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# Vitamin D

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**Dusso, Adriana S., Alex J. Brown, and Eduardo Slatopolsky.** Vitamin D. *Am J Physiol Renal Physiol* 289: F8–F28, 2005; doi:10.1152/ajprenal.00336.2004.—The vitamin D endocrine system plays an essential role in calcium homeostasis and bone metabolism, but research during the past two decades has revealed a diverse range of biological actions that include induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems. Vitamin D itself is a prohormone that is metabolically converted to the active metabolite, 1,25-dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$ ]. This vitamin D hormone activates its cellular receptor (vitamin D receptor or VDR), which alters the transcription rates of target genes responsible for the biological responses. This review focuses on several recent developments that extend our understanding of the complexities of vitamin D metabolism and actions: the final step in the activation of vitamin D, conversion of 25-hydroxyvitamin D to  $1,25(\text{OH})_2\text{D}$  in renal proximal tubules, is now known to involve facilitated uptake and intracellular delivery of the precursor to  $1\alpha$ -hydroxylase. Emerging evidence using mice lacking the VDR and/or  $1\alpha$ -hydroxylase indicates both  $1,25(\text{OH})_2\text{D}_3$ -dependent and -independent actions of the VDR as well as VDR-dependent and -independent actions of  $1,25(\text{OH})_2\text{D}_3$ . Thus the vitamin D system may involve more than a single receptor and ligand. The presence of  $1\alpha$ -hydroxylase in many target cells indicates autocrine/paracrine functions for  $1,25(\text{OH})_2\text{D}_3$  in the control of cell proliferation and differentiation. This local production of  $1,25(\text{OH})_2\text{D}_3$  is dependent on circulating precursor levels, providing a potential explanation for the association of vitamin D deficiency with various cancers and autoimmune diseases.

vitamin D metabolism; vitamin D receptor; calcium homeostasis; transcriptional regulation; rapid steroid actions

VITAMIN D, DISCOVERED AS AN essential nutrient for the prevention of rickets, is required for optimal absorption of dietary calcium and phosphate. Subsequent studies found that rickets could also be prevented by irradiation with UV light, which stimulates formation of vitamin  $\text{D}_3$  by the skin. This ability to produce sufficient amounts of vitamin  $\text{D}_3$  with adequate sunlight exposure indicates that vitamin D is actually not a vitamin. It is now appreciated that vitamin D is metabolized to the steroid hormone 1,25-dihydroxyvitamin  $\text{D}_3$  [ $1,25(\text{OH})_2\text{D}_3$ ] or calcitriol. The earlier observation that metabolites of vitamin D interact with a protein in intestinal extracts led to the identification of the vitamin D receptor (VDR), a member of the steroid receptor superfamily. The VDR is a  $1,25(\text{OH})_2\text{D}_3$ -activated transcription factor that interacts with coregulators and the transcriptional preinitiation complex to alter the rate of target gene transcription. The presence of the VDR in tissues that do not participate in mineral ion homeostasis led to the discovery of a host of other functions for the versatile vitamin D hormone. The ability of  $1,25(\text{OH})_2\text{D}_3$  to inhibit growth and promote differentiation of a variety of cell types has suggested diverse functions in preventing cancers, modulating the immune system, and controlling various endocrine systems. This is in keeping with epidemiological evidence associating vita-

min D deficiency with cancer, autoimmune diseases, hypertension, and diabetes.

The present review focuses on recent developments in our understanding of the vitamin D endocrine system, including 1) mechanisms for facilitated delivery of vitamin D metabolites, 2) the apparent  $1,25(\text{OH})_2\text{D}_3$ -independent effects of the VDR and VDR-independent effects of  $1,25(\text{OH})_2\text{D}_3$ , and 3) the emerging importance of the autocrine/paracrine actions of  $1,25(\text{OH})_2\text{D}_3$ .

## VITAMIN D METABOLISM

### *Vitamin D Bioactivation*

Vitamin D can be obtained from the diet and by the action of sunlight on the skin. Exposure of the skin to the UV rays of sunlight induces the photolytic conversion of 7-dehydrocholesterol to previtamin  $\text{D}_3$  followed by thermal isomerization to vitamin  $\text{D}_3$  (115, 186). Only a few natural food sources contain significant amounts of vitamins  $\text{D}_2$  and  $\text{D}_3$ , but many foods are now fortified with vitamin D. Nonetheless, vitamin D insufficiency persists in most of the world including North America and Europe (48, 236) due to nutritional deficit and perhaps to avoidance of sunlight and the use of sunscreens. This is a major concern because, as discussed below, low vitamin D is associated with many diseases including cancer, autoimmune disease, and hypertension.

The first step in the metabolic activation of vitamin D is hydroxylation of carbon 25, which occurs primarily in the liver (Fig. 1). Several hepatic cytochrome *P*-450s have been shown

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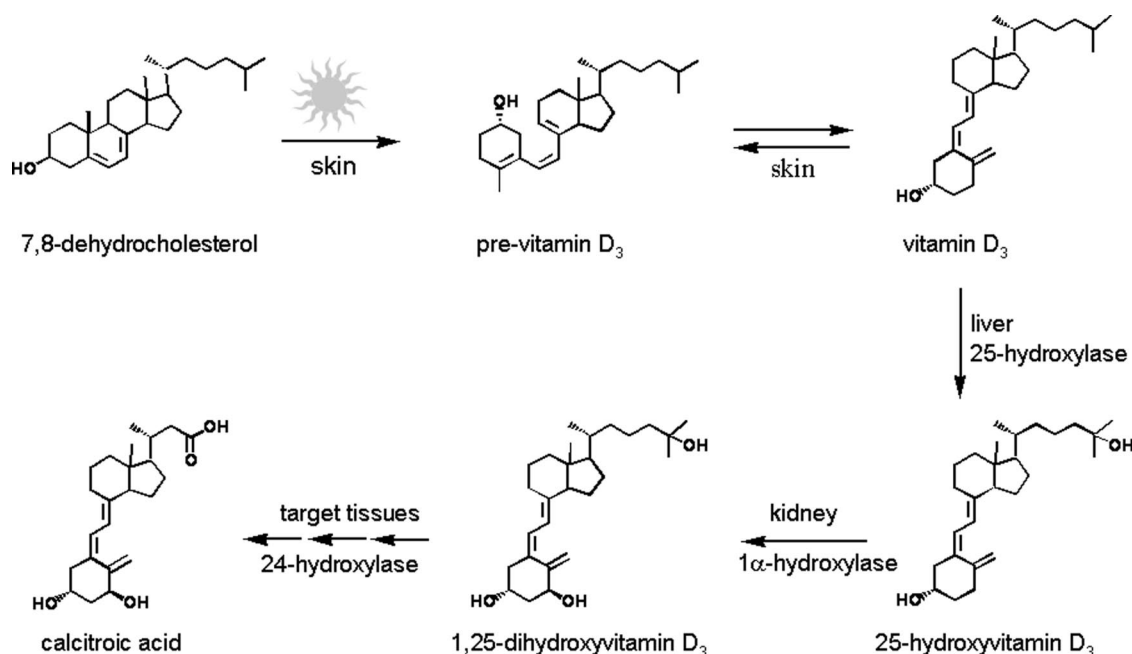


Fig. 1. Vitamin D<sub>3</sub> synthesis, activation, and catabolism. Vitamin D<sub>3</sub> is produced in the skin by the photolytic cleavage of 7-dehydrocholesterol followed by thermal isomerization. Vitamin D is transported to the liver via the serum vitamin D binding protein, where it is converted to 25-hydroxyvitamin D<sub>3</sub>, the major circulating metabolite of vitamin D<sub>3</sub>. The final activation step, 1α-hydroxylation, occurs primarily, but not exclusively, in the kidney (see text), forming 1,25-dihydroxyvitamin D<sub>3</sub>, the hormonal form of the vitamin. Catabolism/inactivation is carried out by 24-hydroxylase, which catalyzes a series of oxidation steps resulting in side chain cleavage.

to 25-hydroxylate vitamin D compounds, but three of these have been excluded as candidates: CYP2C11 is expressed only in males rats (103) and not in humans (204), CYP27A1 knockouts have normal vitamin D levels (201), and CYP3A4 does not hydroxylate vitamin D<sub>3</sub> (99). On the other hand, CYP2R1 appears to be a good candidate. This previously orphan cytochrome *P*-450 was shown to 25-hydroxylate both vitamin D<sub>3</sub> and vitamin D<sub>2</sub>, to be present mainly in the liver and testis (56), and mutations in the 2R1 gene have been identified in a patient with low 25-hydroxyvitamin D levels and rickets (55). Thus CYP2R1 appears to be the critical 25-hydroxylase involved in vitamin D metabolism. The 25-hydroxylation of vitamin D is poorly regulated. The levels of 25(OH)D increase in proportion to vitamin D intake and, for this reason, plasma 25(OH)D levels are commonly used as an indicator of vitamin D status (113).

The second step in vitamin D bioactivation, the formation of 1α,25-dihydroxyvitamin D [1,25-(OH)<sub>2</sub>D] from 25-hydroxyvitamin D, occurs under physiological conditions, mainly in the kidney (86) (Fig. 1), but other cell types can contribute to circulating levels in specific conditions (pregnancy, chronic renal failure, sarcoidosis, tuberculosis, granulomatous disorders, and rheumatoid arthritis). However, the extrarenally produced 1,25(OH)<sub>2</sub>D primarily serves as an autocrine/paracrine factor with cell-specific functions, as discussed below. To date, 1α-hydroxylase has been reported in many cells and tissues including the prostate, breast, colon, lung, pancreatic β cells, monocytes, and parathyroid cells (see Fig. 2) (109).

The 1α-hydroxylase gene has been cloned from several species (90, 169, 220, 225, 233). The cDNA hybridizes solely to chromosomal locus 12q13.1-q13.3, the site to which the defect in patients unable to produce 1,25(OH)<sub>2</sub>D<sub>3</sub> (vitamin D-dependent rickets type I or VDDR-I) has been mapped

(145), and mutations in the coding regions of the 1α-hydroxylase gene have been identified in patients with the disease (90, 136, 258). Targeted ablation of 1α-hydroxylase in mice produced a phenotype consistent with VDDR-I (66, 191).

Renal 1α-hydroxylase activity is highly regulated, in keeping with the potent activity of its product in calcium homeostasis. Dietary calcium can regulate the enzyme directly through changes in serum calcium and indirectly by altering parathyroid hormone (PTH) levels (188). The stimulation of 1α-hydroxylase by hypocalcemia is severely blunted, but not eliminated, by parathyroidectomy (92). Direct suppression of 1α-hydroxylase activity and mRNA by calcium has been

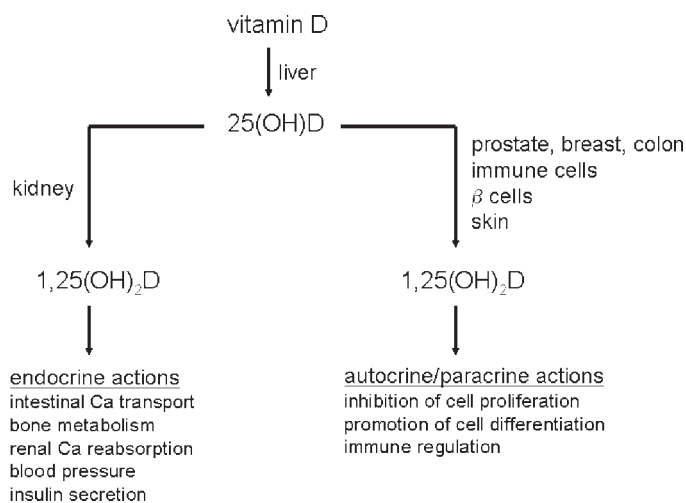


Fig. 2. Renal and extrarenal 1,25(OH)<sub>2</sub>D<sub>3</sub> production serves endocrine, autocrine, and paracrine functions.

demonstrated in a human proximal tubule cell line (27). It is not yet known whether this effect is mediated by the calcium-sensing receptor (CaSR). PTH has been shown to directly regulate  $1\alpha$ -hydroxylase activity (107) and mRNA (220, 225) in renal proximal tubular cells via changes in cAMP (202) through stimulation of  $1\alpha$ -hydroxylase gene transcription (35, 173). Although the promoter for  $1\alpha$ -hydroxylase contains three consensus cAMP response elements (CREs), PTH appears to exert its effect through actions at an element near the transcription start site which lacks a CRE, but contains a binding site for the transcription factor C/EBP $\beta$  (207).

