Vitamin D and Human Health: Lessons from Vitamin D Receptor Null Mice

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The vitamin D endocrine system is essential for calcium and bone homeostasis. The precise mode of action and the full spectrum of activities of the vitamin D hormone, 1,25-dihydroxyvitamin D \([1,25-(OH)_2D]\), can now be better evaluated by critical analysis of mice with engineered deletion of the vitamin D receptor (VDR). Absence of a functional VDR or the key activating enzyme, 25-OHD-1α-hydroxylase (CYP27B1), in mice creates a bone and growth plate phenotype that mimics humans with the same congenital disease or severe vitamin D deficiency. The intestine is the key target for the VDR because high calcium intake, or selective VDR rescue in the intestine, produces a bone and growth plate phenotype. The VDR is nearly ubiquitously expressed, and almost all cells respond to 1,25-(OH)_2D exposure; about 3% of the mouse or human genome is regulated, directly and/or indirectly, by vitamin D hormone. VDR-deficient mice, but not vitamin D- or 1α-hydroxylase-deficient mice, and man develop total alopecia, indicating that the function of the VDR and its ligand is not fully overlapping. The immune system of VDR- or vitamin D-deficient mice is grossly normal but shows increased sensitivity to autoimmune diseases such as inflammatory bowel disease or type 1 diabetes after exposure to predisposing factors. VDR-deficient mice do not have a spontaneous increase in cancer but are more prone to oncogene- or chemocarcinogen-induced tumors. They also develop high renin hypertension, cardiac hypertrophy, and increased thrombogenicity. Vitamin D deficiency in humans is associated with increased prevalence of diseases, as predicted by the VDR null phenotype. Prospective vitamin D supplementation studies with multiple noncalcemic endpoints are needed to define the benefits of an optimal vitamin D status. (Endocrine Reviews 29: 726–776, 2008)

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First Published Online
Abbreviations: AF-2, Activation function-2; APC, antigen-presenting cell; CaBP, calcium binding protein; CAMP, human cathelicidin antimicrobial peptide; CYP, cytochrome P<sub>450</sub>; DBD, DNA binding domain; DBP, vitamin D binding protein; DC, dendritic cell; 7-DHC, 7-dehydrocholesterol; DMBA, 7,12-dimethylbenzanthracene; DRIP, vitamin D receptor interacting protein; EAE, experimental autoimmune encephalomyelitis; EGF, epidermal growth factor; FGF23, fibroblast growth factor 23; HAT, histone acetyltransferase; KO, knockout; LPS, lipopolysaccharide; MHC, major histocompatibility complex; Na/Pi, sodium-dependent phosphate cotransporter; NCX1, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; NFAT, nuclear factor of activated T cells; NRf2, nuclear factor xB; NOD, nonobese diabetic; NR, nuclear receptor; nVDRE, negative VDRE; 1,25-(OH)_2D, 1,25-dihydroxyvitamin D<sub>3</sub> or 1,25-dihydroxyvitamin D<sub>2</sub>; 25-OHD, 25-hydroxyvitamin D<sub>3</sub> or 25-hydroxyvitamin D<sub>2</sub>; FMCA<sub>110</sub>, plasma membrane calcium ATPase; PSA, prostate-specific antigen; RXR, retinoid X receptor; SRC, steroid receptor coactivator; Th, T helper cell; TLR, Toll-like receptor; TRAP, T<sub>r</sub> receptor auxiliary protein; TRPV, transient receptor potential vanilloid; VDR, vitamin D receptor; VDRE, vitamin D responsive element; WT, wild-type.

Endocrine Reviews is published by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
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I. Introduction

A. History of the vitamin D endocrine system

The first extensive description of the clinical picture of rickets, including the name, dates from the 17th century in a Ph.D. thesis equivalent presented at the University of Lug-dunum Batavorum (Leiden, The Low Countries) in 1645 by Daniel Whistler and, briefly thereafter, by Francis Glisson (in collaboration with G. Bate and A. Regemortel) in London in 1650. In addition, a Dutch physician who also lived briefly in London, Ireland, and Paris published in 1649, in his native language, a description of “tabes pectoralis”, the typical thoracic malformation of rickets (1, 2).

The origin of the disease was largely unknown until the discovery of the dual origin of vitamin D at the beginning of the 20th century as a nutritional compound by Mellanby in the United Kingdom and McCollum in the United States (discovery of dietary vitamin D), whereas Huldshinsky, Chick, and Hume, Hess, and Weinstock discovered the curative effects of UV light (3–8). Steenbock discovered, around the same time, that UV irradiation of vegetarian oil produced the antirachitic substance vitamin D2 and patented this discovery.

Rickets, as a disease of children of either rich families (deliberately avoiding exposure to sunshine in countries such as the United Kingdom and India) or very poor families living in the slums of big European industrialized cities, gradually disappeared thereafter due either to better exposure to sunshine or to the oral use of vitamin D-rich cod liver oil. The chemical identification and chemical synthesis of vitamin D earned A. Windaus the Nobel Prize in 1938 (9).

The other major historic steps in the vitamin D saga started with the discovery of the complex metabolism of vitamin D into more than 41 metabolites, especially 25-hydroxyvitamin D (25-OHD) and 1,25-dihydroxyvitamin D [1,25-(OH)2D], and the complex regulation of the renal production of the active end product 1,25-(OH)2D as a true steroid hormone. Subsequently, the transport of vitamin D metabolites outside the cell [by lipoproteins, albumin, and vitamin D binding protein (DBP)] and inside the cell [vitamin D receptor (VDR)], and finally the identification of VDR as a nuclear transcription factor regulating a very large number of genes, further confirmed 1,25-(OH)2D as a classical calciotropic hormone (reviewed in Refs. 10–22).

The nearly ubiquitous presence of VDR, the extrarenal production of vitamin D metabolites, the regulation of multiple genes not involved in calcium metabolism, and finally the careful analysis of the phenotype of VDR-deficient mice and men broadened the scope or spectrum of activities of the vitamin D-VDR endocrine system. The present review will, therefore, primarily focus on the biological action profile of the vitamin D-VDR system, as revealed by studying animals with genetically modified expression of the VDR or other genes involved in vitamin D metabolism or actions. Of course, whenever possible, comparisons with human data will be included, especially those relevant to the (possible) consequences of vitamin D deficiency or resistance for human physiology or disease.

B. Vitamin D metabolism

Vitamin D derives from nutritional origin or is synthesized in the skin under influence of UV-B light. This is a purely photochemical reaction, and no enzymes are involved. However, the reaction requires a sufficiently large concentration of 7-dehydrocholesterol (7DHC) and UV-B 290–315 nm light. This 7DHC is the normal last step in the de novo synthesis of cholesterol and is usually present in low concentrations, unless the activity of 7DHC-Δ7-reductase is high. Information on the regulation of this enzyme is limited, except that total absence causes cholesterol deficiency and Smith-Lemli-Opitz syndrome. Increased activity of this enzyme (e.g., in feline species) eliminates the photoproduction of vitamin D, which then becomes a true vitamin. Vitamin D needs both 25- and 1α-hydroxylation to become the active hormone 1,25-(OH)2D. At least four enzymes, all microsomal cytochrome P450 (CYP) isoforms (CYP2DII, CYP2D25, CYP3A4, and CYP2R1), can accomplish the 25-hydroxylation of vitamin D in human liver cells, but the low-capacity, high-affinity microsomal CYP2R1 is most likely the key enzyme because a homoyzygous mutation was found in a patient with classical rickets and low circulating 25-OHD levels (23). The first putative liver 25-hydroxylase was purified by Russell’s group (24) and later cloned and renamed as CYP27A1. This mitochondrial enzyme is, however, mainly involved in cholesterol and bile acid metabolism and has only a low-affinity high-capacity 25-hydroxylase activity. CYP27A1 null mice and men therefore, exhibit (near) normal 25-OHD concentrations.

Little is known about the regulation of these (putative), 25-hydroxylases, but serum 25-OHD generally reflects the vitamin D nutritional status, and thus little feedback regulation is assumed. By contrast, there seems to be only one 25-OHD-1α-hydroxylase (CYP27B1), which is expressed at the highest concentration in the kidney, where its activity is regulated by calcium and phosphate as well as by their regulating hormones [calcium, PTH, calcitonin, GH, and IGF-I being positive regulators; phosphate, fibroblast growth factor 23 (FGF23), and 1,25-(OH)2D itself being negative regulators] (reviewed in Ref. 13).

Although the kidney seems to be the unique production of...
site of serum 1,25-(OH)₂D as originally described (25), exactly the same enzyme is expressed in many other tissues such as skin, monocytes, placenta, and bone cells, probably to produce 1,25-(OH)₂D for autocrine and paracrine action (reviewed in Refs. 26 and 27). However, the local regulation of the CYP27B1 is quite different because immune stimuli, and not calcium regulating hormones, control monocyte CYP27B1 activity and expression (28). Inactivation of CYP27B1 gene causes a disease previously described in children as vitamin D-resistant rickets type 1 (29). These mice and children have severe rickets unresponsive to normal vitamin D replacement but curable by very high vitamin D or 25-OHD intake or normal 1α-hydroxylated vitamin D replacement doses (30, 31). These diseases clearly demonstrate the essential role of this unique gene product (1α-hydroxylase) in calcium homeostasis. A large number of human mutations in the CYP27B1 gene have been described and can be used as a valuable model to identify the possible active sites (32).

Although a very large number of vitamin D metabolites have been identified in vitro and in vivo (12), only a single multifunctional CYP24A1 gene is responsible for the catabolism of 25-OHD and 1,25-(OH)₂D, resulting in either calcitropic acid after initial 24-hydroxylation or a side chain lactone after initial 23-hydroxylation (32). Deletion of CYP24A1 in mice causes endogenous 1,25-(OH)₂D excess and perinatal lethality, likely due to hypercalcemia in half of the newborns (33, 34). Complete lack of CYP24A1 activity during development in CYP24A1 null pups, born from CYP24A1 null mothers, caused severe mineralization defects in intramembranous bones. This bone defect was normalized by genetic elimination of VDR signaling (34). No human cases of CYP24A1 deficiency have yet been described, but altered CYP24A1 gene expression has been associated with human diseases such as breast cancer (35) and asthma (36). Moreover, alternative gene splicing can produce an inactive enzyme and, therefore, expose cells locally to high 1,25-(OH)₂D concentrations (37). The only noncytotoxic enzyme known to metabolize vitamin D is responsible for the 3-epimerisation of vitamin D₃ or its metabolites. The 3-epimers seem to have lower calcemic effects. Young children have considerably higher 3-epi-25-OHD serum levels than adults; however, the biological consequence of this is yet unknown (38).

Vitamin D and its metabolites are transported in the circulation bound to plasma protein, DBP, which shares many structural and evolutionary similarities with albumin. DBP has at least a dual function, involving the depolymerization and binding of actin (39), in addition to the tight binding of 25-OHD (40). DBP is highly polymorphic in humans, and not a single case of total absence of DBP has been described in men. In DBP null mice, plasma concentrations of 25-OHD and 1,25-(OH)₂D are extremely low, and their metabolic clearance is markedly increased. Consequently, mice lacking DBP are prone to rapidly develop vitamin D deficiency when fed a vitamin D-deficient diet (41). DBP is filtered in the glomerulus of the nephron, but it is reabsorbed together with 25-OHD in the renal tubuli by the bulk carrier transporter megalin. Megalin-deficient mice, therefore, cannot reabsorb DBP or 25-OHD in the nephron and, as a result, develop vitamin D-deficiency rickets (42).

C. Vitamin D receptor

The discovery of the binding of the active vitamin D hormone to a protein (43) coincided with the discovery of the renal origin and chemical identification of the hormone itself (25, 44–46). The molecular cloning of VDR in chicken (47) and later in several other species confirmed that 1,25-(OH)₂D functions like many other steroid hormones, by binding to and activating a nuclear transcription factor. VDR expression has been identified in virtually every human tissue. This universal presence of VDR was already a first, and important, hint for a wide spectrum of activities of the vitamin D-VDR endocrine system. Indeed, nearly all nucleated cells express the VDR, albeit at variable concentrations. The tissue and cell type localization of VDR has been confirmed by binding studies, mRNA in situ hybridization, autoradiography, and protein immunocytochemistry (48–50). The few cells or tissues that have low or absent VDR expression include red blood cells, mature striated muscle, and some highly differentiated brain cells, such as the Purkinje cells of the cerebellum (51). The molecular cloning identification of its structure and functional analysis of its structural domains (Fig. 1) clearly confirmed that the VDR belongs to a class of nuclear transcription factors. There are 48 nuclear receptors (NRs) in the human genome, and many of these have a distant evolutionary origin. These receptors probably started as unliganded DNA binding transcription factors. During evolution, most receptors adopted a specific ligand and segregated into transcription factors that remained homodimers or required heterodimerization [with retinoid X receptor (RXR)] (52–54).

The VDR has been found in mammals, birds, amphibians, and fish with a calcified skeleton (e.g., zebrafish), with a high degree of homology in structure, ligand binding, and functionality. The VDR is undetectable in nonchordate species, and this led to the initial hypothesis that a functional vitamin D endocrine system originated during evolution to allow the accumulation of calcium to build a calcified skeleton. However, the VDR was also detected in jawless primitive fish (lamprey or Petromyzon marinus) (55), indicating that the vitamin D system emerged before the development of calcified structures. In the NR family, the closest structural similarity to the VDR is found in the NR group with metabolic functions, such as the PXR, CAR, and FXR (all bile acid or xenobiotic receptors involved in bile acid homeostasis and detoxification) and the oxysteroid receptors LXR (α and β) (reviewed in Ref. 53). The three closely related receptors (VDR, PXR, and CAR) all share the ability to bind bile acid metabolites and are able to activate CYP enzymes involved in xenobiotic detoxication. One might thus speculate that the VDR originated during early evolution of vertebrates as a transcription factor involved in detoxication and only later became a major player in calcium homeostasis. However, the exact functional role of the VDR in primitive vertebrates or fish has not yet been elucidated.

Analogous to most members of group I NRs (56), the VDR functions through heterodimerization with any of the three RXR isoforms. Both apo RXR (without its specific ligand 9-cis-retinoic acid) and VDR acquire the active confirmation
Fig. 1. Structure and structural domains of VDR. A, Schematic representation of the functional domains in the human VDR. A/B, Aminoterminal region. B, Schematic representation of the regulation of gene transcription by ligand-activated VDR-RXR heterodimers. Transcriptional activation requires the action of many multisubunit coactivator complexes that are recruited in a parallel and/or sequential manner (98).
of the ligand binding domain (LBD) upon binding of 1,25-(OH)₂D to VDR (see Section I.C).

The structure-function analysis of the VDR revealed the expected homology with other NRs containing five functional domains (Fig. 1). For the human VDR, a multitude of different transcripts was found; the transcripts vary in their 5′ untranslated region but mostly use the same translation initiation codon and thereby encode an identical 427 amino acid-long VDR protein (Table 1). A certain proportion of the VDR molecules is alternatively spliced. Of note is the 50 amino acid-extended VDR-B1, which originates from alternative posttranscriptional splicing and the use of a more upstream in-frame start codon and is found in most humans cells (57). This isoform coexists with the previously identified “normal” VDR protein in humans. In kidney cells and in several cell lines, this extended variant constitutes up to 30% of the VDR. Functional differences between these two VDR isoforms have been described, including a difference in transactivation capacity of the promoter of the 24-hydroxylase gene (58) (Table 1). The AB domain of VDR is short, and its function has not been thoroughly defined. A polymorphic variant lacking the first three amino acids at the N terminus has increased transactivation potency due to better interaction with the transcription factor TFIIB (59). The two zinc fingers of the DNA binding domain (DBD) are absolutely essential because many mutations in this region cause VDR resistance in children. The elucidation of the functional domains of VDR by mutational analysis was of course preceded by many biochemical and cellular observations that predicted a specific function for the Zn fingers, activation domains, and interactions between VDR and regulatory DNA elements (reviewed in Refs. 21, 49, 60, and 61). Furthermore, the four VDR null mouse strains all lack either the first or second zinc finger, are fully VDR resistant, and, just like human patients, develop the characteristic phenotype, including alopecia. This region is also important for the nuclear translocation of VDR (62). The hinge region of VDR is 20–50 amino acids longer than in most other NRs and is important for the flexibility of VDR to allow DNA interaction via the DBD, as well as interaction of the LBD with the coactivator proteins. The LBD consists of 12 α-helices and three β-sheets and has a highly flexible unique extension between helix 1 and helix 3. This floppy part of the LBD impairs crystallization, and therefore a LBD without this extra loop was used for crystallization with 1,25-(OH)₂D (63). However, this unique loop seems functionally silent because the loss of this loop in engineered human VDR-LBD or in natural zebrafish VDR has no functional consequences. Nevertheless, a C190W (cystine to tryptophan) mutation in this region caused vitamin D resistance, but this observation was only published in abstract form (cited in Ref. 19). The LBD of VDR is characterized by a large pocket (+/− 700 Å²) in comparison with most other NR LBDs. This is probably related to the long and flexible side chain of the ligand (not present in other steroid hormones) and possibly explains the accommodation of many 1,25-(OH)₂D analogs with a bulky or even a double side chain. Many of the human VDR mutations causing vitamin D resistance rickets are due to mutations in the LBD, thereby impairing or abolishing binding to 1,25-(OH)₂D. When the mutation in the LBD only partially impairs ligand binding, rickets is less severe, and occasionally, hair growth is normal. The binding of ligand to the LBD changes the surface of the LBD necessary for improved heterodimerization with RXR. Mutations or absence of the activation factor-2 (AF-2) domain (Fig. 1) destroy VDR function, and this can best be explained by the function of helix 12 in the “mouse trap” model, whereby the ligand binding induces reorientation of helix 12/AF-2/F domain, which is necessary for the creation of a coactivator platform. As VDR is actively shuttling between the cytosol and the nucleus, genetic mutations impairing this translocation may also impair VDR action.

Mutations causing gene deletion, frameshift, abnormal splice sites, or premature stop codons destroy either the expression or binding activity and, thus, abolish VDR action (19, 64, 65). Vitamin D/VDR resistance with a normal VDR has been described in New World monkeys and in a single human case due to abnormally high expression of an intracellular vitamin D responsive element (VDRE) binding protein (66).

The molecular activity of the VDR as transcription factor is largely based on the model of other NRs. The crystal structure of some holoh- and apo-NRs provided the key information that ligand binding reorients the flexible position of helix 12 away from the core of the LBD into a closer compact structure of the LDB, thereby, together with small repositioning of H₃ and H₁₁, creating a hydrophobic cleft composed of helices 3, 4, and 12 and promoting interactions with the conserved LXLL motif of the coactivators (67). Binding of a full receptor antagonist is thought to induce an alternative conformation in the receptor at H₁₂ such that coactivator binding is compromised. The agonist position of VDR, in complex with 1,25-(OH)₂D or ligands, has been confirmed by direct analysis of the appropriate VDR crystals, whereas the antagonist conformation is based on homology with other NRs and in silico modeling techniques.

