Lack of the Vitamin D Receptor is Associated with Reduced Epidermal Differentiation and Hair Follicle Growth

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The active vitamin D metabolite, 1,25-dihydroxyvitamin D, acting through the vitamin D receptor, regulates the expression of genes in a variety of vitamin D-responsive tissues, including the epidermis. To investigate the role of the vitamin D receptor in mediating epidermal differentiation, we examined the histomorphology and expression of differentiation markers in the epidermis of vitamin D receptor knockout mice generated by gene targeting. The homozygous knockout mouse displayed a phenotype that closely resembles vitamin D-dependent rickets type II in humans, including the development of rickets and alopecia. Hair loss developed by 3 mo after birth and gradually led to nearly total hair loss by 8 mo. Histologic analysis of the skin of homozygous knockout mice revealed dilation of the hair follicles with the formation of dermal cysts starting at the age of 3 wk. These cysts increased in size and number with age. Epidermal differentiation markers, including involucrin, profilaggrin, and loricrin, detected by immunostaining and *in situ* hybridization, showed decreased expression levels in homozygous knockout mice from birth until 3 wk, preceding the morphologic changes observed in the hair follicles. Keratin 10 levels, however, were not reduced. At the ultrastructural level, homozygous knockout mice showed increased numbers of small dense granules in the granular layer with few or no surrounding keratin bundles and a loss of keratohyalin granules. Thus, both the interfollicular epidermis and the hair follicle appear to require the vitamin D receptor for normal differentiation. The temporal abnormalities between the two processes reflect the apparent lack of requirement for the vitamin D receptor during the anagen phase of the first (developmental) hair cycle, but with earlier effects on the terminal differentiation of the interfollicular epidermis. ***Key words: hair follicle/keratinocyte differentiation/vitamin D receptor. J Invest Dermatol 118:11–16, 2002***

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), mediates its action by binding with high affinity to specific vitamin D receptors (VDR) located in the nucleus of target cells (Reichel *et al*, 1989; Darwish and DeLuca, 1993; Bouillon *et al*, 1995). VDR is a member of the nuclear hormone receptor superfamily and acts as a ligand-inducible transcription factor, regulating vitamin D-responsive genes (Mangelsdorf and Evans, 1995). Whereas classic vitamin D target tissues include intestine, kidney, and bone, where $1,25(OH)_2D_3$ acts to maintain serum calcium levels and to build and preserve bone (Bouillon *et al*, 1995), compelling evidence has emerged in recent years that $1,25(OH)_2D_3$ is also critical for the differentiation and proliferation of a large number of normal and malignant cells, including epidermal cells (Bikle and Pillai, 1993). Keratinocytes, the most abundant cells of the epidermis, are the source of 7-dehydrocholesterol, necessary for the photochemical production of the parent vitamin D. They also possess both the 25- and 1α-hydroxylase enzymes, and thus can produce endogenous $1,25(OH)_2D_3$ from the vitamin D (Bikle *et al*, 1986; Lehmann *et al*, 1999). In addition, keratinocytes contain VDR (Stumpf *et al*, 1979; Hosomi *et al*, 1983; Pillai *et al*, 1988) and so can respond to the $1,25(OH)_2D_3$ produced (Hosomi *et al*, 1983; Smith *et al*, 1986; Pillai and Bikle, 1991); however, the function of the VDR in skin *in vivo* is not clear. The alopecia that is variably found in patients with hereditary VDR deficiency (vitamin D-dependent rickets type II), suggests a biologic role for the VDR in the epidermis and particularly in the hair follicle. Hair growth has three defined phases, the first of which is the rapid growth phase (anagen), followed by the apoptosis-driven regression phase (catagen) and the relatively quiescent phase (telogen) (Müller-Röver *et al*, 2001). The regulatory mechanisms governing hair cycling are still not clear, although they can affect the initial development of the hair follicle, or, as is the case for VDR deficiency, the subsequent recycling of the hair follicle. To investigate the physiologic role of VDR in mediating epidermal differentiation and hair follicle growth, we examined the tissue histology and expression of the differentiation markers in the epidermis of VDR knockout (VDRKO) mice during different stages of postnatal development.

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; VDRKO, vitamin D receptor knock-out; PCNA, proliferating cell nuclear antigen.