Dietary phosphate restriction also increases renal  $1\alpha$ -hydroxylase activity (235) and mRNA (220) independently of changes in PTH (120) and calcium (46). The lack of a direct effect of phosphate on  $1\alpha$ -hydroxylase in cell culture suggests that the effect of dietary phosphate may be mediated by a systemic hormone. Likely candidates are the recently discovered phosphaturic factors or phosphatonins, fibroblast growth factor 23 (FGF-23), frizzled-related protein 4 (FRP-4), and matrix extracellular phosphoglycoprotein (MEPE) (reviewed in Ref. 209). FGF-23 reduces renal phosphate reabsorption by inhibition of NPT2. Recent evidence indicates that FGF-23 is increased by phosphate loading, suggesting a role in phosphate homeostasis. Of relevance is that transgenic mice constitutively expressing FGF-23 have reduced  $1,25(\text{OH})_2\text{D}_3$  levels despite low plasma phosphate (219). FRP-4 administration was found to produce hypophosphatemia, but  $1,25(\text{OH})_2\text{D}_3$  levels and  $1\alpha$ -hydroxylase did not increase appropriately, suggesting a suppressive effect of FRP-4 on  $1,25(\text{OH})_2\text{D}_3$  production (22). Similarly, MEPE overexpression in vivo produces hypophosphatemia and reduction of  $1,25(\text{OH})_2\text{D}_3$  levels (203). Thus all of the phosphatonins appear to alter circulating  $1,25(\text{OH})_2\text{D}_3$ , perhaps acting as mediators of phosphate regulation of  $1\alpha$ -hydroxylase.

Feedback regulation by  $1,25(\text{OH})_2\text{D}_3$  limits its circulating levels to minimize the potential for vitamin D intoxication. Although the in vivo effects are due, in part, to increased calcium and phosphate and decreased PTH, direct suppression of  $1\alpha$ -hydroxylase activity has been noted in kidney cell culture (106, 238).  $1,25(\text{OH})_2\text{D}_3$  treatment has been shown to reduce  $1\alpha$ -hydroxylase mRNA (169, 220, 225, 233); however, current evidence indicates that this is not through a direct action of  $1,25(\text{OH})_2\text{D}_3$  and its receptor on the  $1\alpha$ -hydroxylase gene promoter, but inhibition of the PTH (cAMP) induction of promoter activity (35).

Another factor that appears to control renal  $1,25(\text{OH})_2\text{D}_3$  production is the *klotho* gene product. *Klotho*-null mice have elevated  $1,25(\text{OH})_2\text{D}_3$  levels, high plasma calcium and phosphate, and die prematurely due to ectopic calcifications (257). Basal  $1\alpha$ -hydroxylase mRNA is increased despite the hypercalcemia, hyperphosphatemia, and low PTH (239), indicating that the *klotho* gene product is a negative regulator of  $1\alpha$ -hydroxylase. The fact that *klotho* is also induced by  $1,25(\text{OH})_2\text{D}_3$  (239) suggests that it may be involved in the feedback control of  $1,25(\text{OH})_2\text{D}_3$  on its own production.

The regulation of  $1\alpha$ -hydroxylase at extrarenal sites is quite different from that of the renal enzyme, in keeping with the autocrine/paracrine functions of locally produced  $1,25(\text{OH})_2\text{D}_3$ . The rates of  $1,25(\text{OH})_2\text{D}_3$  synthesis and degradation are under the control of local factors, i.e., cytokines and growth factors, that optimize the levels of  $1,25(\text{OH})_2\text{D}_3$  for

these cell-specific actions through mechanisms incompletely understood.

### *1,25(OH)<sub>2</sub>D<sub>3</sub> Metabolism*

The high potency of  $1,25(\text{OH})_2\text{D}_3$  in elevating serum calcium and phosphate levels requires a mechanism to attenuate its activity. This is accomplished within virtually all target cells by the  $1,25(\text{OH})_2\text{D}_3$ -inducible vitamin D 24-hydroxylase, which catalyzes a series of oxidation reactions at carbons 24 and 23, leading to side chain cleavage and inactivation. Mice lacking a functional 24-hydroxylase gene have high serum  $1,25(\text{OH})_2\text{D}_3$  levels due to the decreased capacity to degrade it (224). 24-Hydroxylase is regulated in a reciprocal manner to  $1\alpha$ -hydroxylase. Its activity and expression are increased by phosphate (235, 252) and reduced by PTH (107). The 24-hydroxylase gene contains at least two distinct vitamin D response elements that mediate the effects of  $1,25(\text{OH})_2\text{D}_3$  via its receptor on transcription (54, 185).

$1,25(\text{OH})_2\text{D}_3$  can also be converted to the  $1,25(\text{R})-(\text{OH})_2\text{D}_3-23(\text{S}),26$ -lactone (124). This metabolite has mild antagonist activity toward  $1,25(\text{OH})_2\text{D}_3$  action (123), and more potent lactone analogs have now been developed. Recent studies have demonstrated that the  $3\beta$ -hydroxyl group of  $1,25(\text{OH})_2\text{D}_3$  can be epimerized to the  $3\alpha$  position (26, 39) in a cell-specific manner.  $1,25(\text{OH})_2-3\text{-epi-D}_3$  appears to be catabolized more slowly than the parent hormone and retains significant biological activity. The significance of the 3-epimerase is not clear, but its cell-specific expression suggests that this pathway may function to prolong the activity of  $1,25(\text{OH})_2\text{D}_3$  in cells containing this enzyme. Differential rates of 3-epimerization of vitamin D analogs may provide a mechanism for their selective actions in vivo. In addition, evidence suggests that 3-epimerization of select vitamin D analogs may enhance their proapoptotic activity (174).

### TRANSPORT OF VITAMIN D

Vitamin D metabolites are lipophilic molecules with low aqueous solubility that must be transported in the circulation bound to plasma proteins. The most important of these carrier proteins is the vitamin D binding protein (DBP), which binds the metabolites with high affinity in the order  $25(\text{OH})\text{D} = 24,25(\text{OH})_2\text{D} > 1,25(\text{OH})_2\text{D} > \text{vitamin D}$  (61). Plasma levels of DBP are 20 times higher than the total amount of vitamin D metabolites, and >99% of circulating vitamin D compounds are protein bound, mostly to DBP, although albumin and lipoproteins contribute to lesser degrees. This has a major impact on their pharmacokinetics. DBP-bound vitamin D metabolites have limited access to target cells (61) and, therefore, are less susceptible to hepatic metabolism and subsequent biliary excretion, leading to a longer circulating half-life. Early evidence suggested that only the small fraction of unbound metabolites passively entered target cells to be further metabolized or to exert biological activity. For activated vitamin D compounds [i.e.,  $1,25(\text{OH})_2\text{D}_3$  and its analogs], biological activity was correlated with the concentration of free hormone (23, 37, 73). Thus DBP appears to buffer the free levels of active vitamin D compounds, guarding against vitamin D intoxication (30). DBP levels are not regulated by vitamin D but are reduced by liver disease, nephrotic syndrome, and malnutrition and increased during pregnancy and estrogen



therapy. The concentration of free  $1,25(\text{OH})_2\text{D}_3$ , however, remains constant when DBP levels change, an example of the tight self-regulation of vitamin D metabolism. In fact, DBP-null mice lack any indications of rickets, despite very low total levels of  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$ , supporting the free hormone hypothesis for the actions of  $1,25(\text{OH})_2\text{D}_3$  and its analogs.

On the other hand, it is now clear that  $25(\text{OH})\text{D}$  does not simply diffuse into the proximal tubule cells containing  $1\alpha$ -hydroxylase. Mice lacking the endocytic receptor megalin were unexpectedly found to develop vitamin D deficiency and rickets due to a loss of DBP and its bound vitamin D metabolites in the urine (183, 232). Thus entry of  $25(\text{OH})\text{D}$  into the proximal tubule cells is not by diffusion across the basolateral surface but by receptor-mediated uptake of DBP in the brush border, as depicted in Fig. 3. This mechanism explains the finding that DBP-null mice are resistant to vitamin D intoxication (205). However, because DBP-null mice do not display vitamin D deficiency, DBP-independent uptake of  $25(\text{OH})\text{D}$  must also occur. Megalin is part of a complex of proteins that facilitate endocytosis. Receptor-associated protein (RAP) is also essential as its ablation leads to excretion of DBP (25) as does the removal of cubilin (184), a protein required for sequestering DBP on the cell surface before internalization by megalin.

Once inside the cells, DBP is degraded, apparently by legumain (255), releasing  $25(\text{OH})\text{D}$  for metabolism by  $1\alpha$ -hydroxylase or  $24$ -hydroxylase; however,  $25(\text{OH})\text{D}$  translocation to the mitochondria may also be facilitated rather than passive. It was recently reported that the intracellular COOH terminus of megalin interacts with at least two intracellular vitamin D binding proteins, IDBP-1 and IDBP-3 (2). IDBPs are homologs of heat shock proteins that bind  $25$ -hydroxylated vitamin D compounds as well as estradiol (91) and may serve diverse roles. Overexpression of IDBP-3 in cells expressing megalin increases the movement of  $25(\text{OH})\text{D}$  to the mitochondria for hydroxylation (Fig. 2). In fact, IDBP-3 appears to interact directly with  $1\alpha$ -hydroxylase. On the other hand, IDBP-1 overexpression enhances movement of  $1,25(\text{OH})_2\text{D}_3$  to the VDR. Megalin has also been shown to bind the VDR coactivator SKI-

interacting protein (Skip), suggesting another means of controlling vitamin D action (196).

Megalin levels are increased by  $1,25(\text{OH})_2\text{D}_3$  (155), providing a feed-forward mechanism for  $1,25(\text{OH})_2\text{D}_3$  production. Partial ( $3/4$ ) nephrectomy of rats leads to a gradual fall in megalin expression in the remnant kidney beginning within 2 wk, which is followed later by an increase in  $1\alpha$ -hydroxylase mRNA. This apparently compensates for the reduced megalin levels and the decreased synthetic capacity due to the loss of renal mass (232). This finding further supports the importance of vitamin D supplementation of patients with early renal failure.

### $1,25(\text{OH})_2\text{D}_3$ -VDR ACTIONS

Most of the biological activities of  $1,25(\text{OH})_2\text{D}_3$  require a high-affinity receptor, the VDR, an ancient member of the superfamily of nuclear receptors for steroid hormones. Like the other members of the steroid receptor family, the VDR acts as a ligand-activated transcription factor (36). Figure 4 depicts the domains of the VDR involved in the major steps for VDR control of gene transcription: 1) ligand binding, 2) heterodimerization with retinoid X receptor (RXR), 3) binding of the heterodimer to vitamin D response elements (VDREs) in the promoter of  $1,25(\text{OH})_2\text{D}$ -responsive genes, and 4) recruitment of VDR-interacting nuclear proteins (coregulators) into the transcriptional preinitiation complex, which markedly enhance or suppress the rate of gene transcription by the VDR.