In humans, multiple polymorphisms of the VDR gene have been identified and thoroughly studied (68, 69). These polymorphisms are distributed throughout the complete VDR gene region and have been associated with disorders like cancer and autoimmune diseases. Among the VDR polymorphisms, the FokI single nucleotide polymorphism of the

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table

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<tr>
<th>Molecular mass</th>
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<tr>
<td>K₄</td>
<td>10⁻¹⁰ M</td>
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<td>K₅</td>
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<td>Tissue concentration</td>
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<td>Structural variants</td>
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**Polymorphisms**

- Large number in 5' promoter
- N terminal: Fok I
- C terminal: BsmI, ApaI, TaqI, TraI, polyA microsatellite
translation start site results in a VDR protein shortened by three amino acids (70) and is not linked to any of the other VDR polymorphisms (69). However, the clinical impact of this VDR FokI polymorphism remains unclear. Analysis of the multiple studies assessing the correlation between the VDR FokI polymorphism and genetic predisposition to bone-related disease, risk of cancer, and immune-mediated diseases [such as diabetes (71–81)] fails to reveal solid associations or to provide a clear functional explanation for the observed differences in disease prevalence (reviewed in Ref. 82).

D. VDRE

Upon 1,25-(OH)2D binding, the VDR is phosphorylated (Fig. 1), and its surface conformation is reconfigured, which is thought to result in the release of corepressors (see Section 1.E). Moreover, in response to ligand, the VDR recruits its preferred dimerization partner, RXR, and binds to VDREs composed of two hexameric binding sites arranged either as direct repeats interspaced by a varying number of nucleotides (but generally three) or as inverted palindromes interspaced by nine nucleotides (49, 83). (A/G)G(G/T)TCA is considered to be the consensus sequence for a VDRE half site, although considerable sequence diversity exists. The two receptors interact symmetrically with their LBD (both in helix 12 activated configuration), but their DBD is asymmetrical, with RXR as a more compact and VDR in an extended configuration between the LBD and DBD. This is probably due to the flexibility of the hinge region and allows the correct positioning of both receptors to the helix structure of DNA. Many genes regulated by vitamin D have multiple VDREs in their promoter, sometimes even far away from the DNA. Many genes regulated by vitamin D have multiple VDREs in their promoter, sometimes even far away from the coding region, and chromatin immunoprecipitation analysis has revealed that the receptor complex binds to different VDREs in cyclic waves (84, 85).

E. Corepressors and coactivators

Modulation of gene expression is, however, not mediated directly by binding of VDR/RXR heterodimers to DNA, but rather is dependent upon the ability of this dimer to recruit coregulatory protein complexes, instead of being silenced by corepressors (86, 87). Analogous to other NRs, unliganded VDR is probably kept transcriptionally silent, even when present in the nucleus and bound to chromatin by one or more corepressors [silent mediator for retinoid and thyroid hormone receptors (SMRT), NR corepressors (NCoR), Alien (88) or sin 3] that deacetylase histones (directly or indirectly) and, thus, keep the chromatin in a densely packed configuration that is inaccessible to the transcription protein complex (89).

1. Positive gene regulation. Upon ligand binding-induced reconfiguration of the VDR, surface coactivators are able to bind VDR. Chromatin immunoprecipitation studies indicate that a coactivator exchange occurs in the transcriptional complex on NR-responsive promoters (85). First, coactivators of the CBP/p300 family and of the p160 protein family, including the steroid receptor coactivators (SRCs) (such as SRC-1), are recruited (90–92). These proteins possess intrinsic histone acetyltransferase (HAT) activity and, by acetylating histone tails, open up the chromatin structure, creating a chromatin environment permissive for gene transcription (93). In a second wave, the vitamin D receptor interacting protein (DRIP)/T3 receptor auxiliary protein (TRAP) multimeric complex is recruited, whereby DRIP205/TRAP220 binds directly to VDR/RXR heterodimers through one of two LXXLL motifs (94–96). Subsequently, basal transcription factors, as well as RNA polymerase II, are recruited to the transcription start sites, and as a result, target gene transcription is induced (97, 98). Gene expression can also be affected by rearrangements in the nucleosome array, mediated by ATP-dependent chromatin remodeling complexes among which SWI/SNF-type and ISWI-type complexes and the multiprotein complex WINAC, the latter of which interacts directly with the VDR, via the Williams syndrome transcription factor (99–101). In addition, several other unrelated proteins with coactivator activity have been identified, among which NCoA-62/skinteracting protein and peroxisome proliferator-activated receptor γ coactivator-1α (102, 103).

2. Negative gene regulation. The VDR not only directly activates gene transcription but also directly down-regulates the transcription of the genes encoding PTH, PTHrP, and CYP27B1 (104–108). Negative VDREs (nVDREs), which closely resemble the consensus VDRE sequence, have been mapped both in the human and rat PTH promoter, and in the human PTHrP gene promoter. These nVDREs are bound by either VDR homodimers or by VDR/RXR heterodimers where the VDR occupies the 5′-located half-site (109, 110). A second group of nVDREs, composed of E-box type motifs (5′-CATCTG-3′) was identified in the human CYP27B1, as well as in the human PTH and PTHrP promoters (105, 106). These nVDREs are transcriptionally active, even in the absence of 1,25-(OH)2D, and are bound by VDR-interacting repressor, a basic helix-loop-helix transcription factor that recruits CBP/p300 HAT activity upon phosphorylation by protein kinase A (107). Although the exact mechanisms responsible for transcriptional repression remain to be elucidated, differentiation signal transduction mechanisms have been proposed. In the presence of 1,25-(OH)2D, ligand-induced coregulator switching occurs and is responsible for ligand-induced transcriptional repression (111). Liganded VDR/RXR heterodimers associate with the VDRE-bound VDR-interacting repressor, which results in the replacement of the CBP/p300 HAT coactivator by histone deacetylases and NCoR/SMRT corepressors. The WINAC multimeric complex facilitates chromatin remodeling during this replacement process (112).

Activated NRs can also bind to the binding sites of other transcription factors and, as such, negatively regulate gene transcription in an indirect manner, as was described for the negative regulation of IL-2 and IL-12 gene transcription (113, 114).

F. Genomic and nongenomic actions of the vitamin D hormone

A broad set of microarray studies have been performed to unravel the molecular pathways involved in the biological
action of 1,25-(OH)2D. These studies have used a variety of technology platforms (spotted cDNA arrays, Affymetrix Genechips, Agilent), although it is only in the last couple of years that arrays were used to profile the entire transcriptome. Gene expression profiles after treatment with 1,25-(OH)2D have been generated in classical [bone (115–120), kidney (121), and intestine (122, 123)] and nonclassical [malignant, immune, and smooth muscle cells (124–126)] target tissues and cells. To gain more insight into the antiproliferative and prodifferentiating effect of 1,25-(OH)2D, genomic profiling has been performed in malignant cells such as prostate (127–133), breast (134–136), leukemia (137, 138), colon (139), and ovarian cancer (140) cells, and squamous cell carcinoma cells (141). Although it is difficult to compare such studies due to variations in the experimental design (e.g., cell culture conditions, concentration of 1,25-(OH)2D, and time points after stimulation with 1,25-(OH)2D), the gene expression profiles all agree that 1,25-(OH)2D regulates, directly and/or indirectly, a very large number of genes (0.8–5% of the total genome) and appears to be involved in a variety of cellular functions including growth regulation, DNA repair, differentiation, apoptosis, membrane transport, metabolism, cell adhesion, and oxidative stress.

The mechanisms underlying the immunomodulatory effects of 1,25-(OH)2D have also been investigated using microarray technology on dendritic cells (DCs) (142) and T cells (143, 144), as well as in tissues isolated from mice with autoimmune diseases such as inflammatory bowel disease [colon (145)] and experimental autoimmune encephalomyelitis [spinal cord (146)] after 1,25-(OH)2D treatment. Gene expression profiles were also examined in heart (147) and kidney (121) tissues isolated from VDR null mice and compared with VDR wild-type (WT) mice.

Until now, investigations on 1,25-(OH)2D action using proteomic analysis have been limited. Recently, 10 proteins (out of 270 proteins analyzed) were identified as being differentially expressed between MCF-7 breast cancer cells treated with 1,25-(OH)2D or vehicle using an antibody-based proteomics approach. The function of most of these differentially expressed proteins was consistent with the antiproliferative and proapoptotic effects of 1,25-(OH)2D in breast cancer cells (148).

Steroid hormones and many other ligands of NRs not only exert their function by directly regulating gene transcription but also display a wide variety of rapid nongenomic or nongenotropic actions. This activity includes a number of rapid (seconds to minutes) and usually transient changes in transmembrane transport of ions (such as calcium and chloride) or intracellular signaling pathways (such as changes in cAMP, protein kinase A, protein kinase C, phospholipase C, phosphatidylinositol-3 kinase, and MAPK activities) (reviewed in Refs. 149–152).

The structural requirements of the ligands, which bind to their membrane receptor and induce these rapid actions, differ from those involved in genomic actions (153). The molecular cloning of the putative membrane receptor for 1,25-(OH)2D is long overdue. The picture is further complicated by the involvement of the classical NR, VDR, in these rapid actions because the presence of VDR is necessary in this nongenomic pathway (154). The nongenomic and genomic activities of NR ligands, including 1,25-(OH)2D, may, however, complement each other because (rapid) activation of second messengers (ions or calmodulin-dependent kinase) may activate (e.g., phosphorylate) VDR and amplify its genomic activity (149, 151).

An important tool to analyze the in vivo function of 1,25-(OH)2D was the generation of VDR and CYP27B1 (1α-hydroxylase) null mice. VDR null mice were generated by four independent groups in Tokyo (155), Boston (156), Leuven (157), and Munchen (158) by disruption of the DNA binding site using different targeting constructs and mice strains. One strain of VDR null mice was generated by using a cre-lox system by crossing VDRlox mice with phosphoglycerate kinase-cre mice (157). Two different groups have engineered 1α-hydroxylase null mice by targeting the protein’s heme binding and (part of) the hormone binding domains (159, 160). The study of these VDR or 1α-hydroxylase null mice, or mice with these deletions in combination with other genetic mutations (Table 2), generated a wealth of information regarding the spectrum of activities of the VDR-vitamin D endocrine system (reviewed in Sections II to XI). These studies also revealed that the VDR may have ligand-independent functions, especially in the skin and the immune system.

II. VDR-Vitamin D Endocrine System and Bone and Growth Plate

A. VDR or 1α-hydroxylase inactivation impairs calcium and bone homeostasis

Although the VDR is widely expressed during embryonic development in tissues involved in calcium homeostasis and bone development, VDR null mice were phenotypically normal at birth. Around weaning, they developed hypocalcemia, secondary hyperparathyroidism, and hypophosphatemia. At this age, VDR null mice also became growth retarded and developed severe rickets and osteomalacia (155–158). Mice deficient in 1α-hydroxylase displayed an analogous phenotype, with respect to calcium and bone homeostasis (159, 160). However, they differed in serum levels of vitamin D metabolites. When the VDR was inactivated, serum 1,25-(OH)2D levels were increased due to secondary hyperparathyroidism, increased renal 1α-hydroxylase activity, and decreased 24-hydroxylase activity. Serum 24,25-(OH)2D levels are decreased after weaning. These changes are consistent with the 1,25-(OH)2D/VDR-mediated gene regulation observed in vitro: negative regulation of 1α-hydroxylase, whereas 24-hydroxylase gene expression is positively regulated by 1,25-(OH)2D-induced VDR action (see Section I.B). On the other hand, 1,25-(OH)2D levels were undetectable in 1α-hydroxylase null mice, whereas 25-OHD levels were elevated. Serum 24,25-(OH)2D levels were decreased, comparable to VDR null mice (155, 161). These phenotypic characteristics could be largely corrected by supplementation of dietary calcium (in combination with high-lactose:rescue diet) in both knockout models or 1,25-(OH)2D treatment of 1α-hydroxylase null mice (157, 161–167). These findings confirm previous observations in humans (168–170).
passive calcium absorption occurs in the jejunum. The molecular players regulating this transport also differ between intestine and kidney: TRPV6 is abundantly expressed in duodenum, whereas TRPV5 is the main apical calcium channel in the kidney; CaBP-9k (molecular mass, 9 kDa) is the only cytosolic calcium transporter in the intestine, whereas both CaBP-9k and CaBP-28k (molecular mass, 28 kDa) are present in the kidney; the plasma membrane calcium ATPase (PMCA1b) regulates calcium extrusion in intestine, whereas both Na⁺/Ca²⁺ exchanger (NCX1) and PMCA1b are involved in the kidney (171, 172).

1. Intestinal calcium absorption. As anticipated, intestinal calcium absorption was manifestly (60%) decreased in adult VDR null mice, as measured by accumulation of serum radiolabelled calcium after oral gavage (157) and by the in situ ligated loop technique (167). As already mentioned, hypocalcemia in VDR null mice develops at the time of weaning, which correlates with the observation that before weaning intestinal calcium absorption in rats occurs by a nonsaturable, 1,25-(OH)₂D-independent mechanism (173). This pathway becomes gradually replaced by a 1,25-(OH)₂D-dependent saturable component. The lack of response of the intestine to 1,25-(OH)₂D may be explained by the relative absence of the VDR during early neonatal life because intestinal VDR remains low during suckling and increases to adult levels 1 wk after weaning (174). However, the onset of growth retarda-
tion and bone disease correlates with weaning, even when weaning was accelerated (2 wk) or delayed (4 wk) (155).

The decrease in intestinal calcium absorption was associated with altered expression of several calcium transport proteins in the duodenum of VDR or 1α-hydroxylase null mice. TRPV6 mRNA levels were extremely low in VDR null mice and were not induced by a low-calcium diet or 1,25-(OH)₂D injection, as observed in WT mice (157). Also CaBP-9k mRNA and protein levels were reduced in intestine, albeit at variable degrees in different VDR null strains (155, 175–177). This decrease was already observed in normocalcemic 2-wk-old VDR null mice, which suggests that it was a consequence of VDR deficiency rather than hypocalcemia. Because CaBP-9k mRNA and protein levels decline with age in WT mice, but not in VDR null mice, the difference in CaBP-9k expression between genotypes becomes smaller in aged mice (178). Of note, 1α-hydroxylase null mice also showed a decrease in CaBP-9k expression (159, 160). On the other hand, PMCA₁b gene expression is unaltered in the duodenum of VDR null mice (157). When given a rescue diet (consisting of high lactose and high calcium), TRPV6 and CaBP-9k gene expression in duodenum decreased both in WT and VDR null mice, resulting in comparable levels between the two genotypes (157, 175, 176). These data suggest that, when paracellular calcium transport becomes sufficient to meet dietary calcium needs, the genes involved in active calcium transport are down-regulated by a still uncharacterized mechanism.

The importance of 1,25-(OH)₂D-regulated genes for intestinal calcium absorption was shown for some of the molecular players. During normal calcium intake, both decreased and unaltered intestinal calcium absorption has been reported for TRPV6 null mice, although normal serum calcium levels could be maintained. When switched to a low-calcium diet, TRPV6 null mice were still able to increase intestinal calcium absorption, but not to the same extent as control mice, resulting in hypocalcemia (179, 180). On the other hand, CaBP-9k ablation had no effect on intestinal calcium absorption or serum calcium levels. A likely explanation is that its role is compensated by other known and/or unknown calcium transport proteins (181, 182). Ablation of both TRPV6 and CaBP-9K did not aggravate intestinal calcium absorption when calcium intake was sufficient, but it did aggravate the effect of TRPV6 deletion when switched to a low-calcium diet (179). Taken together, the VDR promotes adequate intestinal calcium absorption by regulating the expression of several known and/or unknown calcium transport proteins in the duodenum (Fig. 2). Recently, the expression of claudin 2 and claudin 12 was shown to be induced by 1,25-(OH)₂D and decreased in the intestine of VDR null mice. These proteins are suggested to form paracellular calcium channels and highlight a new mechanism behind 1,25-(OH)₂D-dependent calcium homeostasis (183).

2. Renal calcium reabsorption. The kidney contributes to the regulation of the extracellular calcium homeostasis by reabsorbing a large fraction of filtered calcium. As mentioned, 1,25-(OH)₂D/VDR action is involved in the active reabsorption of calcium in the distal tubule. In line herewith, VDR null mice showed inappropriately high urinary calcium excretion given the observed hypocalcemia, suggesting renal calcium wasting due to disturbed calcium reabsorption. This was especially evident in normocalcemic VDR null mice on a rescue diet, which exhibited a significant increase in calcium/creatinine ratio compared with WT mice (158, 178).

Expression of CaBP-9k was consistently decreased in the kidney of both VDR and 1α-hydroxylase null mice fed a normal diet. However, CaBP-28k mRNA levels were only moderately decreased in the kidney of VDR null mice, whereas they were significantly decreased in 1α-hydroxylase null mice. A similar pattern was observed for TRPV5 and NCX1: normal in VDR null mice but reduced in 1α-hydroxylase null mice. The expression of PMCA₁b was unaltered, irrespective of the genotype (155, 157, 158, 160, 164, 176). Feeding these mice a rescue diet lowered CaBP-9k expression in WT and VDR or 1α-hydroxylase null mice, leading to comparable levels between all genotypes (157, 164, 167). On the other hand, normalization of 1,25-(OH)₂D serum levels in 1α-hydroxylase null mice resulted in increased expression of renal calcium transport proteins and normalization of serum calcium levels (164). These data indicate that renal calcium reabsorption is impaired when genomic actions of 1,25-(OH)₂D are lacking and that CaBP-9k may be an important component of this process.

The crucial role of 1,25-(OH)₂D-regulated proteins in mediating renal calcium reabsorption was demonstrated in TRPV5 null mice (184). These mice displayed persistent hypercalciuria associated with increased 1,25-(OH)₂D levels and intestinal (hyper)absorption of calcium. This effect on intestinal calcium absorption was thought to be dependent on the increased 1,25-(OH)₂D levels because compound TRPV5/1α-hydroxylase null mice did not show increased intestinal expression of calcium transporters and were severely hypocalcemic (185). However, TRPV5 null mice remained normocalcemic and did not develop rickets, but showed a reduction in bone mass, particularly in cortical thickness.

On the other hand, genetic ablation of the CaBP-28k gene in mice did not affect calcium homeostasis because serum calcium and phosphate levels and urinary calcium excretion were normal. Only subtle effects on bone were observed (both decreased bone mineral density and increased bone...
TRPV5 plays a crucial role in the regulation of several calcium transport proteins among which CaBP-28k is involved in maintaining calcium homeostasis and skeletal mineralization, but the studies also suggest that CaBP-9k can largely compensate for this role (189). Notably, the double null mice on a regular diet were more growth retarded than VDR null mice and died prematurely at 2.5–3 months of age. Compared with VDR null mice, they were as hypocalcemic but showed more severe hypercalciuria, hyperparathyroidism, and pronounced skeletal abnormalities.

On the rescue diet, serum calcium levels were normalized in both VDR null and double null mice; however, in contrast to VDR null mice, the skeletal abnormalities were not completely corrected in the double null mice. Ablation of NCX1 or PMCA1b resulted in embryonic lethality due to their essential function in cell viability by maintaining normal intracellular calcium levels, thereby precluding studies of their contribution to calcium homeostasis (191–194).

Taken together, VDR action is required for adequate calcium reabsorption in the kidney, by regulating the expression of several calcium transport proteins among which TRPV5 plays a crucial role.