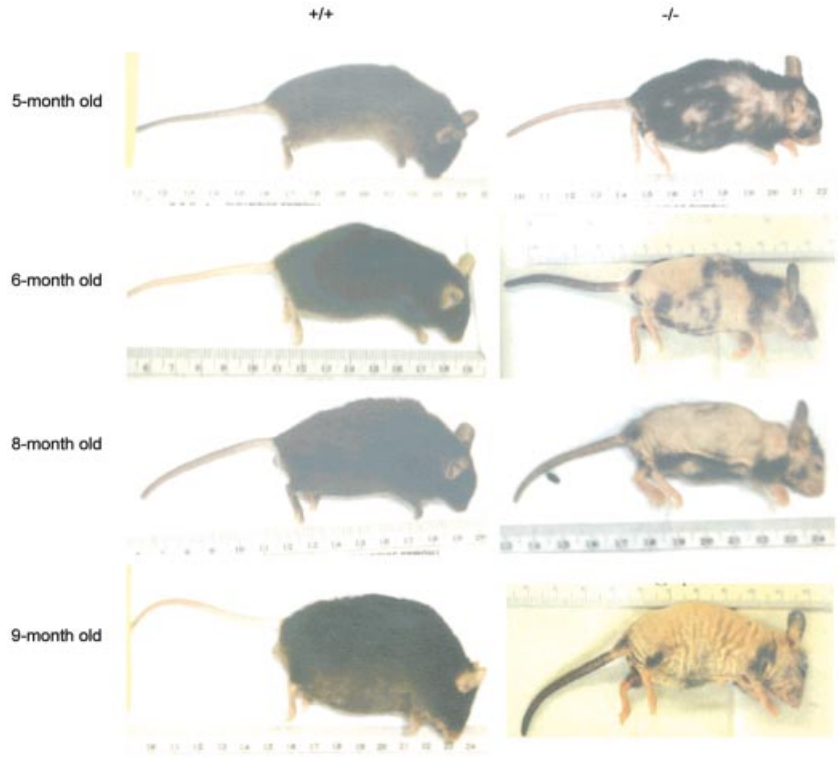

Figure 1. Appearance of VDRKO mice at the age of 5, 6, 8, and 9 mo. +/+, wild-type mice; –/–, VDRKO mice. VDRKO mice demonstrated hair loss starting by 3 mo with progressive loss with age.

MATERIALS AND METHODS

Genotyping of VDR disrupted mice The VDRKO mice (Yoshizawa *et al*, 1997) were genotyped by southern blot analysis (Yoshizawa *et al*, 1997) or polymerase chain reaction. Primers used for polymerase chain reaction include an upper primer (5′-CGGTGCC-CTGAATGAACTGC-3′) and a lower primer (5′-GGGAAGCCAAG-AGGGGAGTC-3′) for amplifying the mutant VDR DNA; an upper primer (5′-GCCACGGGCTTCCACTTCAA-3′) and a lower primer (5′-GGGAAGCCAAGAGGGGAGTC-3′) for amplifying the wild-type VDR DNA.

Immunohistochemistry Affinity-purified rabbit anti-peptide antibodies (BabCo, Berkeley, CA) specific for keratin 10 (K10), involucrin, loricrin, and filaggrin were used. These antibodies were found to recognize both mouse and rat proteins following heat-induced antigen retrieval treatment (in 10 mM citrate buffer; pH 6.0, at 95°C, for 30 min). The primary antibodies were used at a concentration of 4 μg per ml. All the immunoreagents were diluted in 10 mM Tris buffer, pH 7.6, containing 4% bovine serum albumin, 1% teleostean skin gelatin, 0.1% Tween 20, and 500 mM NaCl. The binding of the primary antibodies to the sections was detected by affinity-purified, biotinylated goat anti-rabbit IgG, followed by ABC-peroxidase reagent, both purchased from Vector (Burlingame, CA). Peroxidase activity was revealed with diaminobenzidine substrate (QualTek Laboratories, Santa Barbara, CA) followed by counterstaining with methyl green or hematoxylin. Omitting the first antibodies resulted in no signal, indicating the specificity of the immunodetection.

