Title
The Transient Role for Calcium and Vitamin D during the Developmental Hair Follicle Cycle

Journal
Journal of Investigative Dermatology, 136(7)

ISSN
0022-202X

Authors
Mady, Leila J
Ajibade, Dare V
Hsaio, Connie
et al.

Publication Date
2016-07-01

DOI
10.1016/j.jid.2016.02.813

Peer reviewed

Published in final edited form as:

J Invest Dermatol. 2016 July ; 136(7): 1337–1345. doi:10.1016/j.jid.2016.02.813.

The Transient Role for Calcium and Vitamin D during the Developmental Hair Follicle Cycle

Leila J. Mady[1], **Dare V. Ajibade**[1], **Connie Hsaio**[1], **Arnaud Teichert**[2], **Chak Fong**[2], **Yongmei Wang**[2], **Sylvia Christakos**[1], **Daniel D. Bikle**[2]

[1]Department of Microbiology, Biochemistry and Molecular Genetics, Rutgers University – New Jersey Medical School, Newark, New Jersey, USA

[2]Department of Medicine and Dermatology, Endocrine Unit, Veterans Affairs Medical Center, University of California, San Francisco, California, USA

Abstract

The role for 1,25-dihydroxyvitamin D_3 and/or calcium in hair follicle cycling is not clear despite their impact on keratinocyte differentiation. We found that calbindin-D_{9k} null (knockout) pups generated from calbindin-D_{9k} knockout females fed a vitamin D-deficient, low-calcium (0.47%) diet develop transient alopecia. The pups appear phenotypically normal until 13 days of age, after which the hair progressively sheds in a caudocephalic direction, resulting in truncal alopecia totalis by 20–23 days, with spontaneous recovery by 28 days. Histological studies showed markedly dystrophic hair follicles, loss of hair shafts with increased apoptosis, and hyperplastic epidermis during this time. Ha1 expression is lost during catagen in all mice but recovers more slowly in the knockout pups on the vitamin D-deficient, low-calcium diet. Keratin 1 expression is reduced throughout days 19–28. The expressions of involucrin, loricrin, and cathepsin L is initially increased by day 19 but subsequently falls below those of controls by day 23, as does that of desmoglein 3. Feeding the mothers a high-vitamin D/high-calcium (2%)/lactose (20%) diet lessens the phenotype, and knockout pups fostered to mothers fed a normal diet do not develop alopecia. Our results show that in calbindin-D_{9k} knockout pups, a maternal vitamin D-deficient/low-calcium diet leads to transient noncicatricial alopecia.

INTRODUCTION

The skin has long been known as a target organ for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and calcium (Bikle, 2012). Although $1,25(OH)_2D_3$ and calcium promote differentiation of keratinocytes, the current literature does not suggest a direct role for $1,25(OH)_2D_3$ and/or calcium in hair follicle (HF) cycling, unlike that of the vitamin D receptor (VDR) (Li et al., 1997; Sakai et al., 2001; Xie et al., 2002; Yoshizawa et al.,

Correspondence: Daniel D. Bikle, MD, PhD, VAMC (111N), 4150 Clement Street, San Francisco, California 94121, USA. daniel.bikle@ucsf.edu.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.02.813.

1997). CYP27B1-knockout (KO) mice, which do not synthesize $1,25(OH)_2D_3$, develop rickets like VDR-KO mice, but unlike VDR-KO mice, CYP27B1-KO mice do not develop alopecia (Bikle et al., 2004; Bouillon et al., 2008; Dardenne et al., 2001; Panda et al., 2001). Furthermore, a lactoseenriched, high-calcium rescue diet that normalizes serum calcium effectively rescues the skeletal phenotype of VDR-KO mice but does not prevent the alopecia (Amling et al., 1999; Li et al., 1998). Whether $1,25(OH)_2D_3$ and/or calcium have any effect on HF cycling is, as yet, unresolved.

Calbindin-D_{9k} is a vitamin D-inducible calcium-binding protein in mammalian intestine that is localized primarily in the cytoplasm of the absorptive cells, supporting the proposed role of calbindin in intestinal calcium absorption (Christakos et al., 1989). Although first identified in the intestine (Wasserman et al., 1968), calbindin-D_{9k} is also expressed in the placenta, uterus, brain, and kidney (Bruns et al., 1985; Bruns et al., 1988; Christakos et al., 1989; Sooy et al., 2000; Thomasset et al., 1982; Wernyj et al., 1999). However, calbindin-D_{9k} is not found in skin, although other calcium-binding proteins are (Jin et al., 1997; Schelling et al., 1987). Therefore, it was a surprise to discover that in the absence of calbindin-D_{9k}, maternal deprivation of vitamin D combined with low dietary calcium results in disruption of the HF cycle in preweaned pups, leading to transient alopecia.

