalcitol and doxercalciferol are vitamin D_2 analogs approved for therapeutic use of SHPT. In Japan, falecalcitriol and maxacalcitol are used to treat this disorder. Paricalcitol, maxacalcitol, and falecalcitriol bind directly to the VDR, whereas doxercalciferol needs analogous to alfacalcidol a 25-hydroxylation step in the liver to become active and is independent of renal or extrarenal 1α-hydroxylase.

Seventy-eight patients with end-stage renal disease (ESRD) and receiving hemodialysis were studied in three identical double-blind, placebo controlled, randomized, multicenter trials to evaluate the safety and efficacy of paricalcitol (290). Patients under calcitriol treatment received a 2-wk washout period before weekly measurements of the baseline biochemical parameters of intact PTH, serum calcium, and phosphorus for 2 wk. Biochemical parameters were also measured in patients without calcitriol therapy. After the 2-wk baseline period, patients were randomized in a placebo or paricalcitol (intravenous) treatment group during 12 wk. The dose of paricalcitol was increased as long as PTH did not decrease for more than 30%, serum calcium levels did not increase for more than 11.5 mg/dl, and calcium-phosphorus product was less than 75. PTH levels decreased from 785 $\pm$ 66 to 370 $\pm$ 73 pg/ml during the study of 12 wk in the paricalcitol group, whereas PTH levels did not change in the placebo group. The mean serum calcium levels increased significantly but remained in the normal range at the end of the study (9.56 $\pm$ 0.15 mg/dl), and serum phosphorus levels did not change significantly. There were some episodes of serum calcium levels $\geq$11 mg/dl but very few in number. Limited RCTs are available in which paricalcitol or other vitamin D analogs are compared with calcitriol treatment. Studies in which the dose of paricalcitol was evaluated suggested that for the same suppressive effects, paricalcitol should be dosed at a ratio of 4:1 to calcitriol (289), although other studies indicated a 3:1 ratio (271). A double-blind randomized multicenter study (422) compared the effectiveness of paricalcitol and calcitriol, both injected intravenously in ESRD patients undergoing hemodialysis. Patients with paricalcitol therapy showed a significantly faster $\geq$50% reduction from baseline PTH than did the calcitriol-treated patients, and only the paricalcitol-treated patients had PTH levels in the desired range between 100 and 300 mg/ml. Paricalcitol treatment induced less episodes of hypercal-

Name	*Brand name*	*Indication*	*Structure*	*VDR binding*	*DBP binding*	*CYP24A1 metabolisation*
Paricalcitol (19-nor-1,25$(OH)_2D_2$)	Zemplar® (Abbott)	secondary hyperparathyroidism		1	1	yes
Doxercalciferol (1α-(OH)-D_2)	Hectorol® (Genzyme corp)	secondary hyperparathyroidism		<<1	?	yes
Maxacalcitol (22oxa-1,25$(OH)_2D_3$)	Oxarol® (Chugai Pharmaceutical)	secondary hyperparathyroidism and psoriasis (Japan only)		<1	<0.002	yes
Falecalcitriol (26,27 F6-1,25$(OH)_2D_3$)	Fulstan® (Dainippon Sumitomo) and Hornel® (Taisho Yakuhin)	secondary hyperparathyroidism (Japan only)		<1	<1	resistant
Eldecalcitol (ED71)	Edirol® (Chugai Pharmaceutical)	osteoporosis (Japan only)		<1	4	resistant
Alfacalcidol (1α-(OH)-D3)	Alfarol® (Chugai Pharmaceutical) One-Alpha® (Leo Pharm) …	osteoporosis renal osteodystrophy hyperparathyroidism …		<0.01	<1	yes
Calcipotriol (MC903)	Daivonex®, Dovonex® (LEO Pharma), Sorilux® (Stiefel)	psoriasis		≤1	≤ 0.5	yes
Tacalcitol (1α,24(R)$(OH)_2D_3$)	Curatoderm® (Almirall Hermal), Bonalfa® (ISDIN, Teijin Pharma),…	psoriasis		0.5	0.3	yes

The VDR and DBP binding affinities of 1,25$(OH)_2D_3$ are set to 1.