***In situ* hybridization** Explants were fixed in 4% paraformaldehyde and embedded in paraffin. Digoxigenin-labeled RNA probes to detect involucrin, loricrin, and profilaggrin mRNA were made from linearized rat involucrin cDNA (gift from Robert Rice) and linearized mouse loricrin and profilaggrin cDNA (gifts from S. Yuspa, NIH) as templates, using reagents supplied by Roche (Indianapolis, IN). *In situ* hybridization was performed with probes applied to the sections with hybridization at 45°C for involucrin and 47°C for both loricrin and profilaggrin. The hybridization of digoxigenin-labeled probes to the endogenous mRNA was detected by anti-digoxigenin-alkaline phosphatase with BCIP/NTBT substrate. The sections were counterstained with fast red. Probes of sense orientation served as a control to ensure the specificity of hybridization. The sense control probes resulted in no signal, indicating the specificity of hybridization with the anti-sense probe.

Electron microscopy Skin samples from 1 mo old VDRKO mice and age-matched wild-type littermates were prepared according to previously reported procedures (Yu *et al*, 1996). Briefly, they were fixed in 2.5% glutaraldehyde, osmicated with 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epoxy medium. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Zeiss electron microscope operated at 60 kV. In each section, the entire area of the granular layer was examined, and representative images were photographed for analysis.

RESULTS

Phenotypic abnormalities of VDRKO mice Homozygous VDRKO mice showed normal growth after birth until 3 wk. After 3 wk, the VDRKO mice showed modest growth retardation and developed rickets. The skin and hair appeared grossly normal after birth until about 3 mo, although some animals showed noticeable hair loss by 6 wk. After 3 mo, the VDRKO mouse showed progressive alopecia, which led to nearly total hair loss by 8 mo (**Fig 1**); however, no overt abnormalities were found in the heterozygotes (data not shown).

Hematoxylin–eosin staining shows abnormal hair follicles, proliferating cell nuclear antigen (PCNA) staining shows normal proliferation Hematoxylin–eosin-stained paraffin sections of the skin from homozygous littermates showed normal morphology at birth. In 3 wk old VDRKO mice, dilation of the hair follicles lacking hair shafts and exhibiting thin hair sheaths were observed with formation of dermal cysts. These cysts contained tissue debris and increased in size with age, correlating with the progressive hair loss. As seen in **Fig 2**(***a–d***), the intensity of PCNA staining was well maintained in the epidermis of VDRKO mice at all time points, although a slight but consistent increase was seen at 3 wk. The lining of the cysts in the VDRKO mice was also positive for PCNA staining, indicating ongoing proliferation in these cysts, whereas less proliferation was seen in the hair follicles of the normal mice.

Involucrin, profilaggrin, and loricrin protein levels in epidermis are reduced in VDRKO mice To evaluate the epidermal differentiation in VDRKO mice we examined markers