C. Direct vs. indirect role of 1,25-(OH)2D-VDR action in chondrocytes

The VDR is present in the fetal rat at the time of mesenchymal condensation of skeletal tissues (195). However, embryonic bone development is normal in the absence of 1,25-(OH)2D-VDR action. VDR null pups of heterozygote VDR mothers exhibited normal length, and the morphology and mineral content of long bones was not altered. Growth plate characteristics, including gene expression pattern, were comparable to WT littermates. Of note, VDR null fetuses of heterozygote VDR mothers displayed normal resting and proliferating chondrocyte layers. Accordingly, chondrocyte proliferation and expression of markers of chondrocyte differentiation, including collagen X and osteopontin, were not altered in VDR null mice.

However, after weaning the longitudinal growth of long bones of VDR null and 1α-hydroxylase null mice was impaired and x-ray analysis revealed features of advanced rickets, including widening of the epiphyseal growth plate. Histology showed an increase in width and marked disorganization of the growth plate, including impaired mineralization of hypertrophic chondrocytes (Fig. 3) (155, 156, 158–160). Detailed analysis of the growth plate of VDR null mice demonstrated normal resting and proliferating chondrocyte layers.

By comparing several genetic models with dysregulated phosphate homeostasis, it was recently elucidated that circulating phosphate levels are crucial for hypertrophic chondrocyte apoptosis in vivo. Apoptosis of hypertrophic chondrocytes via activation of the caspase-9-mediated mitochondrial pathway was directly regulated by phosphate levels, as suggested by in vitro studies (198). In line with these findings, prevention of dysregulated mineral homeostasis by dietary intervention or 1,25-(OH)2D administration rescued the rachitic phenotype of VDR or 1α-hydroxylase null mice (161–163, 165, 166). Notably, the morphology and width of the growth plate were indistinguishable from those in WT controls, suggesting that the receptor-dependent actions of 1,25-(OH)2D are not required for normal growth plate development or maturation but that impaired mineral ion, and especially phosphate, homeostasis is the primary cause of the rachitic changes.

In accordance with these findings, chondrocyte-specific inactivation of the VDR did not alter chondrocyte development in the growth plate (199). VDR action in chondrocytes regulated bone development and phosphate homeostasis by inducing expression of paracrine factors. Particularly, vascular endothelial growth factor and receptor activator of nuclear factor κB (NFκB) ligand expression was decreased, leading to impaired vascular invasion and decreased osteoclast number in the metaphysis of long bones of juvenile mice. In addition, FGF23 expression in osteoblasts was re-

Fig. 3. Abnormal growth plate morphology and increased osteoid surface in VDR ablated mice. Goldner staining of tibiae showing abnormal growth plate and increased osteoid volume (dark pink) in VDR null mice compared with VDR WT mice.
duced, resulting in increased serum levels of phosphate and 1,25-(OH)₂D (see **Section II.E.2**). Nevertheless, persistent abnormality of the growth plate has been observed in normocalcemic 1α-hydroxylase null mice or double 1α-hydroxylase/VDR null mice on a rescue diet and has been reported in VDR/RXRδ double null mice (200, 201). A possible explanation is that the correction of the calcium/phosphate homeostasis was quantitatively incomplete, precluding normalization of growth plate morphology. Alternatively, 1,25-(OH)₂D may interact with a novel (unknown) NR in chondrocytes that heterodimerizes with RXRδ.

Taken together, 1,25-(OH)₂D-VDR action influences growth plate morphology, mainly indirectly, by preventing hypophosphatemia that decreases chondrocyte apoptosis. On the other hand, VDR expression in chondrocytes affects trabecular bone mass and serum FGF23 levels during skeletal growth.

**D. Changes in bone metabolism by interference with 1,25-(OH)₂D-VDR action**

After weaning, the characteristic features of osteomalacia became apparent in VDR null and 1α-hydroxylase null mice: the trabecular bone volume was increased due to an increase in the amount of unmineralized bone. The number of osteoblasts lining bone surfaces was increased and was associated with elevated serum levels of alkaline phosphatase. Mineral apposition was, however, markedly impaired. In most VDR null and 1α-hydroxylase null strains, the number of osteoclasts was not significantly altered, although a decrease was reported in one strain. Given the hyperparathyroidism, an increased level of osteoclasts would have been expected (161, 162). Cocultures of osteoblasts and osteoclast progenitors, derived from WT and VDR null mice in different genetic combinations, demonstrated that 1,25-(OH)₂D induces osteoclast formation by VDR-mediated actions in the osteoblasts, but that functionally intact osteoclasts can be formed without 1,25-(OH)₂D action when other inducing agents like PTH are present (202). Biomechanical parameters demonstrated increased bone fragility in the hypoplastic VDR null mice (162).

In VDR null mice fed a rescue diet, none of these parameters was significantly different from those in WT littersmates raised under identical conditions; trabecular parameters and biomechanical competence of cortical bone were normal (162). Detailed dietary studies revealed that the Ca/P ratio in the rescue diet was important for bone mineralization because it affected intestinal calcium and phosphorus transport in VDR null mice (203). A comparable effect was seen in 1α-hydroxylase null mice after feeding a rescue diet or treating with 1,25-(OH)₂D (161, 163). Genetic evidence confirmed the importance of intestinal VDR action for bone homeostasis; introducing VDR specifically in the intestine of VDR null mice reversed the decrease in mineralized bone mass as well as the increase in osteoid volume (R. Masuyama, L. Lieben, R. Bouillon, and G. Carmeliet, unpublished observations). These data suggest that the skeletal consequences of VDR inactivation mainly result from impaired intestinal calcium absorption and/or the associated secondary hyperparathyroidism and hypophosphatemia.

In line with these findings, *in vitro* osteoblast differentiation of bone marrow stromal cells isolated from VDR null mice was comparable to WT controls. No difference was observed in alkaline phosphatase activity, gene expression of osteopontin, bone sialoprotein, and osteocalcin and calcium content of mineralized cell cultures (204). However, the number of bone marrow osteogenic progenitors seemed to decline in normocalcemic aging VDR or 1α-hydroxylase null mice compared with WT mice. This decrease was accompanied by reduced trabecular bone volume, suggesting that (direct) 1,25-(OH)₂D-VDR action in bone may become critical with older age (200). Consistent herewith, mice overexpressing VDR in mature osteoblasts demonstrated increased bone volume due to enhanced cortical bone formation. Trabecular bone volume was also increased in these transgenic mice but was associated with decreased trabecular resorption, suggesting that VDR action in mature osteoblastic cells may inhibit osteoclastic bone resorption, in contrast to its effect in immature osteoblasts (205). However, ablation of VDR enhanced calvarial osteoblast differentiation, suggesting that other endocrine and paracrine factors modulate the effect of VDR on osteoblast differentiation *in vivo* (206).

Whereas the overall beneficial effects of vitamin D on bone have been known for about one century because it can prevent or cure rickets and osteomalacia, the direct effects of the 1,25-(OH)₂D endocrine system are less evident and vary between: 1) beneficial, as demonstrated by increased bone mass and strength in osteocalcin promoter-driven VDR overexpression in osteoblasts (205); 2) neutral, because a simple rescue diet can normalize bone histology, bone mass, and strength (discussed in **Section II.D**); and 3) detrimental for bone, because VDR null bone transplanted into WT mice shows increased bone mass (and vice versa, WT bone transplanted into VDR null mice is more osteopenic) (207). Selective VDR deficiency in chondrocytes also increases bone mass as long as the growth plate remains functional (199). Only detailed phenotypic analysis of mice with cell-specific VDR deletion in osteoblasts, osteoclasts (precursors), or osteocytes will allow this question to be addressed appropriately. Preliminary data on osteoblast-specific VDR deletion suggests that cortical bone of these mice is increased (208).

**E. Interaction of 1,25-(OH)₂D-VDR pathway with phosphate-regulating hormones**

1. **Effects on parathyroid gland.** The hyperparathyroidism observed in VDR null and 1α-hydroxylase null mice was characterized by parathyroid hyperplasia, increased PTH mRNA levels in the parathyroids, and elevated serum PTH levels. Feeding these mice a rescue diet normalized circulating PTH levels, suggesting that ambient serum calcium, rather than 1,25-(OH)₂D-VDR action itself, plays a key role in the pathogenesis of hyperparathyroidism (157, 158, 161, 165). Of note, PTH production is negatively regulated by serum ionized calcium levels acting via the calcium-sensing receptor and by 1,25-(OH)₂D serum levels. However, parathyroid gland hyperplasia persisted in the 1α-hydroxylase null mice fed a rescue diet but was normalized when mice were treated with 1,25-(OH)₂D (200).
To investigate the contribution of PTH in the 1α-hydroxylase null phenotype, double null mice were generated (209). Single PTH null mice developed hypocalcemia, hyperphosphatemia, and low serum 1,25-(OH)_{2}D levels (210). The double null mice died at 3 wk with tetany due to severe hypocalcemia, suggesting that secondary hyperparathyroidism contributes to control calcium homeostasis by its effect on bone and kidney (209). Treating these mice with PTHrP or PTH reduced hypocalcemia, apparently by increasing expression of renal calcium transporters, and additionally enhanced bone formation (211). Administration of 1,25-(OH)_{2}D also increased serum calcium levels, presumably by its action on the intestine and kidney, and also improved bone formation independently of PTH (212).

The hyperparathyroidism, in VDR null or 1α-hydroxylase null mice, may have contributed to the hypophosphatemia, because PTH suppresses apical Na/Pi-2a and Na/Pi-2b cotransporters, which mediate phosphate uptake in the proximal renal tubules and small intestine, respectively (213, 214) (Fig. 4). In addition, 1,25-(OH)_{2}D-responsive phosphate transport in the intestine and kidney has been described, presumably by regulating Na/Pi-2b and Na/Pi-2a protein expression, respectively (215, 216). Accordingly, intestinal phosphate uptake was decreased in VDR null mice. However, feeding VDR null mice or 1α-hydroxylase null mice a low-phosphate diet increased both intestinal Na/Pi-2b and renal Na/Pi-2a protein levels, indicating that expression can also be regulated independently of VDR action (217, 218).

2. Effects on FGF23. Another factor involved in phosphate homeostasis is the circulating phosphaturic factor, FGF23, which is produced by osteoblastic cells and leads to renal phosphate wasting. Like PTH, FGF23 can suppress expression of Na/Pi-2a and Na/Pi-2c cotransporters (219, 220). In addition, FGF23 suppresses the expression of PTH and 1α-hydroxylase, thereby decreasing 1,25-(OH)_{2}D levels (220, 221). The expression of FGF23 is regulated by both 1,25-(OH)_{2}D-dependent and VDR-independent signaling. Stimulation of the 1,25-(OH)_{2}D-VDR pathway induces the expression of FGF23, as evidenced by increased FGF23 levels after 1,25-(OH)_{2}D administration (222). In line with these findings, VDR null mice showed undetectable FGF23 levels (223, 224). In addition, normalization of serum calcium and phosphate levels by dietary means increased FGF23 levels in VDR null mice, indicating that FGF23 expression is also regulated by a VDR-independent pathway, with phosphate being a potent stimulator (223, 224). Moreover, FGF23 expression was inhibited by low serum phosphate levels, which resulted in increased Na/Pi-2a expression and renal phosphate reabsorption (217, 224). To exert its biological function, FGF23 is not dependent on a functional 1,25-(OH)_{2}D-VDR system. Notably, treatment of VDR null mice with FGF23 further decreased hypophosphatemia due to reduced renal and intestinal phosphate absorption, accompanied by decreased Na/Pi (natriuretic-dependent phosphate cotransporter)-2a, Na/Pi-2b, and 1α-hydroxylase expression (223, 225, 226).

As expected, FGF23 null mice showed hyperphosphatemia, moderate hypercalcemia, low PTH levels, and elevated levels of 1,25-(OH)_{2}D. The adult bone phenotype was characterized by a disorganized growth plate lacking hypertrophic chondrocytes and decreased mineralized bone mass with increased osteoid, and it was associated with skeletal nodule formation and soft tissue calcifications (227, 228). Loss of 1,25-(OH)_{2}D activities from FGF23 null mice, by generating compound FGF23/1α-hydroxylase double null mice, resulted in the disappearance of abnormal skeletal nodule formation and soft tissue calcifications, suggesting that at least some of the anomalies found in FGF23 null mice are mediated through increased 1,25-(OH)_{2}D activities. In addition, the severe hyperphosphatemia is reversed into hypophosphatemia, possibly attributable to hyperphosphaturia in compound null mice. The decreased activity of Na/Pi-2a in double mutants may be partly due to the elevated serum PTH levels (228, 229). Comparable effects were observed when FGF23 null mice were crossed with VDR mutant mice and given a rescue diet (230). These data indicate that alterations in mineral metabolism in FGF23 null mice require an intact 1,25-(OH)_{2}D-VDR signaling pathway.

The mineral and bone phenotype of FGF23 null mice is very comparable to the phenotype of Klotho null mice (231). Klotho is a transmembrane protein that enhances FGF23 binding to its receptor complex, implicating Klotho as a cofactor in FGF23-FGF receptor interaction (232). FGF23 serum levels are severely increased in Klotho null mice, but these mice show hyperphosphatemia and hypervitaminosis D, associated with increased expression of Na/Pi-2a and 1α-hydroxylase (229, 233, 234). A significant rescue of this phenotype was obtained when Klotho null mice were fed a vitamin D-deficient diet (234). The altered mineral ion homeostasis, and especially hyperphosphatemia, seemed to be the most important factor causing soft tissue calcifications. Accordingly, Na/Pi-2a null mice displayed hypophosphatemia, associated with secondary increased serum 1,25-(OH)_{2}D and calcium levels leading to renal calcification, which could be rescued by blocking 1,25-(OH)_{2}D activity by genetic means (235).
F. VDR, vitamin D, and tooth development

Teeth contain several types of calcium depositions: the very hard enamel formed by specialized cells that disappear after tooth eruption; dentin, connected with dentinoblasts that survive as long as the living teeth; and finally cementum, generated by cementoblasts, connected with periodontium to the bones of the upper and lower mandible. Thus, each cell type generates a specialized calcified tissue. The cell types responsible for generating these calcified matrices are under the control of vitamin D, as revealed by selective up- or down-regulation of gene expression (e.g., VDR, MSX, osteocalcin, osteopontin, calbindin D proteins, and matrix proteins such as amelogenin) (236, 237). Vitamin D or 1α-hydroxylase deficiency or VDR resistance during tooth development creates abnormal amelogenesis, dentinogenesis, and cementogenesis (238). The maturation and mineralization of enamel are especially decreased, generating a classic lifelong, irreversible enamel dysplasia. The dentin of VDR null mice (age, 70 d) was also clearly undermineralized and thinner, whereas the pulp chamber was enlarged, as revealed by micro-computed tomography scanning. The predentin area was enlarged in VDR null mice and the mineralization front was irregular. These data clearly indicate compromised dentin maturation in VDR null mice (239). Similar tooth abnormalities have been described in vitamin D- or 1α-hydroxylase-deficient or VDR-resistant children. These rachitic teeth are also more prone to periodontal abscesses. The effects of vitamin D status on periodontitis and tooth loss in adults and the elderly are less well evaluated. In a small-scale prospective trial with or without vitamin D and calcium supplementation for the prevention of osteoporosis, tooth loss was substantially lower (odds ratio, 0.4) in the calcium/vitamin D supplemented group (240).

G. Conclusion

VDR and 1α-hydroxylase null mice display severe hypocalcemia, rickets, and osteomalacia. These effects on calcium and bone homeostasis are largely mediated by 1,25-(OH)2D action on intestinal and renal calcium absorption because these processes are severely impaired in VDR-ablated mice. Normalization of serum calcium by genetic or dietary interventions corrects most of these bone abnormalities. Several intestinal and renal 1,25-(OH)2D-responsive genes have been identified, and studies using transgenic mice confirmed a crucial role for TRPV5 in renal calcium reabsorption. Intestinal calcium absorption involves several transporters (TRPV6, CaBP-9k, and PMCA1b), but inactivation of these genes in mice did not impair intestinal calcium absorption, suggesting redundancy or involvement of other molecules. Besides these indirect effects of 1,25-(OH)2D/VDR on bone, direct, although more subtle, positive and negative effects are also observed but still require detailed analysis. In addition, the 1,25-(OH)2D-VDR endocrine system also regulates phosphate balance, mainly by regulation of PTH and FGF23 levels, as shown by hypophosphatemia, secondary hyperparathyroidism, and undetectable FGF23 levels in VDR null mice.

III. VDR-Vitamin D Endocrine System and Skin

The skin is a unique organ in the vitamin D system because it can synthesize vitamin D in response to UV radiation, is capable of metabolic activation of vitamin D into 25-OHD and 1,25-(OH)2D, can inactive these metabolites by activation of CYP24A1, and finally can express the VDR and responds to VDR activation by induction or repression of a multitude of genes. Therefore, the skin is the sole tissue capable of vitamin D synthesis and activation, as well as autocrine/paracrine activation by the vitamin D hormone. Like calcium, 1,25-(OH)2D promotes differentiation of epidermal keratinocytes (241). Both in vitro and in vivo analyses demonstrate that these two pathways act synergistically. Studies in cultured keratinocytes demonstrate that both increases in calcium and treatment with 1,25-(OH)2D decrease keratinocyte proliferation and promote keratinocyte differentiation (241, 242). In vivo analyses in VDR null mice demonstrate a significant abnormality in differentiation of epidermal keratinocytes after the second week of life (243). However, prevention of hypocalcemia in the VDR null mice prevents this phenotype (241), suggesting that normalization of calcium can compensate for the absence of the VDR in this regard. This observation is consistent with studies demonstrating that cultured keratinocytes isolated from VDR null mice fail to differentiate in response to 1,25-(OH)2D but differentiate normally in response to calcium (244). In addition to being expressed in epidermal keratinocytes, the VDR is also present in the outer root sheath and hair follicle bulb, as well as in the sebaceous glands (245, 246).

A. VDR is essential for hair cycling

Humans with VDR mutations have not been reported to have abnormalities in epidermal keratinocyte differentiation, but, like VDR null mice, most kindreds develop alopecia (19). VDR null mice develop hypocalcemia, hypophosphatemia, hyperparathyroidism, and growth retardation by 21 d of age (Section II), followed shortly thereafter by perioral and periorbital hair loss and ultimately alopecia totalis, associated with large dermal cysts (155–158). Whereas the development of hyperparathyroidism and skeletal abnormalities in the VDR null mice is prevented by maintenance of normal mineral ions, alopecia is not (Fig. 5) (165). Furthermore, when WT and VDR null mice are raised under dietary and UV-free conditions that lead to undetectable circulating 25-OHD and 1,25-(OH)2D levels, VDR null mice develop alopecia whereas WT controls do not (247). Thus, the markedly elevated levels of 1,25-(OH)2D cannot be implicated in the pathogenesis of alopecia in the VDR null mice. Furthermore, the absence of alopecia in WT mice with undetectable circulating levels of vitamin D metabolites suggests that the absence of receptor and the absence of ligand have different effects on the hair follicle. In this experimental model, it is unlikely that local vitamin D is produced in the skin and subsequently converted to 1,25-(OH)2D because this process is dependent upon UV radiation. Furthermore, investigations in mice with targeted ablation of CYP27B1 do not exhibit a hair follicle phenotype (159, 160), confirming that the effects of the VDR on the hair follicle do not require 1,25-(OH)2D. However,
these studies do not preclude the possibility that a novel endogenous VDR ligand, synthesized in the skin, could maintain normal hair growth in the absence of vitamin D and 1,25-(OH)\textsubscript{2}D.

