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Vitamin D Metabolism and Function in the Skin

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Abstract

The keratinocytes of the skin are unique in being not only the primary source of vitamin D for the body, but in possessing the enzymatic machinery to metabolize vitamin D to its active metabolite 1,25(OH)₂D. Furthermore, these cells also express the vitamin D receptor (VDR) that enables them to respond to the 1,25(OH)₂D they produce. Numerous functions of the skin are regulated by 1,25(OH)₂D and/or its receptor. These include inhibition of proliferation, stimulation of differentiation including formation of the permeability barrier, promotion of innate immunity, and promotion of the hair follicle cycle. Regulation of these actions is exerted by a number of different coregulators including the coactivators DRIP and SRC, the cosuppressor hairless (Hr), and β-catenin. This review will examine the regulation of vitamin D production and metabolism in the skin, and explore the various functions regulated by 1,25(OH)₂D and its receptor.

Keywords

CYP27B1; differentiation; skin cancer; innate immunity

1 Introduction

The epidermis is the major source of vitamin D for the body. However, the keratinocytes within the epidermis are further capable of metabolizing the vitamin D to its active metabolite, 1,25(OH)₂D. 1,25(OH)₂D, acting through the vitamin D receptor (VDR), regulates epidermal proliferation in the basal layer (stratum basale) and promotes the sequential differentiation of keratinocytes as they form the upper layers of the epidermis. Loss of VDR or loss of the capacity to produce 1,25(OH)₂D (CYP27B1 mutations/deletion) disrupts differentiation of the epidermis and results in hyperproliferation of the basal layers. The keratinocytes lining the outer layer of the hair follicle (the outer root sheath or ORS) also possess VDR. Loss of VDR function either by inactivating mutations or bioengineered deletions leads to loss of hair follicle cycling and alopecia. In this case, it is less obvious that the VDR requires 1,25(OH)₂D for its activity in that deletion of CYP27B1 does not produce alopecia. VDR also functions as a tumor suppressor, a function seen in other epithelial tissues such as the colon, breast and prostate. As for hair follicle cycling, the role of 1,25(OH)₂D in this tumor suppressor function is not clear. The specificity of VDR action within the skin for the different functions it regulates is attributed at least in part to the different coregulators that modulate its genomic actions. In the proliferating keratinocytes of

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the epidermis and hair follicle, the DRIP complex (vitamin D receptor interacting protein complex) also known as Mediator is the dominant coregulator. In the more differentiated keratinocytes of the epidermis, the SRC (steroid receptor coactivator) complexes (SRC 2 and 3) dominate VDR function. In the hair follicle, the coregulator hairless (Hr) plays an important role. For 1,25(OH)₂D regulated VDR actions, Hr acts as a cosuppressor. But its interaction with VDR in regulating hair follicle cycling, a 1,25(OH)₂D independent action of VDR, is less clear. In this review we will examine the production of vitamin D and its subsequent metabolism to 1,25(OH)₂D, then review the different actions of 1,25(OH)₂D and its receptor in the skin, emphasizing the many roles vitamin D signaling plays in regulating epidermal proliferation and differentiation, hair follicle cycling, and tumorigenesis.

2 Vitamin D Production and Metabolism in the Skin

2.1 Vitamin D₃ production

Vitamin D₃ is produced from 7-dehydrocholesterol (7-DHC) (figure 1). Although irradiation of 7-DHC was known to produce pre-D₃ (which subsequently undergoes a temperature rearrangement of the triene structure to form D₃), lumisterol, and tachysterol, the physiologic regulation of this pathway was not well understood until the studies of Holick and colleagues (Holick, et al. 1979, Holick, et al. 1980, Holick, et al. 1981). They demonstrated that the formation of pre-D₃ under the influence of solar or UVB irradiation (maximal effective wavelength between 280–320) is relatively rapid and reaches a maximum within hours. UV irradiation further converts pre-D₃ to lumisterol and tachysterol. Both the degree of epidermal pigmentation and the intensity of exposure correlate with the time required to achieve this maximal concentration of pre-D₃, but do not alter the maximal level achieved. Although pre-D₃ levels reach a maximum level, the biologically inactive lumisterol accumulates with continued UV exposure. Tachysterol is also formed, but like pre-D₃, does not accumulate with extended UV exposure. The formation of lumisterol is reversible and can be converted back to pre-D₃ as pre-D₃ levels fall. At 0°C, no D₃ is formed; however, at 37°C pre-D₃ is rapidly converted to D₃. Prolonged exposure to sunlight would not produce toxic amounts of D₃ because of the photoconversion of pre-D₃ to lumisterol and tachysterol as well as the photoconversion of D₃ itself to suprasterols I and II and 5,6 transvitamin D₃ (Webb, et al. 1989). Thus, stimulation of epidermal D₃ production is a safe way to provide D₃ to the body.

Melanin in the epidermis, by absorbing UV irradiation, can reduce the effectiveness of sunlight in producing D₃ in the skin. This may be one important reason for the lower 25OHD levels in dark skinned individuals living in temperate latitudes (Bell, et al. 1985). Sunlight exposure increases melanin production, and so provides another mechanism by which excess D₃ production can be prevented. The intensity of UV irradiation is also important for effective D₃ production. The seasonal variation of 25OHD levels can be quite pronounced with higher levels during the summer months and lower levels during the winter in areas furthest from the equator. In Edmonton, Canada (52°N), for example, very little D₃ is produced in exposed skin from mid-October to mid-April; Boston (42 °N) has a somewhat longer period for effective D₃ production; whereas in Los Angeles (34 °N) and San Juan (18 °N) the skin is able to produce D₃ all year long (Webb, et al. 1988). Peak D₃ production occurs around noon, with a larger portion of the day being capable of producing D₃ in the skin during the summer than other times of the year. Clothing (Matsuoka, et al. 1992) and sunscreens (Matsuoka, et al. 1987) effectively prevent D₃ production in the covered areas. This is one likely explanation for the observation that the Bedouins in the Middle East, who totally cover their bodies with clothing, are more prone to develop rickets and osteomalacia than the Israeli Jews with comparable sunlight exposure.

2.2 Metabolism of D₃ to its biologically active products

Keratinocytes are not only capable of producing D₃ but of metabolizing D₃ via the vitamin D-25 hydroxylase (CYP27A) and 25OHD-1 α -hydroxylase (CYP27B1) to its active metabolite 1,25(OH)₂D₃ (Bikle, et al. 1986a, Bikle, et al. 1986b, Lehmann, et al. 2001, Matsumoto, et al. 1991, Fu et al. 1997). Keratinocytes are the only cells in the body containing the entire pathway (Figure 1). Most of the circulating 1,25(OH)₂D₃ is produced by the kidney. However, the expression of CYP27B1 is higher in the keratinocyte than in any other cell including the cells of the proximal renal tubule. Presumably the 1,25(OH)₂D₃ produced in the skin is used for autocrine or paracrine purposes.

Parathyroid hormone (PTH) exerts a modest stimulation of 1,25(OH)₂D production by keratinocytes. However, this involves a different mechanism than that resulting in stimulation of 1,25(OH)₂D₃ production by PTH in the kidney. The keratinocyte does not have a classic PTH receptor coupled to adenylate cyclase. Furthermore, these effects of PTH are not reproduced by cAMP or its membrane-permeable derivatives, suggesting that the actions of PTH may be operating through a mechanism independent of cAMP (Bikle, et al. 1986a). The effects of PTH are maximal after a 4-hr incubation of cells with these agents before adding substrate (25OHD₃); that is, the effects are not immediate. In renal cells PTH exerts a more acute stimulation of 1,25(OH)₂D₃ production (Rasmussen, et al. 1972), and cAMP appears to play a second messenger role (Rost, et al. 1981). The mechanism by which PTH stimulates 1,25(OH)₂D₃ production in the keratinocyte remains unclear.