The ligand binding domain (LBD), located in the COOH-terminal portion of the VDR molecule, is responsible for the high-affinity binding of  $1,25(\text{OH})_2\text{D}_3$  ( $K_d = 10^{-10}$  to  $10^{-11}$  M).  $25(\text{OH})\text{D}_3$  and  $24,25(\text{OH})_2\text{D}_3$  bind nearly 100 times less avidly (44, 167). The A ring containing the  $1\alpha$ -hydroxyl group is a critical portion of the  $1,25(\text{OH})_2\text{D}_3$  molecule responsible for VDR binding. However, other domains of the VDR are important, as shown by their capacity to compensate for the lack of a functional  $1\alpha$ -hydroxyl group (194). Ligand binding affinity, however, is not an absolute predictor of the transcriptional activity of ligand-activated VDR. Cell-specific variations in the expression of intracellular binding proteins that mediate the delivery of the ligand to and from the VDR may

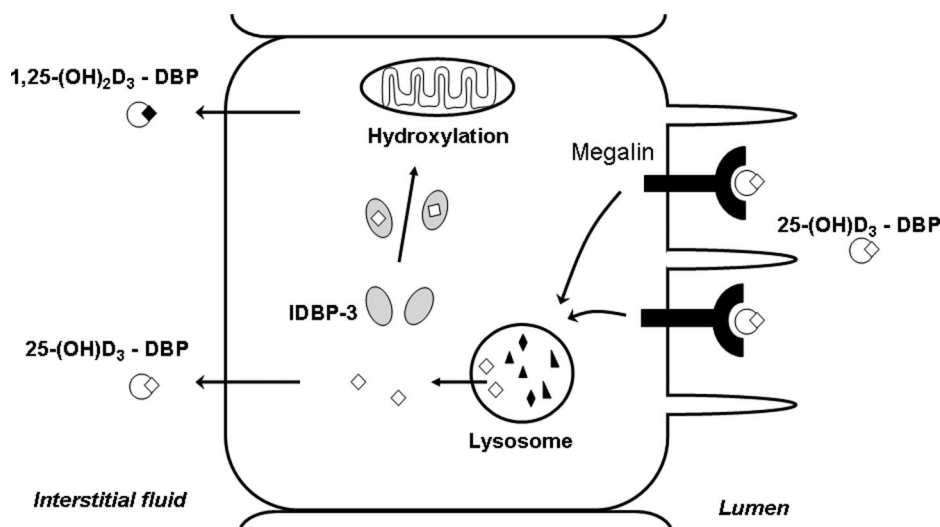


Fig. 3. Roles of megalin and intracellular vitamin D binding protein 3 (IDBP-3) in the delivery and  $1\alpha$ -hydroxylation of  $25$ -hydroxyvitamin D. The majority of circulating  $25$ -hydroxyvitamin D is bound to the vitamin D binding protein (DBP), which is filtered by the kidney and taken up by proximal tubular cells via megalin-mediated endocytosis. The DBP is degraded, and the released  $25$ -hydroxyvitamin D is delivered to the  $1\alpha$ -hydroxylase by IDBP-3 or reenters the circulation bound to DBP. Modified from Ref. 183.

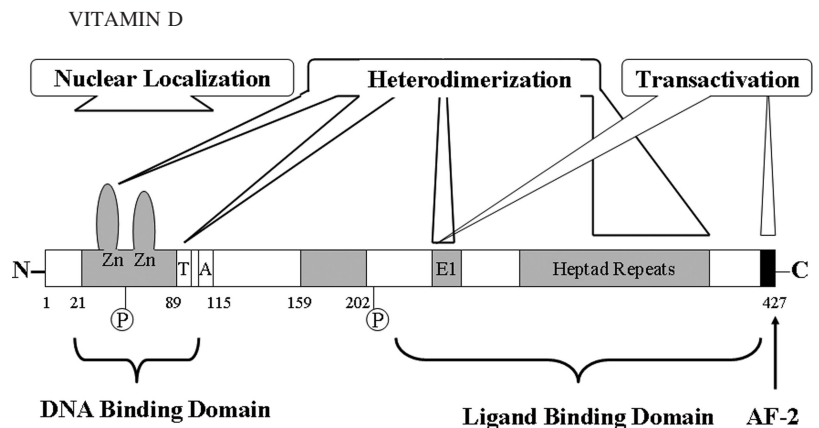


Fig. 4. Functional domains of the vitamin D receptor (VDR). Shown is a schematic representation of the domains of the human VDR molecule mediating ligand and DNA binding, nuclear localization, heterodimerization with the retinoid X receptor (RXR), and transactivation, as well as the 2 major phosphorylation sites. AF-2, activation function-2.

modulate ligand-VDR association/dissociation rates (138) and, consequently, the half-life of the VDR molecule, which is protected from proteosomal degradation (162) through ligand binding (249).

Upon ligand binding, repositioning of helix 12 in the COOH terminus of the VDR ligand binding domain, known as ligand-dependent activation function 2 (AF2), imparts a major conformational change in the three-dimensional structure of the VDR. This activation step appears to be required for the recruitment by the VDR of motor proteins (200), responsible for a rapid translocation of cytoplasmic VDR to the nucleus along microtubules (19). In human monocytes, disruption of microtubular integrity is sufficient to abolish 1,25(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase gene transcription (129). Point mutations in the two nuclear localization signals cause a defective cytoplasmic-to-nuclear translocation and the phenotype of vitamin D-dependent rickets type II (20, 108).

The selective association between the VDR and its protein partner, the RXR, involves dimerization surfaces in three different domains of the VDR molecule and induces a VDR conformation that is essential for VDR transactivating function. An interplay between ligand binding and heterodimerization domains was suggested by two natural mutations (I314S and R391C) in the LBD of the VDR that confer the phenotype of vitamin D resistance by significantly impairing both VDR-RXR heterodimerization and ligand retention (101).

The DNA-binding domain (DBD) of the VDR is highly conserved among nuclear steroid receptors. The DBD is organized into two zinc-nucleated modules, the zinc finger DNA binding motifs, that are responsible for high-affinity interaction with specific DNA sequences in the promoter region of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes, called vitamin D-responsive elements (VDREs). The natural mutations in the zinc finger region of the human VDR result in defective DNA binding and the most severe clinical phenotypes of vitamin D resistance (102). High-resolution crystal structures show the DBD of the VDR bound to the major groove of the hexameric VDRE (217). VDR-RXR binding to VDREs causes a 55° bending of the DNA from the horizontal, but the impact of this DNA bending on VDR-mediated transactivation is unclear (227). The most common VDRE type, designated DR3, contains two variable, six-base half-elements with the general consensus sequence AGGTCA, separated by a spacer of three nucleotides. This sequence directs the VDR-RXR heterodimer to the promoter region of 1,25(OH)<sub>2</sub>D<sub>3</sub>-regulated genes, with the RXR binding the 5' half-site and the VDR occupying the 3'

half-site (102). Another type of VDRE, IP9, consists of two inverted palindromic sequences separated by nine base pairs (210). The VDREs of genes suppressed by the VDR, such as chick PTH and mouse osteocalcin, are similar to the DR3 sequence found in genes in which transcription is induced by vitamin D. This finding raised important questions regarding the mechanisms determining whether gene transcription will be induced or suppressed by 1,25(OH)<sub>2</sub>D<sub>3</sub> (67). The switch from VDR transrepression to activation of the avian PTH gene, induced by changing two bases in the 5'-element of the DR3, suggested that a changed polarity of the VDR/RXR-VDRE complex, with the VDR occupying the 5' half-element, may contribute to VDR-negative regulation of gene transcription (102).

Recently discovered VDR interactions with nuclear coregulator molecules provide new mechanistic insights into positive and negative modulation of VDR-mediated transcription. Two domains of the VDR serve as adaptor surfaces for nuclear proteins necessary for VDR-coregulator interactions (51): one is the RXR heterodimerization domain containing residue 246, which is highly conserved among nuclear receptors, and forms part of the binding interface with transcriptional coactivators. Its alteration severely compromises transactivation. The second region is the previously described AF2 domain, which undergoes a dramatic conformational shift on ligand binding, allowing the recruitment of VDR-interacting proteins including components of the transcription initiation complex, RNA polymerase II, and nuclear transcriptional coactivators that promote chromatin remodeling and gene transcription. Removal of the AF2 domain eliminates 1,25(OH)<sub>2</sub>D<sub>3</sub>-VDR transcriptional activity with little effect on ligand binding or heterodimeric DNA binding. Nuclear coactivators act synergistically with the VDR to markedly amplify 1,25(OH)<sub>2</sub>D<sub>3</sub> gene-transactivating potency. The VDR-nuclear coactivators SRC-1 and CBP/p300 possess histone acetyl transferase activity, which unfolds and exposes the DNA. This allows the recruitment of a second complement of transcriptional coactivators, the DRIP-TRAP complex of ~15 proteins. DRIP205 interacts directly with the VDR. DRIP-TRAP recruitment builds a bridge with the basal transcriptional machinery that favors the assembly of the preinitiation complex to potentiate VDR induction of gene expression (128, 199).

In transcriptional repression by the VDR, such as that of the PTH gene, binding of the VDR-RXR complex to a negative VDRE recruits corepressors of the family of histone deacetylases. These molecules prevent chromatin exposure, and con-

sequently, the binding of proteins (TATA binding protein) mandatory to initiate the transcription of the target gene by RNA-polymerase II (128, 199). In vitro, ligand-specific recruitment of more potent VDR corepressors to the PTH gene promoter appears to mediate a higher transrepression potency for 22-oxa-calcitriol compared with  $1,25(\text{OH})_2\text{D}_3$  (234). In primary cultures of bovine parathyroid cells, however, differences between OCT and  $1,25(\text{OH})_2\text{D}_3$  potency in repressing rat PTH mRNA levels were not apparent (40).

Recent studies suggest a bifunctional role for the VDR comodulator NCoA62/Skip. It can promote transcriptional activation or repression, in a cell-specific manner, depending on the expression of coregulator molecules (147). The coactivator p300 and the corepressors NCoR and SMRT interact with the same  $\text{NH}_2$ -terminal region of the Skip molecule. The relative levels of expression of the nuclear corepressor NCoR and coactivator CBP/p300 in CV-1 and P19 cells dictate whether Skip activates or represses VDR/RXR-dependent transcription (147). More importantly, the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex induces selectively the expression of genes of two important coregulator families, TIF2 from the p160 coactivators and SMRT among corepressors (72). Because SRC1/TIF2 ratios were shown to affect important cellular functions, such as energy metabolism in murine fat tissue (197), the apparent cell specificity of the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex in inducing gene transcription and possibly protein abundance of TIF2 and SMRT suggests that  $1,25(\text{OH})_2\text{D}_3$  itself could modulate the transcriptional competence of target cells.

In addition to Skip, a novel ATP-dependent chromatin remodeling complex containing the Williams syndrome transcription factor potentiates ligand-induced VDR action in both gene transactivation and repression (131).

NCoA62/Skip also mediates a link between transcriptional regulation by the VDR (and other nuclear receptors) and RNA splicing by the spliceosome (262). Skip physically interacts with components of the splicing machinery and nuclear matrix-associated proteins. In fact, expression of a dominant negative Skip interfered with appropriate splicing of transcripts derived from  $1,25(\text{OH})_2\text{D}_3$ -VDR transactivation (262).

In summary, complex cell- and promoter-specific VDR-nuclear coregulator interactions are responsible for VDR regulation of the expression of vitamin D-responsive genes, including VDR coactivator and corepressor molecules.

#### RAPID NONGENOMIC ACTIONS OF $1,25(\text{OH})_2\text{D}_3$

Vitamin D compounds, like other steroid hormones, can also elicit responses that are too rapid to involve changes in gene expression and appear to be mediated by cell surface receptors. The role of the nongenomic actions in most cells remains uncertain. In the chick duodenum,  $1,25(\text{OH})_2\text{D}_3$  stimulates vesicular calcium movement from the lumen to the basolateral surface within minutes (180), but the overall contribution of this pathway is not clear.  $1,25(\text{OH})_2\text{D}_3$  can rapidly stimulate phosphoinositide metabolism (32, 153, 170), cytosolic calcium levels (116, 152, 159, 170, 228), cGMP levels (98, 245), PKC (230), MAP kinases (21, 222), and the opening of chloride channels (260). In chondrocytes, nongenomic actions of both  $1,25(\text{OH})_2\text{D}_3$  and  $24,25(\text{OH})_2\text{D}_3$  alter membrane lipid turnover, prostaglandin production, and protease activity that leads to modification of bone matrix and calcification (33).

The nature of the receptor that mediates the rapid actions remains controversial. At least two distinct receptors have been identified. The better characterized is the membrane-associated, rapid-response steroid-binding protein ( $1,25\text{D}_3$ -MARRS) isolated from chick intestinal basolateral membranes on the basis of  $1,25(\text{OH})_2\text{D}_3$  binding (177). Antibodies to the  $\text{NH}_2$ -terminal domain of  $1,25\text{D}_3$ -MARRS blocked the nongenomic actions of  $1,25(\text{OH})_2\text{D}_3$  (179).  $1,25\text{D}_3$ -MARRS has now been found to be identical to the protein thiol-dependent oxidoreductase ERp57 (178), which plays a role in glycoprotein folding through the formation of disulfide bonds and acts as part of a chaperone complex with calreticulin and calnexin (78). ERp57 ribozyme knockdown reduced membrane binding of  $1,25(\text{OH})_2\text{D}_3$  and rapid responses (178), but how this enzyme mediates the rapid actions of  $1,25(\text{OH})_2\text{D}_3$  remains to be determined. Another  $1,25(\text{OH})_2\text{D}_3$ -binding protein, annexin II, was identified in the plasma membranes of ROS 24/1 rat osteosarcoma cells that do not express the VDR (14). Polyclonal antibodies to annexin II decreased binding of  $1,25(\text{OH})_2$ - $^{14}\text{C}$  $\text{D}_3$  and blocked the increase in cytosolic calcium by  $1,25(\text{OH})_2\text{D}_3$  (16). However, a recent report could not reproduce the  $1,25(\text{OH})_2\text{D}_3$  binding to annexin II (168). The rapid actions of  $24,25(\text{OH})_2\text{D}_3$  in chondrocytes appear to be mediated by a receptor distinct from those for  $1,25(\text{OH})_2\text{D}_3$ , although its identity is not known.