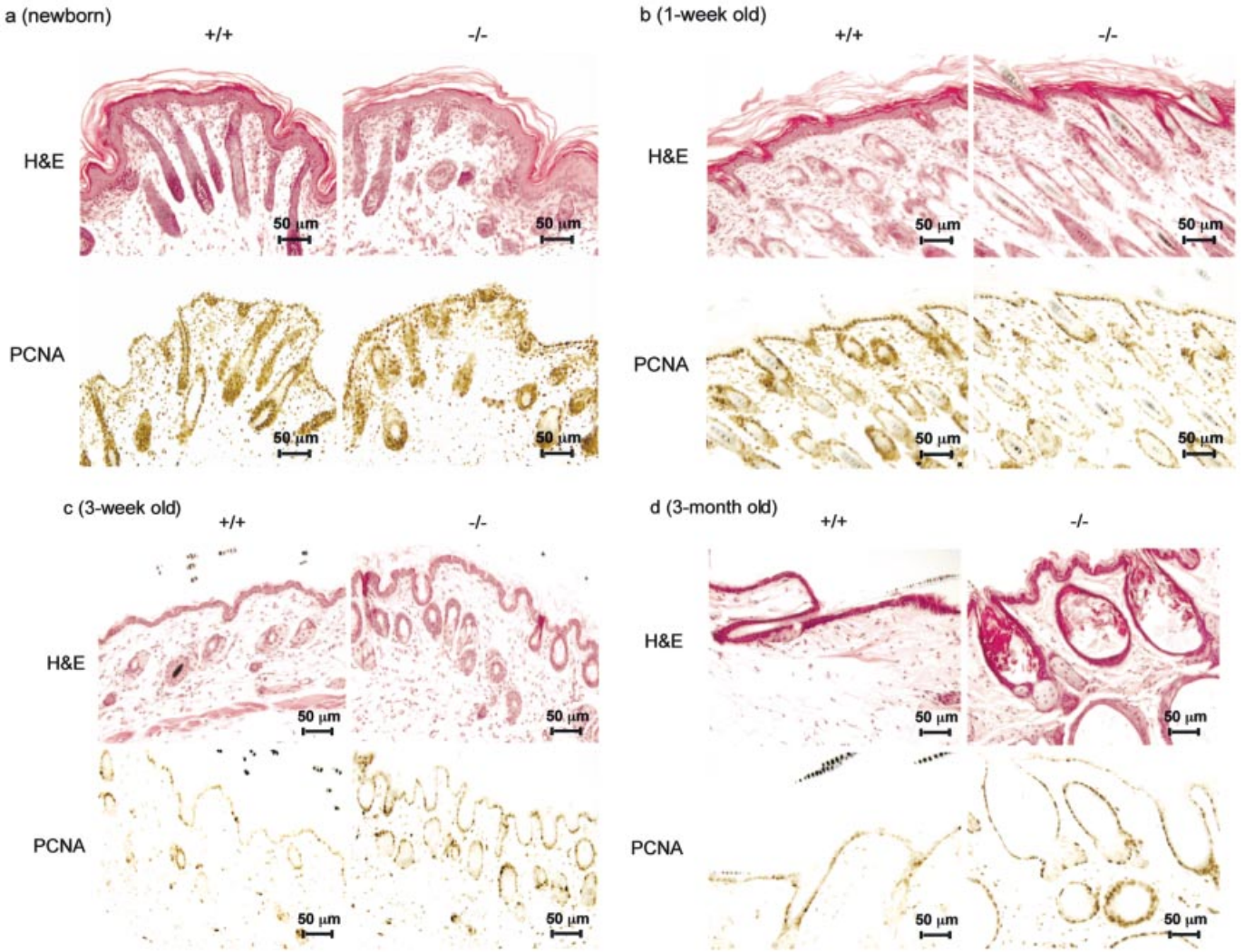

Figure 2. Epidermal morphology and proliferation. Hematoxylin–eosin- and PCNA-stained sections of the epidermis, collected from newborn (*a*), 1 wk (*b*), 3 wk (*c*), and 3 mo (*d*) old VDRKO (–/–) mice and their wild-type (+/+) littermates. VDRKO mice showed abnormal hair follicles. Data presented are from representative samples. Each experiment was repeated with three wild-type mice and three VDRKO mice.

for epidermal differentiation. The epidermis of VDRKO mice at birth showed no difference from wild-type littermates with respect to the expression of involucrin, profilaggrin, and loricrin (**Fig 3*a***). At 1 wk (**Fig 3*b***) and 3 wk (**Fig 3*c***), however, the epidermis of VDRKO mice displayed a marked decrease in the protein expression of these differentiation markers. These differences were less apparent at 3 mo (**Fig 3*d***) as the levels in the wild-type epidermis declined, although profilaggrin expression in the VDRKO mice remained well below that of the wild-type littermates at all time points (**Fig 3*a–d***). Although not a focus of this study, the hair follicles and hair shafts of the normal mice near the epidermis and the cysts of the VDRKO mice also expressed these proteins, which is consistent with previous findings (Limat *et al*, 1994).

Involucrin, profilaggrin, and loricrin mRNA levels in epidermis are reduced in VDRKO mice To confirm these observations, *in situ* hybridization was carried out using specific complementary ribonucleic acid probes for involucrin, profilaggrin, and loricrin RNA. The results showed that the mRNA levels for involucrin and loricrin were reduced in newborn VDRKO mice (**Fig 4*a***). In 1 wk old mice, the mRNA level for profilaggrin was also reduced compared with wild-type littermates (**Fig 4*b***). The levels of these markers in the epidermis of the older animals were reduced to the point that meaningful comparisons between abnormal VDRKO and wild-type epidermis could not be made (data not shown).

Ultrastructural changes in the granular layer of the epidermis of VDRKO mice Skin sections from each group at the age of 4 wk were examined by electron microscopy. In the epidermis, the most consistent changes were found in the granular layer (**Fig 5**). The VDRKO mice had an increased number of small dense granules in the granular layer with few or no surrounding keratin bundles and no apparent mature keratohyalin granules. In contrast, the granules in the wild-type epidermis of the littermates were less dense, irregular in shape, and associated with keratin filament bundles.