1,25(OH)₂D₃ negatively regulates its own levels within the keratinocyte. This negative feedback loop is similar to that observed in the kidney, but it differs from that seen in the macrophage, which lacks this feedback loop. In the keratinocyte, this feedback inhibition is not mediated by an effect on 1,25(OH)₂D₃ production but is due solely to induction of 25OHD 24-hydroxylase (CYP24A) that converts 25OHD₃ and 1,25(OH)₂D₃ to 24,25(OH)₂D₃ and 1,24,25(OH)₃D₃, respectively (Xie et al. 2002b), and then on to further catabolic products. The exquisite responsiveness of CYP24A to 1,25(OH)₂D₃ in keratinocytes may explain why so little 1,25(OH)₂D₃ appears to enter the circulation from the skin when renal production of 1,25(OH)₂D₃ is intact.

The expression and activity of CYP27B1 changes with differentiation (Pillai, et al. 1988a). Enzymatic activity is greatest in the undifferentiated cells. Growing the cells in 0.1 mM calcium, which retards differentiation (Pillai, et al. 1988b), permits the cells to maintain higher activity than when they are grown in 1.2 mM calcium (Bikle, et al. 1989), although acute changes in calcium have little effect on 1,25(OH)₂D₃ production (Bikle, et al. 1986a). These observations in vitro are consistent with the finding that CYP27B1 expression is highest in the stratum basale of the epidermis in vivo (Zehnder, et al. 2001).

Both tumor necrosis factor- α (TNF α) and interferon- γ (IFN γ) stimulate 1,25(OH)₂D₃ production by keratinocytes (Bikle, et al. 1989, 1991b, Morhenn and Wood 1988, Pillai, et al. 1989) (figure 1). These cytokines are stimulated by ultraviolet light (Trefzer, et al. 1993) and barrier disruption (Wood, et al. 1994) and contribute to the increased CYP27B1 expression following such environmental stimuli (Loser and Beissert 2009, Schaubert, et al. 2007).

3 Vitamin D regulation of epidermal proliferation and differentiation

3.1 The differentiation process

The epidermis is composed of four layers of keratinocytes at different stages of differentiation (figure 2). The basal layer (stratum basale) rests on the basal lamina separating the dermis and epidermis. These cells proliferate, providing the cells for the upper

differentiating layers. They are large, columnar cells forming intercellular attachments with adjacent cells through desmosomes. An asymmetric distribution of integrins on their lateral and basal surface may also regulate their attachment to the basal lamina and adjacent cells (Guo, et al. 1991, Marchisio, et al. 1991, Peltonen, et al. 1989). They contain an extensive keratin network comprising principally keratins K5 (58 kDa) and K14 (50 kDa) (Moll, et al. 1982). As these cells migrate outward from this basal layer, they acquire the characteristics of a fully differentiated corneocyte, which is eventually sloughed off.

The layer above the basal cells is the spinous layer (stratum spinosum). These cells initiate the production of the keratins K1 and K10, which are the keratins characteristic of the more differentiated layers of the epidermis (Eichner, et al. 1986). Cornified envelope precursors such as involucrin (Warhol, et al. 1985) also appear in the spinous layer as does the enzyme transglutaminase, responsible for the ϵ -(γ -glutamyl) lysine cross-linking of these substrates into the insoluble cornified envelope (Thacher and Rice 1985). The keratinocyte contains both the soluble (tissue, TG-C, or type II) and membrane-bound (particulate, TG-K, or type I) forms of transglutaminase. It is the membrane-bound form that correlates with differentiation and is thought to be responsible for the formation of the cornified envelope (Thacher and Rice 1985). The granular layer (stratum granulosum), lying above the spinous layer, is characterized by electron-dense keratohyalin granules. These are of two types (Steven, et al. 1990). The larger of the two granules contains profilaggrin, the precursor of filaggrin, a protein thought to facilitate the aggregation of keratin filaments (Dale, et al. 1985). The smaller granule contains loricrin, a major component of the cornified envelope (Mehrel, et al. 1990). The granular layer also contains lamellar bodies, lipid-filled structures that fuse with the plasma membrane, divesting their contents into the extracellular space where the lipid contributes to the permeability barrier of skin (Elias, et al. 1988). As the cells pass from the granular layer to the cornified layer (stratum corneum), they undergo destruction of their organelles with further maturation of the cornified envelope into an insoluble, highly resistant structure surrounding the keratin-filaggrin complex and linked to the extracellular lipid milieu (Hohl 1990). The outer layer of the epidermis provides not only a barrier to water loss (permeability barrier) but a barrier to invasion by infectious organisms via its expression of the innate immune system. In particular disruption of the barrier triggers the induction of defensins such as cathelicidin that provide the initial defense in killing such organisms (Hennings and Holbrook 1983). Calcium forms a steep gradient within the epidermis, with highest concentration in the stratum granulosum (Menon, et al. 1985). Disruption of the permeability barrier by removing the stratum corneum or extracting its lipids leads to a loss of this calcium gradient (Mauro, et al. 1998) resulting in increased lamellar body secretion but reduced expression of the genes for loricrin, profilaggrin, and involucrin (Elias, et al. 2002).

3.2 Role of calcium and 1,25(OH)₂D

Both calcium and 1,25(OH)₂D play important and interacting roles in regulating the differentiation process (figure 3). 1,25(OH)₂D increases the expression of involucrin, transglutaminase, loricrin, and filaggrin and increases cornified envelope formation (Bikle, et al. 1991a, Bikle and Pillai 1993, Hawker, et al. 2007, Hosomi, et al. 1983, McLane, et al. 1990, Pillai and Bikle 1991, Smith, et al. 1986) while inhibiting proliferation. These actions are due at least in part to the ability of 1,25(OH)₂D to increase intracellular calcium levels achieved by induction of the calcium receptor (CaR) (Ratnam, et al. 1999) and the phospholipases C (PLC) (Pillai et al. 1995) that are critical for the ability of calcium to stimulate keratinocyte differentiation (Tu, et al. 2001, Xie and Bikle 1999, 2001). The antiproliferative effects are accompanied by a reduction in the mRNA levels for c-myc (Matsumoto, et al. 1990) and cyclin D1 and an increase in the cell cycle inhibitors p21^{cip} and p27^{kip}. In addition, 1,25(OH)₂D and its receptor regulate the processing of the long

chain glycosylceramides that are critical for permeability barrier formation (Oda, et al. 2009) and induce the receptors, toll like receptor 2 (TLR2) and its coreceptor CD14, that initiate the innate immune response in skin (Schauber, et al. 2007). Activation of these receptors leads to the induction of CYP27B1, which in turn induces cathelicidin resulting in the killing of invasive organisms (Schauber, et al. 2006, Schauber, et al. 2007). Mice lacking the VDR or the enzyme (CYP27B1) producing its ligand 1,25(OH)₂D show defective epidermal differentiation manifesting as reduced levels of involucrin and loricrin and loss of keratohyalin granules (Bikle, et al. 2006, Xie, et al. 2002a), decreased lipid content of the lamellar bodies leading to a defective permeability barrier (Oda, et al. 2009), and a defective response of the innate immune system to wounding (Schauber, et al. 2007).