Formation of hair follicles requires signaling between the mesodermal dermal papilla cells and the epithelial keratinocytes. The dermal papilla cells are thought to send signals to keratinocyte stem cells in the bulge region of the follicle, resulting in proliferation and subsequent differentiation into hair follicle keratinocytes (248). There are three defined stages in the hair cycle: anagen, catagen, and telogen. During anagen, cells proliferate rapidly, generating a mature hair follicle that forms a hair shaft. This is followed by regression (catagen) of the keratinocytes to the level of the hair follicle bulge, and finally the quiescent phase, telogen. It is thought that the approximation of the dermal papilla cells to the keratinocyte stem cells in the bulge, during telogen, enables signaling between these cells, resulting in anagen initiation. Although proliferation of neonatal keratinocytes was unaffected by the absence of the VDR, the VDR null mice were unable to respond to anagen-initiating stimuli after hair follicle morphogenesis is complete (the second week of life), suggesting that the VDR is critical for regulating postnatal hair cycles (244).

Hair reconstitution assays identified the cellular source of the hair follicle defect. In this assay, keratinocytes and dermal papilla cells isolated from neonatal WT and VDR null mice were coimplanted sc into nude mice. Generation of normal follicles was initially observed regardless of the VDR status of the cells used, confirming that the VDR is not essential for hair follicle morphogenesis. However, keratinocytes lacking the VDR were unable to undergo postmorphogenic hair cycles, regardless of the genotype of dermal papilla cells (247). In addition, keratinocyte-restricted VDR expression rescued alopecia, but not abnormalities in mineral ion or skeletal homeostasis, in the VDR null mice (249, 250). Thus, the alopecia in the VDR null mice is due to impaired VDR action in the keratinocyte component of the hair follicle.

Studies using mutant VDR transgenes demonstrated that ligand binding is not required for the VDR to prevent alopecia. The mutation introduced into the LBD of the VDR prevents binding of lithocholic acid, an alternative VDR ligand, as well as that of 1,25-(OH)\textsubscript{2}D, suggesting that the effects of the VDR on the hair follicle are not due to interactions with a novel ligand. However, keratinocyte-specific expression of a VDR with a mutation in the NR coactivator binding domain (AF-2 domain) attenuated but did not prevent the development of alopecia. The VDR null mice with keratinocyte-specific expression of the AF-2 mutant VDR exhibited hair loss (Fig. 5), accompanied by dermal cysts and dilated piliary canals by 8 months of age (251). Thus, the actions of the VDR in the epidermis are independent of ligand binding but require interactions with nuclear factors.

Mutations in the NR corepressor, Hairless, resulted in alopecia strikingly similar to that observed in the VDR null mice (252). Hairless binds to the VDR and represses VDR-mediated transactivation (253, 254); however, neither of the VDR mutants studied in vivo interfered with VDR-Hairless interactions (251). Canonical Wnt signaling has been shown to play an important role in hair follicle development and to participate in postmorphogenic hair cycling (255–257). Interestingly, the dermal cysts and increase in sebaceous glands observed in the VDR null mice and Hairless mice were similar to that observed in mice expressing a keratinocyte-specific Lef1 transgene with a mutation that prevents its interactions with β-catenin (258). Confirming a functional interaction of the VDR with the canonical Wnt signaling pathway, transient gene expression assays in primary neonatal keratinocytes demonstrated that absence of the VDR prevented synergistic activation of a Wnt response element by β-catenin and Lef1 (259). Furthermore, immunoprecipitation studies demonstrated that VDR is present in a complex with β-catenin and Lef1. Hairless has also been shown to promote Wnt signaling (260), suggesting that abnormal ca-
nonical Wnt signaling may be the final common pathway by which VDR and Hairless mutations lead to cutaneous abnormalities (Fig. 6).

Keratinocytes in the bulge region of the hair follicle are thought to contain stem cells that can differentiate into epidermal keratinocytes, hair follicle keratinocytes, and sebaceous cells (261). Colony formation assays were performed to evaluate whether there was a defect in these stem cells in the absence of the VDR. Studies performed in neonatal mice, whose hair follicles are still undergoing morphogenesis, did not exhibit a defect in colony formation, whereas, by 4 wk of age, the keratinocytes of the VDR null mice were unable to form colonies characteristic of those formed by keratinocyte stem cells. Flow cytometric analyses for bulge keratinocytes demonstrated a normal number of keratinocyte stem cells at this age, suggesting a functional abnormality in these cells, based on their inability to form colonies in vitro (259) and their inability to regenerate hair follicles in vivo (244). However, a gradual decrease in the number of bulge keratinocytes was observed with age in the VDR null mice, suggesting a defect in keratinocyte stem cell self-renewal as well. The keratinocyte-specific VDR transgene prevented all these abnormalities in keratinocyte stem cells (259).

Thus, ligand-independent effects of the VDR are required for normal keratinocyte stem cell function. Although these data revealed novel functions for the VDR, they also raised a series of important questions that remain unanswered. The intriguing similarity of the Hairless and VDR mutant phenotypes suggests that these genes act in the same pathway or have similar, yet unidentified, targets in the keratinocyte stem cell. In an analogous fashion, the effects of the VDR and of the canonical Wnt signaling pathway on the hair follicle also suggest that synergistic interactions are essential for hair follicle homeostasis. Identification of the molecular partners of the VDR in keratinocyte stem cells, and of its targets, will undoubtedly reveal novel actions of this NR.

B. CYP27B1 is required for optimal epidermal differentiation

Compared with other tissues, renal tubular epithelial cells and keratinocytes express high levels of CYP27B1, the enzyme responsible for the conversion of 25-OHD to the active metabolite 1,25-(OH)_{2}D. Therefore, several studies used the keratinocyte as a model system to investigate the role of 1α-hydroxylase activity and locally produced 1,25-(OH)_{2}D in cell proliferation and differentiation. Although there was no gross skin phenotype in 1α-hydroxylase null mice, expression levels of the differentiation markers involucrin, profilaggrin, and loricrin were reduced in 1α-hydroxylase null mice (262). Furthermore, a pronounced retardation in the ability to recover normal barrier function after acute perturbation was found in 1α-hydroxylase null mice. These findings suggest that 1α-hydroxylase activity, most likely through the synthesis of active 1,25-(OH)_{2}D, is essential for normal epidermal differentiation. Similar results were obtained by inactivation of both 1α-hydroxylase alleles in a ras-transformed keratinocyte cell line that typically produces squamous carcinoma in nude mice (263). In contrast to tumors from 1α-hydroxylase WT keratinocytes, tumors derived from 1α-hydroxylase null cells were not growth inhibited by locally administered 25-OHD, whereas they remained responsive to the antiproliferative and prodifferentiating effects of 1,25-(OH)_{2}D, demonstrating the importance of 1α-hydroxylase activity in the autocrine regulation of growth and differentiation in keratinocytes. It should be noted, however, that in the absence of locally administered 25-OHD, tumors derived from 1α-hydroxylase null keratinocytes were not larger or heavier than those which originated from WT keratinocytes.
IV. VDR-Vitamin D Endocrine System and Cell Proliferation and Cancer

A. Mechanisms responsible for the antineoplastic effects of 1,25-(OH)2D

In 1981, Colston et al. demonstrated that the doubling time of malignant melanoma cells increased after incubation with 1,25-(OH)2D (264). In that same year, Abe et al. (265) demonstrated the differentiation of HL60 leukemia cells toward the macrophage lineage, after incubation with 1,25-(OH)2D. Since then, the antineoplastic activity of 1,25-(OH)2D was shown both in vitro and in vivo in a wide variety of malignancies, such as leukemia (266, 267) and colon (268, 269), breast (270–272), and prostate cancer (273, 274). In a number of cell types, induction of programmed cell death contributes to the antineoplastic activity of 1,25-(OH)2D, but inhibition of angiogenesis and invasiveness may also contribute to its antitumor activity.

1. Inhibition of proliferation and induction of differentiation. Growth inhibition of keratinocytes and myeloid leukemia cells, after incubation with 1,25-(OH)2D, is accompanied by the induction of a more differentiated phenotype (265). However, regulation of proliferation and differentiation is not always coupled, and the degree of cellular proliferation and/or differentiation is cell type-specific (267, 275). In most cell types that express a functional VDR, incubation with 1,25-(OH)2D results in an accumulation of cells in the G0/G1 phase of the cell cycle (276). The exact sequence of events between VDR-mediated transactivation, or repression of target genes, and the actual G0/G1 arrest is probably cell type-specific and is the subject of intensive research. However, most of the proposed signaling pathways converge at the level of complex formation between the retinoblastoma family of pocket proteins, the E2F family of transcription factors, and the subsequent down-regulation of a large number of E2F-target genes that are required for normal cell cycle progression (276, 277). The pocket proteins, p107 and p130, are crucial to the antiproliferative activity of 1,25-(OH)2D (277). Activation of the cyclin-dependent kinase inhibitors such as p18, p19, p21, or p27, repression of cyclin D1 expression, as well as down-regulation of the activity of complexes between cyclins and cyclin-dependent kinases, have been suggested to be early events, whether or not directly mediated by 1,25-(OH)2D, that occur upstream of the E2F-pathway and may be responsible for the growth-inhibitory effect of 1,25-(OH)2D (276, 278–281). However, 1,25-(OH)2D may also inhibit cell growth by interfering with signaling pathways initiated by TGFβ, epidermal growth factor, IGF, prostaglandins (131), Wnt-ligands (282), as well as by involving in other mitogenic signaling pathways (e.g., ERK/MAPK pathway and c-myc) (269, 270, 281, 283–287) (Fig. 7).

2. Induction of apoptosis. 1,25-(OH)2D induces apoptosis in a number of tumor models, including carcinomas of the breast, colon, and prostate, but the underlying mechanisms of action are only partially elucidated (271, 272, 288). Alterations in the relative expression and/or cellular distribution of the Bcl-2 family of pro- and antiapoptotic proteins and the subsequent release of cytochrome c from the mitochondria have been suggested to contribute to the induction of apoptosis by 1,25-(OH)2D (272, 289). However, depending on the cell type, 1,25-(OH)2D has also been shown to interact with other signaling pathways (e.g., IGF, TNFα) that may lead to the induction of programmed cell death (290, 291).

Interestingly, after cellular stress or UV damage, and depending on the cell type, 1,25-(OH)2D was also able to exert antiapoptotic effects (292–294).

3. Inhibition of angiogenesis and invasiveness. Because angiogenesis is an essential requirement for the growth of solid tumors, the antiangiogenic activity of 1,25-(OH)2D may represent an important mechanism responsible for its tumor suppressive activity. 1,25-(OH)2D was demonstrated to inhibit angiogenesis in both in vitro and in vivo experimental models (295, 296). This antiangiogenic activity was suggested to be mediated by an inhibition of proliferation of tumor-derived endothelial cells, and/or by repression of the release of angiogenic factors, such as vascular endothelial growth factor (e.g., by repression of hypoxia-inducible factor-1 mediated transactivation), TGF-α, and epidermal growth factor (297, 298).

Invasion of tumor cells to secondary sites requires degradation of the extracellular matrix, a process shown to be inhibited by 1,25-(OH)2D. Inhibition of serine proteinases (such as components of the plasminogen activator system), decreased activity of metalloproteinases, increased expression of E-cadherin, and inhibition of tenascin-C might all contribute to the antiantiinvasive effects of 1,25-(OH)2D (268, 299–301).

B. Lessons from animal models: VDR null mice and cancer

1. Hyperproliferation of colonic cells in VDR null mice. VDR and CYP27B1 are expressed in nonmalignant as well as malignant colon tissues from human origin. Interestingly, expression levels of both VDR and CYP27B1 are increased during early colorectal tumor progression, which could suggest a role for 1,25-(OH)2D in the paracrine/autocrine growth inhibition during early stages of colon tumor progression (302, 303). This hypothesis is supported by in vivo findings in Tokyo VDR null mice, which were fed a lactose/calcium-enriched rescue diet to normalize serum mineral homeostasis, bone growth, and body weight. In the distal colon of these mice, complete loss of 1,25-(OH)2D-mediated growth control was accompanied by colonic hyperproliferation, as measured by expression of proliferating cell nuclear antigen, and by cyclin D1 (304, 305). Moreover, direct DNA damage, evaluated by enhanced levels of 8-hydroxy-2′-deoxyguanosine, was enhanced in VDR null mice (304, 305).

2. Skin of VDR null mice is hypersensitive to carcinogenesis. In addition to a role in colon as a physiological growth regulator, 1,25-(OH)2D is thought to exert protective effects in skin transformation. Arguments in favor of this hypothesis are the finding that VDR and CYP27B1 are expressed in normal and malignant keratinocytes and the observation that 1,25-(OH)2D exerts antiproliferative, as well as prodifferentiating, effects on keratinocytes both in vitro and in vivo (243, 306). Skin of Boston VDR null mice maintained on a rescue diet was found to be hypersensitive to tumorigenesis in...
response to oral administration of the carcinogen 7,12-dimethylbenzanthracene (DMBA) (307). Within 60 d of carcinogen exposure, 88% of VDR null mice developed persistent skin tumors, the majority of which were sebaceous, squamous, or follicular papillomas. With aging, skin of VDR null mice not exposed to DMBA became thick and wrinkled and displayed dermoid cysts.

Dysregulated cellular proliferation, as evidenced by basal cell proliferation and epidermal hyperplasia as well as aberrant differentiation in VDR null mice, demonstrated by decreased levels of epidermal differentiation markers such as involucrin, profilaggrin, and loricrin, might be responsible for the enhanced sensitivity to DMBA-induced skin tumorigenesis (243, 307). However, despite the epidermal hyper-
proliferation, spontaneous cutaneous tumors were rarely detected in VDR null mice (307). The molecular basis for predisposing the skin to the development of tumors or basal cell carcinoma may be related to the overexpression of hedgehog signaling in normal and chemocarcinogen stimulated VDR null keratinocytes (308).

3. VDR ablation alters mammary gland morphology and tumorigenesis. VDR is present in all major cell types of the mammary gland including basal and luminal epithelial cells, cap cells, and stromal cells. Within the mammary gland, higher VDR levels are found in differentiated cell populations compared with proliferating populations (309). VDR expression is dynamically regulated throughout the reproductive cycle, with peak levels observed during gestation and lactation (310). These findings suggest that VDR acts as a physiological growth regulator of normal mammary epithelium. This hypothesis is strengthened by observations in Boston VDR null mice kept on a rescue diet. During puberty, glands from VDR null mice displayed increased branching, greater ductal extension, and higher numbers of undifferentiated end bud structures, compared with glands from WT mice. Furthermore, glands from pubertal VDR null mice exhibited enhanced growth in response to exogenous estrogen and progesterone, both in vivo and in organ culture, compared with glands from WT mice (309). During gestation, VDR ablation was accompanied by accelerated glandular development, whereas postlactational involution was delayed in VDR null mice (310). In line with these observations, which suggest a role for 1,25-(OH)2D in the control of mammary gland proliferation and differentiation, enhanced expression of VDR and CYP27B1 was found in breast tumors. However, growth control by 1,25-(OH)2D might be abrogated in breast tumors because the chromosomal region 20q13.2, which contains the VDR, has been amplified in breast tumors (35). In animal models, abrogation of 1,25-(OH)2D signaling by knocking out the VDR was found to lead to an enhanced susceptibility to tumorigenesis. In a first model, Boston VDR null mice were crossed with mouse mammary tumor virus c-neu null mice that specifically express the c-neu protooncogene in the mammary gland. Complete loss of VDR signaling in these mice was accompanied by pathological changes such as dysplasia of the mammary ductal epithelia, atrophy of the associated fat pad, and reduced survival. Although decreased survival of neu/VDR null mice hampered detailed evaluation of tumor development, dysregulated growth of alveolar and ductal cells was consistently observed in these mice. Furthermore, compared with neu-mice that express two intact VDR copies, haploinsufficiency of the VDR was found to accelerate c-neu-driven mammary tumorigenesis (311, 312). In a second model, Boston VDR null mice and their WT littermates were exposed twice to the chemical carcinogen DMBA (313). Compared with WT mice, VDR null mice exhibited an increased incidence of glandular hyperplasia and altered tumor characteristics (hormone-independent tumors with squamous differentiation), but mammary tumor incidence, latency, and multiplicity were not affected by VDR ablation.

4. VDR ablation and other tumors. VDR deficiency not only predisposes to epithelial tumors (colon, skin) but also results in greater susceptibility to chemocarcinogen-induced leukemia (311, 313) (Table 3). Tumor development in ovary, uterus, lung, or liver was not different between VDR WT and null mice. However, lung metastatic cancer growth was extremely reduced in VDR null mice (300). Together, these findings suggest that optimal VDR signaling may contribute to suppression of tumorigenesis.

C. Lessons from epidemiological and intervention studies in humans

1. Vitamin D intake and exposure to sunshine in relation to cancer incidence. The findings that 1,25-(OH)2D inhibits the proliferation of a wide range of cell types and that some cell types in the VDR null mice are characterized by dysregulated cell proliferation and differentiation are in support of the hypothesis that vitamin D levels, obtained either by exposure to sunshine or by vitamin D intake, can be associated with cancer risk in humans. The beneficial effect of sun exposure on cancer incidence was first suggested by Garland and Garland (314), who demonstrated that colon cancer mortality rates in the United States were higher in regions with less UV radiation. Additionally, a large number of studies have pointed to a decreased colon (314, 315), breast (315–319), and prostate (315, 320, 321) cancer risk in regions with high UV radiation. However, the retrospective and subjective assessment of solar exposure may bias the outcome of these studies. Moreover, in both the United States and Europe, UV-B irradiation is shown to be a poor predictor of vitamin D status (322), which suggests that UV light exposure may not represent a surrogate marker of vitamin D status. The relationship between vitamin D intake and cancer risk has been studied extensively. Epidemiological data for breast cancer are sparse but seem to support the conclusion that higher vitamin D intake is associated with reduced risk (318, 319, 323, 324). However, the results for colon cancer are too diverse to support any conclusion (325–330), whereas for pros-
tate cancer, epidemiological data do not support the inverse relationship (331–334). Cross-sectional studies on the relation between nutritional vitamin D intake and cancer thus provide variable results, but this is not surprising because: 1) nutritional intake only partially explains the vitamin D status; 2) nutritional content and intake of vitamin D are notoriously difficult to evaluate, due to variable vitamin D content in a limited number of food items; and 3) there is a confounding factor of variable calcium intake, which may have an independent effect on cancer risk. Therefore, a more accurate and quantitative approach is to investigate whether the concentration of circulating vitamin D metabolites, which accounts for all sources of vitamin D, is associated with cancer risk.

