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Review

The Role of the Vitamin D Receptor in the Epidermal Stem Cell Response to Wounding

Daniel D. Bikle

San Francisco VA Medical Center, University of California San Francisco, San Francisco, CA 94143, USA; daniel.bikle@ucsf.edu

Abstract: Chronic skin wounds are estimated to affect 6.5 million patients in the US, at a cost of over USD 25 billion. Efforts to prevent and/or treat such wounds will result in reduced morbidity and economic losses. This project is focused on the role of vitamin D signaling in the epidermis in the control of stem cell (SC) activation and function during the initial response to the wounding of the skin, a response that, if defective, contributes to poor wound healing or cancer. In this review, I first describe the anatomy of the skin, focusing first on the epidermis, describing the different cell layers which in a spatial way also represent the differentiation process of the interfollicular epidermis (IFE) as it undergoes continuous regeneration. I then describe the other components of the skin, particularly the hair follicle (HF), which undergoes a cyclic pattern of regeneration. Adult SCs residing in these regenerative tissues play essential roles in the maintenance of these tissues. However, when the skin is wounded, the progeny of SCs from all regions of the HF and IFE contribute to the healing process by changing their initial cell fate to take on an epithelial genotype/phenotype to heal the wound. Although earlier lineage tracing studies helped to define the contributions SCs from the different niches made to wound healing, scRNAseq studies have demonstrated a considerably more nuanced picture. The role of vitamin D signaling will be introduced by reviewing the unique role played by the epidermal keratinocyte first in producing vitamin D and then in metabolizing it into its active form 1,25(OH)2D. 1,25(OH)2D is the principal ligand for the vitamin D receptor (VDR), a transcription factor that helps to mediate the genomic changes in the stem cells in their response to wounding. In these actions, the VDR is regulated by coregulators, of which the steroid receptor coactivator complexes SRC 2 and 3 and the mediator complex (MED) play essential roles. The VDR generally acts in association with other transcription factors such as p63 and β-catenin that can colocalize with the VDR in the genes it regulates. Although much remains to be understood, the role of the VDR in the stem cell response to wounding is clearly essential and quite different from its classic roles in regulating calcium metabolism, although calcium is essential for the actions of vitamin D signaling in the skin.

Keywords: keratinocytes; stem cells; vitamin D receptor; wound repair; epidermis; hair follicle

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1. Introduction

The epidermis and its appendages, the hair follicle and the pilosebaceous unit, are regenerative tissues. Normally, the stem cells (SCs) that sustain these tissues are distinct in both their functions and transcriptome, but with wounding, that changes. These stem cells normally maintain a hair follicle and sebaceous gland, but after wounding, all pitch in to heal the wound. Following the repair, they revert back to their prewound state, albeit with some memory, such that subsequent wounding elicits a faster response. The vitamin D receptor (VDR) plays a major role in enabling this response to wounding. In this review, after first describing the anatomy of the skin, including the location of the different SC niches and the normal differentiation processes for the different components of the epidermis as regulated by vitamin D signaling, I will focus on how the various stem cell niches respond to wounding, with as focus on the role the VDR plays in this response.