4 Role of VDR coactivators in epidermal proliferation and differentiation

4.1 VDR coactivator overview

The process of epidermal differentiation is sequential. 1,25(OH)₂D and VDR regulate all steps from the control of proliferation in the SB, to the regulation of K1, K10, involucrin, and transglutaminase production in the SS, to the regulation of loricrin and filaggrin production in the SG, to the synthesis of lipids required for the permeability barrier in the SC, and to the development of the innate immune system (Bikle and Pillai 1993, Hawker, et al. 2007, Schauber, et al. 2007). How does this occur? Although CYP27B1 and VDR are found in highest concentration in the SB, they are both distributed throughout the epidermis (Milde, et al. 1991, Stumpf, et al. 1984, Zehnder, et al. 2001), so this does not provide an obvious explanation for the sequential induction of genes involved in the differentiation process. However, VDR requires the binding of coactivators to stimulate transcription. The two major coactivator complexes in the epidermis are DRIP (Mediator) and SRC (McKenna, et al. 1999, Oda, et al. 2003). We (Oda, et al. 2003) observed that in proliferating keratinocytes DRIP was the major coactivator complex binding to VDR, whereas the SRC complex dominated VDR binding in differentiated keratinocytes (Oda, et al. 2003, Oda, et al. 2007). These results are consistent with our finding that in the epidermis DRIP205 (Med1) is expressed in highest concentration in the SB and SS, whereas SRC3 is expressed in highest concentration in the SG (Oda, et al. 2007, Schauber, et al. 2008). The DRIP complex is anchored to VDR via DRIP205 (Rachez, et al. 1999, Rachez, et al. 2000). The SRC complex is anchored to VDR with one of three homologous proteins, SRC1, 2, and 3 (Leo and Chen 2000), but only SRC2 and 3 are found in keratinocytes (Oda, et al. 2003). These coactivator complexes interact with the C terminal (AF2) domain of VDR following ligand binding via LxxLL motifs (NR boxes). They do not bind to VDR at the same time, competing as they do for the same region of the VDR (Rachez, et al. 2000). The SRC coactivators have 3 NR boxes, whereas DRIP205 has 2. Our recent studies (Teichert, et al. 2009) indicate that VDR binds most strongly to the 2nd and 3rd NR box of SRC1 and 2, the 3rd NR box of SRC3, and the 2nd NR box of DRIP205. Different nuclear hormone receptors differ in the affinity for the different NR boxes of the different coactivators suggesting some degree of specificity (Acevedo, et al. 2004). SRC recruits CREB-binding protein (CBP) or P300 and other histone acetyl transferases (HATs) and methyltransferases (MeTs) to the VDR resulting in a multisubunit complex (Christakos, et al. 2003). The HAT and MeT activity of the SRC complex is thought to destabilize the interaction between DNA and the histone core, enabling transcription to occur. The DRIP complex does not have HAT or MeT activity but functions, at least in part, through recruitment of RNA polymerase II to the transcription start site (Rachez, et al. 2000, Rachez and Freedman 2000). Some studies suggest that the order of coactivator binding to its nuclear hormone receptor is sequential with different kinetics, generally with SRC binding preceding and being required for DRIP binding (Acevedo, et al. 2004). Other studies indicate that the specificity of coactivator binding to VDR depends on the gene being regulated (Carvalho, et al. 2008, Issa, et al. 2002), the ligand being evaluated (Bouillon, et al. 2005), and the cellular context (Maeda, et al.

2002, Peleg, et al. 2003). Our data, summarized below, indicate that the sequential action of $1,25(\text{OH})_2\text{D}$ and its receptor on keratinocyte differentiation is due to the differential expression and distribution of these coactivators according to the differentiation status of the cell coupled with the selectivity of genes regulated by VDR for one or the other of the coactivator complexes.

4.2 Coactivator regulation of VDR function in the skin

DRIP205 is expressed in proliferating keratinocytes, and its expression decreases with differentiation, as the expression of SRC3 is increased (Oda, et al. 2003). Knockdown of DRIP205 using siRNA results in increased keratinocyte proliferation, similar to that seen by knocking down VDR itself, but knockdown of SRC3 does not show such an effect (Oda, et al. 2007). Inhibition of DRIP205 binding to VDR in proliferating keratinocytes blocks VDR transcriptional activity with a VDRE reporter construct, but such inhibition is not seen in differentiated keratinocytes (Oda, et al. 2003). However, this inhibition of transcriptional activity turns out to be gene specific. For example, knockdown of DRIP205 using siRNA methodology has a greater impact on keratins 1, 10, and involucrin expression than does knockdown of SRC3, although depletion of both coactivators profoundly reduces loricrin and filaggrin expression (Hawker, et al. 2007). On the other hand SRC3 knockdown, not DRIP205 knockdown, reduces glucosylceramide production and lamellar body formation similar to that of VDR knockdown (Oda, et al. 2009), and prevents $1,25(\text{OH})_2\text{D}$ induced cathelicidin expression (Schauber, et al. 2008). Thus, our hypothesis is that SRC facilitates the ability of VDR to regulate the more differentiated functions of the keratinocyte, whereas DRIP facilitates the ability of VDR to regulate proliferation and early keratinocyte differentiation, although some overlap in coactivator function is observed.

5 Role of VDR in hair follicle cycling

5.1 The hair follicle cycle

The hair follicle cycle is divided into three main stages: anagen, catagen, and telogen (figure 4). Anagen is the stage of hair follicle growth; catagen is the stage of regression; telogen is the resting stage. Only the proximal or dermal portion of the hair follicle cycles; the distal or epidermal portion does not. The duration of these stages in a given species varies from location to location on the body and between genders. Furthermore, there are two types of cycles: developmental and postnatal. The developmental cycle is initiated during embryogenesis. The follicle develops from specific regions of the epidermis called placodes. The follicle is induced to grow by its interaction with a collection of specialized mesenchymal cells in the dermis called the dermal papilla. Wnt signaling (β -catenin) appears to be necessary to maintain the ability of the dermal papilla to stimulate hair follicle growth (Kishimoto, et al. 2000, Shimizu and Morgan 2004). Following the developmental cycle, which leads to the initial coat of hair, the follicle undergoes repetitive cycling until senescence. The length of the hair is dependent on the duration of anagen. During this stage the follicle grows through the dermis into the subcutaneous tissue. As the follicle develops different cell layers appear. The outer root sheath (ORS) is a direct extension of the stratum basale, and separates the hair follicle from the surrounding connective tissue sheath (CTS). From outside in are found the companion layer, the three layers of the inner root sheath (IRS)—Henle's layer, Huxley's layer, cuticle of the IRS—and the hair shaft itself including the cuticle of the shaft, shaft cortex, and shaft medulla. Stem cells in the bulge are capable of generating all cells in the hair follicle and epidermis (Morris, et al. 2004). The keratins produced by the cells of the IRS and hair shaft differ from those expressed by epidermal keratinocytes (Langbein, et al. 2003). Of particular interest is these hair keratins have β -catenin/lef1 binding sites in their promoters that regulate their expression (Zhou, et al. 1995). Following anagen, the follicle enters catagen during which massive apoptosis occurs

primarily in the cells of the proximal follicle (the dermal portion), and the hair shaft produced during anagen is generally shed. At the end of catagen the follicle enters telogen, the resting phase. Duration of telogen is highly variable. A new cycle then begins with anagen. The juxtaposition of the dermal papilla to the bulge is critical for this process to begin, and it is associated with increased proliferation of stem cells in the bulge with migration of cells from the bulge into the hair bulb to restart the growth of the hair follicle. The regulatory elements that control the transition from one stage to the next are not well understood.