Several studies have indicated that the rapid actions of  $1,25(\text{OH})_2\text{D}_3$  require the presence of the VDR. The apparent nongenomic actions of  $1,25(\text{OH})_2\text{D}_3$  are absent in cells isolated from VDR-null mice (81, 261), and it has been proposed that the VDR mediates these rapid effects. Furthermore, the VDR was recently found to be present in caveolae-enriched plasma membrane fractions (121). However, the ligand specificities of the VDR and the rapid action receptor are very different (29, 182, 263), and  $1,25(\text{OH})_2\text{D}_3$  cannot stimulate transcalcitachia in vitamin D-deficient chick duodenum. This would suggest that the VDR is required for the expression of gene products involved in the rapid, nongenomic response. The exact role of the VDR in the rapid actions remains to be clarified.

Nongenomic events have been proposed to modulate the genomic actions of  $1,25(\text{OH})_2\text{D}_3$  (15, 17, 85), but this remains controversial. Numerous studies have presented evidence that the nongenomic actions may not be critical for  $1,25(\text{OH})_2\text{D}_3$ -mediated gene activation (84, 127, 134, 135, 181, 264) or inhibition of cell proliferation (105, 181). However, nongenomic stimulation of protein kinases could potentially influence the VDR-mediated effects of  $1,25(\text{OH})_2\text{D}_3$ .

#### REGULATION OF $1,25(\text{OH})_2\text{D}_3$ -VDR ACTIONS

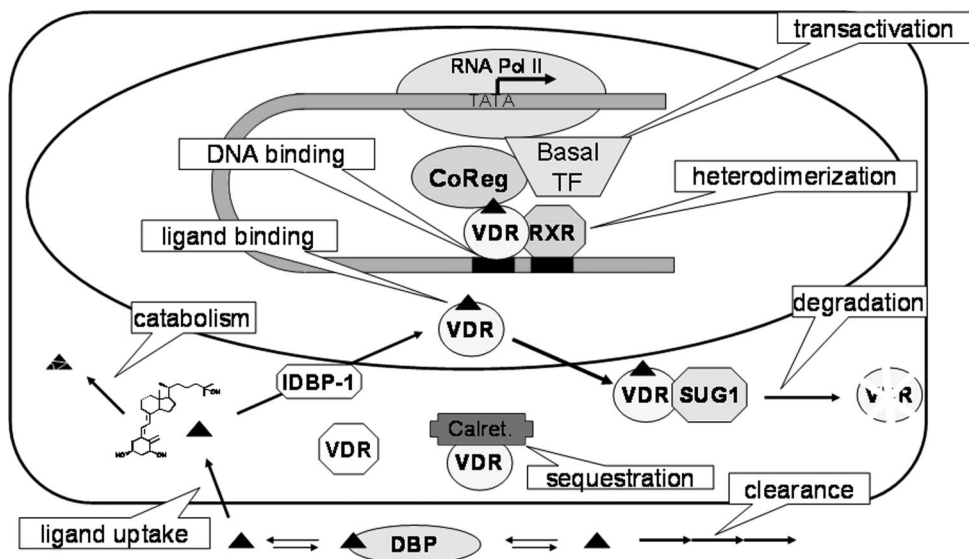
The magnitude of a biological VDR-mediated response to  $1,25(\text{OH})_2\text{D}_3$  is influenced by ligand accessibility to the VDR, cellular VDR content, genetic and posttranslational VDR modifications, and availability and activation state of nuclear coregulators, as depicted in Fig. 5.

##### *Intracellular Ligand and VDR Content*

As described in prior sections, the concentration of ligand in a target cell available for VDR binding is determined by the net balance between the rate of uptake of ligand into the cell and the rate of its metabolic inactivation within the cell. The reduced  $1,25(\text{OH})_2\text{D}_3$  clearance and signs of vitamin D intox-



Fig. 5. Current model for the control of vitamin D receptor (VDR)-mediated actions of  $1,25(\text{OH})_2\text{D}_3$ . The bioactivated vitamin D hormone,  $1,25(\text{OH})_2\text{D}_3$ , circulates bound to the DBP.  $1,25(\text{OH})_2\text{D}_3$  taken up by target cells is either targeted by IDBPs to mitochondrial 24-hydroxylase or to the VDR. The  $1,25(\text{OH})_2\text{D}_3$ -VDR complex heterodimerizes with RXR, and the VDR/RXR heterodimer binds specific sequences in the promoter regions of the target gene. The DNA-bound heterodimer attracts components of the RNA polymerase II (Pol II) preinitiation complex and nuclear transcription regulators, thereby altering the rate of gene transcription. The  $1,25(\text{OH})_2\text{D}_3$ -VDR complex interaction with SUG1 targets the VDR for proteasomal degradation. Calreticulin interaction with the DNA-binding domain of the VDR sequesters the VDR, preventing transactivation. TF, transcription factors.



ication in the 24-hydroxylase-null mice provide conclusive evidence of the critical role of ligand inactivation in the in situ control of the response to vitamin D (226).

In addition to the actual content of intracellular ligand, the levels of intracellular vitamin D interacting proteins (IDBPs) may modulate ligand-VDR association/dissociation rates and therefore ligand-dependent VDR activation, in a cell-specific manner. A differential expression of IDBPs could partially account for the VDR-binding saturation for the vitamin D analog 22-oxacalcitriol. A much lower concentration of the ligand leads to VDR saturation in the parathyroid glands compared with other tissues, all expressing an identical VDR molecule (138).

The intracellular levels of VDR in a target cell are regulated by VDR ligands (homologous regulation) (63) and other hormones and growth factors that do not bind to the VDR (heterologous upregulation), with profound species-, tissue-, and cell-specific variations in VDR regulation (36).

$1,25(\text{OH})_2\text{D}_3$  upregulates VDR mRNA in the kidney and the parathyroid glands through an unknown mechanism, because there is no VDRE in the VDR promoter for direct transactivation of the VDR gene. On the other hand,  $1,25(\text{OH})_2\text{D}_3$  induction of VDR protein occurs in virtually all cell types (9) and involves ligand-dependent stabilization of the VDR from proteosomal degradation. Physical interactions of liganded VDR with SUG1, a component of the proteasome complex, targets the VDR for ubiquitination and subsequent proteolysis (162), thus providing an in situ control of transcriptional responses to  $1,25(\text{OH})_2\text{D}_3$ . In fact, a reduced recruitment of SUG1 to the VDR by  $1,25(\text{OH})_2\text{D}_3$  analogs accounts for their higher transactivating potency (125). It is unclear what targets the liganded VDR to the proteasome or the transcriptional preinitiation complex.

#### VDR Polymorphisms

Many allelic variants (polymorphisms) of the chromosome 12 VDR gene occur naturally in the human population (142, 172), with substantial differences between races and ethnic groups (240). Their expression associates with decreased bone density (76), propensity to hyperparathyroidism

(52, 94), resistance to vitamin D therapy (141), and susceptibility to infections, autoimmune diseases, and cancer (241). The polymorphisms most frequently studied are located in the intron separating exon VIII and IX and were defined by the restriction enzymes *BsmI*, *ApaI*, and *TaqI*. In most epidemiological studies, however, correlations were sought between a single specific polymorphism, or between the *BsmI*-*ApaI*-*TaqI* linkage group and the physiological parameter of interest (248), and lack analysis of the direct influence of allelic variation on VDR protein expression or activity. In fact, *BsmI*, *ApaI*, or *TaqI* alleles have no effect on either the expression levels or the activity of the translated VDR protein. These are important limitations that leave open the possibility that the observed correlations might be due to another nearby site or even to a different gene.

Several functional VDR polymorphisms exist: 1) linked to the *Apa*, *Bsm*, and *Taq* polymorphisms is a microsatellite poly A repeat of variable length [long (L) or short (S)], which may affect mRNA stability; 2) the *FokI* polymorphism is not linked to the others and results in a three amino acid shorter VDR molecule, with higher biological activity to activate the rat osteocalcin gene in fibroblasts (248) and to suppress growth of peripheral blood mononuclear cells (60); 3) a *Cdx2* polymorphism was found in the VDR promoter; its expression results in a VDR gene with a defective binding site for the intestine-specific transcription factor *Cdx2* (8), which causes decreased intestinal VDR levels (254), with the concomitant reduction in  $1,25(\text{OH})_2\text{D}_3$  induction of calcium transport proteins and calcium absorption (83); and 4) a novel VDR B1 isoform was found in several human cell lines (93). VDR B1 is generated by alternative splicing, thereby rendering a VDR with higher transcriptional activity, due to the 50-amino acid  $\text{NH}_2$ -terminal extension of the A/B region with transactivation activity (93).

Importantly, when the functional significance of two unlinked human VDR gene polymorphisms [at a *FokI* restriction site (F/f) in exon II and a microsatellite poly A repeat (L/S)] was examined simultaneously in human fibroblasts spanning >20 genotypes (248), higher transcriptional activity of the F and L biallelic VDR forms was found and statistical significance between genotypes emerged (248).



A more systematic assessment of functional VDR polymorphisms in the population should find clinical application in disease prevention as well as in predicting the response to vitamin D therapy.

#### Posttranslational Modifications of the VDR

Ligand binding to the VDR promotes serine phosphorylation of the receptor. VDR phosphorylation occurs at several loci and is mediated by various kinases including casein kinase II (at serine 208), PKC (at serine 51), and PKA (117) with diverse effects on transcriptional activity. Clearly, nuclear actions of the VDR could be modulated by other hormonal systems acting at the cell surface to activate protein kinase cascades, as demonstrated by inhibition of osteocalcin gene expression through hyperphosphorylation of the VDR (70). As described earlier,  $1,25(\text{OH})_2\text{D}_3$  activation of PKC and other kinases through interaction with cell membrane receptors provides an additional cell-specific mechanism for modulation of the genomic actions of the vitamin D hormone. In fact,  $1,25(\text{OH})_2\text{D}_3$  causes a rapid and sustained activation of ERK in bone cells that results in a cell-specific activation or inhibition of VDR transactivation, which is unrelated to VDR content but dependent on the cellular expression of an RXR isoform (176).

A posttranslational VDR modification is induced by substances from uremic plasma ultrafiltrate (192) that react covalently with the VDR at or near the DNA binding domain (193), thus disrupting VDR-RXR binding to DNA. These interactions may partially account for the resistance to vitamin D commonly associated with chronic kidney disease.

#### Nuclear Levels of Transcriptional VDR Coregulators

As mentioned earlier, the genomic actions of the hormonal form of vitamin D could also be influenced by changes in the nuclear levels or availability of nuclear coregulators of the VDR-RXR complex. Competition between the VDR and transcription factors for other hormonal systems for limiting amounts of common nuclear transcriptional modulators could also affect  $1,25(\text{OH})_2\text{D}_3$ -VDR regulation of gene expression, as demonstrated for estrogen squelching of progesterone-mediated transactivation (221).

Physical VDR-protein interactions compromising the DBD of the VDR and, consequently, gene transactivation were demonstrated for nuclear calreticulin in the parathyroid glands of hypocalcemic rats (214) and with Stat1 (246), the transcription factor mediating most biological responses to  $\gamma$ -interferon, in macrophages. The former prevents  $1,25(\text{OH})_2\text{D}_3$  transrepression of the PTH gene in vitro, and the latter causes hypercalcemia in sarcoidosis. Mechanistically, increased nuclear calreticulin competes with the VDR for DNA binding. Similarly,  $\gamma$ -interferon-activated Stat1 binds the DBD of the VDR, thus inhibiting the ability of excessive serum  $1,25(\text{OH})_2\text{D}_3$  to induce its own catabolism through transactivation of the 24-hydroxylase gene. Extracellular calcium also regulates  $1,25(\text{OH})_2\text{D}_3$ -VDR transcriptional activity in keratinocytes through Ca-dependent recruitment of specific sets of nuclear VDR coactivators (24).