RESULTS

Alopecia development in calbindin-D_{9k}–KO pups generated from calbindin-D_{9k} KO females fed a vitamin D-deficient, low-calcium diet

To verify the null mutation, different tissues (including intestine and kidney) from calbindin-D_{9k}–KO mice were analyzed by reverse transcriptase (RT) PCR and Western blotting. No signal for calbindin-D_{9k} was detected in any tissue in mice homozygous for the targeted mutation (Benn et al., 2008). When fed a standard rodent chow diet, no overt phenotypic differences were observed between wild-type (WT) and calbindin-D_{9k}–KO mice. They have similar fertility, growth rate, serum calcium level, and life expectancy (Benn et al., 2008; Kutuzova et al., 2006). However, a consistent, unexpected HF cycling defect was observed in calbindin-D_{9k}–KO pups generated from calbindin-D_{9k}–KO females fed a vitamin D-deficient, low-calcium (0.47%) (D−/low-Ca) diet 3–4 weeks before mating and during the subsequent pregnancy and lactation. The pups appeared phenotypically normal until 13 days of age, after which the coat began to look ruffled and progressively shed in a caudocephalic direction. By postnatal days 20–23, there was nearly complete hair loss on their trunks, with sparing of the head and the base of the tail (Figure 1). This finding was observed with at least 12 different calbindin-D_{9k}–KO mothers and their respective nursing neonates. All pups in the litter had similar alopecia. Spontaneous recovery began during the fourth week of age. After 28 days of age complete recovery of alopecia was observed whether the pups were fed the standard chow diet or if they were left with the mother (and thus able to eat the D−/low-Ca diet). No subsequent loss of hair was observed. When wild-type mothers were fed a D−/low-Ca diet, a similar alopecia was observed, but unlike the calbindin-D_{9k}–KO mice, the alopecia was not consistently observed. Alopecia was observed in 20% of the litters (n = 10 different wild-type mothers fed a D−/low-Ca diet and their respective nursing neonates), suggesting that the absence of calbindin sensitizes the animal to the factors contributing to

alopecia. Pups from calbindin-D_{9k}–KO mothers fed a vitamin D-replete diet (0.47% Ca; 2,200 IU vitamin D_2/kg) (D+/low-Ca) or a standard chow diet did not have alopecia (n = 7–12 mothers and their respective pups/diet).

Calbindin-D_{9k}–KO pups born to D−/low-Ca diet mothers maintain serum calcium but have undetectable 25(OH)D_3 levels

No significant differences in serum calcium were noted at postnatal days 13–28 between pups born to vitamin D-deficient and those born to vitamin D-replete mothers (serum calcium level of calbindin-D_{9k}–KO pups on postnatal days 13–28 born to vitamin D-deficient mothers = 9.5 ± 0.1 mg/dl; serum calcium level of pups on postnatal days 13–18 born to vitamin D-replete mothers = 9.4 ± 0.1 mg/dl; $P > 0.5$). However, 25(OH)D_3 serum levels of pups born to D−/low-Ca mothers at postnatal days 13–28 were all less than 2.5 ng/ml, compared with an average of 20.7 ± 1.4 ng/ml 25(OH)D_3 levels in serum of pups at these ages from mothers fed a vitamin D-replete diet. Serum calcium and 25(OH)D_3 levels of D−/low-Ca mothers were less than 8 mg/dl and less than 2.5 ng/ml, respectively.

KO pups born to D−/low-Ca diet mothers develop dystrophic HF and hyperplastic epidermis during late catagen but recover

In the skin of calbindin-D_{9k}–KO pups from mothers fed a D−/low-Ca diet, during the late stages of catagen and early telogen (days 19–23) the HFs became markedly dystrophic, and the hair shafts were lost (Figure 2). Moreover, the epidermis became markedly hyperplastic (Figure 2). There was evidence of partial occlusion of the HF with inclusion cysts within the follicle proximal to the obstruction at the follicular outlet. No evidence of parakeratosis was observed, but some orthokeratotic hyperkeratosis was seen. There was no evidence of spongiosis or inflammation. These changes were not observed in pups from mothers with wild-type calbindin-D_{9k} levels, regardless of diet.