5.2 Role of VDR in hair follicle cycling

Alopecia is a well-known part of the phenotype of many patients with mutations in their VDR (Hochberg, et al. 1985, Marx, et al. 1986), a syndrome known as hereditary vitamin D resistant rickets (HVDRR). Vitamin D deficiency per se or CYP27B1 mutations are not associated with alopecia, indicating that the regulation of hair follicle cycling requires VDR but not its principal ligand $1,25(\text{OH})_2\text{D}$. VDR null mice develop their first coat of hair normally, but reinitiation of anagen following the first cycle or after depilation is impaired (Sakai and Demay 2000). Reconstitution of the VDR to the VDR null mouse skin using a keratinocyte specific promoter reverses the defect in hair growth without reversing the metabolic defects of skeletal growth retardation, hypocalcemia, and rickets otherwise associated with the VDR null condition (Chen, et al. 2001, Kong, et al. 2002). On the other hand, correction of the metabolic abnormalities with a high calcium diet prevents the rickets and hyperparathyroidism but does not prevent the alopecia (Li, et al. 1998). Furthermore, it is the lack of VDR in the keratinocyte as opposed to the dermal papilla that is critical. Dermal papilla cells obtained from either VDR null or wildtype mice can initially induce hair growth in a hair reconstitution assay when mixed with epidermal keratinocytes obtained from wildtype or VDR null mice, but if the hair grown with keratinocytes from VDR null mice is then depilated, anagen will not be reinitiated regardless of the source of dermal papilla cells (Sakai and Demay 2000).

5.3 Role of hairless (Hr) in the regulation of hair follicle cycling and its interactions with VDR

Hr mutations in both mice (Panteleyev, et al. 1999) and humans (Ahmad, et al. 1998, Miller, et al. 2001) result in phenocopies of the VDR null mouse and some human VDR mutations, respectively, with regard to the morphologic changes observed in hair follicle cycling. The dissociation of the dermal papilla from the hair bulb by the end of catagen is thought to account for the failure to initiate the subsequent anagen in both Hr mutant and VDR null mice (Bikle, et al. 2006, Panteleyev, et al. 1999). The distal (epidermal) portion of the hair follicle including the sebaceous gland as well as the interfollicular epidermis is less impacted (Bikle, et al. 2006, Panteleyev, et al. 1999, Xie, et al. 2002). We have found VDR and Hr in the nuclei of keratinocytes in the stratum basale and ORS (Bikle, et al. 2006). However, we found little or no VDR in the IRS and hair bulb or cells of the dermal papilla and CTS, whereas we did find Hr in those locations (Bikle, et al. 2006). As will be discussed subsequently, deletion of the transcriptional domain of β -catenin results in a similar phenotype (DasGupta, et al. 2002, Huelsken, et al. 2001).

Hr has characteristics of a coregulator in that it resides in the nucleus; its structure contains a nuclear localization signal, a putative zinc finger, and three LXXLL motifs (Djabali, et al. 2001) like that found in coactivators that interact with nuclear hormone receptors such as VDR as well as $\Phi\text{XX}\Phi\Phi$ motifs (Φ =hydrophobic amino acid) similar to regions in corepressors like SMRT and NCoR responsible for the binding of these corepressors to nuclear hormone receptors. In the brain, Hr has been suggested as a corepressor of the thyroid receptor (THRb) in that Hr can bind to THRb and inhibit its transcriptional activity

(Thompson and Bottcher 1997). However, Hr does not appear to regulate thyroid hormone action in the keratinocyte (Engelhard and Christiano 2004). Rather, VDR appears to be the target (Xie, et al. 2006). Hsieh et al. (Hsieh, et al. 2003) demonstrated that Hr could bind to VDR in COS cells. They noted that Hr bound to VDR in the same region predicted for corepressor binding, and different from the C-terminal region to which coactivators bind. The region of Hr responsible for VDR binding contains one LXXLL motif, but also a Φ XX Φ Φ motif. However, only mutations in the Φ XX Φ Φ motif altered binding to VDR (Hsieh, et al. 2003). Furthermore, when we tested both motifs separately for their binding to VDR, the Φ XX Φ Φ motif had the higher affinity regardless of the presence or absence of 1,25(OH)₂D (Teichert, et al. 2009). We have shown that the endogenous VDR binds to endogenous Hr in keratinocytes (Xie, et al. 2006). Binding of Hr to VDR inhibited 1,25(OH)₂D stimulation of a CYP24A1 (24-hydroxylase) promoter construct containing the vitamin D response element (VDRE) of this vitamin D target gene. Overexpression of Hr blocks the ability of 1,25(OH)₂D to induce differentiation markers in keratinocytes, whereas inhibition of Hr expression enhances the stimulation by 1,25(OH)₂D of these markers (Xie, et al. 2006). The Hr null animal demonstrates upregulation of differentiation markers in the epidermis (Zarach, et al. 2004) consistent with a corepressor role for Hr in vitamin D regulated epidermal differentiation. Antibodies to Hr enhance the binding of VDR to VDREs in vitamin D target genes in gel retardation assays (Xie, et al. 2006) suggesting that Hr binding to VDR blocks its binding to VDREs. 1,25(OH)₂D displaces Hr from VDREs as it recruits the coactivators DRIP205 and SRC3 to these same VDREs (Xie, et al. 2006). Thus at least for 1,25(OH)₂D stimulated actions of VDR, Hr is a cosuppressor.

6 Role of β -catenin as VDR coregulator

6.1 Wnt/ β -catenin signaling pathway

Wnt signaling via activation of β -catenin has a complex role in VDR actions (figure 5). Wnt ligands bind to their seven-transmembrane Frizzled receptors and an LRP5 or LRP6 co-receptor leading to phosphorylation of disheveled (Dvl) resulting in disruption of the axin/APC complex and inhibition of the kinase activity of glycogen synthase kinase-3 β (GSK-3 β), which otherwise phosphorylates the serine(s) within exon 3 of β -catenin facilitating its degradation by the E3 ubiquitin ligase. Thus wnt signaling increases the availability of β -catenin in the cytoplasm, which can then bind to transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to promote expression of genes such as cyclin D1 and c-myc (He, et al. 1998) important for proliferation. β -catenin also forms part of the adherens junction complex with E-cadherin where it may play an important role in keratinocyte differentiation (Xie and Bikle 2007). Tyrosine phosphorylation of β -catenin, as occurs after calcium administration to keratinocytes, promotes the binding of β -catenin and other catenins to the adherens junction complex (Bienz 2005, Xie and Bikle 2007) making it less available for transcriptional activity. Cells differ markedly in the components of the β -catenin signaling pathway utilized. This is well illustrated in the keratinocytes of the hair follicle and interfollicular epidermis (DasGupta, et al. 2002, Huelsken, et al. 2001). LEF1 is the dominant transcription partner for β -catenin in the dermal portion of the hair follicle, which has little E-cadherin. The epidermal keratinocyte, on the other hand, has little LEF1 but a lot of E-cadherin especially in the differentiated layers (Stenn and Paus 2001). Over expression and/or activating mutations in the β -catenin pathway lead to skin tumors, in this case pilomatricomas or trichofolliculomas (hair follicle tumors) (Chan, et al. 1999, Gat, et al. 1998, Xia, et al. 2006) indicative of the hyperproliferative response to β -catenin in these cells. Activating mutations of specific serines within exon 3, or deletion of exon 3, block phosphorylation of β -catenin by GSK-3 β , phosphorylation which otherwise leads to its proteosomal degradation. As a result β -catenin levels increase in the nucleus where its transcriptional activity is exerted in association with members of the LEF/TCF family of transcription factors.