2. Anatomy of the Skin

The interfollicular epidermis (IFE) is made up of four layers of keratinocytes differing in their level of differentiation. The basal layer (stratum basale), resting on the basal lamina separating the dermis and epidermis, contains the IFE stem cells (IFE-SCs) [1]. The function of these SCs is to provide the cells for the upper differentiating layers, as well as maintaining the basal IFE-SC population, which they do by proliferating asymmetrically, with one progeny remaining in the basal layer and the other continuing to proliferate as transient amplifying cells (TAC) that continue to differentiate as they migrate up into the upper layers. Their attachment to the basal lamina and adjacent cells is mediated by an asymmetric distribution of integrins on their lateral and basal surfaces [2]. The keratins K5 (58 kDa) and K14 (50 kDa) are the dominant components of the extensive keratin network within these cells [3], and they attach to adjacent cells via desmosomes and adherens junctions. The layer above the basal cells is the spinous layer (stratum spinosum), which changes the production of keratins from K5 and K14 to K1 and K10 [4]. These cells also begin the production of cornified envelope precursors such as involucrin [5]. In addition, the keratinocytes of the spinous layer express the enzyme transglutaminase K, responsible for the ε-(γ-glutamyl)lysine cross-linking of these substrates into the insoluble cornified envelope [6]. The granular layer (stratum granulosum), lying above the spinous layer, is so named because of the appearance of electron-dense keratohyalin granules. These belong to two types [7]. The larger granules contain profilaggrin, which is further processed into filaggrin, thought to be involved in the aggregation of keratin filaments [8], but also providing a source of amino acids following proteolysis to provide a moisturizing factor [9]. Many cases of atopic dermatitis are associated with mutations in filaggrin [9]. The smaller granules contain loricrin, a major component of the cornified envelope [10]. Also within the granular layer are lipid-filled structures called lamellar bodies that fuse with the plasma membrane, secreting their contents into the extracellular space between the stratum corneum and stratum granulosum, where the lipid contributes to the permeability barrier [11]. In addition to the lipids, the lamellar bodies contain antimicrobial peptides such as cathelicidin that provide protection against invasive organisms [12], providing additional defense against the environment. The final stage occurs as the cells of the stratum granulosum move to the cornified layer (stratum corneum), during which they undergo the destruction of their organelles while achieving final maturation of the cornified envelope into an insoluble, highly resistant structure [13]. During the course of IFE formation, a calcium gradient forms with the highest concentration in the stratum granulosum that helps to maintain these levels of differentiation within the epidermis [14]. The vitamin D receptor (VDR) and the enzyme CYP27B1, which produces the principal VDR ligand 1,25(OH)2D, have their highest concentration in the basal layer. As will be discussed, VDR and CYP27B1 are keyparts of the mechanism regulating epidermal differentiation as well as the stem cell response to wounding. Figure 1 presents a cartoon depicting the different layers of the epidermis and some of their characteristics.

The epidermis also includes the pilosebaceous unit comprising the hair follicle (HF) and the sebaceous gland connected to the interfollicular epidermis (IFE) via the infundibulum, plus the eccrine, apoeccrine and apocrine sweat glands [15,16]. The formation of the HF during development involves the interaction between epidermal cells and a mesodermal condensate that becomes the dermal papilla, a critical structure involved in HF cycling. In mice, the mature HF is formed within two weeks. The bulge region, containing SC critical for HF recycling, forms at this time, just below the sebaceous gland. The HF can conceptually be viewed as the permanent upper portion consisting of the infundibulum and isthmus including the bulge, and the lower portion which cycles. Anagen, catagen, and telogen mark the distinct phases of the hair cycle. Anagen is the growth phase characterized by the proliferation of keratinocytes in the bulge and hair germ, with the formation of the fully elongated HF and hair shaft. This is followed by catagen, characterized by apoptosis of the lower portion of the HF below the bulge. This brings the bulge adjacent to the mesodermal dermal papilla. Telogen is a resting phase that ends when signals from the

dermal papilla initiate a new anagen by stimulating the bulge cells to become secondary germ cells, where they proliferate and differentiate to regenerate the hair shaft [17,18]. The eccrine and apoeccrine sweat glands form from the downward growth of cells from the IFE into the dermis [19]. Apocrine sweat glands, on the other hand, empty their contents into the pilosebaceous duct [20]. These glands are found opposite to the sebaceous glands in the isthmus region of the HF. Within the sebaceous glands is a pool of proliferative sebocytes which maintain the gland as they differentiate [15], ultimately secreting their contents into the hair shaft via the sebaceous duct [21]. Figure 2 shows a cartoon of the HF and location of the stem cell niches in the HF.