Immunohistochemical evaluation of the skin from days 13–28 for markers of epidermal differentiation showed expression of keratin 1 (i.e., K1), involucrin, and loricrin throughout the abnormal utricles and thickened epidermis from days 19–23, with recovery by day 28 (Figure 3). Keratin 6 (i.e., K6), a marker of inflammation, was minimally and not differentially expressed in either the control or diet-deficient pups (data not shown). Use of myeloperoxidase as a marker of inflammatory cells showed very few myeloperoxidase-positive cells in either the controls or the D−/low-Ca KO pups (see Supplementary Figure S1 online). The negative control for these immunohistochemistry assays is shown Supplementary as Figure S2 online.

KO pups born to D−/low-Ca diet mothers show an altered pattern of apoptosis during the HF cycle

To determine whether a D−/low-Ca diet affects cell survival, we performed the TUNEL assay on skin sections from regularly and D−/low-Ca–fed mice at days 13, 19, 23, and 28 (Figure 4 and Table 1). At day 13, in the regular diet group, TUNEL-positive cells were observed in the outer root sheath, central inner root sheath, and in the bulge region (Figure 4a and b). The D−/low-Ca diet increased the number of TUNEL-positive cells in the above areas and the proximal bulb (Figure 4a–c). At day 19, compared with day

13, more TUNEL-positive cells were seen in the bulge/isthmus, but few or none were seen in the central inner root sheath or proximal bulb, whereas numerous TUNEL-positive cells appeared in the epithelial strand (Figure 4c). In the D−/low-Ca diet-fed group, the morphology of the HFs was markedly distorted with a relative paucity of TUNEL-positive cells (Figure 4k). At day 23, very few TUNEL-positive cells were observed in the HFs of the regular-diet group (Figure 4c), although the numbers found in the different regions of the HFs of mice on the D−/low-Ca diet were increased (Figure 4l–m). At day 28, only scattered TUNEL-positive cells were found in the HFs in either the regular diet-fed group (Figure 4n–p) or the D−/low-Ca diet-fed group (Figure 4h). These results are similar to those previously reported (Lindner et al., 1997), at least with respect to late-stage anagen (day 13) and midcatagen (day 19) in the regular diet group, and suggest an earlier onset of catagen with respect to increased apoptosis in the D−/low-Ca group on day 13 not found again with recovery on day 28. No TUNEL-positive cells were seen in the dermal papilla at any stage.

Expression of epidermal differentiation markers was altered in KO pups born to D−/low-Ca diet mothers, as was that of cathepsin L and desmoglein 3

To quantify the expression of these epidermal markers, we used PCR of RNA from whole skin biopsy samples (Figure 5). The marker of hair shaft development, Ha1, was not expressed during catagen (days 19–23) in either the D−/low-Ca KO pups or the pups raised on a normal diet. However, the recovery of Ha1 expression was delayed in the D−/low-Ca KO pups on day 28. Involucrin expression was greater at day 13 than at other times of the cycle. Expression of involucrin in the D−/low-Ca KO pups was greater than that in the pups on the regular diet at day 19 but was reduced by day 23. The increase at day 19 may reflect the initial expression of involucrin in the hyperplastic epidermis and the dystrophic HFs that is decreased subsequently. These results are consistent with immunohistochemistry showing apparent reduction of involucrin in the epidermis between KO pups on the different diets on days 23 and 28. Loricrin, on the other hand, showed maximal expression on day 23 in the KO pups on the normal diet, far greater than the expression seen in the D−/low-Ca KO pups, which had their peak expression at day 19. As for involucrin, the greater expression of loricrin in the D−/low-Ca KO pups on day 19 may reflect the expression of loricrin in the hyperplastic epidermis and dystrophic follicles. These results confirm the reduction in loricrin on day 23 in the KO pups on the D−/low-Ca diet apparent in the immunohistochemistry testing results. The expression of cathepsin L (Ctsl) was assessed, because its deletion is associated with periodic hair loss (Roth et al., 2000). Its expression in the D−/low-Ca diet-fed mice was substantially greater than in controls on day 19 but fell below the expression level in controls by day 23. Similarly, the expression of desmoglein 3 (Dsg3) was assessed because it anchors the telogen hair in the follicle (Koch et al., 1998). Its expression in the D−/low-Ca diet-fed mice was comparable with that in controls on days 13–19 but was reduced relative to controls on day 23. Immunohistochemistry staining of Ctsl and Dsg3 expression was consistent with the messenger RNA data, although Dsg3 expression appeared to be greater at 19 days in the D−/low-Ca mice than in controls (see Supplementary Figure S3 online). Thus, neither Ctsl nor Dsg3 could explain the hair loss caused by the D−/low-Ca diet.