The following section on biological actions of vitamin D presents the current understanding of cell-specific molecular events that translate into the most relevant calcitropic and noncalcitropic actions of the vitamin D hormone. These in-

clude 1) VDR interactions with the transcriptional machinery at a particular gene promoter resulting in direct regulation of gene transcription, 2) VDR-independent actions of  $1,25(\text{OH})_2\text{D}_3$ , and 3)  $1,25(\text{OH})_2\text{D}_3$ -VDR actions on critical signaling pathways unrelated to the vitamin D endocrine system.

#### BIOLOGICAL ACTIONS OF VITAMIN D

##### Classic Vitamin D-Responsive Tissues

The vitamin D endocrine system is an essential component of the interactions among the kidney, bone, parathyroid gland, and intestine (summarized in Fig. 6) that maintain extracellular calcium levels within narrow limits, a process vital for normal cellular physiology and skeletal integrity. This section discusses the exclusive vs. redundant actions of the  $1,25(\text{OH})_2\text{D}_3$ -VDR system compared with those of extracellular calcium in modulating skeletal and mineral homeostasis, based on the evidence obtained from targeted deletion of the genes encoding  $1\alpha$ -hydroxylase, the VDR, or both.

**Intestine.** Vitamin D is essential to enhance the efficiency of the small intestine to absorb dietary calcium and phosphate. The studies in mice lacking the VDR (150),  $1\alpha$ -hydroxylase, or both (190) corroborated the evidence from patients with vitamin D-dependent rickets type I and II that both  $1,25(\text{OH})_2\text{D}_3$  and the VDR are required for optimal intestinal calcium absorption.  $1,25(\text{OH})_2\text{D}_3$  induces active cellular calcium uptake and transport mechanisms, as depicted in Fig. 7. Calcium uptake requires the epithelial calcium channel TRPV6 (also known as CaT1 or ECaC2) and, to a lesser extent, TRPV5 (ECaC1). Thereafter, calbindin D transports calcium across the cell, and the plasma membrane  $\text{Ca}^{2+}$  ATPase, PMCA1b, and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX1) mediate the final delivery of calcium to the bloodstream (31).

The initial calcium uptake is the rate-limiting step in intestinal calcium absorption and highly dependent on vitamin D (244). The expression of TRPV5 and TRPV6 channels is reduced in the VDR-null mice (31, 244). In contrast, the mRNA levels for both channels are upregulated on calcitriol supplementation in wild-type mice (243). The higher potency

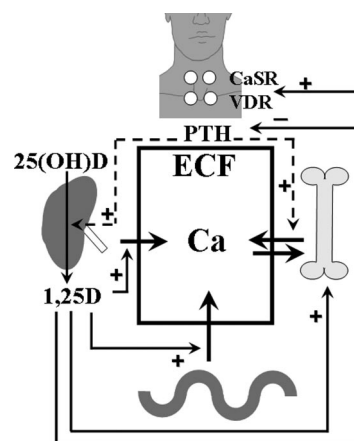


Fig. 6. Role of  $1,25(\text{OH})_2\text{D}_3$  in calcium homeostasis.  $1,25(\text{OH})_2\text{D}_3$  produced in the kidney induces intestinal calcium absorption, controls bone remodeling, suppresses parathyroid function [parathyroid hormone (PTH) synthesis and cell growth], and renal calcium reabsorption to maintain calcium in the extracellular fluid (ECF) within the narrow limits essential for normal cell physiology and skeletal integrity. CaSR, calcium-sensing receptor.

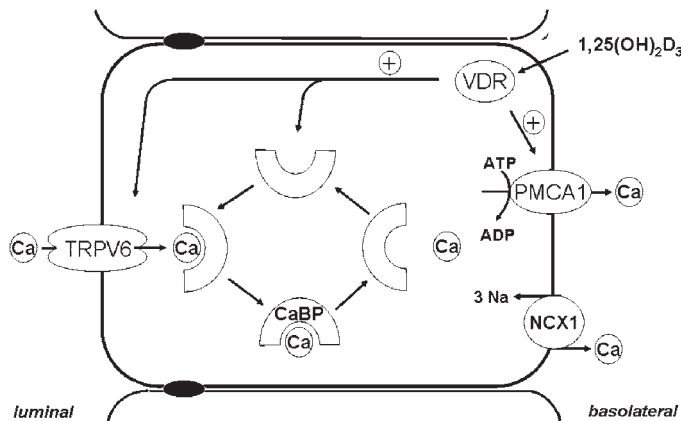


Fig. 7. Regulation of epithelial calcium transport by  $1,25(\text{OH})_2\text{D}_3$ . Epithelial calcium transport is stimulated by  $1,25(\text{OH})_2\text{D}_3$  by induction of 1) the apical calcium channel (TRPV6 or TRPV5) that enhances calcium entry, 2) the cytosolic calcium binding protein (CaBP; calbindin) that facilitates calcium movement across the cell, and 3) the basolateral plasma membrane calcium ATPase (PMCA1) that pumps calcium from the cell. Modified from Ref. 112. NCX1,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger.

of calcitriol compared with its analog,  $19\text{nor-}1,25(\text{OH})_2\text{D}_2$ , in upregulating the expression of these channels could account for the reduced calcemic action of the latter in the intestine (38). Intestinal TRPV5 and TRPV6 expression confers calcium influx with properties identical to those observed in native distal renal cells, including high  $\text{Ca}$  selectivity and negative feedback regulation to prevent calcium overload during trans-epithelial calcium transport (110).

The calcium-transporting proteins TRPV6, calbindin  $\text{D}_{9\text{K}}$ , and PMCA1b are increased by high dietary calcium in the duodenum of  $1\alpha$ -hydroxylase-null mice, thus demonstrating a  $1,25(\text{OH})_2\text{D}_3$ -independent upregulation (243).

Rapid nongenomic effects of  $1,25(\text{OH})_2\text{D}_3$  appear to mediate the increase in both vesicular and paracellular pathways for intestinal calcium absorption. There is controversy, however, on the actual contribution of these nongenomic pathways to intestinal calcium absorption in vivo.

$1,25(\text{OH})_2\text{D}_3$  also increases active phosphate transport through stimulation of the expression of the  $\text{Na-Pi}$  cotransporter (253) and changes in the composition of the enterocyte plasma membrane (144) that increase fluidity and phosphate uptake. Little is known, however, concerning the molecular mechanisms involved in the extrusion of phosphate across the basolateral membrane into the circulation.

**Skeleton.** Vitamin D is essential for the development and maintenance of a mineralized skeleton. Vitamin D deficiency results in rickets in young growing animals and osteomalacia in adults. The rescue of the skeletal phenotype of rickets in the VDR-null mice with a high-calcium, high-P, and high-lactose diet ("rescue diet") rendered vitamin D dispensable in skeletal growth, maturation, and remodeling (150). Clinical studies in patients with vitamin D-dependent rickets type II supported these findings. Their abnormalities in bone mineralization were completely resolved by calcium infusion. However, the dispensable role of vitamin D in bone metabolism has been challenged by the comprehensive work by Panda and collaborators (190). In these studies, the phenotype resulting from simultaneous deletion of the genes encoding  $1\alpha$ -hydroxylase and the VDR was compared with those of individual knockout

mouse models that reproduced vitamin D-dependent rickets type I and II, respectively. In addition, in all three mice genotypes, the responses resulting from defective  $1,25(\text{OH})_2\text{D}_3$ -VDR were unmasked from those caused indirectly by hypocalcemia or high serum PTH, through dietary manipulations with using the rescue diet or  $1,25(\text{OH})_2\text{D}_3$  administration. These studies conclusively demonstrated that, except for cartilage and skeletal mineralization, normalization of serum calcium cannot entirely substitute for defective  $1,25(\text{OH})_2\text{D}_3$ -VDR in skeletal homeostasis. Growth plate development requires coordinated calcium and  $1,25(\text{OH})_2\text{D}_3$  actions, whereas optimal osteoblastic bone formation and osteoclastic bone resorption demand both  $1,25(\text{OH})_2\text{D}_3$  and the VDR. Specifically, all three mouse genotypes presented the characteristic rachitic changes in long bones, such as enlarged and distorted cartilaginous growth plates with a widened hypertrophic zone. The abnormalities were less severe in the VDR-null mice compared with the  $1\alpha$ -hydroxylase knockout mice.  $1,25(\text{OH})_2\text{D}_3$  administration to  $1\alpha$ -hydroxylase-null mice could not normalize the growth plate if hypocalcemia was not corrected. Furthermore, elimination of hypocalcemia with the rescue diet did not completely normalize the growth plate. Thus only the combination of high calcium and  $1,25(\text{OH})_2\text{D}_3$  could normalize chondrocyte growth, the latter apparently through a novel VDR-independent mechanism.

Furthermore, the  $1,25(\text{OH})_2\text{D}_3$ -VDR system was revealed to be critical for the normal coupling of bone remodeling (190). Both osteogenesis and osteoclastogenesis were impaired in the three  $1,25(\text{OH})_2\text{D}_3$ -VDR-defective mutants. As expected from the anabolic effects of high levels of PTH that result from hypocalcemia, marked increases in osteoblast number, serum alkaline phosphatase, bone formation, and bone volume were observed in the three  $1,25(\text{OH})_2\text{D}_3$ -VDR-defective mutants. However, the defective osteoblastogenesis was unmasked only after correction of hypocalcemia and secondary hyperparathyroidism with the rescue diet. The three "rescued" genotypes elicited reduced osteoblast number, mineral apposition rates, and bone volume compared with wild-type mice in vivo and decreased production of mineralized colonies ex vivo. Taken together, these findings suggest that the  $1,25(\text{OH})_2\text{D}_3$ -VDR system may exert an "anabolic" effect necessary to sustain bone-forming activity, which is unmasked only when the defective  $1,25(\text{OH})_2\text{D}_3$ -VDR system exists in the presence of normal PTH.

Similarly, an intact  $1,25(\text{OH})_2\text{D}_3$ -VDR system is critical for both basal and PTH-induced osteoclastogenesis. In the three mouse genotypes, osteoclast numbers were inappropriately low (i.e., not different from the normocalcemic, vitamin D-replete wild-type controls with normal PTH) considering the high serum PTH. Defective control of receptor activator of NF- $\kappa\text{B}$  ligand (RANKL)-receptor activator of NF- $\kappa\text{B}$  (RANK) interactions by an altered  $1,25(\text{OH})_2\text{D}_3$ -VDR system contributes to abnormal coupling in bone turnover. Figure 8 shows the RANK/RANKL-osteoponterin (OPG) interactions between osteoblasts and osteoclasts that integrate bone remodeling (34, 133). Osteoblasts express a surface ligand, RANKL, which can bind either RANK or an osteoblast-derived soluble decoy receptor, OPG. The binding of RANKL to RANK induces a signaling cascade that results in differentiation and maturation of osteoclasts.  $1,25(\text{OH})_2\text{D}_3$  as well as PTH and prostaglandins stimulate RANKL expression (137), but  $1,25(\text{OH})_2\text{D}_3$  also

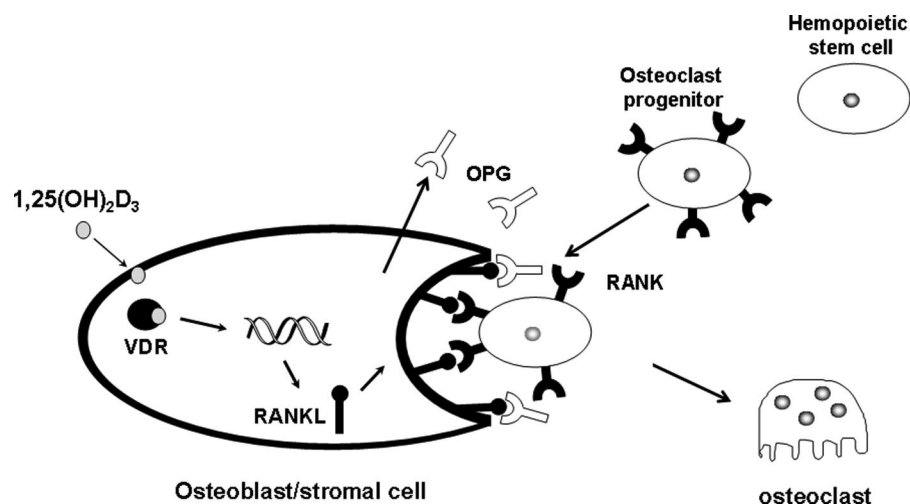


Fig. 8.  $1,25(\text{OH})_2\text{D}_3$  regulates osteoclastogenesis by reciprocal regulation of receptor activator of NF- $\kappa\text{B}$  (RANK) ligand (RANKL) and osteoprotegerin (OPG).  $1,25(\text{OH})_2\text{D}_3$ -VDR increases the expression of RANKL on the surfaces of the osteoblast. RANKL interaction with its receptor, RANK, promotes maturation of osteoclast progenitor cells to mature osteoclasts, the bone-resorbing cells.  $1,25(\text{OH})_2\text{D}_3$ -VDR also represses the expression of OPG, a decoy receptor that binds RANKL and prevents RANK-mediated osteoclastogenesis. Modified from Refs. 95 and 175.

inhibits OPG production (140), with a corresponding increase in osteoclastogenesis and osteoclast activity.