2. Plasma 25-OHD concentrations in relation to cancer risk. Numerous studies have addressed the question as to whether high levels of circulating vitamin D metabolites are associated with reduced cancer risk. Most reports agree that serum levels of 1,25-(OH)2D are poorly associated with cancer incidence (335–337). It is, however, generally accepted that, due to its relatively long half-life of approximately 2 to 3 wk, circulating 25-OHD level constitutes a better indicator of vitamin D status (12). Given the almost universal expression of CYP27B1, it is assumed that most cells are able to produce their own active 1,25-(OH)2D from 25-OHD, whereby the amount of locally produced active 1,25-(OH)2D is dependent on the serum concentration of its precursor (338). The relationship between serum 25-OHD and cancer incidence has been studied extensively for colorectal (314, 335, 337, 339–345), prostate (346–352), and breast (336, 353–355) cancer, with conflicting results. Different study outcomes may be related to divergent median 25-OHD in the investigated populations. Because it is suggested that the inverse association between serum 25-OHD levels and cancer incidence is more pronounced in the deficiency region (356), it is possible that, in populations with elevated circulating 25-OHD levels, no beneficial effects on cancer incidence are observed. Recent meta-analysis of studies addressing the association between 25-OHD levels and colorectal cancers revealed that individuals with serum 25-OHD levels equal to or greater than 82 nmol/liter had a 50% lower incidence of colorectal cancer than those with relatively low levels (≤30 nmol/liter) (357–359). The inverse association between colorectal cancer mortality and serum 25-OHD levels was confirmed by the results of the Third National Health and Nutrition Examination Survey (360). The association between plasma 25-OHD and prostate or breast cancer is more mixed because most studies show a negative relation, but a substantial number of studies show no relation, and a few even indicate a positive relation between (higher) 25-OHD levels and prostate cancer (357, 358).

The final question is, of course, whether serum 25-OHD is a predictor or has a causative relation with the overall cancer risk. An association between total cancer mortality and baseline serum 25-OHD levels was not supported by a large survey (360). A few reports suggested that higher 25-OHD concentrations were associated with an increased cancer incidence (352, 361, 362). Some of the study results may be biased, due to the fact that only single measurements of 25-OHD are used in the studies, and these may not reflect long-term vitamin D status. Therefore, intervention studies in which vitamin D supplements are administered and cancer incidence is monitored may be more informative.

3. Intervention studies

a. Effect of vitamin D supplementation on cancer risk. Recently, two randomized, double-blind, placebo-controlled trials have been completed in which the effect of vitamin D plus calcium supplementation on cancer risk was prospectively studied. In the Women’s Health Initiative (WHI) study, postmenopausal women received calcium (1 g) plus vitamin D (400 IU) or a matching placebo on a daily basis for 7 yr (363). Although a significant inverse relationship was found between baseline levels of serum 25-OHD and colorectal cancer risk, the incidence of invasive colorectal cancer did not differ significantly between women assigned to calcium plus vitamin D supplementation and those assigned to placebo. In a second, much smaller, 4-yr study in postmenopausal women, higher doses of calcium (1.4–1.5 g) and vitamin D (1100 IU) were administered, which raised serum 25-OHD to levels above 80 nmol/liter (364). Comparable to the WHI study, higher circulating 25-OHD levels were associated with a decreased cancer risk. However, in this study, improvement of the calcium and vitamin D nutritional status did significantly reduce overall cancer risk in postmenopausal women. In view of the small number of cancer deaths and a major confounding factor of calcium intake, which by itself seems to have contributed more than vitamin D intake, a much larger prospective study with substantial vitamin D supplementation is essential.

b. Application of vitamin D metabolites in the treatment of prostate cancer. The effect of oral vitamin D3 (2000 IU daily) on prostate-specific antigen (PSA) levels was investigated in a small number of prostate cancer patients (n = 15) in whom local treatments had failed (365). In 9 of 15 patients, PSA levels decreased or remained stable after the initiation of vitamin D3 supplementation. Moreover, in 14 of 15 patients, a prolongation of PSA doubling time was observed. In a small pilot trial in which 1,25-(OH)2D was administered on a daily basis to patients with rising PSA after radiation or prostatectomy, PSA doubling times tended to decrease (366). However, increased dosing of 1,25-(OH)2D was not possible due to hypercalcemia. Therefore, alternative dosing schedules have been evaluated. Weekly instead of daily administration of 1,25-(OH)2D allowed significant dose escalation, with minimal calcemic effects (367). Moreover, combination therapy with dexamethasone, estramustine, or taxotere plus dexamethasone was shown to be safe and well tolerated (368–371). In a setting of metastatic androgen-independent prostate cancer, addition of weekly high dose 1,25-(OH)2D to a regimen of docetaxel and dexamethasone had favorable effects on PSA response in a small patient cohort (30 of 37 patients achieved a PSA response) (368). Recently, the safety and activity of high dose 1,25-(OH)2D in combination with docetaxel plus dexamethasone, was compared with placebo and docetaxel plus dexamethasone in a double-blinded randomized study in a large cohort of patients with androgen-independent prostate cancer (369). Ad-
dition of high dose 1,25-(OH)₂D to docetaxel-based therapy proved to be safe and was associated with improved survival. However, no significant differences in PSA responses were observed.

These intervention studies illustrate the possible therapeutic application of 1,25-(OH)₂D in cancer treatment. However, analogs of 1,25-(OH)₂D with good systemic bioavailability, an enhanced potency to inhibit cell proliferation, and reduced calcemic effects may have a greater impact on the suppression of malignant cell growth. Vitamin D analogs have already been shown to be effective in the treatment of the hyperproliferative disorder psoriasis (372). Moreover, the potential clinical usefulness of vitamin D analogs in cancer has been demonstrated in different animal models of breast, prostate, and colon cancer (reviewed in Refs. 373 and 374). The vitamin D analog seocalcitol (EB1089), which showed therapeutic anticancer potential in various animal models, was evaluated in phase I (375) and phase II (376, 377) clinical trials. However, at the maximal tolerable dosage, no objective antitumor activity of seocalcitol was observed in patients with advanced disease. To enhance the applicability of vitamin D analogs, several strategies have been suggested. To limit the calcemic activity, analogs with a greater dissociation of antiproliferative and calcemic activity might be used, but combination with compounds that limit bone resorption may also offer a valuable option. Alternatively, coadministration of chemotherapeutic cytotoxic agents and vitamin D analogs may have synergistic effects on cell growth, tumor angiogenesis, and metastasis and augment the efficacy of vitamin D analogs in the treatment of cancer.

**D. Conclusion**

Nearly all cell types express a functional VDR, and their growth is inhibited upon exposure to pharmacological doses of 1,25-(OH)₂D, the active metabolite of vitamin D. However, under physiological conditions, 1,25-(OH)₂D is suggested to regulate normal cell growth because VDR null mice are predisposed to develop cancer when challenged with carcinogenic agents (Table 4). In parallel, epidemiological studies suggest that a poor vitamin D status is associated with a higher incidence of human cancer, further supporting the notion that 1,25-(OH)₂D is an important regulator of normal cell proliferation. However, in a large study cohort, vitamin D supplementation (400 IU/d) was not associated with a decreased colorectal cancer risk. Therefore, long-term prospective studies with vitamin D (>1000 IU/d) are essential to evaluate the potential preventive effect of a better vitamin D status. In addition, selective VDR modulators, alone or in combination therapy, may constitute a better alternative for the treatment of existing cancers.

A clear example of the potential clinical applications of the antiproliferative activity of 1,25-(OH)₂D or its analogs is the now widespread use of calcipotriol or related analogs in the topical treatment of hyperproliferative skin diseases, such as psoriasis (378–380), although part of the clinical activity may also be due to their immune prospectives (381).

**V. VDR-Vitamin D Endocrine System and Immunology**

**A. Vitamin D physiology and the immune system**

The VDR is expressed in most cells of the immune system, including activated CD4⁺ and CD8⁺ T lymphocytes, as well as in antigen-presenting cells (APCs) such as macrophages and DCs (382). VDR expression in B lymphocytes is more controversial, with positive and negative reports (382, 383). 1α-Hydroxylase or CYP27B1 is also expressed in macrophages, DCs, and even T and B lymphocytes (144, 338, 383–385). The 1α-hydroxylase present in immune cells is identical to the renal enzyme, but regulation of its expression and activity is different (Fig. 8). Whereas the renal enzyme is under control of calcemic and bone signals [such as PTH and 1,25-(OH)₂D itself], the macrophage enzyme is primarily regulated by immune signals, with IFNγ and Toll-like receptor (TLR) agonists being powerful stimulators (28, 385–390). Importantly, and in contrast with 1α-hydroxylase regulation in kidney, 1α-hydroxylase production in immune cells is not subjected to negative feedback signals from 1,25-(OH)₂D itself (384, 385). This explains the massive local production of 1,25-(OH)₂D by disease-associated macrophages that is seen in patients with granulomatous diseases (sarcoidosis and tuberculosis), and the consequent possible spillover in the general circulation, eventually leading to systemic hypercalcemia. Up-regulation of 1α-hydroxylase, and therefore 1,25-(OH)₂D synthesis, by immune cells and especially DCs typically occurs at later stages of immune activation, thus providing a late negative feedback loop, down-regulating immune responses. In parallel, VDR is down-regulated in macrophages and DCs at later stages of immune activation (391, 392). It is of interest that a defect in up-regulation of 1α-hydroxylase in response to immune stimuli had been reported in nonobese diabetic (NOD) mice, an animal model of type 1 diabetes that spontaneously develops this autoimmune disease (385).

The 25-hydroxylase and 24-hydroxylase enzymes are also expressed in immune cells (144, 363, 384, 393). Whereas the expression of 25-hydroxylase is poorly regulated, the presence of 24-hydroxylase in immune cells is highly regulated and dependent on the differentiation status of the cell. Undifferentiated monocytes are highly susceptible to 1,25-(OH)₂D-mediated 24-hydroxylase induction, whereas differentiated/activated macrophages are resistant due to an interplay between IFNγ and 1,25-(OH)₂D-mediated effects.

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**Table 4. VDR-vitamin D endocrine system and cancer: summary**

<table>
<thead>
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<th>1. 1,25-(OH)₂D is a potent inhibitor of cell cycle progression</th>
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<td>Mainly as inhibitor of progression beyond G₀/G₁ stage</td>
<td>Coordinated action on many cell cycle pathways</td>
<td>Occurring in most normal and malignant cells</td>
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<td>Variety of escape mechanisms in more malignant cell types</td>
<td>2. VDR null mice are predisposed to carcinogen/oncogen induced cancers</td>
<td>3. Large number of cross sectional and prospective epidemiological data link vitamin D deficiency with higher cancer incidence</td>
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<tr>
<td>4. Some selective VDR modulators inhibit tumor growth in rodent tumor models</td>
<td>5. Proof of concept of therapeutic value of Vitamin D supplementation: inconsistent results</td>
<td>Active vitamin D metabolites/analogs: ongoing studies</td>
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STAT1α, involved in IFNγ-signaling, interacts with the DBD of the VDR, thereby prohibiting binding of the 1,25-(OH)₂D/VDR/RXR-complex to the 24-hydroxylase promoter and induction of transcription (393). This impaired ability to initiate its own breakdown also contributes to the persistent overproduction of 1,25-(OH)₂D in macrophages of patients with granulomatous diseases (386).

The regulated expression of all these components of the vitamin D endocrine system (VDR, and 25-, 1α-, and 24-hydroxylase) within immune cells strongly suggests a physiological role for 1,25-(OH)₂D in the immune system.

B. VDR and the immune system

1. Signaling through the VDR and the immune system. The discovery that the VDR is widely expressed in the immune system led to the recognition of a central immunomodulatory role for 1,25-(OH)₂D (394, 395). Here, as in other 1,25-(OH)₂D-responsive tissues, the 1,25-(OH)₂D/VDR complex can directly interact with VDREs in the promoter region of 1,25-(OH)₂D target genes. Studies reporting a 1,25-(OH)₂D-mediated increase in TNFα production by bone marrow cells demonstrated that this was partially mediated by direct binding of the liganded VDR-RXR complex to a VDRE in the promoter region of the TNFα gene (396). More recently, a VDRE was discovered in the promoter of the human cathelicidin antimicrobial peptide (CAMP) gene (397). An example of a negative VDRE was found in the promoter region of the IFNγ gene. Presumably IFNγ production is inhibited by binding of the liganded VDR-RXR complex to this negative VDRE, as well as by interacting with a crucial upstream enhancer element (398).

In a unique manner, suppression of the granulocyte/macrophage recruiter, granulocyte macrophage colony stimulating factor, by 1,25-(OH)₂D is achieved by binding of ligand-bound VDR monomers to functional repressive complexes in the promoter region of the cytokine gene (399, 400).

Additionally, liganded but also unliganded VDR can interfere with signaling by other transcription factors, a mechanism by which 1,25-(OH)₂D exerts most of its immune ef-
fected. The VDR/1,25-(OH)₂D complex dose-dependently interferes with the signaling of transcription factors [such as nuclear factor of activated T cells (NFAT), NFkB, and activating protein-1 (AP-1)] that play a crucial role in regulating immunomodulatory genes (400–402) and whose dysregulation is suggested to be involved in the pathogenesis of different autoimmune diseases (403). As such, 1,25-(OH)₂D impedes the activation of NFkB as well as its binding to consensus sequences, thus resulting in 1,25-(OH)₂D-induced down-regulation of IL-8 and IL-12 (114, 404). In DCs and many other cell types, this NFkB pathway has been proven to be very important in 1,25-(OH)₂D-mediated effects (402, 405–407). Notably, inhibition of phosphorylation (and subsequent ubiquitination and degradation) of the cytosolic inhibitor of NFkB, inhibition of NFkB nuclear translocation, binding (and therefore retention) of NFkB to VDR in the nucleus, and interference with DNA binding of the NFkB-complex are targets of 1,25-(OH)₂D actions. Moreover, a VDRE has been found in the promoter of RelB, one of the NFkB family members, contributing to the 1,25-(OH)₂D-mediated inhibition of NFkB-mediated maturation of DCs (408). Constitutive association of (unliganded) VDR with the RelB promoter is enhanced by ligand-binding but decreased by lipopolysaccharide (LPS). This process is controlled by chromatin remodeling by means of histone deacetylase 3 activity. Studies in mouse embryonic fibroblasts isolated from VDR null mice confirmed the direct involvement of VDR in the regulation of NFkB activity (409). VDR null cells exhibited reduced inhibitor of NFkB levels, leading to increased nuclear translocation of p65. Moreover, because of the lack of VDR, the physical interaction between VDR and p65 in the nucleus is absent, which may free p65 and again increase its activity. Together, these combined changes lead to the marked increase of NFkB activity in VDR null fibroblasts.

Similarly, the NFAT and AP-1 transcription factor pathways are involved in the 1,25-(OH)₂D-mediated inhibition of IL-2 and IL-4 (113, 410, 411).

2. VDR and CYP27B1 polymorphisms and the immune system. A vast amount of information has been collected over the years regarding the association of VDR polymorphisms with susceptibility to different autoimmune diseases and infection (412). The results obtained so far are conflicting, and the role of VDR polymorphisms remains unresolved. However, many immune and regulatory genes are located in close vicinity of the VDR gene on human chromosome 12q13.11. In type 1 diabetes, recent genome searches indicate the possible existence of additional disease susceptibility genes in this region. Although the VDR is a potentially good candidate diabetes susceptibility gene and an association between some VDR gene polymorphisms and type 1 diabetes susceptibility has been shown in different populations, a recent meta-analysis could not find any evidence for an association between VDR polymorphisms and type 1 diabetes risk in either case-control or family-transmission studies (413). In contrast, susceptibility to type 1 diabetes is associated with polymorphisms in the CYP27B1 gene (414), as well as the 25-hydroxylase (CYP2R1) gene (415). These findings of an association between vitamin D metabolism and diabetes risk are concordant with the association of vitamin D (deficiency) levels and type I diabetes.

Also, for many other autoimmune diseases (multiple sclerosis, rheumatoid arthritis, Graves’ disease, systemic lupus erythematosus, hepatitis, inflammatory bowel diseases, and thyroiditis), as well as for tuberculosis infection, the association with VDR polymorphisms has been investigated with conflicting results. A recent meta-analysis for tuberculosis risk demonstrated that the data gathered to date are inconclusive and studies are underpowered (416). One recent study reports an association of improved renal allograft survival with the FokI T allele (417), which gives rise to the long form of VDR, which has been shown to be less immunologically active (418). Indeed, we described that the shortened VDR FokI C allele exhibits higher transcriptional activity of immune-specific transcription factors, leading to changes in proliferation and cytokine synthesis of different immune cell types, correlating with a more activated immune system and thus, possibly predisposing to autoimmune diseases or graft rejection.

3. Absence of the VDR and the immune system. Because the immune effects of 1,25-(OH)₂D are mediated through the VDR, the immune system of VDR null mice became a focus of thorough investigation. Myelopoiesis, as well as cellular number and composition in all immune organs of VDR null mice, was shown to be normal (419, 420). In humans with a mutation in the VDR gene resulting in vitamin D-resistant rickets type II, normal myelopoiesis was observed (421). Experiments performed with VDR null mice fed a normal diet revealed some intrinsic defects in T cell proliferation (to anti-CD3, a calcium dependent signal) and macrophage function (chemotaxis), which could all be remediated by normalizing calcium levels by a high-calcium, high-lactose rescue diet (419). These calcium-related effects include in vivo resistance to multiple low-dose streptozotocin-induced diabetes [MLDS-DM, a model of immune-mediated toxin-induced diabetes (422)] in hypocalcemic but not in normocalcemic VDR null mice. Other studies did, however, report some immune abnormalities in normocalcemic VDR null mice, each focusing on different cell types. When analyzing the cytokine production by splenic T cells, a defect in T helper 1 cell (Th1)-related IFN-γ production was observed, presumably related to defective IL-18 production by macrophages and decreased STAT4 expression in T cells themselves (420). A study focusing on the phenotype of DCs in normocalcemic VDR null mice found some abnormalities in DCs from skin-draining lymph nodes (423). These lymph nodes were enlarged, with a higher proportion of more mature DCs (observed as higher major histocompatibility complex (MHC) II and CD40, CD80/86 expression). Yet another study demonstrated an accelerated maturation and increased responsiveness to IgE-mediated stimulation of mast cells from VDR null mice, the main Th2-type effector cells releasing histamine, upon stimulation mainly by IgE (424).

When comparing the studies investigating the prevalence of different autoimmune disease models in VDR null mice, some apparent contradictions surfaced. In different models of inflammatory bowel disease (whether chemically induced, induced by the transfer of CD45RB<sup>high</sup> pathogenic T cells,
or occurring spontaneously in IL-10 null mice), exacerbated disease severity was always observed in VDR null mice (425–427). This was accompanied by increased expression of inflammatory and Th1-related cytokines and chemokines in the intestinal tract (e.g., IL-1, TNFα, IFNγ, IL-12, and MIP1α). In contrast, VDR null mice on a high calcium diet were found to be less susceptible to experimentally induced autoimmune encephalomyelitis, a multiple sclerosis model (428). More recently, we described the effects of lack of functional VDR in the animal model for type 1 diabetes, the NOD mouse (429). Analysis of the immune system revealed severe immune defects considered to be crucial in the development of NOD diabetes, but the VDR null NOD mice, fed a normal diet that did not rescue the hypocalcemia, did not display an enhanced susceptibility to diabetes. A clear decline in the number of immature TCR-α/β+CD4+CD8+ NKT cells and regulatory CD4+CD25+ T cells was observed in immune organs of VDR null NOD mice. A relative deficiency of TCR-α/β+CD4+CD8+ NKT cells and regulatory CD4+CD25+ T cells is postulated to contribute to an inability to maintain peripheral tolerance and lead to the development of diabetes in regular NOD mice (430, 431). Moreover, inactivation of VDR in NOD mice resulted in disturbed cytokine and chemokine profile with extremely low IL-1, IL-6, and CCL2 transcripts reflecting a maturational defect of macrophages in NOD mice (432), in agreement with similar observations in vitamin D-deficient NOD mice (433, 434). The thymus and lymph nodes of VDR null NOD mice contained even fewer mature CD11c+ DCs than WTs, and this could contribute to a defective elimination of diabetogenic T cells (435). The association of VDRR II and early onset type 1 diabetes has been reported once (436).