K10 protein levels in the epidermis of VDRKO mice Having shown the reduction in keratin bundles detected by electron microscopy, we examined the K10 protein levels in these mice at different ages, as K10 is one of the major keratins forming these bundles. As seen in **Fig 6**, K10 levels were well maintained in the epidermis of VDRKO mice at all time points, although a modest reduction was seen at 3 wk.

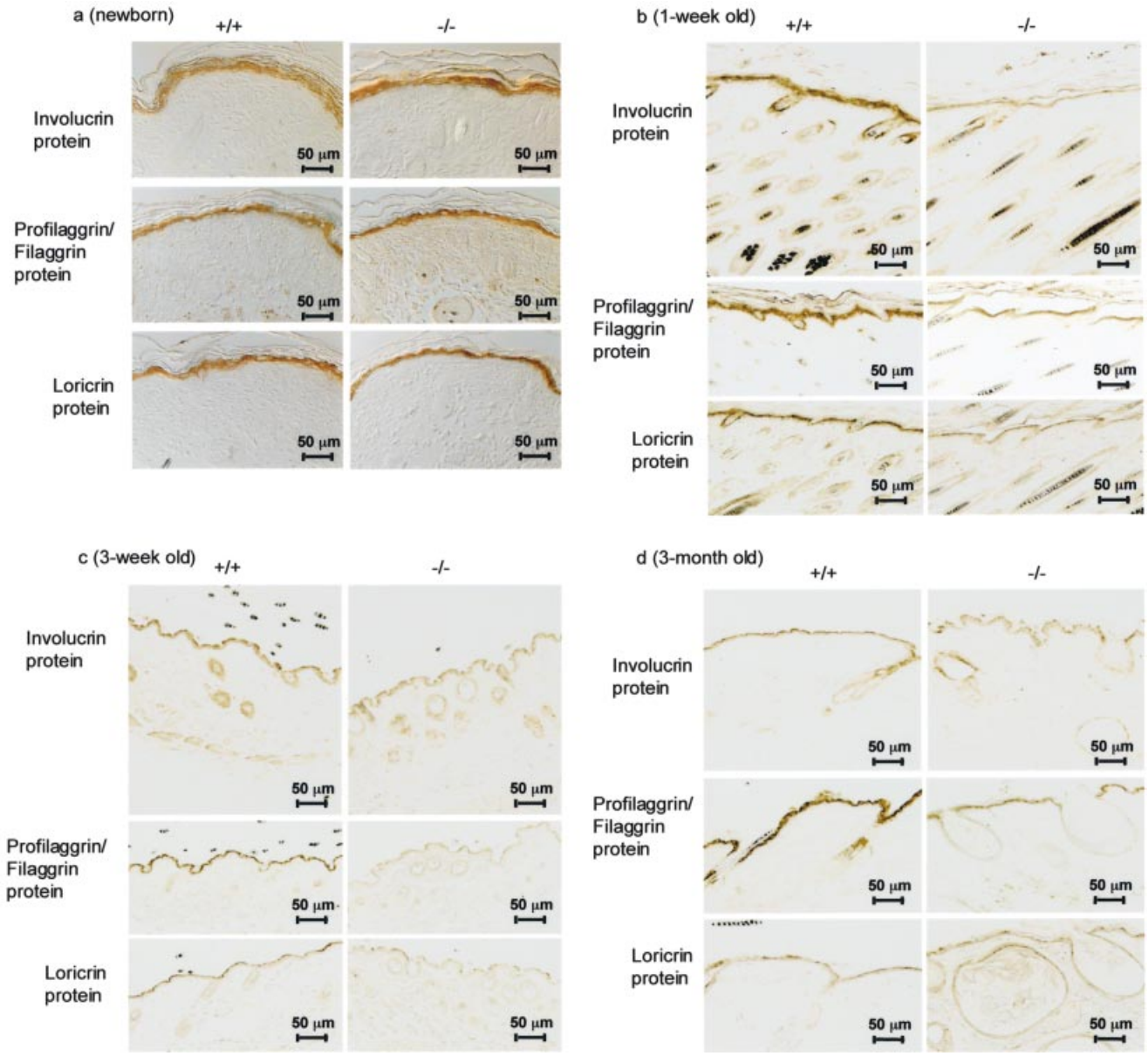

Figure 3. Reduced protein levels of involucrin, profilaggrin, and loricrin in epidermis from VDRKO mice. Involucrin, profilaggrin, and loricrin proteins as shown by the brown signal were detected in the epidermis from VDRKO (–/–) mice and wild-type (+/+) littermates at newborn (*a*), 1 wk (*b*), 3 wk (*c*), and 3 mo (*d*) by immunocytochemistry, as described in *Materials and Methods*. Data presented are from representative samples. Each experiment was repeated with three wild-type mice and three VDRKO mice.