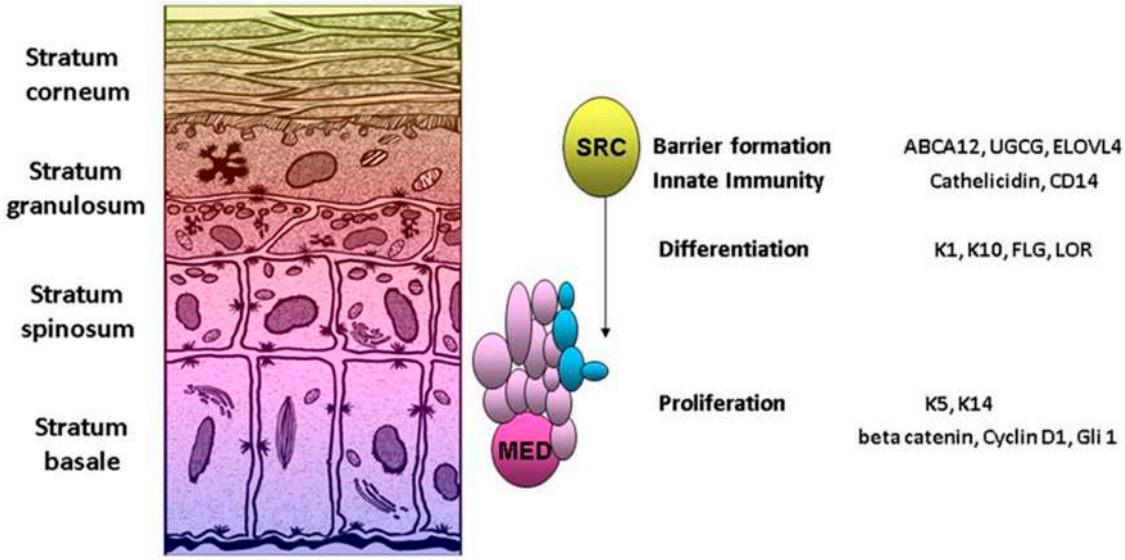

Figure 1. The four layers of the epidermis. The basal layer of the epidermis (stratum basale) contains the stem cells and transient amplifying cells from which cells in the upper layers are derived. These cells express the keratins K5 and K14 and markers of proliferation such as β-catenin (CTNNB), cyclin D1, and GLI1. As these cells migrate into the spinous layer, they begin the production of different keratins, namely K1 and K10, as well as precursors and enzymes involved in cornified envelope formation, namely involucrin and transglutaminase-K. Further migration into the stratum granulosum is marked by the expression of filaggrin and loricrin, proteins also contributing to the cornified envelope. These cells also express enzymes involved in lipid production, lipids that are packaged into lamellar bodies and subsequently injected into the intercellular spaces between the stratum granulosum and stratum corneum to waterproof the permeability barrier. Moreover, lamellar bodies also contain antimicrobial peptides such as cathelicidin produced in the stratum granulosum, providing protection against invasive organisms. VDR and CYP27B1 expression is highest in the stratum basale. Also shown in this figure is the asymmetric distribution of two major coregulators of VDR action—Med1, which is expressed primarily in the stratum basale and spinosum, facilitates the VDR's regulation of proliferation and early stages of differentiation; SRC3, found in highest concentration in the stratum granulosum, facilitates the VDR's regulation of terminal differentiation.

Underlying the epidermis is the dermis. This can be considered as two not well demarcated layers. The papillary layer next to the epidermis consists of loose connective tissue through which course a network of capillaries that service the epidermis, whereas the lower reticular layer comprises a denser network of elastin and collagen fibers giving the skin its elasticity and strength [16]. Fibroblasts within the papillary layer form the dermal papillae critical for HF cycling, as well as the erector pili muscle [22]. Below the dermis (hypodermis) is the fat layer. The cells within this layer are remarkably active, producing a number of adipokines and cytokines that help to initiate the proliferation and migration of stem cells during wound repair [23,24].