KO pups born to D−/low-Ca diet mothers when fostered by mothers on a normal diet did not develop alopecia

When calbindin-D_{9k}–KO females, fed the D−/low-Ca diet before mating and during pregnancy, were fed a D−/high-Ca (2%), high-lactose (20%) diet during lactation (from birth to postnatal day 28), a less severe phenotype was observed in the pups on days 19–23, when only partial alopecia was observed (Figure 6A). In addition, when the pups from calbindin-D_{9k}–KO females fed the D−/low-Ca diet were fostered to mothers fed a diet containing vitamin D3 and calcium (1%), complete rescue of the alopecia phenotype was observed (Figure 6B). These findings suggest a maternal component for the HF cycling defect in the KO pups that is triggered by the effects of both low calcium and vitamin D3 deficiency.

DISCUSSION

In this study we show that calbindin-D_{9k}–KO pups generated from calbindin-D_{9k}–KO females fed a D−/low-Ca diet develop transient alopecia during the first postnatal catagen. In contrast to VDR-KO mice, there is permanent recovery of HF cycling after weaning. Postmorphogenetic HF development involves catagen (regression; beginning at postnatal day 14–17 in the mouse and extending for about 3 days), followed by telogen (quiescence), exogen (shedding), and anagen (period of growth) generally around 4 weeks (Muller-Rover et al., 2001; Paus, 1998; Stenn and Paus, 2001). In our study the pups from calbindin-D_{9k}–KO mothers fed a D−/low-Ca diet had total truncal alopecia by postnatal day 20–23 but recovered by day 28. Histology of the affected skin throughout the postnatal time course indicated that the major defect was in the first postnatal catagen/telogen. During this period the mice on the D−/low-Ca diet showed increased apoptosis relative to their littermates on the regular diet. Cyclic alopecia has also been observed in other transgenic mice including Ctsl-KO mice, Dsg3-KO mice, in mice with deletion of the calcineurin gene specifically in keratinocytes, and in hematopoietic and endothelial cell-specific peroxisome proliferator-activated receptor-γ KO mice (Koch et al., 1998; Mammucari et al., 2005; Roth et al., 2000; Wan et al., 2007). Ctsl is a ubiquitously expressed lysosomal cysteine protease that, when deleted, alters HF cycling with periodic loss of hair and epidermal hyperplasia. Although murine hair is not typically shed during telogen, Dsg3-null mice display telogenic hair loss and failure of adhesion between telogen club cells and the outer root sheath, indicating a role for Dsg3 (a cell-cell adhesion molecule) in anchoring the telogen hair to the outer root sheath of the follicle (Koch et al., 1998). In the calcineurin-KO mice, gene array studies showed that the expression of certain Notch-responsive genes involved in HF structure and/or adhesion are suppressed (Mammucari et al., 2005). In the Dsg3- and Ctsl-KO mice and in the mice with keratinocyte-specific calcineurin deletion, at least two complete cycles of hair loss and regrowth were noted. In our study and in mice with targeted deletion of peroxisome proliferator-activated receptor-γ (Wan et al., 2007), the defect is observed only in the first postnatal HF cycle. Moreover, we found a reduction in Ctsl and Dsg3 expression only on day 23, after the loss of hair. For the alopecia observed in pups from both peroxisome proliferator-activated receptor-γ and calbindin-D_{9k}–null mothers there is rescue of the phenotype after weaning and with foster mothers. This suggests a maternal component to the defect in HF cycling unlike in the alopecia observed in other KO

mice. In mice with targeted deletion of peroxisome proliferator-activated receptor-γ, it was suggested that the alopecia is caused by inflammatory lipids in the milk, which result in the accumulation of inflammatory lipids in the skin of the pups (Wan et al., 2007). However, we did not detect evidence of an inflammatory component. In our study, substituting a high-calcium (2%), high-lactose diet for the D−/low-Ca diet during lactation (birth to postnatal day 28) resulted in a less severe phenotype in the pups. Also, pups from calbindin-D_{9k}–KO mothers fed a 2,000-IU vitamin D2/kg, 0.47%-calcium diet or a chow diet did not develop alopecia.