6.2 β -catenin interactions with VDR

In colon cancer cells VDR has been shown to bind to β -catenin, and reduce its transcriptional activity in a ligand dependent fashion (Palmer, et al. 2001). Furthermore, in these cells $1,25(\text{OH})_2\text{D}$ has been shown to increase E-cadherin expression, such that β -catenin is redistributed from the nucleus to the plasma membrane where it forms a complex with E-cadherin and other catenins at adherens junctions (Shah, et al. 2003). However, the suppression of β -catenin signaling by $1,25(\text{OH})_2\text{D}$ does not necessarily require E-cadherin (Shah, et al. 2006). Rather β -catenin binds to VDR in its AF-2 domain, binding that enhances the ability of $1,25(\text{OH})_2\text{D}$ to activate the transcriptional activity of the VDR (Shah, et al. 2006) but blocks the transcriptional activity of β -catenin. Mutations in the AF-2 domain of VDR that block coactivator binding do not necessarily block β -catenin binding (Shah, et al. 2006). Whether β -catenin binding alters DRIP205 or SRC3 binding to this same region has not been determined. Palmer et al. (Palmer, et al. 2008) evaluated the interaction between VDR and β -catenin in transcriptional regulation in keratinocytes, and identified putative response elements for VDR and β -catenin/LEF in a number of genes. These interactions were either positive or negative, depending on the gene being evaluated. The hypothesis put forward is that genes in which the interaction is positive (ie. stimulated transcription) benefit from β -catenin acting as a coactivator for VDR on VDREs, whereas in situations where the interaction is negative (ie. suppression of transcription) VDR prevents β -catenin from binding to TCF/LEF required for transcription of those genes. We (Y Oda and D Bikle, unpublished) have found in keratinocytes that knockdown of VDR reduces E-cadherin expression and formation of the β -catenin/E-cadherin membrane complex resulting in increased β -catenin transcriptional activity, whereas $1,25(\text{OH})_2\text{D}$ administration has the opposite effect. This was associated with increased (with VDR and DRIP205 knockdown) or decreased (with $1,25(\text{OH})_2\text{D}$ administration) keratinocyte proliferation and cyclin D1 expression, respectively. Other studies suggest that VDR potentiates, not inhibits, β -catenin transcriptional activity. Cianferotti et al. (Cianferotti, et al. 2007) found a reduction in proliferation of keratinocytes in the dermal portion of the hair follicle (below the bulge) in VDR null mice, and no stimulation of proliferation when β -catenin was overexpressed in these cells in contrast to the stimulation of proliferation in control animals. Thus VDR/ β -catenin interactions can be positive or negative, depending on the gene/cell/function being evaluated.

7 Conclusions

The skin is unique in being not only the source of vitamin D for the body but in being capable of producing and responding to the active metabolite of vitamin D, $1,25(\text{OH})_2\text{D}$. $1,25(\text{OH})_2\text{D}$ and its receptor have many roles in the skin. Some of these roles-- induction of genes required for differentiation, suppression of genes involved with proliferation-- appear to require both $1,25(\text{OH})_2\text{D}$ and VDR and synergize with the actions of calcium. Other roles such as the regulation of hair follicle cycling require VDR but do not require $1,25(\text{OH})_2\text{D}$. Different coactivator complexes including DRIP and SRC modulate the actions of VDR, and the choice of coactivator complex in many cases is gene specific. Regulation of proliferation is dependent on DRIP, whereas more differentiated functions including innate immunity and permeability barrier formation are SRC dependent. Hr is a coregulator with profound actions in hair follicle cycling. Although Hr blocks $1,25(\text{OH})_2\text{D}$ regulated VDR functions, its role in VDR regulated hair follicle cycling is less clear. β -catenin interactions with VDR function can enhance VDR induction of some genes, whereas VDR can suppress β -catenin induction of other genes. Many genes in the skin contain both putative VDREs and LEF/TCF sites. At this point we are a long ways away from understanding how $1,25(\text{OH})_2\text{D}$ and VDR regulate all the various functions in the skin that they impact. But what we have learned indicates that

the skin is a fertile area for understanding the mechanisms by which vitamin D signaling regulates so many different physiologic processes.

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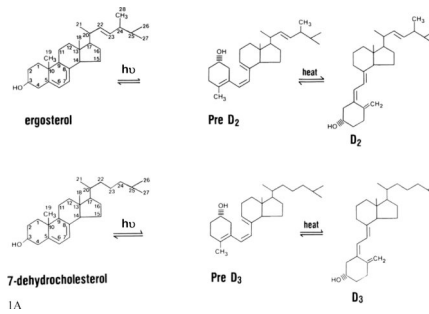
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Vitamin D Metabolism in the Keratinocyte