VDR null mice are resistant to experimentally induced airway inflammation and asthma (437, 438). This was proposed to be due to a failure of the lung environment to respond to inflammation and attract pathogenic immune cells (Th2 cells and eosinophils), and not to defects in the priming and lung homing of the immune cells themselves. A robust Th2 response, characterized by IL-5 and IL-13 production, along with elevated IgE levels, could be induced in VDR null mice. The failure of trafficking of immune cells to the lung is in concordance with the recent rediscovery that 1,25-(OH)2D plays an important role in migration of immune cells. In addition to the long-known defects in macrophage chemotaxis caused by vitamin D deficiency, it was discovered more recently that 1,25-(OH)2D plays a role in micro-environmental positioning of T cells by altering their expression pattern of chemokine- and homing receptors (144). Increased expression of the skin-homing chemokine receptor, CCR10, and decreased expression of gut-homing CCR9 and α4β7 directed T cells toward the skin after 1,25-(OH)2D exposure. This tissue tropism of lymphocytes is proposed to be based on a model in which DCs process and “interpret” locally produced metabolites to program T cell homing and microenvironmental positioning with gut-tropism linked to vitamin A processing and skin-tropism linked to vitamin D processing (439). This altered T cell homing behavior could play a role not only in the resistance of VDR null mice to allergy but also in their increased susceptibility to inflammatory bowel disease.

VDR null mice are more resistant to parasitic Leishmania major infection (440). This infection model depends on the microbial function of IFNγ-activated macrophages, a host defense mechanism inhibited by 1,25-(OH)2D, as part of a negative feedback loop controlling inflammatory responses (441). In VDR null mice, this 1,25-(OH)2D-mediated inhibition is absent, resulting in continuous activation of macrophages and, consequently, enhanced microbial activity. This is in sharp contrast with the association between vitamin D deficiency and increased susceptibility for most nonparasitic infections. This discrepancy might be related to parasitic infections being more closely linked with Th2 defense mechanisms, whereas the innate immune system and Th1 arm of the immune system deals with other infections.

C. Vitamin D ligand deficiency and the immune system

In previous centuries, the medical world had already linked the presence of immune abnormalities to a deficiency in vitamin D, the precursor of the active VDR ligand. The oldest known effects of vitamin D deficiency on immune function are at the level of macrophage function (442) and resistance to infections, especially tuberculosis. A correlation between susceptibility to and disease progression of tuberculosis and serum vitamin D levels has been repeatedly observed (443–447). Moreover, oral administration of vitamin D has been shown to markedly improve tuberculosis outcome (448, 449). A recent study on healthy tuberculosis contacts demonstrated that vitamin D treatment increases their monocytes’ capacity to mount a protective response to the bacillus Calmette-Guérin vaccine (450). It has been suggested that under conditions of low systemic 25-OHD levels, as is the case in vitamin D deficiency, only limited 1,25-(OH)2D can be produced locally by macrophages from its precursor 25-OHD, impeding the normal antimicrobial and antiinflammatory function attributed to 1,25-(OH)2D. This hypothesis is confirmed by the lower induction of the CAMP by monocytes incubated with 25-OHD-deficient serum from sunlight-deprived African-Americans (451). Defects in immune functions indispensable for antimicrobial activity have been observed in vitamin D-deficient mice (433, 452). In addition, a disturbed delayed type hypersensitivity response has been reported in mice lacking vitamin D (453).

Furthermore, it has been suggested that in vitamin D deficiency the 1,25-(OH)2D-mediated attenuation of pathologic Th1 immune responses is impaired, thus explaining the increased risk for Th1-mediated autoimmune diseases (454) such as inflammatory bowel disease (455, 456), rheumatoid arthritis (457, 458), systemic lupus erythematosus (459, 460), multiple sclerosis (461–463), and type 1 diabetes (464). Many epidemiological data link vitamin D deficiency and increased prevalence of these autoimmune diseases with, for example, a 3-fold increase in type 1 diabetes when vitamin D deficiency was present in early life (464). Another strong argument linking vitamin D levels to immune function is the correlation between areas with low vitamin D supply (due to insufficient sunlight exposure time or nutritional vitamin D uptake) and increased incidences of different autoimmune diseases (465–468). The seasonal variation in onset and exacerbations of autoimmune diseases, with a peak in late
winter/early spring when serum vitamin D levels are lowest, strengthens this correlation (469, 470). In a very large prospective, nested case-control study among more than 7 million U.S. military personnel, a clear association between serum 25-OHD levels and risk of multiple sclerosis was observed (463). The risk of multiple sclerosis dose-dependently decreased with increasing serum 25-OHD levels, with the highest risk for serum 25-OHD levels below 75 nmol/ml, a marginally reduced risk for serum 25-OHD levels of 75–100 nmol/ml, and a significant 51% reduction of multiple sclerosis risk for serum 25-OHD levels above 100 nmol/ml.

In animal models of autoimmune diseases, aggravated disease presentation has also been demonstrated during vitamin D deficiency. In NOD mice, vitamin D deficiency during early life results in a more aggressive manifestation of the disease, with an earlier onset and a higher incidence (433, 434). The thymus of vitamin D-deficient NOD mice had decreased CD8⁺ T cell and increased immature CD4⁺CD8⁺ T cell numbers, possibly pointing toward a T cell maturation problem. Moreover, numbers of regulatory CD4⁺CD62L⁺ T cells, already low in NOD mice, were further decreased in both thymus and peripheral lymph nodes. Besides these T cell abnormalities, vitamin D deficiency in NOD mice caused severe defects in macrophage function with lower respiratory burst capacity and a disturbed cytokine profile (increased IL-15 and extremely low IL-1 and IL-6). This aberrant inflammatory behavior of resident macrophages might, besides playing a role in antimicrobial activity, impair their migratory capacity, thus trapping these macrophages in the pancreas and leading to nonspecific damage to pancreatic β-cells, eventually triggering a β-cell destructive cascade. Accordingly, a highly inflamed state has been previously demonstrated in islets of NOD mice (471). Moreover, excessive expression of proinflammatory cytokines has been found in the islets of vitamin D-deficient NOD mice, indicative of a higher activation status of the infiltrating immune cells and thus a more aggressive presentation of the disease (433). Experimental autoimmune encephalomyelitis and inflammatory bowel disease are also accelerated and aggravated in vitamin D-deficient animals (465, 472, 473). Whereas vitamin D deficiency is strongly correlated with human immune abnormalities (454), the effects of supplements of vitamin D or 25-OHD in autoimmune diseases are less clear. Much depends on the initial vitamin D levels before supplementation—whether supplementation reverses existing vitamin D deficiency or adds extra vitamin D to already vitamin D-sufficient levels. For type 1 diabetes, multiple studies have evaluated the impact of vitamin D intake during pregnancy and/or early life and the prevalence of diabetes or diabetes-related autoimmunity (474, 475). 25-OHD levels at onset of disease were also investigated (476–478). Supplementing already vitamin D-sufficient NOD mice or BB rats with 1000 IU of regular vitamin D at an early age does not protect from autoimmune diabetes (479, 480). However, the insulin content in the islets of normal as well as diabetic NOD mice was increased. In the experimental autoimmune encephalomyelitis (EAE) model, vitamin D supplements fed to vitamin D-deficient mice conferred protection from disease (473).

The relation between vitamin D deficiency and asthma and allergy is more controversial. One study, however, demonstrated an inverse correlation between vitamin D levels and allergy or asthma (481). A link could be made not only to immune function but also to the development of the lung in utero and later on to its function. Some epidemiological data show that (higher) maternal vitamin D intake during pregnancy or early in life is inversely correlated with asthma and allergy later on (482, 483). However, other studies report a detrimental effect of vitamin D supplementation on the development of allergy (484–486).

**D. Discrepancy between loss of VDR and vitamin D deficiency in the immune system**

The discrepancy between the consequences of absence of the NR vs. absence of ligand on autoimmune disease presentation is largely unexplained. Vitamin D-deficient animals and humans have an increased sensitivity to mycobacteria, probably related to deficient macrophage function (451), and consequent inability to induce CAMP expression (487). In contrast, macrophage chemotaxis, phagocytosis, and respiratory burst capacity were normal in VDR null NOD mice. Moreover, the DC phenotype was abnormal in VDR null mice on different backgrounds (419, 429), whereas DCs from vitamin D-deficient mice did not present abnormalities (E. van Etten, G.B. Ferreira, A. Giuletti, and C.

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**Table 5. Comparison of the absence of VDR and vitamin D in the immune system: effects of the absence of the VDR, or vitamin D deficiency and the administration of pharmacological doses of the active 1,25-(OH)₂D or analogs in different immune settings**

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<tr>
<th>Autoimmune diseases</th>
<th>VDR KO</th>
<th>Vitamin D deficiency</th>
<th>1,25-(OH)₂D or analogs</th>
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<td>Human data</td>
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=, No change in disease presentation; +, aggravation; −, protection; nd, no data available; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; SLÉ, systemic lupus erythematosus.
Mathieu, unpublished results). Changes in T lymphocyte behavior were observed in VDR null NOD mice, but not in vitamin D-deficient mice.

Not only are the effects of lacking ligand or lacking the NR dependent on the tissue, but they also affect the presentation of different diseases in different ways (Table 5). As stated before, in NOD mice only vitamin D deficiency (433) and not lack of VDR (429) aggravated diabetes presentation (higher incidence and earlier onset). In the multiple sclerosis model, experimental autoimmune encephalomyelitis, VDR null mice were protected from disease (428), whereas vitamin D deficiency accelerated disease development (472). In contrast, in models of acute experimental inflammatory bowel disease, more severe colitis was present in the absence of either vitamin D or its receptor (427). A clear explanation for these diverse disease phenotypes is lacking, but involvement of different immune cells in respective target organs that are affected in a different way by ligand or receptor loss may be responsible.

This discrepancy of effects between lack of receptor and lack of ligand has also been observed for other NRs such as the thyroid receptor/thyroid hormone and the estrogen receptor/estradiol system (488). Repression by unliganded thyroid receptor has more profound effects on gene expression than absence of the thyroid receptor. Similarly, in the immune system, vitamin D deficiency often has more impact on disease outcome than absence of the VDR (Table 5). On the contrary, in the skin, absence of the VDR generates a more profound phenotype (alopecia) than absence of the ligand.

E. Pharmacological effects of 1,25-(OH)2D and analogs in the immune system

1. In vitro. 1,25-(OH)2D directly inhibits proliferation of human and murine T cells and inhibits the expression of several T cell cytokines (IL-2, IFNγ, and TNFα) (394, 395, 466, 489–491). The greatest immunomodulating effects of 1,25-(OH)2D have been found in APCs, especially DC. Multiple in vitro studies using either human peripheral blood monocytes or murine bone marrow cells as precursors revealed that 1,25-(OH)2D can potently inhibit DC differentiation (492–494). Also, the in vitro and in vivo maturation process of DCs is seriously impaired by 1,25-(OH)2D, with decreased surface expression of MHC class II, costimulatory molecules (CD40, CD80, CD86), and other maturation-induced surface markers. Even 1,25-(OH)2D treatment can redirect already differentiated DCs toward a CD14+ cell type. In addition, 1,25-(OH)2D treatment of differentiating DCs disturbs their migratory capacity in response to inflammatory and lymph node homing chemokines, although the expression of the cognate chemokine receptors was unaffected (492). Furthermore, the cytokine expression pattern of DCs is altered by 1,25-(OH)2D with strong inhibition of IL-12 and up-regulation of IL-10 expression (493, 494).

Because DCs are, as professional APCs, the predominant cells linking innate to adaptive immune responses, the effect of 1,25-(OH)2D on DCs inevitably has major impact on T cell behavior (Fig. 9). Although 1,25-(OH)2D has direct effects on T cells, it is mainly by this indirect way that 1,25-(OH)2D influences T cell responses. By altering DC cytokine expression (suppression of Th1-directing IL-12 and up-regulation of counteracting IL-10), 1,25-(OH)2D skews the T cell differentiation toward a Th2 phenotype. More importantly, by modulating DC maturation and activation, 1,25-(OH)2D gives rise to tolerogenic DCs, thus contributing to the induction of regulatory T cells or even T cell anergy and apoptosis (495). This 1,25-(OH)2D-mediated induction of regulatory T cells has been shown in vitro and in vivo. When cultured together with 1,25-(OH)2D-modulated DCs, naive as well as committed autoreactive T cells showed complete hyporesponsiveness, as determined by decreased proliferation and IFNγ secretion, and even underwent antigen-dependent apoptosis (493, 494, 496).

2. In vivo. High supraphysiological doses of 1,25-(OH)2D are needed to obtain immunomodulatory effects in vivo. Concomitant calcemic side effects are, therefore, observed, such as hypercalcemia, hypercalciuria, renal calcification, and increased bone resorption, prohibiting the clinical use of 1,25-(OH)2D as immunomodulator. To overcome this limitation, structural analogs of 1,25-(OH)2D have been developed showing higher immunomodulatory potency but lower calcemic activity (12, 497). The therapeutic potential of treatments including 1,25-(OH)2D or its analogs has been explored in vivo in several animal models of autoimmune diseases, organ transplantation, and infection (reviewed in Refs. 394, 395, 491, and 498–500).

3. Autoimmune diseases. 1,25-(OH)2D and its analogs prevent diabetes and insulinis (the histological lesion in pancreatic islets caused by infiltrating immune cells and preceding the clinical presentation of diabetes) in NOD mice when treatment is started at an early age, before the onset of insulinis (501–503). The beneficial effects of 1,25-(OH)2D and analogs, in this model, are a consequence of 1) the restoration of defective suppressor cell activity; 2) the enhanced clearance of autoreactive T cells, by restoring apoptosis sensitivity in the central and peripheral immune system; and 3) a shift from a Th1 to a Th2 cytokine expression profile locally in the pancreas and in the pancreas-draining lymph nodes (504, 505). 1,25-(OH)2D induces this immune shift in response to autoantigens but not to disease-irrelevant self or foreign antigens (505). When administered in mice of older age when autoimmune β-cell destruction is already taking place, analogs of 1,25-(OH)2D could still block diabetes progression, either alone or in combination with a short induction course of cyclosporin A (506, 507). After syngeneic islet transplantation, although analogs of 1,25-(OH)2D in monotherapy can delay autoimmune diabetes recurrence in NOD mice, better protection from disease recurrence and less toxicity were obtained with combinations of 1,25-(OH)2D and standard immunosuppressants (cyclosporin A, IFNβ) (508, 509). For combinations with cyclosporin A, graft survival persisted, even after withdrawal of therapy, suggesting a reinduction of self-tolerance (508).

Many other examples of animal autoimmune disease models exist in which treatment, including 1,25-(OH)2D or its analogs, prevents or attenuates disease, such as systemic lupus erythematosus (510), experimental autoimmune encephalomyelitis (511–516), collagen-induced arthritis (517,
518), inflammatory bowel disease (465), prostatitis (519), thyroiditis (520), and different forms of nephritis (521, 522). A few clinical trials even demonstrated disease-ameliorating effects of 1,25-(OH)2D analogs in patients suffering from multiple sclerosis or rheumatoid arthritis (523, 524). For the prostate, which is one of the known extrarenal sites of 1,25-(OH)2D synthesis and VDR expression, prostatitis (519) and prostate cancer (see Section IV) are not the only possible applications for 1,25-(OH)2D3. Recently, an analog of 1,25-(OH)2D3 proved to be protective in animal models of benign prostate hyperplasia, a complex syndrome comprising prostate overgrowth, urinary irritative syndrome, and an inflammatory component, by targeting prostatic growth, bladder function, and inflammation (525, 526). A multicenter, double-blind, randomized, placebo-controlled phase IIa clinical study confirmed the inhibitory effects on prostate growth (527).

The mechanisms behind the 1,25-(OH)2D-induced protection from these different autoimmune diseases has not yet been fully elucidated. In EAE, the induction of Th2 responses, inhibition of Th1 action and effects on the blood-brain barrier could be involved (528–532). In the model of inflammatory bowel disease the protective effects of 1,25-(OH)2D and its analogs are partly mediated through inhibition of Th17 cells, together with an increase in regulatory T cells (533). Th17 cells are a recently defined helper T cell population, along with Th1, Th2 and regulatory T cells, which play a proinflammatory role in autoimmunity and tissue inflammation (534).

4. Transplant survival. 1,25-(OH)2D and its analogs can also prolong the survival of heart, aorta, kidney, liver, small bowel, pancreatic islet, and skin allografts (535–540). These effects of 1,25-(OH)2D and analogs were generally modest when used in monotherapy but are more robust when used in combination with subtherapeutic doses of classical immunosuppressants (cyclosporin, tacrolimus, sirolimus, mycophenolate, and glucocorticoids). Adding 1,25-(OH)2D3 or analogs to low doses of classical immunosuppressants resulted in a clear synergistic immunomodulation, as measured in vitro and in vivo (514). When 1,25-(OH)2D3 or analogs were administered in transplant recipients, an increased re-

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FIG. 9. Overview of the pharmacological effects of 1,25-(OH)2D in the immune system. 1,25-(OH)2D inhibits the surface expression of MHC II-complexed antigen and of costimulatory molecules, as well as the production of the cytokine IL-12 in APCs (such as DC), thereby shifting the polarization of T cells from an (auto-)aggressive effector (Te) toward a protective or regulatory (Tr) phenotype. 1,25-(OH)2D exerts its immunomodulatory effects also directly on the level of T cells. Together, these immunomodulatory effects of 1,25-(OH)2D onto players of the adaptive immune system can lead to the protection of target tissues in autoimmune diseases and transplantation. In the innate immune system on the other hand, 1,25-(OH)2D strengthens the antimicrobial function of monocytes and macrophages, for example through enhanced expression of the CAMP, eventually leading to better clearance of pathogenic microorganisms.
sistance to opportunistic infections was observed (541). Moreover, whereas none of the standard immunosuppressants is able to prevent chronic rejection, treatment with analogs of 1,25-(OH)\(_2\)D does have an impact. Chronic rejection is caused by immunological and nonimmunological factors and is characterized by perivascular inflammation, fibrosis, and vascular narrowing, due to smooth muscle cell proliferation. In an aortic allograft model, which mimics the vascular lesions seen in human chronic allograft rejection, treatment with a 1,25-(OH)\(_2\)D analog prevented chronic aortic allograft rejection and attenuated vascular damage (542, 543).

5. Infections. Exposing monocytes and macrophages to 1,25-(OH)\(_2\)D improves their chemotactic and phagocytic capacity, features that are indispensable for their tumor cell cytotoxicity and microbacterial activity (544). Monocytes and macrophages are, in addition to their role as APCs in adaptive immune responses, key players in mounting innate immune responses against various infectious agents, detecting dangerous microbial invaders by means of their pattern-recognition receptors (e.g., TLRs), and subsequently producing antimicrobial peptides such as defensins and cathelicidins (545, 546). 1,25-(OH)\(_2\)D is able to induce the expression of human CAMP in human primary cells (monocytes, keratinocytes, neutrophils, and macrophages) and cell lines through direct interaction with VDRE sequences present in the promoter of the CAMP gene (397, 487, 547, 548). In human monocytes, triggering of TLR2/1 results in the conversion of 25-OHD into active 1,25-(OH)\(_2\)D and the consequent induction of CAMP (451), whereas at lower 25-OHD levels (for example, in serum of African-Americans), the induction of CAMP and subsequent antimicrobial capacity is reduced. In human keratinocytes, at sites of injury, the induction of CAMP by 1,25-(OH)\(_2\)D requires SRC3 and is influenced by histone acetylation (549). In contrast with the human CAMP gene, no VDRE were detected in the promoter region of the mouse calcidiol gene (548). Consistent with these findings, no differences in CAMP expression were observed between WT and VDR null mice. At later stages of infection (after 72 h), 1,25-(OH)\(_2\)D suppresses the expression of TLR2 and TLR4 in human monocytes by a VDR-dependent mechanism (550), possibly representing a negative feedback mechanism, so as to prevent excessive TLR-activation and eventually sepsis.