The VDR and DBP binding affinities of the analogs (see Bouillon et al., 53).

FIGURE 10. Overview of clinically approved vitamin D analogs.

cemia and increased calcium-phosphorus product compared with calcitriol therapy. Also in chronic hemodialysis patients with SHPT paricalcitol intravenous therapy seemed to be superior compared with calcitriol therapy in a single-center randomized open label study (2). Moreover, paricalcitol has a small effect on intestinal calcium absorption compared with calcitriol in hemodialysis patients (273).

Doxercalciferol is mainly used for the treatment of moderate to advanced CKD (353). Intermittent intravenous or oral doxercalciferol therapy in hemodialysis patients with SHPT (152, 301) decreased PTH levels. The study consisted of a washout period (8 wk), an open-label treatment with doxercalciferol (16 wk), and a randomized, double-blinded treatment with doxercalciferol or placebo (8 wk). Baseline PTH levels (897 ± 52 pg/ml) decreased by 20 ± 3.4% by week 1 and by 55 ± 2.9% at week 16. PTH levels returned to baseline during placebo treatment but remained suppressed with doxercalciferol therapy. During open-label treatment, serum calcium levels were 9.2 ± 0.84 to 9.7 ± 1.05 mg/dl and phosphorus levels

were 5.4 ± 1.10 to 5.9 ± 1.55 mg/dl. During double-blinded treatment, serum calcium levels were slightly increased with doxercalciferol compared with the placebo group, but phosphorus levels did not differ. A RCT with doxercalciferol in patients with SHPT associated with CKD stages 3 and 4 (94) demonstrated a decrease of ≥30% in PTH levels in 74% of the treated patients after 24 wk of oral therapy compared with 7% of the patients in the placebo group. No significant differences were observed between both groups in mean serum calcium or phosphorus levels or incidence of hypercalcemia or hyperphosphatemia. A prospective clinical trial was done in South Asian patients with CKD stage 4 and confirmed that doxercalciferol is effective in suppressing SHPT with an acceptable risk of hypercalcemia and hyperphosphatemia (116). A prospective study in 60 pediatric patients with CKD on peritoneal dialysis and doxercalciferol or calcitriol therapy for 8 mo (in combination with either calcium, carbonate, or sevelamer) demonstrated the same potency of both agents to reduce PTH levels, bone turnover, and FGF23 levels (485). Limited studies compared the efficacy of doxercalciferol with paricalcitol. High-dose paricalcitol (160 μg) was compared with high-dose doxercalciferol (120 μg) in a prospective study of 13 hemodialysis patients (212), and the degree of PTH suppression was similar in both groups; however, the serum phosphorus levels increased faster and higher in the doxercalciferol-treated patients compared with the paricalcitol treated patients. A dose equivalency study was performed on chronic hemodialysis patients treated with a stable dose of paricalcitol for at least 3 mo and were randomized to be treated with a dose of doxercalciferol equivalent to either 35, 50, or 65% of the paricalcitol dose for 6 wk. Serum PTH, calcium, phosphorus, and albumin were determined at baseline and monitored every 2 wk. A linear regression analysis of percent change in PTH values by dose group was performed to determine the conversion factor (527). In patients on a maintenance dose of paricalcitol, dosing doxercalciferol at 55–60% of the paricalcitol dose resulted in comparable inhibition of PTH. Paricalcitol and doxercalciferol are used in the United States to treat SHPT, whereas falecalcitriol and maxacalcitol are applied in Japan. Comparative studies with maxacalcitol and calcitriol are equally potent in reducing PTH levels and are equally safe (339, 438). Maxacalcitol (intravenous) and paricalcitol (intravenous) were recently tested for 12 wk in a double-blind controlled study in 255 Japanese CKD patients with SHPT on hemodialysis. Both intravenous paricalcitol (27.7%) and maxacalcitol (30.5%) were equally effective in reducing PTH without the presence of hypercalcemia during treatment (10).