In all three mouse genotypes, osteoclast size was reduced and RANKL expression was low. The rescue diet corrected osteoclast size to that of wild-type mice but could not normalize osteoblastic RANKL content.  $1,25(\text{OH})_2\text{D}_3$  administration could not correct either osteoclast size or osteoblastic RANKL levels in the absence of the VDR. Consequently, the  $1,25(\text{OH})_2\text{D}_3$ -VDR system appears necessary for maximal PTH-induced osteoclast production. This validates prior studies using an *in vitro* osteoclast-generating model, in which osteoblasts from VDR-null mice stimulated osteoclast production in response to PTH but could not sustain osteoclastogenesis from normal spleen precursors in response to  $1,25(\text{OH})_2\text{D}_3$  (231).

**Parathyroid glands.** The vitamin D endocrine system is a potent modulator of parathyroid function. Whereas vitamin D deficiency results in parathyroid hyperplasia and increased PTH synthesis and secretion,  $1,25(\text{OH})_2\text{D}_3$  administration inhibits PTH synthesis and parathyroid cell growth, thus rendering  $1,25(\text{OH})_2\text{D}_3$  therapy effective in treating the secondary hyperparathyroidism of chronic kidney disease (75).

In addition to direct transrepression of the PTH gene by the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex,  $1,25(\text{OH})_2\text{D}_3$  regulates both parathyroid levels of VDR and the response of the parathyroid gland to calcium.  $1,25(\text{OH})_2\text{D}_3$ -induced increases in parathyroid VDR result from increases in mRNA levels, possibly secondary to increases in serum calcium (42), as well as through ligand-dependent protection of proteosomal VDR degradation (249). In fact, in rats with kidney failure, a strong direct correlation exists between serum  $1,25(\text{OH})_2\text{D}_3$  levels and parathyroid VDR protein content (69). Furthermore, prophylactic  $1,25(\text{OH})_2\text{D}_3$  administration prevents the decrease in parathyroid VDR expression that accompanies the progression of kidney disease.

$1,25(\text{OH})_2\text{D}_3$  modulation of the parathyroid gland response to calcium involves direct  $1,25\text{D}$ -VDR induction of CaSR gene transcription (41) at the two VDREs in the CaSR promoter (49). Importantly, the rescue diet corrects the high serum PTH levels in the VDR- and  $1\alpha$ -hydroxylase-null mice and in vitamin D-deficient rats (139), suggesting that neither the VDR nor  $1,25(\text{OH})_2\text{D}_3$  is essential but are cooperative with calcium in controlling PTH synthesis.

New insights into the mechanisms underlying  $1,25(\text{OH})_2\text{D}_3$  suppression of parathyroid hyperplasia emerged after the characterization of the mitogenic signals that trigger the switch of a normally quiescent parathyroid cell to a proliferating one. Increases in parathyroid expression of the growth promoter transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and its receptor, the epidermal growth factor receptor (EGFR), mediate the parathyroid growth induced by kidney disease and aggravated by low calcium or high P intake in rats (64, 74). The mechanisms by which  $1,25(\text{OH})_2\text{D}_3$  arrests parathyroid cell growth include 1) prevention of the increases in parathyroid expression of the highly mitogenic TGF- $\alpha$ /EGFR growth loop (64, 74); 2) enhancement of parathyroid expression of the cyclin-dependent kinase inhibitors p21 and p27 (64, 74, 237), which are known suppressors of cellular growth; and 3) downregulation of cell membrane and nuclear growth signals from TGF- $\alpha$ -activated EGFR (62).

A critical role of  $1,25(\text{OH})_2\text{D}_3$  in the control of parathyroid cell growth emerged from studies in  $1\alpha$ -hydroxylase-null mice. Normalization of serum calcium corrected serum PTH levels but could not suppress parathyroid hyperplasia (190). A novel VDR-independent mechanism appears to mediate the antiproliferative properties of high serum  $1,25(\text{OH})_2\text{D}_3$  in the parathyroid glands, because calcium normalization effectively arrests parathyroid growth in VDR knockout mice, which elicit supraphysiological circulating levels of  $1,25(\text{OH})_2\text{D}_3$ .

Intraparathyroid (percutaneous) injection therapy with the  $1,25(\text{OH})_2\text{D}_3$  analog 22-oxacalcitriol regressed parathyroid hyperplasia and induced apoptosis in patients with secondary hyperparathyroidism resistant to intravenous 22-oxacalcitriol (218). It is unclear whether this is attributable to the very high concentration of 22-oxacalcitriol acting on the diminished VDR levels or due to a VDR-independent mechanism.

As expected from  $1,25(\text{OH})_2\text{D}_3$  upregulation of parathyroid VDR and CaSR expression, prolonged  $1,25(\text{OH})_2\text{D}_3$  deficiency in chronic kidney disease leads to markedly reduced parathyroid VDR and CaSR levels, thereby requiring higher serum calcium or  $1,25(\text{OH})_2\text{D}_3$  doses to suppress PTH synthesis or to arrest growth (75).

**Kidney.** The most important endocrine effect of  $1,25(\text{OH})_2\text{D}_3$  in the kidney is a tight control of its own homeostasis through simultaneous suppression of  $1\alpha$ -hydroxylase and stimulation of  $24$ -hydroxylase and very likely



through its ability to induce megalin expression in the proximal tubule (155).

1,25(OH)<sub>2</sub>D<sub>3</sub> involvement in the renal handling of calcium and phosphate continues to be controversial due to the simultaneous effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on serum PTH and on intestinal calcium and phosphate absorption, which affect the filter load of both ions. 1,25(OH)<sub>2</sub>D<sub>3</sub> enhances renal calcium reabsorption and calbindin expression and accelerates PTH-dependent calcium transport in the distal tubule (87), the main determinant of the final excretion of calcium into the urine and the site with the highest VDR content. ECaC (or TRPV5) is an important target in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated calcium reabsorption. Several putative VDR binding sites have been located in the human promoter of the renal ECaC. Decreases in circulating levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations resulted in a marked decline in the expression of the channel at the protein and mRNA levels (111).

The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> in improving renal absorption of phosphate in the presence of PTH may not be due to a direct action of the sterol on the kidney.

A renoprotective effect for 1,25(OH)<sub>2</sub>D<sub>3</sub> therapy was suggested in the rat model of kidney disease. 1,25(OH)<sub>2</sub>D<sub>3</sub> administration attenuates the development of glomerulosclerosis and the progression of albuminuria through PTH-independent antiproliferative actions (213). 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced decreases in podocyte loss and podocyte hypertrophy (143) may also contribute to the less pronounced albuminuria and glomerulosclerosis.

#### Nonclassic Vitamin D Actions

Compelling genetic, nutritional, and epidemiological evidence links abnormalities in the vitamin D endocrine system with disorders unrelated to calcium homeostasis, ranging from hypertension and disturbed muscle function to susceptibility to infections, autoimmune diseases, and cancer (114, 266). This section presents the current understanding of the mechanisms underlying the noncalcitropic actions of vitamin D most critical in disease prevention.

**Suppression of cell growth.** The seminal observations by Abe and collaborators (1) in 1981 that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits clonal proliferation of a variety of human leukemia cell lines and promotes the differentiation of normal and leukemic myeloid precursors toward more mature, less aggressive phenotypes, rendered 1,25(OH)<sub>2</sub>D<sub>3</sub> potentially useful in the treatment of leukemias and other myeloproliferative disorders.

The protective role of vitamin D in cancer was suggested by a strong epidemiological association between prostate, breast, and colon cancer and vitamin D deficiency and was confirmed by nested controlled studies in the case of colorectal and prostate cancer (266). The 1,25(OH)<sub>2</sub>D<sub>3</sub>-VDR system arrests the cancerous cell cycle at the G1-G0 transition through multiple mechanisms. 1) 1,25(OH)<sub>2</sub>D<sub>3</sub> induces gene transcription of the cyclin-dependent kinase inhibitor p21 (154), which appears to be sufficient to arrest growth and promote differentiation in cells of the monocyte-macrophage lineage. 2) 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the synthesis and/or stabilization of the cyclin-dependent kinase inhibitor p27. The former involves VDR-Sp1 interactions at the p27 promoter (148), and the latter occurs through VDR-induced reduction of CDK2 activity and Skip2 abundance, the main factors responsible for p27 degra-

dation. In hepatocarcinomas, 1,25(OH)<sub>2</sub>D<sub>3</sub> also stabilizes p27 through induction of PTEN, a phosphatase that dephosphorylates p27, thus preventing its proteosomal degradation (160). 3) In tumors in which growth is driven by TGF- $\alpha$ /EGFR overexpression, 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the sequestering of ligand-activated EGFR into early endosomes, thus reducing growth signals from ligand-activated EGFR at the cell membrane, and EGFR transactivation of the cyclin D1 gene in the nucleus (62). 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibition of the TGF- $\alpha$ /EGFR growth loop could contribute to the efficacy of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the treatment of hyperplastic keratinocyte growth in psoriasis, because psoriatic keratinocytes overexpress TGF- $\alpha$ . 4) In the monocytic cell line HL60 (126) and in osteoblasts (100), 1,25(OH)<sub>2</sub>D<sub>3</sub> induces C/EBP $\beta$  expression, a protein recently identified as a potent suppressor of the oncogenic-cyclin D1 signature in human epithelial tumors (146). In contrast, the dominant negative C/EBP $\beta$  isoform (LIP), which lacks the transactivation domain, potentiates cyclin D1 induction of cellular growth. In EGFR-driven cancers, prevention of increases in LIP partially accounts for the potent antiproliferative properties of EGFR-tyrosine kinase inhibitors (12). Thus in human tumors, the intracellular C/EBP $\beta$ -to-LIP ratio correlates inversely with proliferating activity (259). A similar induction of C/EBP $\beta$  expression by 1,25(OH)<sub>2</sub>D<sub>3</sub> in cell types other than osteoblasts and monocyte/macrophages should contribute to higher C/EBP-to-LIP ratios and, consequently, to reduced proliferation rates. 1,25(OH)<sub>2</sub>D<sub>3</sub> induction of C/EBP $\beta$  in HL-60 cells promotes the differentiation of these immature myeloid precursors to normal macrophages through C/EBP $\beta$  interactions with phosphorylated retinoblastoma protein (126). 5) 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the levels of HSPA20, a novel phosphoprotein that enhances growth and survival in the prolactin-dependent rat Nb2T lymphoma, a valuable model of tumor progression in hormone-dependent cancers (130).

At least some of the antiproliferative actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> may be autocrine rather than endocrine. 25-Hydroxyvitamin D<sub>3</sub>, at concentrations too low to activate the VDR, arrests growth in cells expressing 1 $\alpha$ -hydroxylase (18, 212), whereas targeted deletion of 1 $\alpha$ -hydroxylase in keratinocytes (119) abolishes antiproliferative properties of 25-hydroxyvitamin D<sub>3</sub>. An additional consideration for cancer prevention comes from the observation that in prostate cancer cells, the expression of 1 $\alpha$ -hydroxylase decreases with the progression of malignancy, thereby reducing the potency for autocrine 1,25(OH)<sub>2</sub>D<sub>3</sub> production to arrest growth (212). The apparently higher antiproliferative efficacy of autocrine vs. exogenously administered 1,25(OH)<sub>2</sub>D<sub>3</sub> could result from a differential induction of 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism. Higher 1,25(OH)<sub>2</sub>D<sub>3</sub> availability should result from exclusive local induction of 24-hydroxylase compared with the systemic activation of catabolism that follows exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> administration. In fact, in human cancers an inverse association was reported between constitutive expression of 24-hydroxylase in the tumor and the efficacy of vitamin D therapy (65).