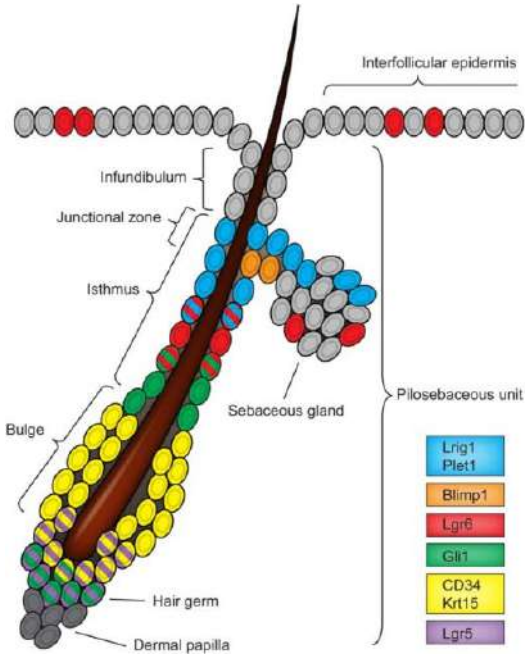

Figure 2. The hair follicle and its stem cell niches. The hair follicle can be divided into the infundibulum merging into the IFE, the junctional zone in the upper portion of the isthmus which separates the infundibulum and isthmus, the bulge region below the isthmus, and the hair germ adjacent to the dermal papilla in the dermis. The sebaceous gland attaches to the hair shaft in the junctional zone/isthmus. These regions contain different stem cell niches. Several markers of the different stem cell niches are shown. The IFE in this cartoon is marked by Lgr6, which is also expressed in other stem cell niches including the sebaceous gland and isthmus. Lrig1 and Plet1 are markers of stem cells in the isthmus and sebaceous gland. Gli1 is a marker for the stem cells in the upper portion of the bulge, with CD34 and Krt15 also marking the bulge stem cells. Lgr5 marks cells of the lower bulge and hair germ. See the text for a more complete description of these stem cell markers.

3. Stem Cell Niches in the Epidermis

In a recent review, Oak and Cotsarelis [25] compiled a table of stem cell markers for the IFE, infundibulum, isthmus, bulge, sebaceous gland, and secondary hair germ. Markers for IFE-SCs include the keratins K5 and K14, the integrins β1 and α6, and the surface marker Lgr6. Lrig1 marks the SCs of the infundibulum. Lgr6 and Lrig1, as well as the transcription factor Gli1, mark the isthmus. The bulge SCs express the keratins K15 and K19, the surface markers CD34 and CD200, and a number of transcription factors such as Gli1, SOX9, Lhx2, TCF3, TCF4, and NFATc1. The label-retaining cells (LRC) of the bulge, thought to be the true SCs, are CD34+/Lgr5−, whereas the CD34+/Lgr5+ are the proliferative cells in the hair germ [26]. Sebaceous gland SCs express Lgr6, Lrig1, and the transcription factor Blimp 1. The secondary hair germ SCs express many of the same markers as those of the bulge, including K15, K19, CD200, Lgr5, and the transcription factors Gli1 and Lef1. The utilization of single-cell RNA sequencing (scRNAseq) has demonstrated that within these niches, substantial heterogeneity exists [27–29], which will be discussed further in the next section.

4. The Response of Stem Cells to Wounding

When the skin is wounded, the progeny of SCs from all regions of the HF and IFE contribute to repair, at least initially [30,31]. Lineage tracing studies demonstrated that cells from the bulge contribute about 25% of the newly formed epidermis [32]. However, these cells do not persist. Other studies, including those from our lab, showed that SCs from other SC niches in the HF also contributed to epidermal regeneration, and tended

to remain longer [33,34]. The main point is that upon wounding, all stem cells in the IFE and HF redirect their function to closing the wound in a process involving changes in transcription to adopt an epidermal genotype described as lineage infidelity [35]. The study by Ge et al. [35] used ATAC to identify changes in chromosome accessibility during the response to wounding that enabled HFSCs of the bulge to acquire an epidermal phenotype and migrate to the wound to help reform the epidermis. They focused on two transcription factors, Klf5 and SOX9, that were unique to the IFE and HFSC niches, respectively. HFSCs that migrated to the wound gained expression of Klf5, with gradual loss of SOX9 with re-epithelialization. HFSCs initially comprise 26% of the wound epithelium on day 8 post wound, but that number is reduced to 3.5% by day 20 [32]. On the other hand, Lgr6 cells from the isthmus last much longer in the epithelium, though not indefinitely [36]. That said, SCs from the HF are not required for re-epithelialization, as healing takes place in mice lacking HF [37] or in the hairless epidermis, such as the paw.

