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The 1,25-dihydroxyvitamin D₃-independent actions of the vitamin D receptor in skin

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Abstract

The vitamin D endocrine system plays important but poorly understood roles in the skin and in hair follicle cycling. Rare, human genetic disorders and knockout mouse models highlight essential roles and potentially novel mechanisms of the vitamin D endocrine system in the skin. Vitamin D receptor knockout mice express a hair follicle cycling defect and a hyperproliferative phenotype resulting in disordered skin structure, epidermal thickening, and alopecia. In contrast, ligand knockout mice (i.e., mice with a disrupted *CYP27B1* gene that encodes the 25-hydroxyvitamin-D₃ 1 α -hydroxylase) have normal hair follicle function and a comparatively modest skin phenotype. These disparate models indicate that VDR may function independently of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in regulating hair follicle cycling and skin biology. Recent studies highlight this concept and provide key support for this hypothesis. While VDR knockout mice are highly susceptible to chemically-induced skin tumorigenesis, *CYP27B1* knockouts are resistant. These studies reveal a second global physiological process in the skin that may be regulated by VDR in a 1,25(OH)₂D₃-independent fashion, namely, genoprotection against carcinogenic mutagens. Key cellular and molecular data supporting this mechanism were published recently showing a keratinocyte-selective transactivation activity mediated by VDR that is independent of the 1,25(OH)₂D₃ ligand. Thus, evidence is building to support a potentially novel, 1,25(OH)₂D₃-independent mechanism through which VDR functions in keratinocytes and perhaps within stem cell populations in the follicle to regulate genoprotection and other key developmental processes in the skin.

Keywords

vitamin D receptor; skin carcinogenesis; keratinocyte; cholecalciferol; transcription regulation

1. The Vitamin D Endocrine system

The bioactive metabolite of vitamin D is 1,25- dihydroxyvitamin D₃ (1,25(OH)₂D₃). It is the hormone of the vitamin D endocrine system and the high affinity ligand of the vitamin D

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receptor (VDR). The vitamin D endocrine system is part of a multi-hormonal system that directs mineral homeostasis, protects skeletal integrity, and modulates cell growth and differentiation in bone as well as in a diversity of other cell types (1). Indeed, rickets and osteomalacia are hallmarks of the vitamin D deficiency state and of hereditary hypocalcemic vitamin D resistant rickets (HVDRR), a rare genetic disorder caused by inactivating mutations in the VDR (2). Importantly, select HVDRR patients also exhibit profound, total alopecia highlighting a critical, though uncharacterized, role for VDR in normal hair follicle cycling and function. The $1,25(\text{OH})_2\text{D}_3$ hormone is generated from sequential hydroxylations of vitamin D_3 (3). The first hydroxylation occurs in the liver and is catalyzed by vitamin D-25-hydroxylase (25-OHase) to produce 25-hydroxyvitamin D_3 ($25(\text{OH})\text{D}_3$), the major circulating form of vitamin D in mammals. The second hydroxylation takes place in the kidney where the renal $25(\text{OH})\text{D}_3$ -1 α -hydroxylase (1 α OHase) encoded by the *CYP27B1* gene, results in the production of $1,25(\text{OH})_2\text{D}_3$. The renal 1 α OHase enzyme is stimulated by low serum calcium, phosphorus and by parathyroid hormone. Moreover, the expression of the *CYP27B1* gene is negatively regulated by high levels of $1,25(\text{OH})_2\text{D}_3$ and this completes a classic endocrine feedback system to control serum levels of $1,25(\text{OH})_2\text{D}_3$ (3). Inactivation, or catabolism, of vitamin D metabolites is initiated by the ubiquitous enzyme 25-hydroxyvitamin D_3 -24-hydroxylase (encoded by the *CYP24A1* gene) to generate either $24,25(\text{OH})_2\text{D}_3$ or $1,24,25(\text{OH})_3\text{D}_3$. The 24-hydroxylated metabolites are further degraded and eventually excreted as either calcitroic acid or 23-carboxyl derivatives. Catabolism of the hormone is also carefully regulated. $1,25(\text{OH})_2\text{D}_3$ stimulates *CYP24A1* expression to control overproduction the hormone. Indeed, *CYP24A1* is one of the most highly induced genes in cells treated with $1,25(\text{OH})_2\text{D}_3$ and the promoter of this gene has been studied extensively to understand the cellular and molecular processes involved in $1,25(\text{OH})_2\text{D}_3$ -activated transcriptional processes in vitamin D target cells.