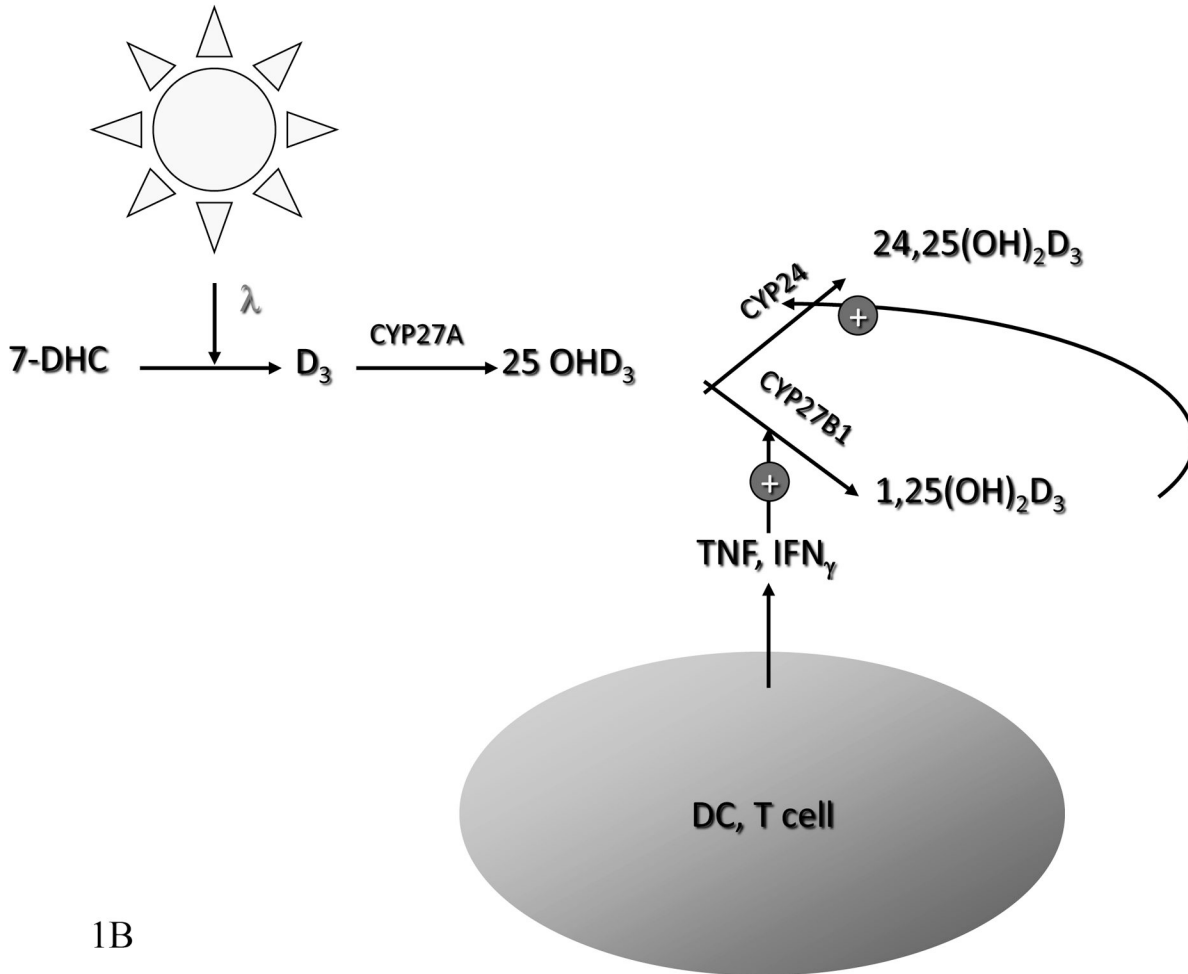


Figure 1. The production of vitamin D and its subsequent metabolism

A. Vitamin D production. Sunlight (the ultraviolet B component) breaks the B ring of the precursor sterol to form pre-D₂ or pre-D₃ from ergosterol or 7-dehydrocholesterol, respectively. In a temperature dependent step the A ring is rotated around the C5 to C6 double bond so that the 3β-hydroxyl group is positioned below the plane of the A ring to form D₂ and D₃.

B. Metabolism of Vitamin D in the keratinocyte. In the rest of the body the liver converts vitamin D to 25OHD. The kidney converts 25OHD to 25OHD and 1,25(OH)₂D. Regulation of CYP27B1 in the kidney is exerted by calcium, phosphorus, parathyroid

hormone, FGF23, and 1,25(OH)₂D itself. However, in the keratinocyte, vitamin D can be directly metabolized to 25OHD and further metabolized to 25OHD and 1,25(OH)₂D. Cytokines such as TNF and IFN- γ are the principal regulators of CYP27B1 in the keratinocyte.

Coactivators VDR Function Target genes

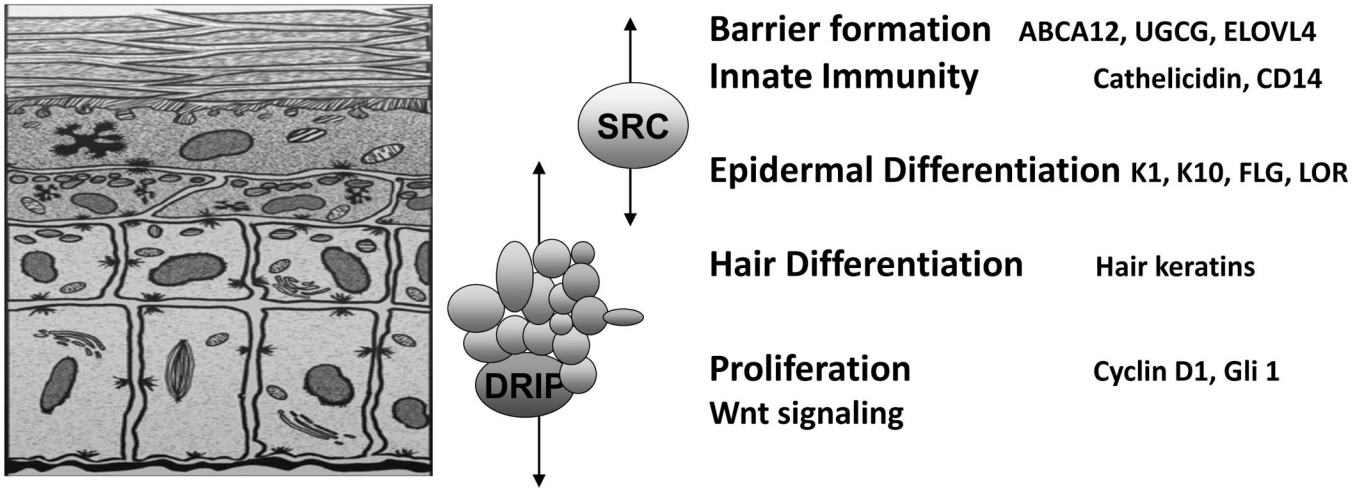


Figure 2. The different layers of the epidermis, and the functions within those layers regulated by VDR and its coactivators

The basal layer of the epidermis (stratum basale) contains the stem cells that through proliferation provide the cells for the upper layers. As the keratinocytes leave the basal layer differentiation takes place with K1, K10, involucrin, and transglutaminase being expressed in the stratum spinosum, filaggrin and loricrin being expressed in the stratum granulosum. Lamellar bodies forming in the stratum granulosum inject their lipid content into the intercellular spaces between the stratum granulosum and stratum corneum to provide the water proofing for the permeability barrier. DRIP205 is most highly expressed in the stratum basale and spinosum where it participates with VDR in regulating proliferation in partnership with the wnt/ β -catenin signaling pathway. Cyclin D1 and Gli 1 (a transcriptional factor for the hedgehog pathway) are regulated by VDR, DRIP205, and β -catenin. SRC3 on the other hand is found in highest concentration in the stratum granulosum where it participates with VDR in the regulation of terminal differentiation. SRC3 is critical for lipid processing and lamellar body formation required for formation of the permeability barrier as well as $1,25(\text{OH})_2\text{D}$ induction of cathelicidin and CD14 required for innate immunity.