More recent scRNAseq studies of the skin during the healing process have demonstrated a considerably more nuanced picture. In particular, 15 clusters in unwounded skin and 14 clusters after wounding have been identified by Haensel et al. [38], including 4 basal cell subclusters, 2 spinous subclusters, and 4 HF subclusters. Of the basal cell subclusters, one was marked by SC genes including Col 17a and ΔNp63, indicative of early proliferation, a second with early response gene markers such as fos, jun, and Id1, and a third cluster with growth arrest genes such as Cdkn1a, Irf6, Ovo1, and Sfn. In the wounded skin, the growth arrest cluster-type genes were predominant in the leading edge of the re-epithelialization process where differentiation into the upper layers of the epidermis begins, whereas Col17a1 and ΔNp63 were found in the proliferation zone behind the leading edge. As noted above, the SCs from the bulge change from expressing markers of the bulge SCs such as CD34 as they migrate into the epidermis during wound healing [38] and begin expressing the markers of the IFE-SCs. In confirmation of the lineage tracing studies mentioned above, Joost et al. [39] labeled the infundibulum SCs and bulge SCs with Lgr6 and Lgr5, respectively, and noted the more rapid movement of the Lgr6+ cells into the wound than the Lgr5+ cells, but in both cases, the cells started expressing the IFE transcriptome as they migrated into the wound.

5. Vitamin D Metabolism in the Skin

Vitamin D is produced in the skin with the photo/thermal conversion of 7-dehydrocholesterol (7-DHC) to previtamin D, which is then isomerized to vitamin D. This step is well known. Less well known is the production of 7-DHC, a step in the Kandutsch–Russell pathway for cholesterol synthesis. 7-dehydrocholesterol reductase (DHCR7) converts 7-DHC to cholesterol, so its activity dictates how much 7-DHC is available for vitamin D production [40]. There is another pathway for cholesterol synthesis. Most tissues express the Bloch pathway, which does not go through 7-DHC. Thus, these tissues cannot form vitamin D, even if exposed to sunlight. Keratinocytes are further capable of producing 1,25(OH)2D as they express one of the 25-hydroxylases (CYP27A1) [41] and CYP27B1 [42], the 25OHD 1 hydroxylase that produces 1,25(OH)2D. Moreover, keratinocytes express CYP11A1 (the side chain cleavage enzyme critical for steroidogenesis), which can convert vitamin D to 20(OH)D and other vitamin D metabolites [43] with biologic activity overlapping the roles of 1,25(OH)2D [44]. The expression and activity of CYP27B1 in keratinocytes are linked to differentiation, decreasing as the keratinocytes differentiate. Moreover, the hormonal regulation of CYP27B1 activity in keratinocytes differs from that in renal cells. Both tumor necrosis factor (TNF) and interferon-gamma (IFNγ) bind to their receptors on keratinocytes [45,46] and stimulate 1,25(OH)2D production [47,48]. These cytokines do not regulate CYP27B1 in the kidney. On the other hand, parathyroid hormone via its stimulation of cyclic AMP production increases the CYP27B1 activity in renal cells, but not in keratinocytes [49]. Although 1,25(OH)2D regulates its own production in both keratinocytes and renal cells, the mechanisms are different. In renal cells, this is achieved at the genomic level, whereas in keratinocytes, this occurs via the induction of CYP24A1, the

enzyme that catabolizes 1,25(OH)2D [50]. These differences in the regulation of 1,25(OH)2D production in different cells provide one mechanism for cell specificity. The differential regulation of VDR function, discussed next, is a second mechanism.