The biological effects of $1,25(\text{OH})_2\text{D}_3$ are mediated through the VDR, a member of the nuclear receptor superfamily (4, 5). Binding of $1,25(\text{OH})_2\text{D}_3$ induces conformational changes in VDR that lead to altered transcription of select target genes, such as *CYP24A1*. $1,25(\text{OH})_2\text{D}_3$ binding to the VDR promotes its heterodimerization with retinoid X receptor (RXR), a common heterodimeric partner for class II nuclear receptors (6). The liganded VDR-RXR heterodimer binds with high affinity to vitamin D response elements in the promoters of target genes. Ligand binding also alters the interaction of numerous nuclear receptor comodulator proteins that interact directly with VDR and other nuclear receptors (7). Corepressors such as NCoR, SMRT, and Hairless interact with unliganded nuclear receptors to mediate active repression of select genes. Ligand binding promotes the dissociation of some corepressors from nuclear receptor- DNA complexes and the subsequent recruitment of coactivator proteins such as SRCs, Mediator, and CBP/P300. Coactivators are essential for ligand-activated nuclear receptor-mediated transcription. The coactivator and corepressor are part of large protein complexes that include the basal transcriptional machinery and histone modifiers to stimulate or repress expression of VDR-regulated genes. Thus, $1,25(\text{OH})_2\text{D}_3$ -triggered associations of various protein-protein and protein-DNA complexes involving VDR are an essential aspect of canonical transcriptional regulation in target cells comprising the mineral-regulating activities of the vitamin D endocrine system. Importantly, skin keratinocytes and the stem cell niche within the hair

follicle may represent intriguing exceptions to this canonical $1,25(\text{OH})_2\text{D}_3$ -triggered function of the VDR.

2. Mouse knockout models point to novel mechanisms of VDR in the skin

The development of mouse models in which the two key components of the vitamin D endocrine system (namely, the receptor and the ligand) have been genetically disrupted has led to a vastly improved insight into the significance of vitamin D in mammalian biology. There are four published mouse strains with targeted deletions in the *VDR* gene (8–11) and two strains targeting *CYP27B1* (12, 13). The VDR knockout (VDRKO) mice present with a phenotype that is remarkably similar to HVDRR. The VDRKO mice are viable and develop normally until they are weaned onto standard laboratory chow diets. Within weeks after weaning, VDRKO mice show signs of hair loss and growth retardation. Serum mineral homeostasis is dramatically altered as hypocalcemia, hypophosphatemia, and hyperparathyroidism develop. These mineral imbalances result in severe skeletal defects, including decreased bone mineral density, thinned bone cortex, and widened under-mineralized growth plates. Importantly, providing the VDRKO mice with a calcium- and phosphorus-rich diet (the so-called rescue diet) normalizes serum calcium and PTH levels and the bones of these mice develop normally without major abnormalities (14, 15). Thus, the bone phenotype in VDR null mice may be secondary to the malabsorption of calcium in the intestine. The alopecia phenotype in the VDRKOs reflects critical roles for VDR in keratinocytes and in hair follicle cycling (16–18). The alopecia is not reversed or prevented by the calcium rescue diet, emphasizing a direct role for VDR in this process (15). As one might have suspected, the rachitic phenotype of the *CYP27B1* knockout ($1\alpha\text{OHaseKO}$) skeleton is remarkably similar to the VDRKO phenotype (12, 13). Similarly, the rachitic phenotype of the $1\alpha\text{OHaseKO}$ is rescued by the high calcium diet (19). However, a striking difference between the VDRKOs and the $1\alpha\text{OHaseKO}$ models is that the $1\alpha\text{OHaseKO}$ animals do not express a hair follicle defect (12, 13). Humans with a genetic defect in the $1\alpha\text{OHase}$ gene also do not manifest alopecia. The basis for this difference may reflect a ligand-independent role for VDR in hair follicle cycling. The lack of hair cycling defects in wild-type mice fed a vitamin D-deficient diet for up to 5 generations and that lack detectable levels of serum $1,25(\text{OH})_2\text{D}_3$ supports this hypothesis (18). Moreover, transgenic expression of a mutant VDR (L233S) that is incapable of binding $1,25(\text{OH})_2\text{D}_3$ and of transactivating VDR, is fully capable of rescuing the alopecia phenotype when it is expressed in keratinocytes of the VDRKO mouse (20). Together, these data indicate that the role of VDR in hair follicle cycling may be independent of the $1,25(\text{OH})_2\text{D}_3$ ligand. These findings focus renewed attention on the actions of VDR in controlling cell function in select target organs. Thus, in the absence of $1,25(\text{OH})_2\text{D}_3$ VDR may repress a subset of target genes in a manner analogous to other nuclear receptors through corepressor interactions (21–23). Such VDR-RXR repressed genes could be involved in negatively regulating hair follicle cycling. Consequently, these studies introduce a novel and potentially significant concept in VDR biology, identifying target genes and establishing molecular mechanisms that govern the function of VDR, independent of the $1,25(\text{OH})_2\text{D}_3$ ligand, in keratinocytes.