**Regulation of apoptosis.** 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced apoptosis is an important contributor to the growth-suppressing properties of the sterol in hyperproliferative disorders. In breast cancer cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> induces apoptosis through reciprocal modulation in Bcl2 and Bax content (247). It also increases intracellular calcium (216), which activates the calcium-dependent proapoptotic proteases microcalpain and caspase 12, with a

major role for microcalpain (163).  $1,25(\text{OH})_2\text{D}_3$  also increases the antitumoral and proapoptotic properties of ionizing radiation in MCF7 breast tumor xenografts in nude mice and TNF- $\alpha$ -induced apoptosis in MCF7 cells through caspase-dependent and -independent mechanisms (229). In contrast to the proapoptotic actions of  $1,25(\text{OH})_2\text{D}_3$  in glioma (77), melanoma, and mammary cancer (242), the sterol elicits no proapoptotic effects in normal astrocytes, melanocytes (208), and mammary cells. More importantly,  $1,25(\text{OH})_2\text{D}_3$  protects keratinocytes from the apoptosis initiated by UV radiation or chemotherapy (71), and primary melanocytes from that initiated by TNF- $\alpha$  and UV irradiation, the latter through induction of sphingosine 1-phosphate (208).

$1,25(\text{OH})_2\text{D}_3$  induction of calbindin D (28K) appears to mediate the protection from apoptotic cell death, through direct inhibition of caspase 3, in various cell types including 1) presenilin-1 proapoptotic signals in neural cells; 2) PTH/PLC induction of apoptosis in renal cells; 3) cytokine- and free radical-mediated destruction of pancreatic  $\beta$  cells; and 4) TNF- $\alpha$ - and glucocorticoid-induced apoptosis in osteoblastic and osteocytic cells (59).

Importantly, in normal tissues,  $1,25(\text{OH})_2\text{D}_3$  proapoptotic properties are critical in controlling hyperplastic growth. In normal mammary tissue,  $1,25(\text{OH})_2\text{D}_3$ -VDR control of apoptosis through caspase 3 and Bax induction is required during pregnancy, lactation, and postlactational involution (265). In the absence of hypocalcemia, VDR-null mice exhibit accelerated lobuloalveolar development and premature casein expression during pregnancy, as well as delayed postlactational involution.

Taken together, these findings demonstrate proapoptotic as well as antiapoptotic effects of vitamin D, important in normal tissue development and function, as well as in the induction of growth arrest in cancer and noncancerous hyperproliferative disorders. The association of certain VDR alleles with susceptibility to cancer (241) suggests the involvement of the VDR. In fact, in mammary epithelial tumor cell lines generated in wild-type and VDR-null mice,  $1,25(\text{OH})_2\text{D}_3$  inhibits growth and induces apoptosis exclusively in the VDR-containing cells of wild-type mice (242).

In addition, in human colorectal cancer, the transcription factor Snail is expressed and recruited to the native VDR promoter, thereby reducing VDR and, consequently,  $1,25(\text{OH})_2\text{D}_3$ -VDR induction of E-cadherin, which influences cell fate during colon cancer progression (189). High levels of Snail causing low VDR content in higher grade colorectal cancers limit the efficacy of  $1,25(\text{OH})_2\text{D}_3$  therapy (189). However, VDR content is not the only determinant of the efficacy of  $1,25(\text{OH})_2\text{D}_3$  in controlling tumor growth. In prostate cancer, altered levels of expression of the steroid receptor corepressor SMRT or defective nuclear VDR localization, but not reduced VDR levels, is responsible for the resistance to  $1,25(\text{OH})_2\text{D}_3$  antiproliferative properties (132). Interestingly, recent studies showed reversal of the resistance to  $1,25(\text{OH})_2\text{D}_3$  therapy caused by decreased VDR and increased NCoR1 content in the breast cancer cell line MDA MB231 using the histone deacetylation inhibitor trichostatin A (13).

Importantly, micromolar concentrations of  $1,25(\text{OH})_2\text{D}_3$  arrest growth and induce apoptosis in mammary epithelial tumor cell lines generated from VDR-null mice (242), suggesting the existence of VDR-independent mechanisms.

*Modulation of immune responses.* The efficacy of the vitamin D endocrine system in controlling infection, autoimmune diseases, and tolerance in transplantation can be attributed to  $1,25(\text{OH})_2\text{D}_3$  prodifferentiating effects on monocyte-macrophages, antigen-presenting cells, dendritic cells (DC), and lymphocytes. This section integrates the evidence from *in vivo* studies in humans and animals lacking either VDR function or vitamin D with that from *in vitro* findings to assemble a model for the functional regulation of the immune system by  $1,25(\text{OH})_2\text{D}_3$ .

A causal relationship exists between  $1,25(\text{OH})_2\text{D}_3$ -VDR function and innate and adaptive immunity to infections: recurrent infections are commonly associated with vitamin D-deficient rickets (104), and an impaired immune defense mechanism often accompanies the  $1,25(\text{OH})_2\text{D}_3$  deficiency of chronic renal failure (10). Subtle changes in VDR function, as the result of the expression of certain VDR alleles, affect the susceptibility or resistance to mycobacterial or viral infection.  $1,25(\text{OH})_2\text{D}_3$  also functions as a vaccine adjuvant (68). Mechanistically,  $1,25(\text{OH})_2\text{D}_3$  induction of p21 and C/EBP $\beta$  could mediate the enhancement of monocyte-macrophage immune function. As mentioned earlier,  $1,25(\text{OH})_2\text{D}_3$  induction of p21 is sufficient to direct monocyte differentiation toward mature macrophages (154). C/EBP $\beta$  is a transcription factor critical for macrophage antibacterial, antiviral, and antitumoral activities and for the synthesis of IL-12, the cytokine mediating potent Th1 responses. In fact, severe impairment of all of these macrophage properties occurs in macrophages from C/EBP $\beta$ -null mice (96). Thus  $1,25(\text{OH})_2\text{D}_3$  induction of C/EBP $\beta$  expression in cells of the monocyte-macrophage lineage (126) could contribute to  $1,25(\text{OH})_2\text{D}_3$ -mediated enhancement of monocyte differentiation to macrophage, immune function, host defense against bacterial infection, and tumor cell growth.

Local  $1,25(\text{OH})_2\text{D}_3$  production by the disease-activated macrophage could explain the association between vitamin D deficiency and increased susceptibility to mycobacterial infections. Recent studies have provided important insights into cytokine regulation of macrophage  $1,25(\text{OH})_2\text{D}_3$  production.  $\gamma$ -Interferon, the cytokine elevated in proportion to the severity of tuberculosis, is a powerful inducer of macrophage  $1\alpha$ -hydroxylase gene expression and, therefore,  $1,25(\text{OH})_2\text{D}_3$  production. Interestingly,  $\gamma$ -interferon transactivates macrophage  $1\alpha$ -hydroxylase through a C/EBP $\beta$ -mediated process (82) similar to that described in cAMP induction of the renal enzyme. In the presence of increased  $\gamma$ -interferon levels, neither is macrophage  $1\alpha$ -hydroxylase subjected to feedback inhibition by  $1,25(\text{OH})_2\text{D}_3$  nor can  $1,25(\text{OH})_2\text{D}_3$  induce  $24$ -hydroxylase and, therefore, its own catabolism. Macrophage-produced  $1,25(\text{OH})_2\text{D}_3$  not only induces macrophage antibacterial function but synergizes with the Stat1-mediated actions of  $\gamma$ -interferon (246), the most potent macrophage-activating cytokine. Interactions between the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex and  $\gamma$ -interferon-activated Stat1 prevent Stat1 deactivation, thus prolonging Stat1 transactivation of  $\gamma$ -interferon-responsive genes, including C/EBP $\beta$ , which greatly enhance macrophage immune function. The potency of the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex in controlling mycobacterial infections *in vivo* contradicts the *in vitro* data of  $1,25(\text{OH})_2\text{D}_3$  strong suppression of IL-12 and  $\gamma$ -interferon synthesis, as well as Th1 responses. The role of the VDR in the development of Th1 responses is evident from studies in VDR-null mice which showed impaired pro-

duction of the Th1-promoting factor IL-18, decreased Th1 proliferative responses to CD3 and CD28 stimulation in the presence of exogenous IL-12, and decreased expression of Stat4, a Th1 transcription factor (187).

Studies on the function of  $1,25(\text{OH})_2\text{D}_3$  as a vaccine adjuvant suggest that local high levels of macrophage-produced  $1,25(\text{OH})_2\text{D}_3$  could induce T cell responses, including CD4-Th2 cell-mediated and mucosal antibody responses to cutaneous antigens in vivo, an integral component of the host defense protection mechanism against colonization with infectious agents (104).

In contrast to its stimulatory effects on monocyte-macrophages,  $1,25(\text{OH})_2\text{D}_3$  acts as an immunosuppressive agent in lymphocytes (164). Several cytokines involved in T cell functions are direct targets for  $1,25(\text{OH})_2\text{D}_3$  actions, including IL-2, which is suppressed via  $1,25(\text{OH})_2\text{D}_3$ -VDR impairment of NF-AT complex formation at the distal NF-AT site of the IL-2 promoter (6).  $1,25(\text{OH})_2\text{D}_3$  also promotes the development of Th2 cells through direct effects on naive CD4<sup>+</sup> cells.

A recently appreciated  $1,25(\text{OH})_2\text{D}_3$  action is the maintenance of DC in a state of immaturity. DC are highly specialized antigen-presenting cells that can prime naive T cells in either a tolerogenic (immature) or an immunogenic manner, depending on the nature of the processed antigen and the state of DC maturation. In either monocyte-derived DC or bone marrow-derived DC in vitro, treatment with  $1,25(\text{OH})_2\text{D}_3$  results in reduced expression of the costimulatory molecules DC40, DC80, DC86, and IL-12 and increases in IL-10 (195).  $1,25(\text{OH})_2\text{D}_3$  also upregulates the ILT3 receptor in DC cells, which associates with tolerance induction and modulation of chemokine production (53). The combination of effects prompts T cells with tolerogenic properties that favor suppressor T cell enhancement.

In relation to  $1,25(\text{OH})_2\text{D}_3$  efficacy in the establishment and or maintenance of immunological self-tolerance, seminal studies demonstrate  $1,25(\text{OH})_2\text{D}_3$  inhibition of disease induction in experimental autoimmune encephalomyelitis (EAE), thyroiditis, insulin-dependent diabetes mellitus, inflammatory bowel disease (IBD), systemic lupus erythematosus, and both collagen-induced arthritis and Lyme arthritis, as reviewed by Hayes et al. (104) and Adorini et al. (3). Differences exist, however, between EAE and IBD in vitamin D responsiveness. While vitamin D deficiency accelerates the development of EAE, less severe EAE occurs in VDR-null mice (166). This raises some questions as to the relative roles of  $1,25(\text{OH})_2\text{D}_3$  and the VDR in regulating immune responsiveness. In contrast, the susceptibility to IBD is markedly enhanced in VDR-null mice (89), raising the interesting possibility that vitamin D regulation of autoimmunity differs between the gastrointestinal tract and the central nervous system. The seasonal variations in the onset and severity of multiple sclerosis provide an important insight into the importance of vitamin D status from sunlight exposure in immune function because serum  $25(\text{OH})\text{D}$ , but not  $1,25(\text{OH})_2\text{D}_3$ , varies seasonally (79). The ability of DC cells to synthesize  $1,25(\text{OH})_2\text{D}_3$  (88) supports a role for local  $1,25(\text{OH})_2\text{D}_3$  production in mounting a tolerogenic response while sensitizing proinflammatory DC to apoptosis (223).

$1,25(\text{OH})_2\text{D}_3$  also inhibits rejection of transplanted tissue. In experimental heart transplantation in rats,  $1,25(\text{OH})_2\text{D}_3$  is more efficacious than cyclosporin in prolonging allograft survival, without increasing susceptibility to fungal or viral infec-

tion (122). In renal transplantation,  $1,25(\text{OH})_2\text{D}_3$  prolongs the function of the transplanted kidney by decreasing intragraft fibrosis (11). The cross talk between  $1,25(\text{OH})_2\text{D}_3$ -VDR and TGF- $\beta$ /Smad3 interactions appears to mediate this process (256).