6. The Vitamin D Receptor (VDR)

The VDR is a member of the nuclear receptor superfamily. It was initially identified in the intestine, where it mediates the actions of 1,25(OH)2D on intestinal calcium and phosphate transport [51]. However, the VDR is expressed in most, if not all, tissues in the body, including those of the skin, where it is found at its highest concentrations in the basal cells of the epithelium and stem cell niches of the HF [52]. As we [53] recently reviewed, the human VDR contains 427 amino acid residues. Following a short N terminal region is the DNA binding domain (DBD), comprising two zinc fingers held in a tetrahedral configuration by four cysteine residues. A hinge region then separates the DBD from the ligand binding domain (LBD). The hinge region is critical for the binding of the VDR/RXR heterodimer, essential for the binding of the VDR to its DNA binding sites. Most of these sites involve two stretches of six reasonably well-conserved nucleotides separated by three nonspecific nucleotides (a DR6), but other configurations are also known. Variations in these sequences can alter the configuration of the heterodimer affecting the ligand and the coactivator binding, providing both cell and gene specificity. The LBD comprises 12 α-helices (H1–12). After ligand binding, the C terminal H12 closes over the ligand, forming a binding pocket, and along with H3 and H4, it provides an interface to which coactivators with LXXLL motifs can bind, the AF2 domain. In the epidermis, the major coactivators are the steroid receptor coactivator (SRC) complexes 2 and 3 and the mediator complex [54] (Figure 1). The mediator complex is found primarily in the basal cells and enables the VDR to regulate proliferation and the early stages of differentiation [55]. The mediator complex marks super enhancer regions of the genes involved in cell fate determination [56], critical for the stem cell response to wounding, where the VDR, in association with other transcription factors, exerts its transcriptional effects on stem cell fate [34]. The SRC complexes are expressed in the more differentiated cells of the epidermis and enable VDR to regulate the terminal stages of differentiation [57].

7. VDR Mediation of the Wounding Response

Wound healing is a multistage process, with inflammation and activation of the innate immune system triggering the activation and proliferation of stem cells that ultimately close the wound [31]. Mice lacking the VDR, the calcium receptor, or the capacity for 1,25(OH)2D production are limited in these early responses, including a reduction in stem cells with reduced β-catenin signaling and failure of these cells to form E-cadherin/catenin complexes, important both for the migration of the stem cells and the re-epithelialization of the wound [52,58,59], as discussed below. Furthermore, TGF signaling is impaired in VDR-null mice, associated with a reduction in macrophage recruitment and granulation tissue formation, impacting the dermal component of the wound [60].

As noted earlier, we and others have shown that IFE and HFSCs express high levels of the VDR [52,61]. The critical role of the VDR in HF stem cells is demonstrated by the failure of hair follicles in mice lacking the VDR (VDRKO) to reinitiate anagen after the first developmental cycle [62], leading to alopecia. As shown initially by Cianferotti et al. [63], this action of the VDR does not require its ligand 1,25(OH)2D. A similar phenomenon likely accounts for the reduction in the response of these SCs to re-epithelialize a wound. Several mechanisms have been proposed for this failure of the SCs to respond in VDRKO mice. One mechanism involves the loss of proliferative potential in HFSCs lacking the VDR [63], which our studies with epidermal-specific VDRKO mice confirm [52]. A second explanation proposes that the progeny of HFSCs lacking VDR fail to migrate out of their niches, delaying the wounding response, suggesting a loss of activation and/or migration of the progeny [64]. Our data support this concept as well [52]. Thus, the delay in the wounding response in VDRKO mice appears to be due to decreased activation/proliferation

of the SCs plus reduced migration to the wound site, which then are blunted in their ability to differentiate and so to re-establish an intact epithelium. A critical event in mediating a number of these events is the formation of the E-cadherin (Cdh1)/catenin (Ctnn) complex, which is blocked in VDRKO keratinocytes. This complex maintains SCs in their niches [65], regulates the extent to which SC division is symmetrical (maintaining SC numbers) or asymmetrical (initiating differentiation) [66], and enables the migration of keratinocytes as a sheet to re-epithelialize the wound [67]. This complex also performs a key signaling function for calcium and vitamin D in stimulating differentiation of the regenerating epidermis [68].