One of the most pronounced effects of $1,25(OH)_2D_3$ in the mammalian intestine is increased synthesis of the calcium binding protein calbindin-D_{9k} (Christakos et al., 1989). It has recently been reported that prolactin, a lactogenic polypeptide hormone of the anterior pituitary, cooperates with $1,25(OH)_2D_3$ in regulating calbindin-D_{9k} in the intestine (Ajibade et al., 2010). Prolactin levels are markedly elevated during lactation, when there is increased calcium requirement for the neonate (Meites et al., 1972). In addition, it has been shown that intestinal calbindin-D_{9k} is induced during late pregnancy and lactation (Van Cromphaut et al., 2003; Zhu et al., 1998). Although previous studies in calbindin-D_{9k}–KO mice indicate that calbindin-D_{9k} is not required for intestinal calcium absorption under nonpregnant, nonlactating conditions (Akhter et al., 2007; Benn et al., 2008), this study suggests that calbindin-D_{9k} may be an important physiological mediator of intestinal calcium absorption during pregnancy and lactation at a time when calcium demands are increased.

Although pups from vitamin D-deprived or vitamin D-deficient mothers have been reported to be relatively protected with regard to calcium deficiency compared with their mothers (Boass et al., 1981; Halloran and DeLuca, 1979), pups nursed by vitamin D-deprived mothers fed a low calcium diet may have a more marked effect on normal homeostasis in the absence of calbindin-D_{9k}, resulting in the consistent alopecia observed in the nursing neonates. Keratin 1, involucrin, and loricrin—early, middle, and late differentiation markers, respectively—showed decreased expression in the pups with alopecia except for involucrin and loricrin at day 19, when expression may have been increased relative to controls by the abnormal expression of these genes in the dystrophic follicles (utricles) and hyperplastic epidermis. Ha1, a late marker of HF differentiation, was as expected not expressed during catagen in either controls or the diet deficient pups. However, its re-expression by 28 days was reduced in the diet-deficient KO pups. These data are consistent with abnormalities in both epidermal and HF differentiation. K6, a marker of inflamed skin, and myeloperoxidase, a marker of inflammatory cells, were not increased in the epidermis of the pups with alopecia. The regulation of involucrin and loricrin by $1,25(OH)_2D_3$ and calcium are consistent with our findings of decreased involucrin and loricrin expression in the skin of pups from vitamin D-deprived calbindin-D_{9k}–null mothers fed a low-calcium diet. Mice deficient in CYP27B1 (which do not synthesize $1,25(OH)_2D_3$) but raised on a high-calcium/lactose (rescue) diet do not show alopecia (Bikle et al., 2004; Dardenne et al., 2001; Panda et al., 2001). Similarly, mice lacking the calcium-sensing receptor in the epidermis do not develop alopecia (Tu et al., 2012). Our findings indicate that to develop alopecia, the nutritional defect needs to occur during lactation. Previous studies have shown low serum levels of $25(OH)D_3$ in neonates during the suckling period compared with levels after weaning, suggesting impaired milk transfer of $25(OH)D_3$ (or its precursor) (Mendelsohn

and Haddad, 1975). Thus, the suckling period may be a particularly vulnerable time for pups regarding regulation of calcium homeostasis, and this may affect HF cycling. We (Oda et al., 2015) have recently evaluated the impact of the combined disruption of vitamin D and calcium signaling on wound healing, which like HF cycling requires activation of stem cells, their proliferation, migration, and differentiation. In those studies we found poor wound healing associated with a loss of HF stem cells and a failure of their activation in response to wounding in mice lacking the vitamin D receptor in their keratinocytes when fed a low-calcium diet. However, the nutritional defect in vitamin D and calcium in the calbindin-D_{9k}–KO mothers may affect other factors transferred in the milk to the pup that not obviously involved in calcium homeostasis but affect signaling events in the HF, which contribute to the aberrant postnatal HF development. Clearly additional studies are required to determine the mechanism for this transient disruption of HF cycling.