DISCUSSION

Mammalian epidermis is a stratified squamous epithelium, with a basal layer of proliferating cells surmounted by several layers of progressively differentiating, postmitotic suprabasal cells. The differentiation of keratinocytes in the epidermis is a complex process involving the sequential, well-orchestrated expression of structural proteins (Fuchs, 1990; Eckert *et al*, 1997). Involucrin, a component of the cornified envelope, is a suprabasal marker of keratinocyte differentiation that is expressed in the late spinous layer and throughout the granular layer. Profilaggrin, the precursor of filaggrin, is found in larger, less dense granules. Filaggrin is thought to facilitate the aggregation of keratin filaments into bundles. Loricrin is stored in smaller, more electron dense granules and like involucrin is incorporated into the cornified envelope. Profilaggrin and loricrin are expressed in the granular layer of the epidermis where they are stored in keratohyalin granules. All three differentiation markers showed decreased expression in VDRKO mice from birth until 3 wk. This reduction in protein expression was associated with loss of mature keratohyalin granules in the cytoplasm of granular layer keratinocytes from VDRKO mice and loss of keratin filament bundles. As K10 is one of major keratins expressed in the suprabasal layers of the epidermis, we determined its level to see if a lower K10 level could account for the loss of keratin filament bundles. The normal K10 levels suggest that the loss of keratin bundles may reflect decreased bundling of keratin due to decreased filaggrin rather than decreased keratin production; however, decreased levels of other keratins, not measured in this study, could be postulated. Similar morphologic changes have been reported in flaky tail mice lacking filaggrin (Presland *et al*, 2000) and transgenic mice overexpressing keratinocyte growth factor (Guo *et al*, 1993).

These data indicate that the lack of VDR is associated with reduced epidermal differentiation during the first month of life. The differences in differentiation between homozygous knockouts