3. VDR and skin, potential interplays with Hairless

A candidate protein that may be central to the mechanism of gene repression by apo-VDR is Hairless (Hr), a transcriptional repressor that interacts with nuclear receptors including the VDR (24, 25). Previous studies indicate that hair loss in VDRKO mice is associated with generalized atrichia, development of deep dermal cysts and utriculi (17, 26, 27, Bikle, 2006 #1298). Notably, mutations in the *Hairless* gene also leads to alopecia in humans and mice (28–30). Zinser et al. highlighted the strong correlation between the skin phenotype in aging VDRKO mice and that of the severe form of the hairless gene mutation known as the rhino mouse (27). Both the rhino and VDRKO mice exhibit excessive amounts of thickened, wrinkled waxy skin secondary to the formation of large utriculi. A similar skin phenotype of progressive alopecia, degeneration of hair follicles and dermal cysts is also evident in mice with targeted ablation of RXR α (31), the obligate nuclear receptor heterodimeric partner of the VDR. One possible explanation for the similarities in these three knockout models is that *Hairless* is a target gene for VDR-RXR regulation. However, *Hairless* expression is generally unaffected by VDR or RXR α ablation (18, 31), but more recent studies indicate that regulation may occur at defined windows of the developing follicle (32). Alternatively, these 3 genes may be mechanistically linked by regulating a common pathway in hair follicle function. Along these lines, the hairless gene product was identified as a nuclear receptor corepressor that interacts with histone deacetylases to suppress target gene expression (33). Importantly, the hairless protein was shown to interact with the VDR and dramatically repress both basal and 1,25(OH) $_2$ D $_3$ -stimulated VDR-mediated transactivation in transient gene expression assays (24, 25). Furthermore, *Hairless* is expressed in the same hair follicle cells as the VDR suggesting that the physical and functional interaction of *Hairless* and VDR occurs *in vivo* (24, 32). Indeed, the ligand binding L233S VDR mutant that rescues alopecia in the VDRKO mouse retains strong interactions with the hairless protein (20). Cumulatively, these data point to a mechanism through which the unliganded VDR-RXR heterodimer interact with the hairless and/or other corepressors to repress genes that are involved in negatively regulating the normal hair follicle cycle in mice and humans (Fig. 1). Thus, ablation of VDR, but not the 1,25(OH) $_2$ D $_3$ ligand, would lead to enhanced expression of this putative inhibitor and a blockade in normal hair follicle cycling. Alternative mechanisms are also possible including transactivation of positive regulators of the hair follicle cycle by VDR-RXR heterodimers via mechanisms that do not require the 1,25(OH) $_2$ D $_3$ ligand (Fig. 1).