The treatment of CKD with vitamin D derivatives (calcitriol etc.) or vitamin D analogs has primarily focused on the outcome of biochemical parameters [PTH, calcium, phosphorus, $25(OH)D_3$, or $1,25(OH)_2D_3$ levels] (231) but also intermediate (vascular calcifications, bone density, and histology) or hard clinical end points (cardiovascular disease, mortality, fractures. . .) should be investigated in prospective RCTs. There have been several observational studies which demonstrated a survival benefit in CKD patients on dialysis (314); however, a recent meta-analysis of RCTs evaluating the effects of oral vitamin D supplementation (cholecalciferol, doxercalciferol, paricalcitol, alfacalcidol) compared with placebo on overall all-cause mortality (RR = 0.84; 95% CI = 0.47, 1.52), cardiovascular mortality (RR = 0.79; 95% CI = 0.26, 2.28) and serious adverse cardiovascular events (RR = 1.20; 95% CI = 0.49, 2.99) in CKD patients did not show convincing evidence (284). The studies included in this meta-analysis showed a large variation in the doses used and the treatment period. A meta-analysis of RCTs investigating the effect of vitamin D therapy in nondialysis CKD patients on proteinuria and the progression of CKD together with adverse events such as hypercalcemia (497) showed that this treatment decreased proteinuria and had no negative influence on renal function. However, there was an increased risk of developing hypercalcemia compared with the control patients. Moreover, there was no superiority found for newer vitamin D analogs (paricalcitol, doxercalciferol, maxacalcitol, falecalcitriol) versus established vitamin D compounds (ergocalciferol, cholecalciferol, 25D3/calcidiol, calcitriol). The VITAL double-blind, placebo-controlled randomized-controlled study (98) included patients with CKD stages 2–4 and demonstrated in a post hoc analysis that paricalcitol dose-dependently reduced PTH levels but with a modest increase in calcium and phosphate. Moreover, paricalcitol reduced bone-specific alkaline phosphatase which may be beneficial for inhibiting vascular calcification. In the PRIMO trial, 227 CKD stage 3 and 4 patients with mild left ventricular hypertrophy but with normal systolic function were randomized to paricalcitol or placebo treatment (443). The primary end point was a change in ventricular mass over 48 wk, but the paricalcitol treatment was not significantly different from the placebo group, although the risk of hospitalizations was lower in the paricalcitol group (1.1 vs. 8.8 per 100 persons years). Paricalcitol was able to suppress PTH to 30% of baseline in 85.7% of patients compared with 16.5% in the placebo group, but there was an increase in serum calcium and phosphorus.

In conclusion, a lot of clinical studies with vitamin D analogs were performed, and for some of them it is not yet completely clear if these compounds are indeed superior to calcitriol to diminish hypercalcemia and hyperphosphatemia. For this reason, more elaborate RCTs should be performed with several vitamin D analogs together such as maxacalcitol, paricalcitol, doxacalciferol, and compared with calcitriol while taking into account biochemical parameters but also patient-centered end points (CKD progression, fractures, mortality, cardiovascular disease).

2. Osteoporosis

Calcium (1 g) and vitamin D (cholecalciferol, 800 IU) supplementation is given to prevent or treat osteoporosis, but also pharmacological intervention with bisphosphonates, selective estrogen receptor modulators (raloxifene), denosumab, strontium relanate, and PTH peptides are fre-