HF cycling has been reported to be sensitive to many factors including nutritional, hormonal, and environmental factors (Paus, 1998; Stenn and Paus, 1999, 2001), which may be produced locally in the skin (Slominski et al., 2013). Our results show that, in the absence of calbindin-D_{9k}, maternal deprivation of vitamin D combined with low dietary calcium intake consistently results in noncicatricial alopecia in nursing neonates, suggesting for the first time a role for calcium and possibly $1,25(OH)_2D$ or other vitamin D metabolites such as 20 hydroxyvitamin D (Slominski et al., 2014a; Slominski et al., 2014b) in postnatal HF cycling.

MATERIALS AND METHODS

Animals

Calbindin-D_{9k}–KO mice were generated and genotyped as previously described by Lee et al. (2007), maintained on a chow diet ad libitum (Rodent Laboratory Chow 5001, Ralston Purina Co., St. Louis, MO, USA), with incandescent lighting on a 12-hour light, 12-hour dark cycle. KO mice were backcrossed with C57BL/6J mice for 8 or more generations. The Rutgers University New Jersey Medical School Animal Care and Use Committee approved all animal experiments conducted.

Experimental design

Calbindin-D_{9k}–KO female mice were fed either a D− (0.47% calcium, 0.3% phosphate; Teklad diet TD 89123, Harlan Teklad, Madison, WI, USA) or a D+ diet (2,200 IU/kg vitamin D, 0.47% calcium, 0.3% phosphate; Teklad diet TD 07370, Harlan Teklad) for 3–4 weeks before mating to a calbindin-D_{9k}–KO male and during the subsequent pregnancy and lactation. Pups from these mice were compared with pups from calbindin-D_{9k}–null parents fed a chow diet. To determine the effect of increased maternal calcium intake during lactation on alopecia of the pups, a diet containing 2% calcium and 20% lactose (without vitamin D; Teklad diet TD 97340, Harlan Teklad) was substituted for the D−/low-Ca diet from the day of birth of the pups. This diet was maintained throughout lactation. For foster mother experiments, pups from mothers fed a D−/low-Ca diet were fostered on the day of birth to mothers on a normal chow diet. Two pups were left with the original mother. (Foster pups and original pups were distinguished by ear punching).

Immunohistochemistry

During the period of alopecia, skin samples from the affected skin were selected. Details of the procedure can be found in the Supplementary Materials online.

Apoptosis assay

Apoptotic cells in the sections were detected with the terminal TUNEL assay. Details of the procedure are found in the Supplementary Materials.

Serum calcium and 25OHD measurements

The serum concentration of calcium was determined by Sigma Diagnostic Reagents. The 25OHD levels were determined by Heartland Laboratories.

PCR

Total RNA was isolated from mouse skin using RNA STAT-60 kit (Tel-Test “B”, Inc. Friendswood, TX, USA), and messenger RNA levels were determined by quantitative PCR as previously described (Oda et al., 2012; Tu et al., 2012). The primers used are tabulated in the Supplementary Materials and Supplementary Table S1 online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Support was provided by NIH Grant DK38961-22 (SC), NIH Grant AR050023 (DB), VA Merit Review grant (DB), DOD Grant CA110338 (DB), and a United Negro College Fund Merck Graduate Student Research Dissertation Fellowship (DVA). We acknowledge helpful discussions with and assistance of Dr. Puneet Dhawan and Dr. Peter Elias in certain aspects of this investigation. The Calbindin-D9k KO mice were provided to Dr. Christakos by Drs. EB Jeung and GT Oh.

Abbreviations:

1,25(OH)2D3	1,25 dihydroxyvitamin D3
Ctsl	cathepsin L
D−/low-Ca	vitamin D-deficient/low-calcium
D+/low-Ca	vitamin D-replete/low-calcium
Dsg3	desmoglein 3
HF	hair follicle
KO	knockout
VDR	vitamin D receptor

REFERENCES

Ajibade DV, Dhawan P, Fechner AJ, Meyer MB, Pike JW, Christakos S. Evidence for a role of prolactin in calcium homeostasis: regulation of intestinal transient receptor potential vanilloid type 6, intestinal calcium absorption, and the 25-hydroxyvitamin D(3) 1alpha hydroxylase gene by prolactin. Endocrinology 2010;151:2974–84. [PubMed: 20463051]

Akhter S, Kutuzova GD, Christakos S, DeLuca HF. Calbindin D9k is not required for 1,25-dihydroxyvitamin D3-mediated Ca2+ absorption in small intestine. Arch Biochem Biophys 2007;460:227–32. [PubMed: 17224126]