5. VDR protects against chemically-induced skin tumorigenesis

VDRKO mice are also highly sensitive to DMBA-induced skin tumorigenesis (27, 34). VDRKO mice develop skin tumors with 100 % penetrance and a mean latency of approximately 10 weeks following oral DMBA administration. This rapid onset in the VDRKO animals is contrasted with the wild-type animals that do not develop skin tumors for periods up to 30 weeks post-DMBA treatment. Like the wild-type animals, the 1 α OHaseKO mice are also completely resistant to skin tumor formation induced by oral DMBA (34). These studies suggest a potential connection between enhanced sensitivity to chemically-induced skin tumorigenesis and defective hair follicle cycling in the VDRKO mice. Interestingly, most mouse models that have mutations leading to abnormalities in hair

follicles and hair follicle cycling also display enhanced sensitivity to topical two-stage skin carcinogenesis. While the precise nature of this connection is unresolved, it likely involves the follicular stem cell compartment located in the bulge region of the hair follicle. Regardless, the dramatically enhanced sensitivity of the VDRKO mouse and profound resistance of the $1\alpha\text{OHaseKO}$ mouse to chemically-induced skin tumorigenesis clearly indicates that the genoprotective roles of VDR in the skin are primarily independent of the $1,25(\text{OH})_2\text{D}_3$ ligand. Thus, in addition to maintaining normal hair follicle cycling, $1,25(\text{OH})_2\text{D}_3$ -independent actions of VDR can now be extended to a second critical physiological process in the skin: protecting the keratinocyte genome against damaging agents that lead to carcinogenesis.

6. VDRKO mice are highly susceptible to UV-induced skin tumorigenesis

While chemical carcinogenesis is a useful paradigm to examine *in vivo* skin carcinogenesis in laboratory animals, the major environmental risk factor in human skin carcinogenesis is exposure to ultraviolet light (35). In particular, UVB light (i.e., wavelengths between 290 and 320 nm) induces DNA damage through the formation of cyclobutane pyrimidine dimers (36) and (6–4) photoproducts (37). Failure to adequately repair these genetic lesions or to remove a cell containing these lesions may result in mutated cells with the potential to clonally expand and become tumorigenic. Based on the profound sensitivity of VDRKO mice to chemical carcinogens, we tested whether these mice showed enhanced tumor development in response to chronic UVB exposure (34). Indeed, these studies showed that VDR is essential for protection against UV-induced skin carcinogenesis in mice. VDRKO mice developed tumors in response to thrice weekly exposure to UVB with 100% penetrance and a mean latency of approximately 30 weeks. Wild-type controls were markedly delayed and showed limited penetrance. Mechanisms underlying the genoprotective role of VDR have not been fully defined. However, our initial studies point to critical roles of VDR in UV-induced cell cycle arrest, repair of thymine dimer damage, and apoptotic processes that ultimately remove damaged cells from UV-exposed skin (34). In addition, VDRKO skin shows reduced epidermal thickening in response to repeated UV exposure, indicating that VDRKO skin does not mount an appropriate protective response to UV, leaving basal keratinocytes more exposed to penetrating UV rays. The requirement for the $1,25(\text{OH})_2\text{D}_3$ ligand in protecting against UV-induced tumorigenesis remains an open question.

7. Cellular and molecular evidence for $1,25(\text{OH})_2\text{D}_3$ -independent actions of VDR selectively in keratinocytes