quently used for osteoporosis treatment (221). Both alfacalcidol and calcitriol are used in some countries including Japan for the treatment of osteoporosis. Alfacalcidol is metabolized to calcitriol by its 25-hydroxylation in the liver. Both compounds have been proven to be active in increasing bone mineral density (BMD) and in reducing vertebral and nonvertebral fractures in prospective, randomized, and placebo-controlled trials (335). In a randomized, double-blind, double-dummy, parallel group study alfacalcidol was superior to vitamin D plus calcium in increasing BMD in postmenopausal osteoporosis. Lumbar BMD in the alfacalcidol arm increased 2.33% compared with baseline levels after 12 mo and 2.87% after 18 mo, whereas in the vitamin D plus calcium group there was only an increase of 0.7% at both time points. At the end of the study, serum calcium concentration was slightly higher in the alfacalcidol group versus vitamin D plus calcium (9.56 ± 0.48 vs. 9.45 ± 0.49 mg/dl) but was still within the safety margin (335). Eldecalcitol is a vitamin D analog with a hydroxypropyloxy group at the carbon 2β-position of $1,25(OH)_2D_3$ which is approved in Japan since 2011 for the treatment of osteoporosis. In a placebo-controlled randomized-controlled trial, eldecalcitol suppressed in a dose-dependent manner bone turnover and increased lumbar spine and total hip BMD in osteoporosis patients with sufficient vitamin D supply. A lower incidence of hypercalcemia was observed with 0.75 μg/day eldecalcitol compared with 1.0 μg/day (297). In a phase III randomized, double-blind comparative study, the efficacy of eldecalcitol (0.75 μg/day) was compared with alfacalcidol (1.0 μg/day) over 3 years in osteoporotic patients receiving 400 IU vitamin D if serum $25(OH)D_3$ was less than 20 ng/ml (296). Eldecalcitol was more potent to increase BMD and decrease the frequency of vertebral and wrist fractures compared with alfacalcidol treatment. The incidence of hypercalcemia above 11.5 mg/dl was very low in both groups (2 in eldecalcitol and 0 in alfacalcidol group), but the increase of serum and urinary calcium was significantly higher with eldecalctiol therapy.

A vitamin D analog which possesses bone anabolic effects and could possibly be used for the treatment of osteoporosis is 2-methylene-19-nor-20(*S*)-$1,25(OH)_2D_3$ (2MD). In ovariectomized rats 2MD restored trabecular and cortical bone mass and strength and enhanced bone-mobilizing activity (226). A randomized, double-blind, placebo-controlled trial in postmenopausal women with osteopenia demonstrated that a daily oral treatment for 1 year with 2MD (220 or 440 ng) increased bone resorption and bone formation markers; however, it did not change bone mass (109). In this study 2MD suppressed serum PTH levels dose-dependently without elevating serum calcium levels at the lowest dose of 220 ng/day. Recently a dose-finding study with 2MD in postmenopausal women confirmed the PTH suppressive effects of 2MD without calcemic side effects (509). A distribution study performed in rats showed that radioactive 2MD was accumulated in the classical target tissues of vitamin D (bone, intestine) but was most strongly localized in the thyroid/parathyroid (509). In a 5/6 nephrectomy rat model of renal failure, 2MD suppressed PTH dose-dependently, and a side-to-side experiment with paricalcitol demonstrated that the effective dose of 2MD was 40 times lower than paricalcitol without increasing serum calcium levels (509). Currently 2MD is under evaluation in phase 2 study in dialysis patients.

C. Analogs Related to the Nonclassical Effects of $1,25(OH)_2D_3$

1. Psoriasis

The epidermis has a pivotal position in the vitamin D system since it is the site of photosynthesis of vitamin D which can be further converted locally to $25(OH)D_3$ (CYP27A1 and CYP2R1 enzyme) and finally to active $1,25(OH)_2D_3$ (CYP27B1 enzyme). Moreover, the VDR is present in the skin and $1,25(OH)_2D_3$ can induce differentiation at 10^{-10} M whereas it has a dual effect on the proliferation of keratinocytes. At physiological doses (picomolar), $1,25(OH)_2D_3$ promotes proliferation and at supraphysiological concentrations $1,25(OH)_2D_3$ inhibits proliferation. Psoriasis is a chronic autoimmune inflammatory skin disorder, and patients have erythematous scaling plaques that are the result of keratinocyte hyperproliferation and abnormal differentiation (397). Calcipotriol, tacalcitol, and the more recent approved maxacalcitol are used either as monotherapy or in combination with topical steroids to treat psoriasis. The vitamin D analogs exert prodifferentiating and antiproliferative effects on keratinocytes and also possess important anti-inflammatory properties (418). Topical calcipotriol increased the expression of VDR on epidermal keratinocytes which enhances its potent effects on cell proliferation and differentiation (378). However, not all patients with psoriasis respond well to vitamin D analog treatment, and the responsiveness can be correlated to the increase in VDR mRNA in treated skin areas (77). Pro-inflammatory cytokines TNF-α, IFN-γ, IL-2, and IL-8 play a critical role in the T-cell-mediated inflammatory process in the psoriatic skin (397). Thirty patients with moderate plaque-type psoriasis treated with calcipotriol or vehicle showed an increase in IL-10 levels (pg/mg protein) and a decrease in IL-8 levels (pg/mg protein) in skin plaques when treated with calcipotriol compared with baseline levels at the start of the treatment (220), whereas both cytokines were unaffected by vehicle treatment. Moreover, calcipotriol suppressed the Th17 cytokine-mediated production of psoriasin and koebnerisin, two antimicrobial chemoattractant peptides that amplify psoriatic inflammation (185). The cytokine lymphopoietin which induces Th2 differentiation and inhibits IL-12/23 production was upregulated by topical calcitriol or calcipotriol treatment as well as the antimicrobial peptide cathelicidin (393). Calcipotriol has a very short half-life, and it has been proven that it is very effective and safe in the topical treatment of psoriasis (451). Maxa-