F. Conclusion

The regulated presence in many immune cells of the key enzymes involved in the metabolism of 1,25-(OH)\(_2\)D and the presence of VDR in almost all immune cells suggests a physiological role for the VDR-vitamin D endocrine system in immune homeostasis. Besides solid epidemiological evidence linking vitamin D deficiency to autoimmune diseases and increased susceptibility to infections, genetic studies also point toward associations between immune behavior and the different components of the VDR-vitamin D endocrine system. Intervention studies with pharmacological doses of 1,25-(OH)\(_2\)D and some of its analogs have until now only been performed in animal models. These clearly demonstrate prevention of several autoimmune diseases, as well as prolongation of graft survival. All these data support the concept that the VDR-vitamin D endocrine system plays a role in the etiology of autoimmune diseases, such as type 1 diabetes, being involved in the genetic (genetic variations in CYP27B1), environmental (vitamin D deficiency), and immunological aspects [potent immunomodulatory effects of 1,25-(OH)\(_2\)D of their etiology. Moreover 1,25-(OH)\(_2\)D can stimulate the native immune system and thus may offer a novel strategy to cope with (drug-resistant) infections.

The main practical conclusion from the studies on the VDR-vitamin D endocrine system and immunology to date is that avoiding vitamin D deficiency is important, not only for calcium and bone issues but also for a healthy immune system.

VI. VDR-Vitamin D Endocrine System and Glucose Homeostasis

Calcium fluxes and regulation of intracellular calcium stores are essential in the regulation of insulin secretion by the β-cells of the islets of Langerhans in the pancreas. This makes 1,25-(OH)\(_2\)D, the central hormone in calcium regulation, a potential candidate for influencing normal β-cell function. Since the early observations in 1980 by Norman et al. (551) that pancreatic insulin secretion is inhibited by vitamin D deficiency, several reports have demonstrated an active role for vitamin D in regulating the function of the endocrine pancreas, especially the insulin-producing β-cells. The VDR is present in β-cells (552), and intriguing observations suggest the presence of a receptor localized in the membrane (553–555).

A. Vitamin D deficiency and VDR null mice

The earliest studies focused mainly on the effects of vitamin D deficiency in animal models and humans on insulin secretion and glucose tolerance (551, 556–565). Vitamin D deficiency clearly impairs secretion of insulin (but not the other pancreatic hormones) and induces glucose intolerance, whereas vitamin D reverses the abnormalities (551, 556, 559, 560, 562–564). Data from VDR null mice are conflicting, with some groups reporting impaired glucose tolerance (565) while others report normal glucose metabolism (419, 429). As in other systems, the background of the mouse in which the VDR deletion is introduced seems to be of critical importance. Recent in vitro data on β-cell function in islets from VDR null mice did not show any abnormalities (419, 429). The pancreatic β-cell may, therefore, represent another target tissue with discrepancy between effects of the vitamin D ligand and the VDR itself. In NOD mice, calcium levels are essential in maintaining normal β-cell function because islets isolated from vitamin D-deficient, normocalcemic, NOD mice had normal insulin synthesis and secretion in response to glucose (433). Whether the effects of vitamin D deficiency and repletion or vitamin D resistance on glucose homeostasis in vivo are direct or indirect effects of vitamin D cannot be resolved by such studies because vitamin D-deficient animals do not have a normal food intake, thus losing weight and being unable to maintain normal plasma calcium levels.
Moreover, these metabolic changes, per se, directly impair calcium handling in the β-cell and provoke β-cell dysfunction and glucose intolerance (556, 564).

B. Effects of vitamin D metabolites in vitro

Convincing data come from in vitro studies where islets isolated from vitamin D-deficient animals show impaired insulin release when cultured in vitro and challenged with glucose (566, 567), whereas these abnormalities can be prevented by culturing the islets in the presence of high concentrations of 1,25-(OH)₂D (557, 566–568). Moreover, high concentrations of 1,25-(OH)₂D increase insulin synthesis and release upon glucose challenge in isolated pancreatic islets from normal animals (557, 559, 568–577). The mechanism by which 1,25-(OH)₂D affects insulin secretion probably involves a significant rise in cytosolic calcium levels after incubation with 1,25-(OH)₂D, resulting in insulin secretion. Controversy still exists as to whether this higher intracellular calcium comes from an influx of calcium via voltage-dependent calcium channels, or whether mobilization of calcium from intracellular organelles and activation of receptor-potentiating systems via protein kinase C/protein kinase A pathways are implicated (553, 566, 567).

In the pathogenesis of type 1 diabetes, β-cell damage by cytokines and other inflammatory agents might play an important role. Several studies demonstrate a protective effect of 1,25-(OH)₂D (incubation periods ranging between 48 and 72 h) or its analogs on cytokine-induced β-cell dysfunction and death (572, 575, 577). A shorter exposure to 1,25-(OH)₂D (24 h) did not confirm these data (574). We could not find protective effects of 1,25-(OH)₂D on β-cell death but clearly demonstrated altered expression of chemokines in the β-cells, thus potentially altering their fate in type 1 diabetes (578) (see Section V).

C. Effects of vitamin D metabolites in vivo: clinical implications for type 2 diabetes

Vitamin D-deficient humans have reduced insulin secretion, whereas, as expected, vitamin D treatment (and calcium supplementation) restores glucose tolerance (559, 570, 573, 579). Administration of vitamin D supplements or high doses of 1,25-(OH)₂D, to vitamin D-replete patients with impaired glucose tolerance or frank type 2 diabetes yields conflicting results, with some reporting improvement (573) and others no effect (580), and one study even showed worsening of type 2 diabetes. Notably, Taylor and Wise (581) reported that vitamin D supplementation in three cases of British Asians with vitamin D deficiency and type 2 diabetes led to increased insulin resistance and deterioration of glycemic control.

Uremic patients are 1,25-(OH)₂D-deficient and also show insulin resistance. Allegra et al. (569) clearly demonstrated that insulin resistance in uremic patients decreased after 1,25-(OH)₂D therapy but that glucose intolerance was not completely reversed.

The relationship between vitamin D status and the metabolic syndrome has been evaluated in some small-scale studies, as well as in the large-scale Third National Health and Nutrition Examination Survey. 25-OHD levels above 19 ng/ml, and especially levels above 39 ng/ml, were associated with a significant lower odds ratio for the presence of metabolic syndrome and especially of abdominal obesity (582). Vitamin D deficiency is also associated with higher cardiovascular risk factors (see Section VII). A small-scale post hoc analysis of a bone study revealed that daily calcium (500 mg) and vitamin D (700 IU) supplementation for 3 yr prevented a further rise in fasting blood glucose in a subgroup with impaired fasting blood glucose (100–125 mg/dl) at baseline (583).

VII. VDR-Vitamin D Endocrine System and Cardiovascular System

Many cells that play a crucial role in the cardiovascular system express the VDR and respond to 1,25-(OH)₂D with cell-specific gene regulation and functional consequences. These cells include vascular endothelial cells, cardiomyocytes, vascular smooth muscle cells, and monocytes/phagocytes (584) (Fig. 10). The juxtaglomerular cells of the nephron,
hypertrophy and fibrosis, suggesting that 1,25-(OH)2D also
VDR null strain, and no systemic hypertension. Even in the
cant increase in plasma renin activity in the same Boston
ibility). Another group (588), however, found a nonsignifi-
cardiac repercussions (hypertrophy and decreased contract-
native mechanism contributing to cardiac hypertrophy in
which produce renin, are also 1,25-(OH)2D-sensitive (Fig.
Renin is a protease that cleaves angiotensinogen to an-
giotensin I, which is subsequently converted to angiotensin
II. Angiotensin II activates specific receptors and regulates
electrolyte, volume, and blood pressure homeostasis. In VDR
null mice, increased renal renin mRNA levels have been
observed, even after normalization of calcium homeostasis
by a rescue diet (585). This leads to high plasma angiotensin
levels, systemic hypertension, and ultimately results in
cardiac hypertrophy (586). The hypertensive effects of VDR
deletion are blocked by angiotensin-converting enzyme
inhibitors. A VDRE has been identified in the promoter of
the renin gene, and direct inhibition of renin expression by 1,25-
(OH)2D has been confirmed in vitro (586) and in vivo (121,
587). In 1α-hydroxylase-deficient mice, renal renin expres-
sion was also increased. Renin, therefore, is likely to be neg-
atively and directly regulated by 1,25-(OH)2D in mice, with
cardiac repercussions (hypertrophy and decreased contract-
ability). Another group (588), however, found a nonsignifi-
cant increase in plasma renin activity in the same Boston
VDR null strain, and no systemic hypertension. Even in the
absence of hypervitaminosis, VDR null mice developed cardiac
hypertrophy and fibrosis, suggesting that 1,25-(OH)2D also
has direct effects on prevention of cardiomyocyte hypertrophy
(588). Cardiac myocytes from VDR null mice showed accel-
 rated rates of contraction and relaxation, compared with WT mice, and 1,25-(OH)2D directly affected contractility in
WT but not in VDR null cardiac myocytes (589). An alter-
native mechanism contributing to cardiac hypertrophy in
these VDR null mice could be increased extracellular matrix
production and subsequent fibrosis, due to deficient expres-
sion of tissue inhibitors of metalloproteases-1 and -3 (147).

The VDR-vitamin D effects on the renin-angiotensin sys-
tem may also be involved in the renoprotective effects of
1,25-(OH)2D. VDR null mice exhibit an earlier onset and
more severe nephropathy after streptozotocin-induced dia-
betes (590). This more severe diabetic nephropathy is char-
acterized by increased proteinuria; higher renin, angiotensin,
and angiotensin receptor expression; more mesangial scle-
rosis; lower nephrin expression; and decreased podocyte number. These renoprotective effects of the vitamin D en-
docrine system were also observed in patients with chronic
renal failure because vitamin D analogs were able to reduce
proteinuria (591).

In normotensive (592, 593) and hypertensive patients (594,
595), blood pressure is inversely correlated with plasma 1,25-
(Oh)2D and renin concentration. Vitamin D and calcium
supplementation decreased blood pressure in short-term
studies (596), and 1α-hydroxylated vitamin D therapy also
reduced blood pressure in hypertensive patients (597).

The VDR-vitamin D endocrine system has several other
beneficial effects on cells of the cardiovascular system. In
cultured monocytic cells, 1,25-(OH)2D has anticoagulant ef-
effects of hypocalcemia), platelet aggregation was increased
and LPS-induced, multiorgan thrombosis was also en-
hanced. This was consistent with increased tissue factor ex-
pression in liver and kidney and decreased thrombomodulin
expression in several tissues (600).

In a LPS-induced disseminated intravascular coagulation
rat model, administration of 1,25-(OH)2D reduced thrombo-
sis (601). In a phase II human trial of intermittent high-dose
1,25-(OH)2D therapy to delay metastatic prostate cancer
growth, a significant reduction of thromboembolic events
was reported (602). Vascular smooth muscle cells, as well as
endothelial cells, are responsive to 1,25-(OH)2D. Plasmino-
gen activator inhibitor 1 and thrombospondulin 1 gene ex-
pression is indirectly suppressed by 1,25-(OH)2D (345, 603).
In addition 1,25-(OH)2D enhances smooth muscle cell relax-
ation and cardiac myocyte contractility (345, 604). Based on
in vitro effects of 1,25-(OH)2D and phenotypic analysis of
VDR null mice, the combined effects of 1,25-(OH)2D on cells
of the vascular wall and on cardiomyocytes provide syner-
gistic beneficial effects on cardiovascular function.

Nevertheless, it is well known that vitamin D toxicity is
associated with ectopic calcification, especially of the vas-
cular wall. This has been observed in rodents and patients
exposed to high doses of exogenous vitamin D, as well as in
mice with endogenous overproduction of 1,25-(OH)2D due
to targeted gene silencing of CYP24A1 (33, 34), FGF23 (227),
and Klotho (231) (Table 6). These ectopic calcifications are
also observed in the kidney and other soft tissues, leading to
organ failure and early lethality. The ectopic calcifications
are partially a passive phenomenon due to oversaturation of
Ca × P concentration, but in some tissues (e.g., vascular wall)
a true cellular transformation of mesenchymal cells into os-
teoblast-like cells is induced by excess 1,25-(OH)2D or excess
serum phosphate and membrane phosphate transporter Pit
1 (605). Of course, atherosclerotic intimal and medial artery
as well as valvular calcifications can also be induced by a
wide variety of factors, including exposure to oxidized LDL,
inflammatory cytokines, and other proteins that enhance net calcium deposition. Treatment of secondary hyperparathyroidism with active vitamin D metabolites is frequently associated with increased risk for ectopic and, especially, vascular calcifications, because of potential direct effects of 1,25-(OH)2D on vascular calcifications and indirect effects on increasing the Ca × P product. Although this risk is real and the Ca × P product should be carefully monitored, treatment of severe secondary hyperparathyroidism with large doses of vitamin D may actually decrease vascular calcifications (606).

Several clinical studies link mild vitamin D deficiency with increased cardiovascular risk (factors) such as hypertension, obesity, type 2 diabetes, and metabolic syndrome and even vascular calcifications (607–611). The largest prospective intervention trial (WHI) with calcium (1 g/d) and vitamin D (400 IU/d), however, revealed no change in coronary or cerebrovascular risk after 7 yr of follow-up (612). Patients with chronic renal failure on maintenance hemodialysis are highly vulnerable to diffuse vascular calcifications and are frequently deficient in both 25-OHD and 1,25-(OH)2D. Very large retrospective studies in Japanese and U.S. chronic renal failure patients, subsequently confirmed in smaller studies in other populations, revealed a survival benefit, especially cardiovascular, from treatment with a VDR agonist (613, 614). Apart from the genomic VDR-mediated effects, several rapid nongenomic effects of 1,25-(OH)2D on muscle cells have been reported, with rapid transient changes in intracellular calcium concentrations and other intracellular signaling pathways (151, 618). These findings may be relevant for the myopathy associated with severe vitamin D deficiency of rickets or osteomalacia and in chronic renal failure, is associated with muscle weakness or myopathy. It is unclear whether this is a direct or indirect consequence of lack of vitamin D action (616). This question has been addressed in VDR null mice on both normal and rescue diets (617). Muscle fibers (type I and type II) of all striated muscle are significantly smaller in VDR null mice (−20%), suggesting effects during a late stage of muscle development. This was confirmed by gene and protein expression analysis. Markers of early muscle development (myogenic transcription factors such as myf5, E2A, and myogenin) are still present in striated muscle of 3-wk-old VDR null mice, but not in WT muscle. Similarly, neonatal and embryonic myosin heavy chain isoforms remain much longer expressed in the cytoplasm of small muscle fibers of VDR null mice. These observations were present in normocalcemic VDR null mice, indicating a direct vitamin D–VDR effect. These genes (myf5, myogenin, and neonatal myosin heavy chain isoform) were all down-regulated by 1,25-(OH)2D in a myocyte cell line (617).

Myf-5/myo D double null mice show complete absence of skeletal muscle, and myogenin/MRF4 double null mice have a major deficit in the formation of mature multinucleated myotubes. It is unclear why VDR null muscle cells express more Myf-5 and myogenin and yet have decreased muscle mass and size, but this nevertheless indicates that the vitamin D–VDR endocrine system is important for striated muscle. Apart from the genomic VDR-mediated effects, several rapid nongenomic effects of 1,25-(OH)2D on muscle cells have been reported, with rapid transient changes in intracellular calcium concentrations and other intracellular signaling pathways (151, 618). These findings may be relevant for the myopathy associated with severe vitamin D deficiency of rickets or renal osteodystrophy patients. Sarcopenia, or progressive loss of muscle mass and strength, is frequently seen in the elderly in association with vitamin D deficiency (619–621). Vitamin D supplementation of vitamin D-deficient elderly subjects has a modest effect on muscle function, body sway, and frequency of falls (622–626). The overall effect of vitamin D on falls is, however, modest and inconsistent (627) and cannot be fully dissociated from the frequent concomitant administration of calcium supplements. The combined supplementation with vitamin D and calcium clearly reduces the risk of fractures, according to several meta-analyses (628, 629).

In conclusion, severe vitamin D deficiency in patients with rickets, osteomalacia, or renal osteodystrophy is frequently associated with myopathy. Milder vitamin D deficiency in the elderly is associated with decreased muscle mass and
strength, and predisposes to falls. *In vitro* studies, especially muscle phenotyping of VDR null mice, suggest a direct effect of the VDR-vitamin D endocrine system on the later stages of muscle cell development and thus could potentially explain the observations in vitamin D-deficient patients. However, the molecular and cellular details of the mode of action of 1,25-(OH)$_2$D on striated muscle are scarce.

**IX. VDR-Vitamin D Endocrine System and Brain**

VDR and key enzymes of vitamin D metabolism are expressed in nearly all regions of the rodent brain (630). The functionality of the vitamin D endocrine system has been demonstrated *in vitro* using neuronal or glial cell culture because 1,25-(OH)$_2$D is a potent inducer of nerve growth factor, glial cell line-derived neurotropic factor, and neurotropin 3 (631–633). VDR null mice show abnormal behavior, especially muscle and motor behavior, but normal cognition, as evaluated by a wide variety of more than 40 behavioral screening tests (634). Because these animals were raised on a normal calcium intake and therefore developed hypocalcemia and growth delay, an indirect effect of hypocalcemia and muscle weakness on motor behavior cannot be excluded. Another extensive set of experiments, performed in Tuomimaa’s laboratory, revealed that VDR null mice on a normal calcium diet have an abnormal grooming pattern (higher frequency of grooming, but with incorrect transition and increased interruption of grooming behavior) (635, 636). They confirmed impaired motor performance (637) and suggested that VDR null mice display an increased anxiety behavior (638). Most other behavioral tests including mating behavior were normal.

Simple vitamin D deficiency during early life also has neurobehavioral effects because brains of newborn rats born to vitamin D-deficient mothers are larger, with larger lateral ventricles, increased brain cell proliferation, and decreased cortical brain thickness (639). At a molecular level, the expression of nerve growth factor and glial cell line-derived neurotrophic factor was decreased in vitamin D-deficient newborn rats. These effects were not transient because at 10 wk of age, the lateral ventricles were still enlarged and the expression of genes involved in neuronal function or structure (*e.g.*, nerve growth factor, neurofilament, and γ-aminobutyric acid-A) were lower in brains of rats that were transiently vitamin D deficient during early development (640, 641). A detailed proteomics analysis of prefrontal and hippocampus areas of brains from rats exposed to transient pre- and perinatal vitamin D deficiency identified dysregulation of more than 30 proteins involved in a variety of cellular functions. Half of these proteins are known to be misexpressed in schizophrenia or multiple sclerosis (642). A more detailed functional analysis of late consequences of developmental vitamin D deficiency in rats suggests persistent mild learning and memory dysfunction (643) and drug-induced hyperlocomotion (an animal model of schizophrenia) (644). Other mild behavioral abnormalities were found in two different strains of mice born to vitamin D-deficient mothers raised on a vitamin D-replete diet after weaning (645). The neuroprotective effects of 1,25-(OH)$_2$D have also been demonstrated in rodent models of stroke and 6-hydroxy-dopamine toxicity (646, 647). The rodent data thus indicate that pre/perinatal VDR-vitamin D deficiency can result in long-lasting changes in gene and protein expression in the brain and may be associated with variable, subtle, structural and behavioral abnormalities.