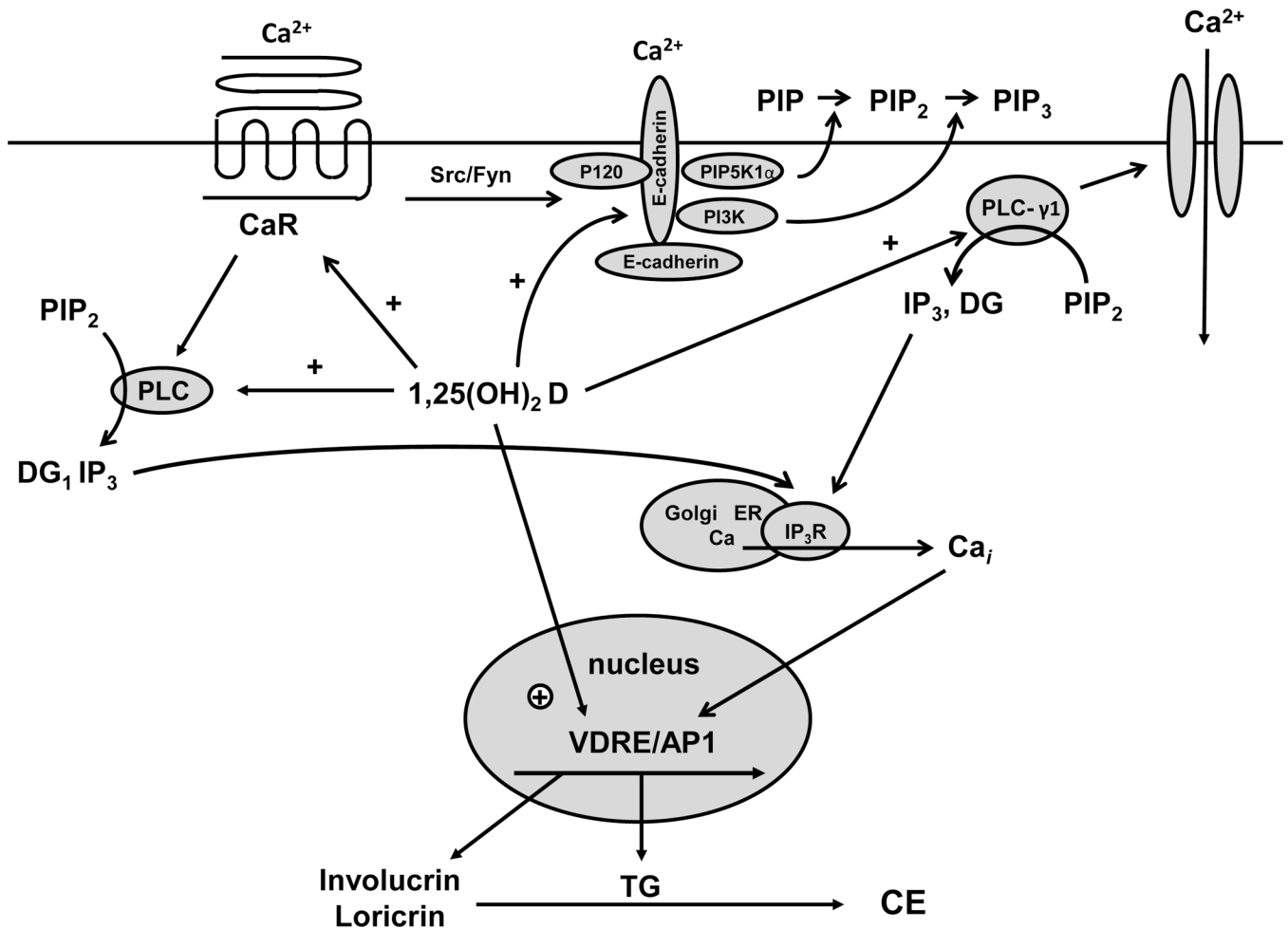


Figure 3. Calcium and 1,25(OH)₂D interactions in the regulation of keratinocyte differentiation
 Critical steps in calcium induced keratinocyte differentiation involve activation of the calcium sensing receptor (CaR) and formation of the E-cadherin complex at the membrane. This complex includes a number of catenins and phospholipid modifying enzymes (PI3K, PIP5K1 α) that are critical for subsequent differentiation events. In particular the activation of phospholipase C- γ 1 (PLC- γ 1), the enzyme largely responsible for maintaining increased levels of intracellular calcium (Ca_i) through its effects on both plasma membrane calcium channels and in the production of IP₃ from PIP₂ for stimulation of calcium release from intracellular stores, requires the formation of the E-cadherin complex. Hydrolysis of PIP₂ by PLC- γ 1 also leads to diacylglycerol production (DG) and activation of protein kinases C, that also play an important role in keratinocyte differentiation. Within hours of the calcium switch keratinocytes change from making the basal keratins K5 and K14 to making keratins K1 and K10 followed, subsequently, by increased levels of profilaggrin, involucrin and loricrin. Loricrin, involucrin and other proteins are cross linked into the insoluble cornified envelope by the calcium sensitive, membrane bound form of transglutaminase, which like involucrin and loricrin increases within 24 hours after the calcium switch. The induction of these proteins represents a genomic action (likely indirect) of calcium as indicated by a calcium induced increase in mRNA levels and transcription rates. The CaR by regulating phospholipase C (PLC) activity controls the production of inositol tris phosphate (IP₃) and diacyl glycerol (DG). PLC- β is activated directly by CaR via a G protein coupled mechanism, whereas PLC- γ 1 is activated by phosphatidyl inositol tris phosphate (PIP₃),

levels that are maintained in the membrane by phosphatidylinositol 3 kinase (PI3K) bound to the E-cadherin complex. CaR regulates E-cadherin complex formation through src/fyn tyrosine kinases that phosphorylate the catenins and PI3K essential for their binding to E-cadherin. Extracellular calcium (Ca_o) activates these processes through the CaR and by stabilizing E-cadherin membrane localization. $1,25(OH)_2D$ modulates calcium regulated differentiation at several steps. First, $1,25(OH)_2D$ increases CaR expression, thus making the cell more responsive to calcium. Second, $1,25(OH)_2D$ induces all the PLCs, as does calcium, again increasing the responsiveness of the cell to calcium. Third, the VDR is required for calcium induced formation of the E-cadherin complex in the membrane, in part through induction of E-cadherin as well as CaR. Finally, $1,25(OH)_2D$ induces the transcription of genes such as involucrin and transglutaminase and possibly the other differentiation markers in addition to PLC and CaR.