calcitol was more potent to diminish erythema and scaling compared with calcipotriol in a double-blind, placebo-controlled comparator study (33). Topical tacalcitol ointment was very potent to reduce the psoriasis area severity index (PASI) without calcemic side effects (461). The adverse side effect noted for all vitamin D analogs is skin irritation. Combination therapies targeting immune-mediated inflammation together with epidermal changes provided improved efficacy and safety versus monotherapy (451).

2. Cancer

More than 3,000 vitamin D analogs were developed worldwide and several analogs demonstrated more potent antiproliferative and prodifferentating effects on cancer cell lines compared with $1,25(OH)_2D_3$. Moreover, some analogs demonstrated a dissociation between anti-cancer and calcemic side effects in several mice models of cancer (xenografts, chemically induced). In view of these promising results, a limited number of analogs have been tested in cancer patients. Seocalcitol was the first analog that has been explored in advanced breast and colon cancer (177) and inoperable pancreatic cancer (138); however, no clear anticancer activities were observed. A very small effect was seen with seocalcitol in inoperable hepatocellular carcinoma in which 2 of 33 patients showed a complete response (105). Some of the analogs that are approved for SHPT have also been tested on their anti-tumor capacity in urogenital neoplasms. Doxercalciferol (265) or paricalcitol (399) was mainly tested in patients with androgen-independent prostate cancer, but only doxercalcifereol could stabilize the disease in 6 of the 20 patients for over 6 mo in a phase II study (265). Doxercalciferol was also evaluated in localized prostate cancer and high-grade prostatic intraepithelial neoplasia, but no beneficial effects were noticed in serum and tissue markers (159). The anti-psoriatic vitamin D analog calcipotriol was applied topically at a dose of 100 μg/day in patients with locally advanced or cutaneous metastases from breast cancer. One study reported no response after 3 mo of treatment (338), while in the other study, three patients showed a 50% reduction in the diameter of treated lesions after 6 wk (56). The 16-ene-23-yne-$1,25(OH)_2D_3$ (ILX23-7553) analog showed in in vitro and in vivo animal models potent antitumor properties with diminished calcemic side effects (333) and was recently investigated in 16 patients with advanced solid tumors, but no objective response was observed (208). Since the monotherapeutic approach was not really successful, several clinical trials were set up to combine vitamin D analogs with established protocols in cancer therapy (chemotherapy, radiotherapy, ...). The 14-epi-19-nor-23-yne-$1,25(OH)_2D_3$ analog inecalcitol was given orally to patients with hormone-refractory prostate cancer in combination with docetaxel for maximum 18 wk. This study had a response rate of 85% based on a PSA decline of at least 30% within 3 mo of treatment. This analog has now successfully been tested in a phase II study in chronic lymphocytic leukemia (CLL) at a dose of 2 mg daily after which 52% of patients experienced stabilization or decrease of blood leukemic lymphocytes counts. Based on the promising results of inecalcitol in CLL a phase II study is planned with 4 mg/day given to chronic myeloid leukemia patients under treatment with oral imatinib (website Hybrigenics). A placebo-controlled study with oral doxercalciferol (10 μg/day) in combination with weekly docetaxel for 4 wk did not enhance PSA response rate or survival (25). Oral paricalcitol was recently shown to be safe in women with metastatic breast cancer receiving taxane-based chemotherapy (249).