The disparate hair follicle and skin tumor sensitivities of the VDRKO and $1\alpha\text{OHaseKO}$ mice strongly suggest that VDR has $1,25(\text{OH})_2\text{D}_3$ -independent actions in skin. Until recently however, cellular and molecular data supporting this hypothesis have been lacking. We explored the transcriptional activation potential of VDR in a well-defined primary human keratinocyte cell culture system using the highly responsive 24-OHase promoter-luciferase reporter gene construct. Importantly, this is a serum-free cell system using defined media lacking endogenous vitamin D compounds. VDR-dependent activation of this promoter was observed in this defined system that was independent of vitamin D

compounds including $1,25(\text{OH})_2\text{D}_3$ (38). Strikingly, this activity was apparent only in primary human and mouse keratinocyte cultures. Primary dermal fibroblast cultures as well as immortalized keratinocytes and numerous other cell types lacked this activity. Protein-protein interaction analysis suggested that an enhanced association of VDR and RXR occurred in keratinocytes compared to dermal fibroblasts and this may drive the formation of an active VDR-RXR heterodimeric complex in the absence of the $1,25(\text{OH})_2\text{D}_3$ ligand. Indeed, forced expression of RXR resulted in strong $1,25(\text{OH})_2\text{D}_3$ -independent transactivation by the VDRL233 ligand binding mutant providing a potential explanation for the ability of this mutant to rescue the hair follicle phenotype of the VDRKO mouse. These *in vitro* data support the concept that some aspects of VDR-mediated transcription in keratinocytes are independent of the $1,25(\text{OH})_2\text{D}_3$ ligand.

8. Summary

The stark phenotypic differences between the receptor and ligand knockout mice indicate that the actions of VDR and $1,25(\text{OH})_2\text{D}_3$ are uncoupled in the skin. The data obtained in primary human and mouse keratinocyte cultures (38) provide important cell-based, *in vitro* evidence supporting the *in vivo* phenotypic differences characterized in the VDRKO and $1\alpha\text{OHaseKO}$ skin. They further indicate that VDR may control gene expression in keratinocytes independent of the $1,25(\text{OH})_2\text{D}_3$ ligand. Several possibilities exist to explain the apparent uncoupling of VDR and $1,25(\text{OH})_2\text{D}_3$ in both hair follicle cycling and in protection against chemically-induced skin tumorigenesis. First, other lipid ligands may exist that activate the VDR selectively in keratinocytes. These ligands may be other related vitamin D metabolites or completely novel keratinocyte ligands. Lithocholic acid is a known alternative ligand for the VDR (39) and numerous other lipids are being identified that can activate VDR (40). High circulating levels of 25-hydroxyvitamin D_3 exist in VDRKO mice that could potentially activate the VDR, but such a mechanism would need to be preferential in keratinocytes compared to other mineral-regulating sites. Second, VDR may function as an unliganded transcriptional repressor, silencing the expression of inhibitory genes (Fig. 1, gene X) that block hair follicle cycling or suppress DNA repair or apoptotic responses. Ablation of VDR leads to high level expression of these inhibitory factors and subsequent disruptions in hair follicle cycling and DNA repair pathways. Third, it is possible that an apo-VDR can activate a subset of genes that are required for hair follicle cycling and resistance to carcinogens in keratinocytes (Fig. 1, gene Y). Perhaps, keratinocyte-selective signaling pathways activated from the cell surface impinge on VDR and/or RXR via phosphorylation or another post-translation process to drive VDR-activation of select keratinocyte genes (Fig. 1, asterisks). For example, okadaic acid, a protein phosphatase inhibitor, increases both $1,25(\text{OH})_2\text{D}_3$ -dependent and independent activation of VDR by promoting interaction between VDR and the VDR-interacting protein 205 coactivators (41). Additional studies addressing these various possibilities may produce novel strategies of targeting VDR pharmacologically in the skin.

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Fig. 1.

Hypothetical mechanisms in 1,25(OH)₂D₃-independent keratinocyte gene regulation by VDR. The upper schematic depicts the suppression of a tonic inhibitor (gene X) of the hair follicle cycle and of DNA repair processes by an unliganded VDR-RXR-corepressor complex. Ablation of VDR leads to increased expression of the X inhibitor and suppression of hair follicle cycling and processes needed for efficient genoprotection and repair. The lower model evokes a mechanism in which the unliganded VDR-RXR heterodimer activates genes (gene Y) that promote hair follicle cycling and genoprotective pathways. One possible means through which this may occur is post-translational modification of VDR and/or RXR (asterisks) that facilitates coactivator interaction in the absence of the 1,25(OH)₂D₃ ligand.