The human equivalent of vitamin D effects on early brain development has not been fully explored. Low levels of 25-OHD in pregnant mothers has been associated with increased risk of schizophrenia of their children (648). Retrospective analysis of the effects of vitamin D supplements during the first year of life in northern Finland revealed that regular or intermittent vitamin D supplements (especially ≥2000 IU/d) were associated with a reduced risk (RR, 0.23) of schizophrenia in males but not in females (at the age of 31 yr) (648). The absolute number of cases was too small to allow firm conclusions about the association with vitamin D deficiency, and thus additional studies are required. Low vitamin D status is also frequently observed in patients with Alzheimer’s disease and schizophrenia and in elderly subjects with cognitive dysfunction (649).

Thus, the rodent and human data on the effect of the VDR-vitamin D endocrine system on brain and behavioral function are far from conclusive in terms of a causal relationship, but nevertheless are sufficiently hypothesis generating to warrant further cellular, animal, and human studies.

**X. VDR-Vitamin D Endocrine System and Reproduction**

Not only did the first strain of VDR null mice develop the expected bone and hair phenotype, but female mice also showed uterine hypoplasia and impaired ovarian folliculogenesis (155). The fertility of vitamin D-deficient female rats was decreased by 75% (650). This reduced fertility persisted in vitamin D-deficient, yet calcium-repleted rats, suggesting a direct effect of vitamin D rather than an indirect effect mediated by hypocalcemia (651).

A possible mechanism may be the direct stimulatory effect of 1,25-(OH)$_2$D on the aromatase gene expression in reproductive tissues of female and male mice (652, 653). It is notable that male fertility was also reduced, as demonstrated by reduced sperm number and mobility and histological changes in testicular morphology (652). LH and FSH serum levels were increased in these mice, indicating primary hypergonadotropic hypogonadism. Supplementation with estradiol corrected the reproductive phenotype of VDR null mice, whereas partial correction of calcium homeostasis by a high-calcium diet was only partially effective (652). These data all indicate a direct effect of the VDR-vitamin D endocrine system on female and male reproduction. These observations are consistent with the presence of the VDR in reproductive tissues, including human sperm (654). Subsequent studies using the same Tokyo VDR null mice confirmed a lower reproductive efficacy in mice fed a normal or low-calcium diet, whereas on a high-calcium/lactose diet reproduction normalized (655), implying that hypocalcemia is the major driving factor for reduced fertility. The reduced conception rate (−90%) in VDR null mice was also confirmed...
in the Boston strain, but again, a rescue diet normalized the conception rate (196).

The effects of maternal VDR deficiency on fetal and postnatal development have been evaluated in vitamin D- and 1α-hydroxylase-deficient animals and VDR null mice. On a normal calcium diet, fetuses from these animals are growth retarded and usually, but not always, have abnormal serum calcium levels and skeletal mineralization defects (196, 656–658). When abnormalities were observed in fetuses born from vitamin D-deficient or VDR null mothers, they were rescued by normalization of serum 1,25-(OH)2D and/or calcium levels (657, 658). A more detailed analysis revealed that VDR null pups from VDR null mothers showed a more normal development than heterozygote VDR pups. These heterozygous pups responded to excess of 1,25-(OH)D with decreased bone mineralization and early postnatal mortality (659).

The human equivalent of VDR or 1α-hydroxylase deficiency on reproduction is poorly studied. The effects of severe or even mild vitamin D deficiency on human reproduction are also poorly studied. However, vitamin D deficiency is quite frequent in pregnancy, frequently persists during lactation, and thus is not an absolute obstacle to successful reproduction.

Vitamin D deficiency during pregnancy has also been associated with an increased risk of preeclampsia (660, 661), based on a large nested case-control study. This is reminiscent of the effects of VDR or vitamin D deficiency on systemic hypertension (see Section VII). Therefore, further studies are desirable in pregnant VDR or 1α-hydroxylase null mice, as well as intervention studies with vitamin D supplementation in pregnant women, especially in view of the high prevalence of vitamin D deficiency during pregnancy (662–664). Moreover, the long-term consequences of the prenatal and early postnatal effects of vitamin D deficiency might be very serious because developmental vitamin D deficiency is associated with decreased bone mass (665), enhanced risk for autoimmune disease (such as type 1 diabetes; see Section V), and brain dysfunction (see Section IX).

XI. VDR-Vitamin D Endocrine System and Adipocytes

Fat cells are very active endocrine cells that express the VDR and could, therefore, be potential targets for vitamin D action. VDR null mesenchymal cells from bone marrow express increased mRNA levels of peroxisome proliferator-activated receptor γ and other markers of adipogenic differentiation when cultured under adipogenic conditions (204). This is not totally unexpected because mesenchymal stem cells are preferentially oriented toward the osteoblastic lineage when exposed to 1,25-(OH)2D, whereas adipogenesis is impaired (666, 667). This 1,25-(OH)2D-VDR effect on adipogenesis is mediated by down-regulation of C/EBPβ, DKK1, SFRP2 and, thus, enhancement of the canonical Wnt signaling pathway.

The in vivo effects of VDR or CYP27B1 deficiency on total body composition (muscle/fat/bone) independent from serum calcium homeostasis has not yet been fully explored.

XII. General Conclusions and Perspectives

The VDR-vitamin D endocrine system originated during the evolution of vertebrates, shortly before their skeletons became calcified. Its best known action is the regulation of extracellular calcium and phosphate homeostasis, especially by increasing the absorption or reabsorption of calcium at nearly all epithelia involved in calcium transport. The consequences of vitamin D deficiency or loss of vitamin D action (by a deficit in its supply, metabolic activation, or inactivation of its NR) generates a bone, growth plate, and tooth phenotype known as rickets (before end of puberty) or osteomalacia. The bone/growth plate/tooth phenotype of mice with genetically engineered lack of vitamin D activation (1α-hydroxylase or CYP27B1 null mice) or activity (VDR null mice) is, in all aspects, identical to the phenotype of humans with similar disorders or severe vitamin D deficiency. The growth plate of VDR or 1α-hydroxylase-deficient animals or men is structurally abnormal by widening of the hypertrophic chondrocytes and deficient mineralization of metaphyseal bone. Low levels of extracellular calcium and phosphate impair bone mineralization, whereas osteoclast and especially osteoblast activity is altered, resulting in undermineralization of bone and teeth. This explains, to a large extent, the clinical picture of rickets, characterized by stunted growth, enlargement of wrists and ribs, and decreased bone strength with bowing of weight bearing bones. The molecular and cellular mechanisms involved in the etiology of this bone/growth plate/tooth development are complex. However, normalization of this phenotype in mice or men with 1α-hydroxylase or VDR deficiency by a high calcium and lactose intake or by selective rescue of the VDR in the intestine of VDR null mice provides very strong arguments for the intestinal epithelium as the primary target for vitamin D action. Several gene products involved in transepithelial calcium transport are strongly vitamin D-dependent, but it seems that the three major candidates (TRPV5/6; CaBP-9/28k and PMCA pumps) cannot fully explain the molecular mechanism of 1,25-(OH)2D-VDR dependent calcium transport. The hunt for the key vitamin D-dependent genes for the most important vitamin D action is therefore still ongoing.

The VDR-vitamin D endocrine system is also functional in other calcium transporting or calcium-sensing tissues such as the kidney, all bone cells including growth plate chondrocytes, and parathyroid glands, with less convincing or absent effects on calcium transport in the placenta or mammary gland (Table 7). Due to the dominant effect of VDR/1α-hydroxylase deficiency on intestinal calcium and phosphate transport, it will require the generation and phenotypic analysis of tissue-specific transgenic animals to define the possible direct effects of the vitamin D endocrine system on other tissues involved in global calcium homeostasis (e.g., selective deletion or rescue of VDR in bone, kidney, or parathyroid cells). Selective VDR deletion in growth plate chondrocytes already demonstrated its redundancy for the growth plate itself but revealed a potential endocrine function of the growth plate VDR on bone and kidney.

The widespread presence of VDR in nearly all cells and tissues, the nearly equally widespread presence of vitamin D-metabolizing enzymes, and the very large number of
genes (estimated at about 3% of the mouse or human genome) that are directly and/or indirectly responsive to VDR-1,25-(OH)2D all point toward a role for this endocrine system well beyond extracellular calcium and phosphate homeostasis (Table 7). This is, in fact, not the exception but the rule because nearly all ligands for NRs have a wide spectrum of activities beyond their primary targets (e.g., estrogens, androgens, glucocorticoids, thyroid hormones, retinoids, and peroxisome proliferator-activated receptor y ligands).

The skin is the most obvious “noncalcemic” target for the VDR-vitamin D endocrine system because it was known from human vitamin D-resistant rickets (type II due to VDR mutations) that acquired generalized alopecia is part of the phenotype. This was confirmed in all VDR null mouse strains. Their hair cycle phenotype is purely dependent on VDR and not on the vitamin D ligand because it cannot be reproduced by vitamin D or 1α-hydroxylase deficiency. This is a first clear example of discordance between VDR and 1,25-(OH)2D deficiency and reminiscent of other examples in the NR field (e.g., thyroid hormone receptor). The molecular mechanisms that lead to alopecia are only partially understood and involve protein-protein interactions between VDR, RXR, hairless, and components of the wnt signaling.

Although normocalcemic VDR and 1α-hydroxylase null mice do not develop a skin or epidermis phenotype beyond alopecia, they show, nevertheless, mild defects in the repair of the essential barrier function of their skin after acute skin injury.

The vitamin D hormone, 1,25-(OH)2D, is a potent inhibitor of cell cycle progression, with predominant effects on G0/G1,

### Table 7. Overview of the VDR null mouse phenotype

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Total VDR deletion</th>
<th>Total VDR deletion on rescue diet</th>
<th>Tissue-specific deletion/transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>Impaired (active)</td>
<td>Not available</td>
<td>VDR-calbindin rescue, tissue-specific KO: rescue of bone phenotype in progress</td>
</tr>
<tr>
<td></td>
<td>calcium absorption</td>
<td></td>
<td>Chondrocyte-specific KO: no growth plate phenotype increased bone mass</td>
</tr>
<tr>
<td>Growth plate</td>
<td>Ricketic hypertrophy</td>
<td>Normal</td>
<td>Osteoblast specific KO: phenotype analysis in progress</td>
</tr>
<tr>
<td>Bone</td>
<td>Impaired mineralization</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Relative hypercalcemia</td>
<td>Hypercalciuria</td>
<td>Not available</td>
</tr>
<tr>
<td>Parathyroid gland</td>
<td>Hyperparathyroidism</td>
<td>Normal</td>
<td>Tissue-specific KO: phenotype analysis in progress</td>
</tr>
<tr>
<td>Skin</td>
<td>Epithelium</td>
<td>Impaired differentiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impaired barrier function (CYP27B1 KO)</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Hair follicle</td>
<td>Total alopecia</td>
<td>Total alopecia</td>
<td>Keratinocyte-specific VDR rescue: normalization of hair growth</td>
</tr>
<tr>
<td>Immune system</td>
<td>Innate (monocytes)</td>
<td>Increased activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acquired</td>
<td>Resistance to LDS-induced diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal susceptibility to type 1 diabetes</td>
<td>Resistance to Leishmania infection</td>
<td></td>
</tr>
<tr>
<td>Immune system</td>
<td>Increased activity</td>
<td>Increased resistance to EAE</td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>Not available</td>
<td>Resistance to airway inflammation</td>
<td></td>
</tr>
<tr>
<td>Allergy/asthma</td>
<td>Not available</td>
<td>Increased risk of chemocarcinogen or oncogen mediated cancer</td>
<td></td>
</tr>
<tr>
<td>Cell proliferation/cancer</td>
<td>Not available</td>
<td>More susceptible to IBD</td>
<td></td>
</tr>
<tr>
<td>Mammary gland</td>
<td>Not available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>High renin hypertension</td>
<td>Idem</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cardiac hypertrophy</td>
<td>Idem</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>Impaired maturation of striated muscle</td>
<td>Idem</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Subtle behavioral modification (grooming, maternal behavior, etc.)</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>Reproduction</td>
<td>Males</td>
<td>Reduced fertility</td>
<td>(Near) normal</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>Reduced fertility</td>
<td>Impaired fertility in Tokyo KO</td>
</tr>
</tbody>
</table>

LDS, Low-dose streptozotocin; IBD, inflammatory bowel disease; idem, phenotype identical to phenotype of total VDR deletion without a reserve diet.
cell cycle arrest. This antiproliferative effect is coordinated by direct and indirect control of a very large number of genes involved in DNA replication and cell cycle progression. VDR null mice should, therefore, be more prone to develop cancer and, indeed, they develop more tumors or premalignant lesions when exposed to oncogenes or chemocarcinogens. Moreover, there are strong epidemiological links between vitamin D deficiency and a wide variety of common cancers in humans, especially of colon, breast, and prostate. All the cells (APCs and monocyte/macrophages, T and B cells, and mast cells) of the immune system express VDR either in the basal state or after appropriate immune stimuli. Some immune cells (especially mononuclear cells) express CYP27B1 after exposure to combined immune stimuli. In the basal state, VDR or 1α-hydroxylase null mice do not show a major immune phenotype at least after normalization of serum calcium homeostasis, although there are many subtle cellular and molecular defects in their immune system. VDR- and/or vitamin D-deficient mice are, however, more prone to develop autoimmune diseases such as inflammatory bowel disease or type 1 diabetes. In contrast to the hyperreactive acquired immune system, the native immune defense system seems to be hyporeactive in vitamin D-resistant or -deficient animals and humans, and this may predispose to (bacterial) infections. Little is known about the immune abnormalities in humans with genetic defects in vitamin D action, but there are many suggestions for an increased prevalence of major autoimmune diseases (especially type 1 diabetes and multiple sclerosis) in vitamin D-deficient subjects, especially when deficiency is present during development (diabetes) or before adulthood (multiple sclerosis).

A wide spectrum of observations links vitamin D deficiency or resistance with increased cardiovascular risks. VDR and 1α-hydroxylase-deficient mice overexpress renin, and this leads to systemic hypertension and cardiac hypertrophy. VDR null mice are also more prone to diabetes-induced renal damage. VDR null mice also develop more inflammation-induced multiorgan thrombosis, probably related to an increased expression of prothrombotic and reduced expression of antithrombotic or fibrinolytic factors. 1,25-(OH)2D exerts beneficial effects on several cells of the vascular wall such as endothelial and smooth muscle cells. In line with these animal data, vitamin D deficiency in humans is associated with increased incidence of cardiovascular risk factors and metabolic syndrome, but causality has yet to be demonstrated by appropriate intervention studies. Nevertheless, vitamin D toxicity either due to exogenous vitamin D (metabolite) overdosing or by endogenous overproduction (e.g., by deletion of CYP24A1, FGF23, or Klotho gene) is mainly due to ectopic and, especially, vascular and valvular calcifications and subsequent organ failure. Therefore, vitamin D deficiency, as well as vitamin D excess, has negative cardiovascular effects, demonstrating the need for carefully optimizing the exposure to vitamin D.

VDR null mice, even when normocalcemic, show signs of impaired development of striated muscle. This may be reminiscent of the effects of severe vitamin D deficiency on muscle weakness, as seen in patients with rickets, osteomalacia, or chronic renal failure. VDR is expressed in most brain areas, but VDR null mice do not have an obvious neurological phenotype. However, VDR null mice display a variety of subtle behavioral abnormalities such as abnormal grooming pattern and maternal behavior. Finally, VDR null mice have impaired male and female reproduction, which is correctable by a high-calcium diet in some, but not all, knockout (KO) strains.

The comparison of the actions of VDR and its natural ligands [1,25-(OH)2D and maybe lithocholic acid] revealed, as for other NRs, that VDR has some ligand-independent actions (e.g., alopecia in VDR but not in 1α-hydroxylase null mice; differences in immune effects between VDR and vitamin D deficiency). Moreover, some VDR-vitamin D endocrine effects may be limited to specific time periods, such as the action on thymic T cell or brain cell programming during late embryonic development, whereas for other tissues VDR becomes only activated at the end of weaning (e.g., intestinal calcium absorption). The generation of tissue- or cell-specific but also developmental stage-specific VDR transgenic mice may help to elucidate such phenomena.

The bone/growth plate/tooth phenotypes of human and mouse VDR or 1α-hydroxylase null mice are perfectly overlapping. This raises the question whether this also applies to the spectrum of noncalcemic effects of the VDR-vitamin D endocrine system. This is certainly the case for the acquired total alopecia observed in case of VDR resistance. There are many similarities between the immune, cardiovascular, and muscle phenotype of VDR-resistant mice and observational data in humans with vitamin D deficiency. This also applies to the antiproliferative effects of 1,25-(OH)2D on most benign and malignant cells and the possible link between chronic vitamin D deficiency and subsequent cancer risk. Only large-scale and long-term prospective studies can reveal the clinical implications of (mild) vitamin D deficiency for major human diseases such as cancer, autoimmune disorders, and muscle or brain function. The VDR null phenotype and human epidemiological data are certainly sufficiently hypothesis generating to warrant such studies.

Acknowledgments

The authors thank all co-authors of their previous publications who contributed to the original data recorded here, with special thanks to Femke Baeke, Guy Eelen, Conny Gysemans, Ritsuko Masuyama, Lut Overbergh, and Sophie Van Cromphaut (Laboratory for Experimental Medicine and Endocrinology, Leuven, Belgium), and special thanks also to Yanchun Li, Cindy Chen, Kristi Skorjica, Yoshiyuki Sakai, Megan Cox, and Luisella Cianferotti (Boston Laboratory, Boston, MA).

Received January 30, 2008. Accepted July 8, 2008.

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The work in the authors' laboratories was supported by the University of Leuven (GOA/04/10, EF/05/007), Flanders Research Foundation [Fonds voor Wetenschappelijk onderzoek Vlaanderen (FWO)], Grants G.0084.02, G.0233.04, G.0508.05, G.0552.06, G.0553.06, and G.0649.08], the Juvenile Diabetes Research Foundation (JDRF; Grant 4-2005-1237), and the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgian State (Grants IUAP P5/17 and P6/34). C.M. is holder of a Clinical Research Fellowship (FWO, Belgium), E.V.E. holds a Postdoctoral Fellowship (JDRF, 3-2006-33), and L.L. is a fellow of the IWT-Flanders. The work in Boston was supported by National Institutes of Health Grant DK46974.
Disclosure Statement: G.C., L.V., E.v.E., A.V., H.F.L., L.L., C.M., and M.D. have nothing to disclose. R.B. received consulting fees (Lilly, Tilibet, BioXell) and lecture fees (Merck & Co, Amgen) and has intellectual property rights on a university patent on vitamin D analogs licensed to Hybrigenix, France.

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