Vitamin D analogs possess potent anti-cancer effects in cancer cell cultures and in animal cancer models, but these effects could not be confirmed in human studies. In contrast to chemotherapeutics, vitamin D analogs are not cytotoxic and should therefore be combined with established cancer therapies.

D. Mechanism of Action

The mechanisms of action of the analogs with increased antiproliferative, prodifferentiating effects and/or decreased calcemic side effects are not yet fully understood and are probably based on a combination of several phenomena. Differences in pharmacokinetics may contribute to lower calcemic activity because most of these analogs display a low affinity for DBP and therefore their free concentration approaches their total plasma concentration which results in rapid extracellular clearance. Moreover, altered pharmacokinetics may also explain the enhanced antiproliferative effects since the analog will quickly reach high peak levels in target tissues compared with the slow rise and drop of $1,25(OH)_2D_3$. Maxacalcitol has a short half-life due to 500 times lower affinity to DBP (341). Eldecalcitol on the contrary has a fourfold stronger affinity for DBP than $1,25(OH)_2D_3$ (381) which results in longer sustained plasma levels than $1,25(OH)_2D_3$. A recent study manually docked calcitriol and eldecalcitol in the crystal structure of DBP (236) and demonstrated that calcitriol binds to DBP via three hydrogen bonds, whereas eldecalcitol binds to DBP via three additional hydrogen bonds that interact with the 3-hydroxypropyloxy group.

Also, the intracellular metabolism might be different from that of $1,25(OH)_2D_3$ due to chemical modifications (side chain fluorination, 20-epimerization) that make the analogs more resistant to catabolism by CYP24A1. Hence, the cells are exposed for a longer period to the analog (213). Eldecalcitol binds more weakly to VDR [less than half of that of $1,25(OH)_2D_3$] but is more potent to induce mRNA expression of CYP24A1 than calcitriol. However, eldecalcitol is not metabolized by CYP24A1, which may also prolong the activity of this analog in target tissues (381). At the cellular level, the antiproliferative activity seems to correlate well with the ability of an analog to promote interaction between VDR and coactivator proteins (130). The elucidation of the crystal structure of the ligand-binding domain of

VDR and the determination of the exact interaction of $1,25(OH)_2D_3$ with amino acids in the ligand-binding pocket demonstrated an expansion of this binding pocket near the position of carbon 2 of the A-ring of the ligand (384). Vanhooke et al. (465) crystallized the ligand-binding domain of the VDR with the carbon 2 analog 2MD in complex with a LXXLL containing coactivator peptide and showed that the LBD bound to 2MD was unchanged compared with the VDR-LBD-$1,25(OH)_2D_3$ complex. On the other hand, rat VDR-LBD studied in solution by NMR spectroscopy showed different chemical shifts when bound to 2MD compared with $1,25(OH)_2D_3$ (413). The 14-epi analog inecalcitol was cocrystallized with VDR-LBD (129) and the 17-methyl D-ring analog CD 578 with VDR-LBD together with an SRC-1 coactivator peptide with second LXXLL-motif containing NR box (128). Both studies indicated that the analogs did not induce major differences in the protein conformation upon binding with the LBD compared with binding with $1,25(OH)_2D_3$ but induce rather subtle differences such as closer or additional contacts to certain amino acid residues allowing more stable interactions with coactivators. Approximately 10-fold lower doses of inecalcitol are needed, compared with $1,25(OH)_2D_3$, to acquire the same amount of coactivator interactions (129). The crystal structures of VDR-LBD bound to 20-epi vitamin D analogs [KH1060, 20-epi-$1,25(OH)_2D_3$], seocalcitol, or calcipotriol also demonstrated that all compounds are anchored to the same residues in the LBD via the hydroxyl groups of the A-ring and the side chain, therefore they are locked in identical positions and form the same hydrogen bonds (447, 448). New insights were gathered by the synthesis of the Gemini analog which has two identical side chains branching at carbon 20 and is characterized by less VDR binding affinity compared with $1,25(OH)_2D_3$ but equal or more transactivation potency (332). The VDR-Gemini complex can switch from agonist to an inverse agonist confirmation in the presence of an excess of corepressor N-Cor (172). The Gemini-VDR complex revealed that the binding of a ligand with 25% increased volume did not change the overall structure of the LBD including the position of helix H12 compared with VDR-LBD-$1,25(OH)_2D_3$ complex; however, an extra channel was opened to accommodate the second side chain (91). Several derivatives (two different side chains) of the original Gemini analog were developed to enhance its biological potency (251), for example, by decreasing CYP24A1 metabolic degradation via carbon-23 unsaturation or 26,27-fluorination. The crystal structures of such Gemini derivatives (Gemini-0072, -0097) with zebrafish zVDR-LBD and a GRIP1/TIF2-coactivator peptide containing LXXLL motif, showed that the second side chains induced an extra cavity within the LBD comparable to the original Gemini and that additional interactions of the side chain fluorine atoms stabilize helix H12 allowing increased interactions with coactivator proteins (201).