The ability of  $1,25(\text{OH})_2\text{D}_3$  to reduce rejection in pancreatic islet and liver grafts was attributed to the reduction in the levels of costimulatory molecules in DC cells as well as T suppressor cells.  $1,25(\text{OH})_2\text{D}_3$  reduced intragraft levels of IL-2 and IL-12 while increasing IL-4 and IL-10 concentrations, thereby signaling a possible shift to Th2-mediated responses (97).

In summary, the mechanisms underlying  $1,25(\text{OH})_2\text{D}_3$  immune actions could be attributed to a paracrine feedback loop that resolves inflammation or influences the differentiation fate of activated CD4 T cells and/or the enhancement of suppressor T cell function, or a combination of these.

*Control of differentiation and function in the skin.* Vitamin D was used to treat a variety of skin diseases including psoriasis in the 1930s. However, it was not until the mid-1980s that the therapeutic potential of vitamin D in skin diseases reemerged. A dramatic improvement was seen in the psoriatic lesions in a patient receiving oral  $1\alpha$ -hydroxyvitamin  $\text{D}_3$  to treat severe osteoporosis (171). As mentioned earlier,  $1,25(\text{OH})_2\text{D}_3$  antiproliferative properties in psoriatic keratinocytes overexpressing TGF- $\alpha$  could result from  $1,25(\text{OH})_2\text{D}_3$  efficacy in inhibiting mitogenic signals from the TGF- $\alpha$ /EGFR growth loop (62). The immunosuppressive properties of  $1,25(\text{OH})_2\text{D}_3$  on Langerhans cells, the antigen-presenting cells of the epidermis, could also mediate the efficacy of the sterol in treating psoriasis, melanoma, and scleroderma.

The  $1,25(\text{OH})_2\text{D}_3$ -VDR complex is also essential for normal hair and skin development. The critical role of the VDR, but not  $1,25(\text{OH})_2\text{D}_3$ , in the hair cycle was conclusively demonstrated by the development of alopecia in patients with hereditary vitamin D-resistant rickets and in VDR-null mice, whereas alopecia is absent in  $1\alpha$ -hydroxylase-null mice or in patients with vitamin-deficient rickets type I. Importantly, unlike other phenotypic features of vitamin D resistance in mice lacking VDR, normalization of serum calcium fails to correct the alopecia, dilated hair follicles, and dermal cysts (150). Further studies on the role of the VDR in the hair cycle allowed the identification of the hairless (Hr) gene as a potent repressor of VDR-mediated transcription. Unlike other VDR corepressors, Hr does not interact with the AF2 domain of the VDR (118).

In normal keratinocytes, locally produced  $1,25(\text{OH})_2\text{D}_3$  induces a number of proteins directly involved in differentiation. In addition, autocrine effects of  $1,25(\text{OH})_2\text{D}_3$  include increases in CaR and phospholipase C gene expression, which are required for modulation of keratinocyte differentiation by calcium (24). Recent studies in  $1\alpha$ -hydroxylase-null mice indicate that  $1,25(\text{OH})_2\text{D}_3$  is mandatory for the maintenance of a steep calcium gradient within the epidermis that affects normal keratinocyte function, the integrity of the permeability barrier, and the recovery when the permeability barrier is disrupted. As mentioned earlier, extracellular calcium induces a differential association of the VDR with two distinct family of coactivator molecules (DRIP205 and SRC3), thus controlling  $1,25(\text{OH})_2\text{D}_3$ -VDR transcriptional activity as well as Ca/ $1,25(\text{OH})_2\text{D}_3$  cooperative regulation of keratinocyte differentiation and function.



**Control of the renin-angiotensin system.** The renin-angiotensin system plays a central role in the regulation of blood pressure, electrolytes, and volume homeostasis. Several epidemiological and clinical studies suggested an association between inadequate sunlight exposure or low serum  $1,25(\text{OH})_2\text{D}_3$  with high blood pressure and/or high plasma renin activity (149, 151).  $1,25(\text{OH})_2\text{D}_3$  acts as a negative endocrine regulator of the renin-angiotensin system. In VDR-null mice, marked increases in renin expression and plasma angiotensin II production caused hypertension, cardiac hypertrophy, and increased water intake. Corroborative studies in wild-type mice demonstrated that inhibition of  $1,25(\text{OH})_2\text{D}_3$  synthesis increased renin expression and that  $1,25(\text{OH})_2\text{D}_3$  administration suppressed renin production through a VDR-mediated mechanism unrelated to changes in serum calcium.

**Control of insulin secretion.** In experimental animals, vitamin D deficiency associates with an earlier and more aggressive onset of diabetes, probably related to abnormalities in immune function, and impaired glucose-mediated insulin secretion that can be reversed by calcitriol repletion (57).  $1,25(\text{OH})_2\text{D}_3$ , through a VDR-mediated modulation of calbindin expression, appears to control intracellular calcium flux in the islet cells, which, in turn, affects insulin release (58).

In the  $1,25(\text{OH})_2\text{D}_3$  deficiency of chronic kidney disease, there is abnormal insulin secretion, a blunted response of the pancreatic  $\beta$  cells to glucose challenge, and insulin resistance (5, 7, 158).  $1,25(\text{OH})_2\text{D}_3$  deficiency produces abnormal regulation of insulin secretion independently of alterations in VDR levels in pancreatic  $\beta$  cells. Also,  $1,25(\text{OH})_2\text{D}_3$  administration corrects the abnormal insulin secretion independently of changes in serum levels of calcium or PTH (4, 198). The finding of  $1\alpha$ -hydroxylase activity in pancreatic cells (211) raises the possibility of an autocrine control of insulin secretion by  $1,25(\text{OH})_2\text{D}_3$ .

**Control of muscle function.** Skeletal muscle weakness and atrophy, with electrophysiological abnormalities in muscle contraction and relaxation, occur in vitamin D deficiency, in  $1,25(\text{OH})_2\text{D}_3$  deficiency due to chronic kidney disease, and with the prolonged use of anticonvulsant drugs that decrease serum 25-hydroxyvitamin D levels. Although these defects were originally attributed to low calcium, there is evidence from studies in VDR-null mice of direct  $1,25(\text{OH})_2\text{D}_3$  action on skeletal muscle growth and differentiation (28, 80).

In the heart,  $1,25(\text{OH})_2\text{D}_3$  controls hypertrophy in cardiac myocytes (251) and the synthesis and release of atrial natriuretic factor (250). In end-stage renal disease, therapy with 25-hydroxyvitamin D or  $1,25(\text{OH})_2\text{D}_3$  improves both left ventricular function in patients with cardiomyopathies and skeletal muscle weakness. The mechanisms involved are unclear. In vitro, vitamin D analogs elicit a differential potency to regulate muscle cell metabolism and growth (215), which suggests a therapeutic role in ameliorating the myopathies associated with chronic kidney disease.

**Control of the nervous system.**  $1,25(\text{OH})_2\text{D}_3$  actions in the nervous system include induction of VDR content (VDR is expressed in the brain and on several regions of the central and peripheral nervous system) (43), the conductance velocity of motor neurons, and the synthesis of neurotrophic factors, such as nerve growth factors and neurotrophins, that prevent the loss of injured neurons (47, 50).  $1,25(\text{OH})_2\text{D}_3$  also enhances the expression of glial cell line-derived neurotrophic factor, a

potential candidate for treatment of Parkinson's disease (206). In addition to increased nerve growth factor, combined treatment with  $1,25(\text{OH})_2\text{D}_3$  and  $17\beta$ -estradiol in rats elicits neuroprotective effects after focal cortical ischemia induced through the phototrombosis model (157).

$1,25(\text{OH})_2\text{D}_3$  influences critical components of orderly brain development (165). In the embryonic rat brain, the VDR increases steadily from day 15 to day 23, and  $1,25(\text{OH})_2\text{D}_3$  induces the expression of nerve growth factor and stimulates neurite outgrowth in embryonic hippocampal explants and primary cultures. Low prenatal vitamin D in utero leads to increased brain size, altered brain shape, enlarged ventricles, and reduced expression of nerve growth factor in the neonatal rat.

The association of vitamin D deficiency and abnormal brain development makes vitamin D an attractive candidate for treatment of schizophrenia, a disorder resulting from gene-environment interactions that disrupt brain development (161). Also, transient prenatal vitamin D deficiency in rats induces hyperlocomotion in adulthood with severe motor abnormalities (45).

## CONCLUDING REMARKS

Critical findings in the last five years have improved our understanding of vitamin D bioactivation and actions and the relevance of the vitamin D endocrine system in disease prevention.

In relation to vitamin D bioactivation to  $1,25(\text{OH})_2\text{D}_3$ , CYP2R1, a nonexclusively hepatic cytochrome P-450, was identified as the critical 25-hydroxylase, suggesting that hepatic and extrahepatic 25-hydroxyvitamin D synthesis could contribute to vitamin D status. In the kidney, 25-hydroxyvitamin D uptake by proximal tubular cells was shown to require receptor-mediated endocytosis by megalin, a protein not ubiquitously distributed and induced by  $1,25(\text{OH})_2\text{D}_3$ , whereas intracellular vitamin D binding proteins mediate the final transport of  $25(\text{OH})\text{D}$  to mitochondrial  $1\alpha$ -hydroxylase. Cell-specific differences in the expression of the proteins mediating active substrate uptake and delivery to renal  $1\alpha$ -hydroxylase in kidney disease and to the extrarenal  $1\alpha$ -hydroxylases of DC and numerous other cell types should affect not only serum  $1,25(\text{OH})_2\text{D}_3$  levels but local  $1,25(\text{OH})_2\text{D}_3$  production and, therefore,  $1,25(\text{OH})_2\text{D}_3$  autocrine control of immune responses, cell growth, differentiation, and secretory function.

Of great importance in  $1,25(\text{OH})_2\text{D}_3$ -VDR regulation of gene transcription were the findings that the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex controls the gene expression of critical nuclear coregulator molecules and splicing by the spliceosome, thus regulating not only the cell competence in inducing or repressing VDR transcriptional activity but also the splicing of transcripts of vitamin D-responsive genes.

Several VDR-dependent and -independent mechanisms were characterized as critical in bone remodeling and renal and intestinal calcium uptake, or parathyroid and chondrocyte growth, respectively. Also, a novel cross talk emerged between extracellular calcium and  $1,25(\text{OH})_2\text{D}_3$ -VDR. The  $1,25(\text{OH})_2\text{D}_3$ -VDR complex not only controls extracellular calcium levels but the cellular responses to calcium as well, through modulation of the expression of the calcium sensor in the parathyroid glands and in several other tissues, including the kidney. In turn, extracellular calcium modulates the re-

sponse to  $1,25(\text{OH})_2\text{D}_3$ -VDR, in a cell-specific manner, through the selective recruitment to the nuclear VDR of co-regulator molecules that affect VDR transactivation potency.

The link between a defective vitamin D endocrine system and hypertension, cancer, and noncarcinogenic hyperproliferative disorders, autoimmune diseases, and susceptibility to infections has been further characterized. The  $1,25(\text{OH})_2\text{D}_3$ -VDR complex was found to 1) suppress the renin-angiotensin system; 2) induce C/EBP $\beta$ , a potent suppressor of the cyclin D1 oncogene in epithelial carcinomas in humans, and an inducer of macrophage differentiation and immune function; 3) promote and/or prevent apoptosis as required for normal tissue development; 4) inhibit growth signals from activated EGFR in EGFR-driven carcinomas and secondary hyperparathyroidism; and 5) modulate the immunogenic or tolerogenic state of DC, the main determinants of the susceptibility to infections, autoimmune diseases, or the development of tolerance after transplantation.

The strong epidemiological association between vitamin D status [rather than serum  $1,25(\text{OH})_2\text{D}$  levels] and susceptibility to infections, autoimmune diseases, and cancer suggests an advantage for local  $1,25(\text{OH})_2\text{D}_3$  production over systemic  $1,25(\text{OH})_2\text{D}_3$  administration in growth arrest, immunomodulation, and cell secretory functions. Future studies in this area should help optimize the use of the vitamin D endocrine system in disease prevention.

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