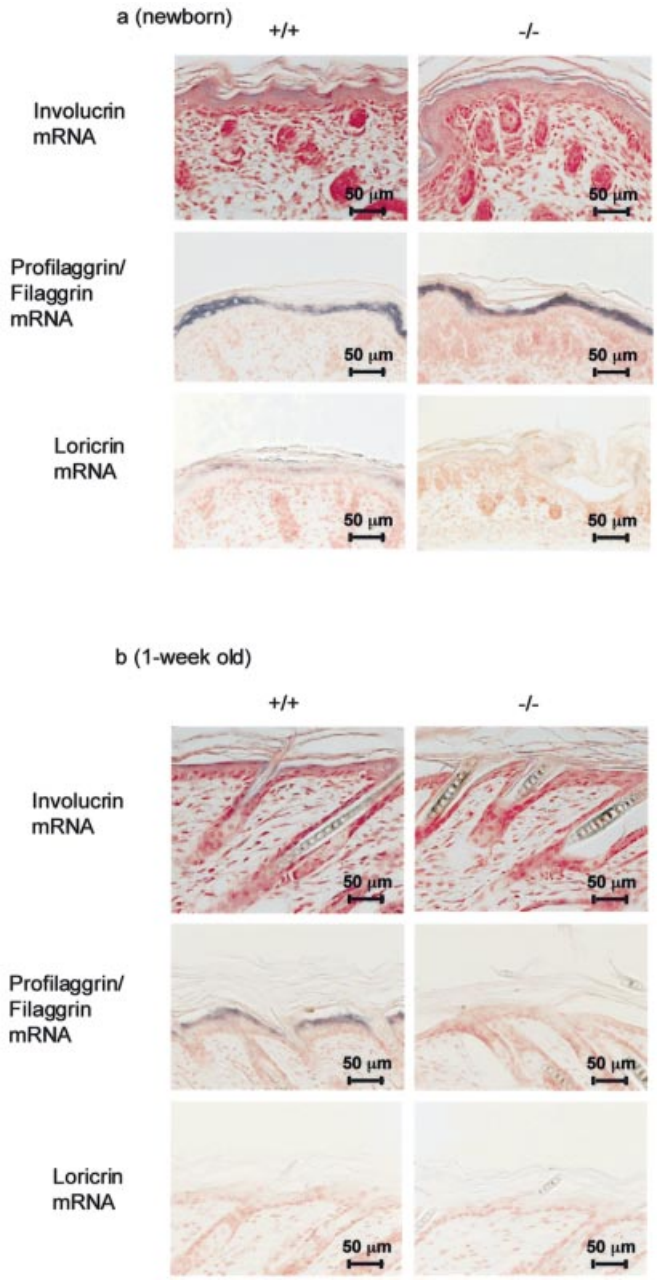

Figure 4. Reduced mRNA levels of involucrin, profilaggrin, and loricrin in epidermis from VDRKO mice. Involucrin, profilaggrin, and loricrin mRNA as shown by the blue signal were detected in the epidermis from VDRKO (–/–) mice and wild-type (+/+) littermates at newborn (*a*) and 1 wk (*b*) by *in situ* hybridization, as described in *Materials and Methods*. Data presented are from representative samples. Each experiment was repeated with three wild-type mice and three VDRKO mice.

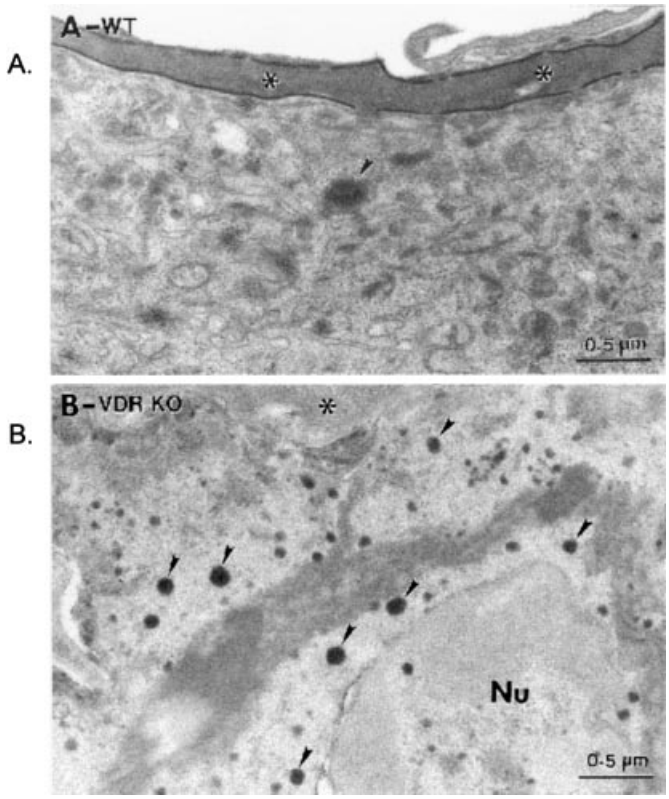

Figure 5. Abnormal granules in the cytoplasm of granular layer keratinocytes from VDRKO mice. Ultrastructural analysis of granular keratinocytes from age-matched wild-type (WT) mouse skin (*A*) shows mature keratohyalin granules and numerous keratin filament bundles. Asterisks indicate portions of a corneocyte. Ultrastructural analysis of granular layer keratinocytes from VDRKO mouse skin (*B*) shows numerous small, electron dense granules in the cytoplasm and few keratin filament bundles.