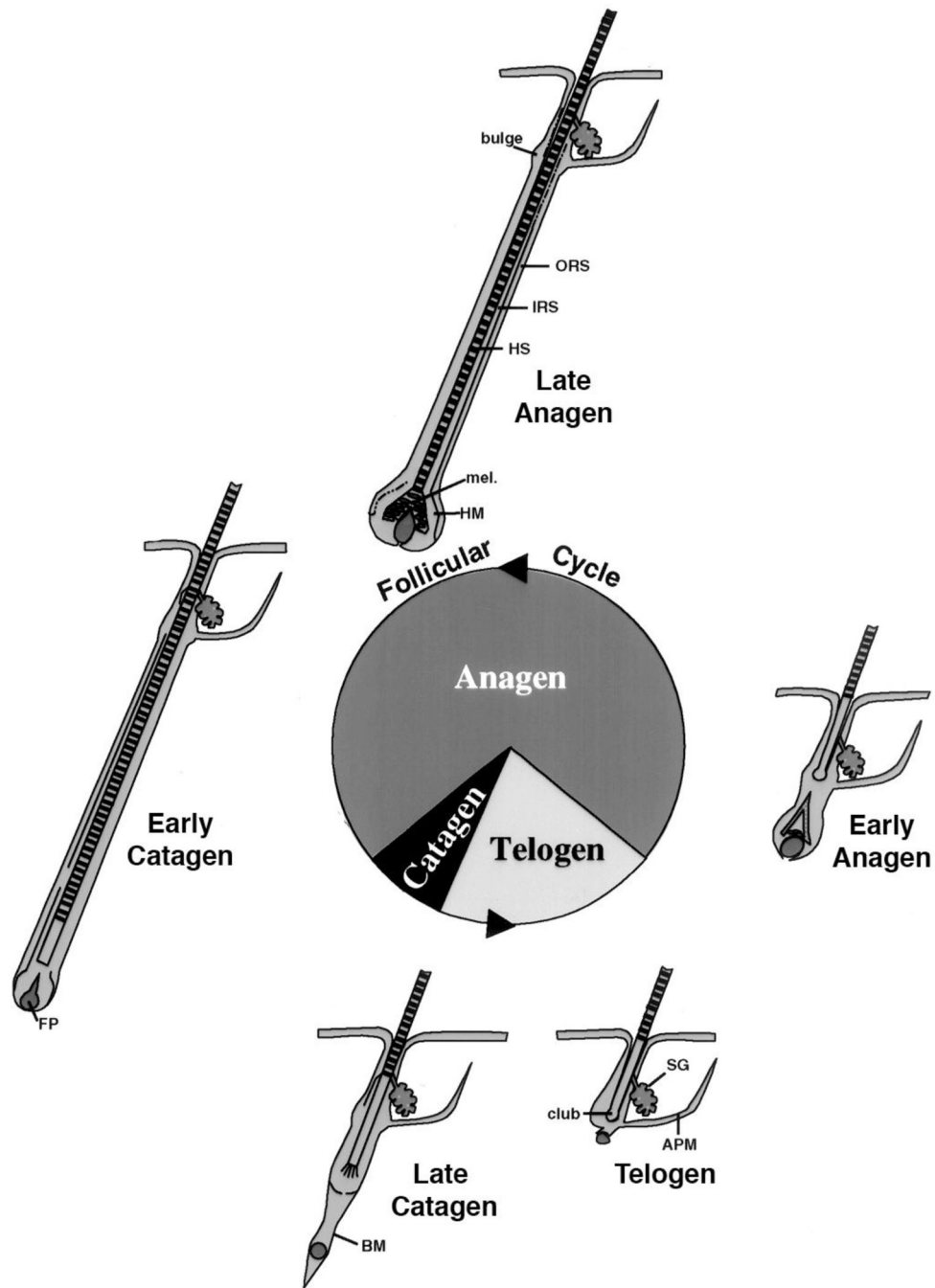


Figure 4. Hair follicle cycling

The initial developmental phase of hair follicle development terminates with catagen and the first telogen, after which repetitive cycles of anagen (the growth phase), catagen (the regression phase), and telogen (the resting phase) occur throughout the life span of the animal. In general the hair follicle spends most of its time in anagen. But cycle duration varies according to location, gender, age, species. The bulge is the source of stem cells for the regenerating hair follicle, responding to signals from the dermal or follicular papilla (FP). Although VDR is not required for the developmental phase of the hair follicle, it is essential for subsequent hair follicle cycling. ORS: outer root sheath, IRS: inner root sheath, HS: hair shaft, mel: melanin for the hair shaft, HM: hair matrix, BM: basement membrane,

SG: sebaceous gland, APM: arrector pili muscle. Figure adapted from figure 1 in KS Stenn and R Paus, *Physiol Rev*:81:449–494, 2001.

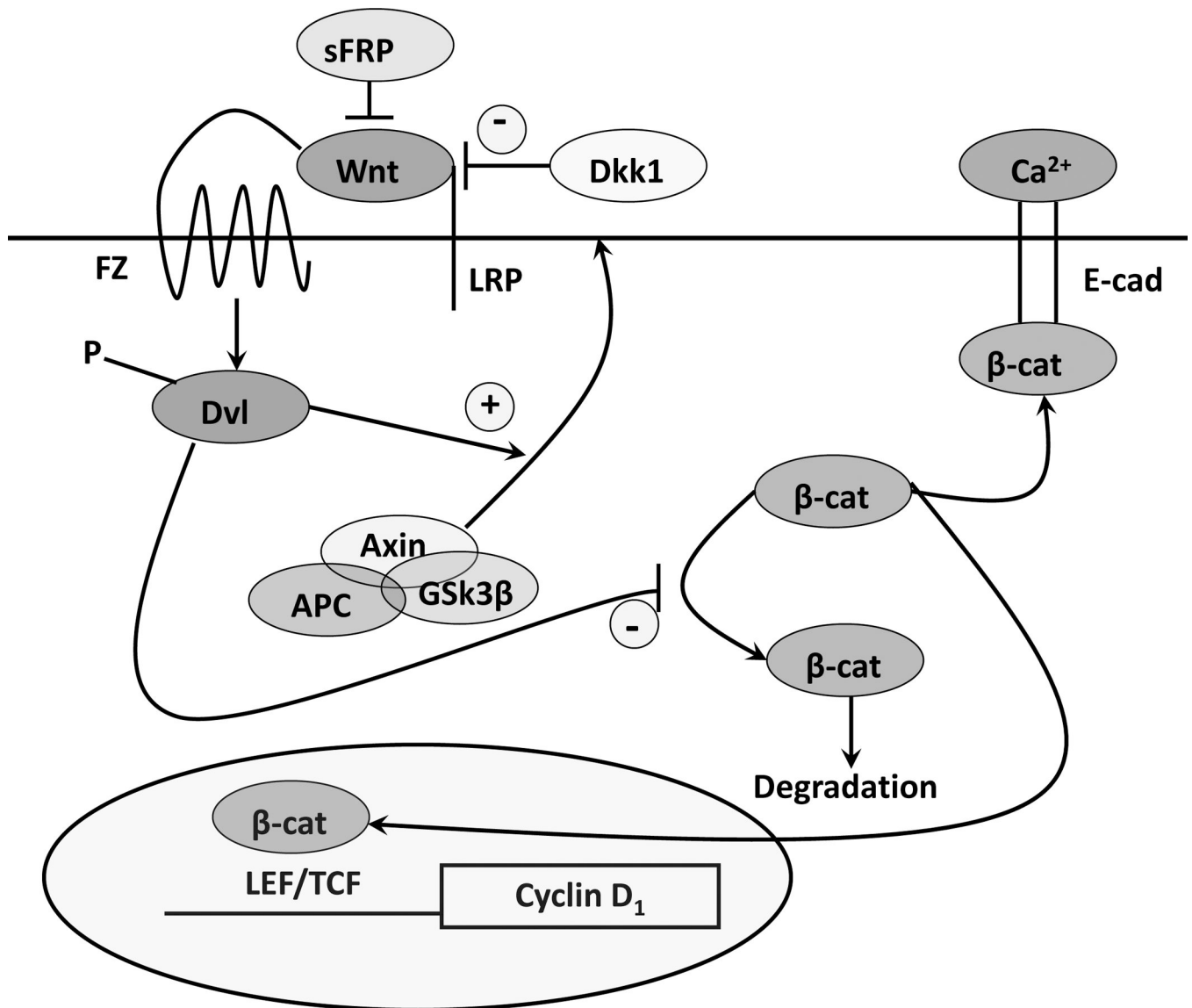


Figure 5. The canonical wnt signaling pathway

Wnts bind to their frizzled receptors (FZ) and coreceptors LRP in the membrane. This binding can be blocked by dickkopf (Dkk) or soluble frizzled related proteins (sFRP). Activation of FZ by wnt results in phosphorylation of disheveled (Dvl), which induces the disruption of the axin/APC/GSK-3 β complex and recruitment of axin to the membrane. This complex when active phosphorylates β -catenin, leading to its proteosomal degradation. However, following wnt stimulation β -catenin is no longer degraded and can enter the nucleus where in combination with members of the LEF/TCF family can induce expression of its target genes such as cyclin D1. β -catenin also binds to the E-cadherin complex in the plasma membrane, binding which promotes differentiation. 1,25(OH)₂D/VDR interact with wnt signaling at multiple points. 1,25(OH)₂D/VDR binds β -catenin reducing its transcriptional activity. 1,25(OH)₂D/VDR promotes the formation of the E-cadherin/catenin complex in the membrane also serving to reduce translocation of β -catenin into the nucleus.