The VDR does not act alone in promoting the wounding response. As already alluded to, calcium is an important partner, as dietary calcium deficiency or lack of the calcium sensing receptor can likewise retard the wounding process [61]. Other transcription factors can influence VDR activity. As noted previously, the RXR is a frequent partner with the VDR, regulating genomic actions. A second transcription factor, p63, induces the VDR by directly binding to the *VDR* promoter [69]. Like *Vdr*, the deletion of *p63* blocks epidermal differentiation [70]. In our analysis of human keratinocytes, we [34] identified colocalization of the VDR and p63 in 99 sites in the absence of 1,25(OH)2D, increasing to 799 sites after 1,25(OH)2D administration. The top Gene Ontology (GO) biologic processes for genes with the colocalization of p63 and VDR binding sites in their promoter regions included the regulation of wound healing, the regulation of SC differentiation, adherens junction organization, DNA replication, and the response to calcium [34]. Although most p63 binding sites are not of known relevance to keratinocyte differentiation, among those that are, the VDR is one of the partnering transcription factors [71], and these sites are enriched in super enhancers considered important for SC fate determination [56]. p63 has several isoforms due to alternative splicing—the full-length Tap63, containing all 14 exons, and ΔNp63, lacking the first 3 exons [72]. Both bind to the same DNA element, but it is the ΔNp63 isoform that is most highly expressed in the basal layer of the epidermis and is critical for keratinocyte differentiation [73]. ΔNp63 and the VDR are expressed in the same regions of the HF [74], and as noted in the scRNA seq studies discussed earlier, it is expressed in the basal proliferating SC cluster along with Col17a [38]. When ΔNp63 is knocked down in these cells, wound healing is impaired [74]. Moreover, human keratinocytes lacking ΔNp63 have a blunted response to 1,25(OH)2D [34].

Beta-catenin is likely also to have a major role in cooperating with the VDR in the wounding response. Stem cell activation is associated with increased β-catenin signaling [75], and this increased signaling is dependent on the presence of the VDR [76,77]. Beta-catenin binds to the VDR in its AF2 domain like other coactivators, likely increasing its transcriptional activity [78]. Palmer et al. [79] identified putative response elements for the VDR and β-catenin in a number of genes, including those of the hedgehog pathway, which likely have a role in the response to wounding. Moreover, we [34] identified a β-catenin response element (TCF4) in close association with both p63 and VDR response elements in the Fos gene, suggesting that β-catenin and p63 may collaborate with the VDR in controlling cell fate genes directing stem cell response to wounding.

8. Summary and Conclusions

In this review of the role of the VDR in the stem cell response to wounding, I have presented the concept that the epidermis is a dynamic structure continuously undergoing regeneration, whether this is ongoing, as in the epithelium and pilosebaceous unit, or cyclic, as in the lower portion of the HF. Critical for this regeneration are the stem cells that occupy different niches in the basal layer of the IFE and HF. Each niche has its own instructions for the regeneration of the tissue for which it is responsible. But when the skin is wounded, these distinct stem cells change fates and move into the wound to regenerate the epithelium, adopting the transcriptome of the epithelial cell. This change in cell fate, labeled as genomic infidelity, is reversible to a large if not complete extent when the wound is closed, as some residual memory appears to remain. Vitamin D signaling plays an important role in this

process. First of all, the keratinocyte, the major cell in the epidermis, has the machinery to make 1,25(OH)2D, the principal VDR ligand, without outside substrates. The keratinocyte expresses the VDR at the highest concentration in these stem cell niches. The VDR is central to vitamin D signaling, but not all actions of VDR require 1,25(OH)2D, including hair follicle cycling [63]. Moreover, the VDR does not act alone. The mediator complex has a major influence in regulating the proliferative and early differentiating actions of the VDR, whereas SRC complexes enable the subsequent actions on terminal differentiation. p63 and β-catenin also enable at least some of the actions of the VDR, principally in the early stages of proliferation and differentiation. Thus, the VDR, facilitated by its coregulators and associated transcription factors, enables the stem cells to heal the wound and regenerate the epidermis.

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