More recently, ChIP studies are performed to examine VDR binding sites throughout the genome after treatment with $1,25(OH)_2D_3$ or analogs. Such a study compared the binding sites of the VDR in intestinal tissue after $1,25(OH)_2D_3$ or 20-epi-$1,25(OH)_2D_3$ analog treatment and showed that both compounds induce VDR binding to *CYP24A1* and *TRPV6* loci in the intestine, but the analog elicited a prolonged VDR binding to these genes leading to its superagonistic characteristics such as hypercalcemia in vivo (512). In osteoblast cell models, 2MD bound to the VDR was able to bind VDREs at lower concentrations compared with $1,25(OH)_2D_3$ (500). ChIP and Re-ChIP assays revealed that eldecalcitol mediates dissociation of Williams syndrome transcription factor (WSTF) from the aromatase *CYP19A1* promoter and thereby decreases the gene expression of aromatase in MCF-7 cells (274). Microarray studies demonstrated that $1,25(OH)_2D_3$ as well as vitamin D analogs regulate the expression of genes involved in several signaling pathways in a cell type and tissue specific manner (241). Moreover, on the same tissue, $1,25(OH)_2D_3$ or vitamin D analogs up or downregulate the expression of the same set of genes (241).

In conclusion, several analogs are used to treat SHPT, osteoporosis, or psoriasis; however, most of the clinical trials testing vitamin D analogs for cancer treatment were not really convincing. The concept that vitamin D analogs can be used for cancer therapy should be further explored by combining analogs with standard protocols for cancer therapies as well as by examining the correct duration and timing of administration. The exact working mechanism is not completely unraveled, but new findings that vitamin D can induce epigenetic modulations should be further explored.

VII. CONCLUSIONS AND FUTURE DIRECTIONS

New target genes involved in the multiple actions of $1,25(OH)_2D_3$ in numerous different systems will undoubtedly be identified. New insight will be obtained with regard to regulatory pathways, novel transcription factors, and epigenetic modifications involved in mediating these diverse biological responses. We will also obtain an increased understanding of the structure of VDR in the presence of protein partners thus facilitating selective modulation of $1,25(OH)_2D_3$ action in different target tissues. It is likely that future studies will reveal that posttranscriptional mechanisms are also an important mechanism controlling the expression of vitamin D target proteins. Further studies are also needed using null and transgenic mice to increase our understanding of vitamin D biology. The main function of vitamin D is increased intestinal calcium absorption. However, we are far from understanding the mechanisms involved. To identify new therapeutic approaches to sustain calcium balance, the multiple mechanisms by which $1,25(OH)_2D_3$ acts in both proximal and distal segments of the intestine need further definition. Although there is compelling evidence from the laboratory of extraskeletal beneficial effects

of $1,25(OH)_2D_3$, conclusive clinical data for the use of vitamin D and analogs for the treatment or prevention of a number of disease processes are not yet available. Although there are many differences between the human condition and mouse models, since many genes function similarly in animals and humans, results in animals models may unravel complex signaling pathways similarly affected in humans. These findings could lead to the identification of novel targets for chemoprevention and chemotherapy.

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