and wild-type littermates became less apparent in mice older than 3 wk, suggesting that with time compensatory mechanisms develop. Although our data indicate that the RNA levels for the differentiation markers are reduced at birth, Sakai and Demay (2000) have shown similar levels of keratin 1, involucrin, and loricrin mRNA in cultured keratinocytes and epidermis isolated from 4 d old VDRKO and wild-type mice. These observations suggest that in culture, keratinocyte differentiation is less dependent on the VDR, although their failure to see a reduction in the mRNA levels of involucrin and loricrin extracted from skin is less readily understood. Our *in vitro* studies have shown that $1,25(OH)_2D_3$ induces keratinocyte differentiation, although differentiation can occur in the absence of $1,25(OH)_2D_3$ (Bikle and Pillai, 1993). Both involucrin and transglutaminase are responsive to $1,25(OH)_2D_3$, and a vitamin D responsive element has recently been identified in the involucrin promoter (Bikle *et al*, submitted for publication) but these differentiation markers also respond to calcium in the absence of $1,25(OH)_2D_3$. In contrast, previous studies have not shown induction of the keratins by $1,25(OH)_2D_3$ (Blumenberg *et al*, 1992; Tomic *et al*, 1992; Lu *et al*, 1994). Thus, our *in vivo* data are consistent with these *in vitro* observations in that expression of these differentiation markers does not have an absolute dependence on $1,25(OH)_2D_3$ and VDR. $1,25(OH)_2D_3$ inhibits cell proliferation as well as stimulates differentiation. Our data, however, do not show major changes in epidermal proliferation as detected by PCNA staining or routine histology in VDRKO mice, similar to the observations of Sakai and Demay (2000), although the modest increase in PCNA staining in VDRKO mice is consistent with a minor loss of proliferative regulation in these mice.

VDR is not only expressed in epidermal keratinocytes, but also in the outer root sheath keratinocytes and dermal papilla cells of hair follicles (Stumpf *et al*, 1984; Berger *et al*, 1988; Reichrath *et al*, 1994). Furthermore, the enzyme for synthesizing $1,25(OH)_2D_3$, 25-hydroxyvitamin D-1α-hydroxylase, is expressed in both the basal layer of the epidermis and the matrix cells of hair follicles in the dermis (Zehnder *et al*, 2001), suggesting that keratinocytes in hair follicles make as well as respond to their own $1,25(OH)_2D_3$. Our data showed that the reduced expression of epidermal differentiation markers was seen by 1 wk after birth, whereas

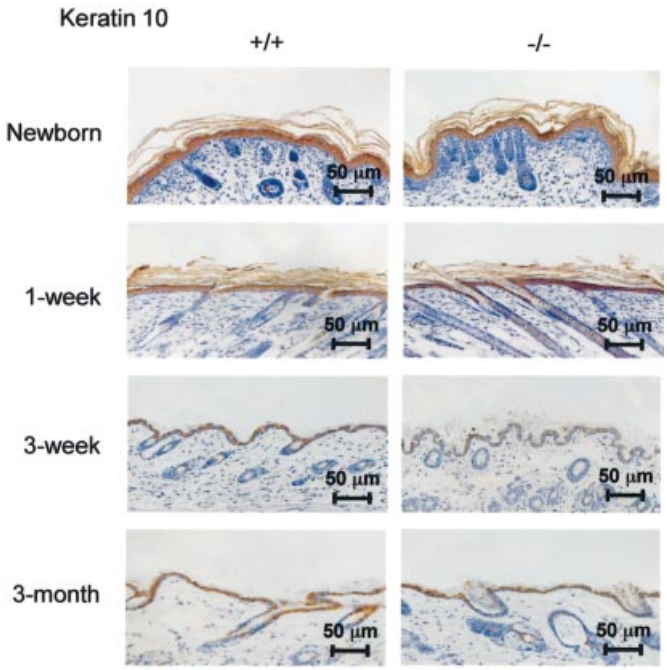

Figure 6. K10 protein levels in epidermis from VDRKO mice. The K10 protein as shown by the brown signal was detected in the epidermis from VDRKO (–/–) mice and wild-type (+/+) littermates at newborn (*a*), 1 wk (*b*), 3 wk (*c*), and 3 mo (*d*) by immunocytochemistry, as described in *Materials and Methods*. Data presented are from representative samples. Each experiment was repeated with three wild-type mice and three VDRKO mice.

changes in the hair follicles were not seen before 3 wk. The timing of these changes indicates that prenatal development of the epidermis and the first (developmental) hair growth cycle does not require the VDR, whereas the development of the adult skin and the onset of the second hair growth cycle do. These data suggest that VDR is required for a critical stage of secondary hair follicle development. The development of alopecia in the case of VDR deficiency but not vitamin D deficiency remains unexplained, however, suggesting that VDR has ligand-independent roles critical for hair follicle cycling. The similarity of the hair loss pattern and the dermal cysts between VDRKO mice and the rhino mutation/hairless mice suggests a similar pathway for these two zinc finger transcription factors, VDR and hairless, in mediating hair growth cycles (Miller *et al*, 2001; Sundberg and King, 2001). This opens an exciting new area of investigation for vitamin D in the skin.

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