Amling M, Priemel M, Holzmann T, Chapin K, Rueger JM, Baron R, et al. Rescue of the skeletal phenotype of vitamin D3 receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. Endocrinology 1999;140:4982–7. [PubMed: 10537122]

Benn BS, Ajibade D, Porta A, Dhawan P, Hediger M, Peng JB, et al. Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 2008;149:3196–205. [PubMed: 18325990]

Bikle DD. Vitamin D3 and the skin: physiology and pathophysiology. Rev Endocr Metab Disord 2012;13:3–19. [PubMed: 21845365]

Bikle DD, Chang S, Crumrine D, Elalieh H, Man MQ, Choi EH, et al. 25 Hydroxyvitamin D3 1 alpha-hydroxylase is required for optimal epidermal differentiation and permeability barrier homeostasis. J Invest Dermatol 2004;122:984–92. [PubMed: 15102089]

Boass A, Ramp WK, Toverud SU. Hypocalcemic, hypophosphatemic rickets in rat pups suckling vitamin D-deprived mothers. Endocrinology 1981;109: 505–12. [PubMed: 6972864]

Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, et al. Vitamin D3 and human health: lessons from vitamin D3 receptor null mice. Endocr Rev 2008;29:726–76. [PubMed: 18694980]

Bruns ME, Kleeman E, Mills SE, Bruns DE, Herr JC. Immunochemical localization of vitamin D-dependent calcium-binding protein in mouse placenta and yolk sac. Anat Rec (Hoboken) 1985;213:514–7, 532–515.

Bruns ME, Overpeck JG, Smith GC, Hirsch GN, Mills SE, Bruns DE. Vitamin D-dependent calcium binding protein in rat uterus: differential effects of estrogen, tamoxifen, progesterone, and pregnancy on accumulation and cellular localization. Endocrinology 1988;122:2371–8. [PubMed: 3286225]

Christakos S, Gabrielides C, Rhoten WB. Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations, and molecular biology. Endocr Rev 1989;10:3–26. [PubMed: 2666110]

Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R. (2001) Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. Endocrinology 2001;142:3135–41.

Halloran BP, DeLuca HF. Vitamin D3 deficiency and reproduction in rats. Science 1979;204:73–4. [PubMed: 432628]

Jin L, Miyamoto O, Toyoshima T, Kobayashi R, Murakami TH, Itano T. Localization of calbindin-D28k in normal and incised mouse skin: immunohistochemical and immunoblot analysis. Arch Dermatol Res 1997;289:578–84. [PubMed: 9373717]

Koch PJ, Mahoney MG, Cotsarelis G, Rothenberger K, Lavker RM, Stanley JR. Desmoglein 3 anchors telogen hair in the follicle. J Cell Sci 1998;111(Pt 17):2529–37. [PubMed: 9701552]

Kutuzova GD, Akhter S, Christakos S, Vanhooke J, Kimmel-Jehan C, Deluca HF. Calbindin D(9k) knockout mice are indistinguishable from wild-type mice in phenotype and serum calcium level. Proc Natl Acad Sci USA 2006;103:12377–81. [PubMed: 16895982]

Lee GS, Lee KY, Choi KC, Ryu YH, Oh GT, Jeung EB. Phenotype of a calbindin D9k gene knockout is compensated for by the induction of other calcium transporter genes in a mouse model. J Bone Miner Res 2007;22:1968–78. [PubMed: 17696760]

Li YC, Amling M, Pirro AE, Priemel M, Meuse J, Baron R, et al. Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but

not alopecia in vitamin D3 receptor-ablated mice. Endocrinology 1998;139:4391–6. [PubMed: 9751523]

Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, et al. Targeted ablation of the vitamin D3 receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci USA 1997;94:9831–5. [PubMed: 9275211]

Lindner G, Botchkarev VA, Botchkareva NV, Ling G, van der Veen C, Paus R. Analysis of apoptosis during hair follicle regression (catagen). Am J Pathol 1997;151:1601–17. [PubMed: 9403711]

Mammucari C, Tommasi di Vignano A, Sharov AA, Neilson J, Havrda MC, Roop DR, et al. Integration of Notch 1 and calcineurin/NFAT signaling pathways in keratinocyte growth and differentiation control. Dev Cell 2005;8:665–76. [PubMed: 15866158]

Meites J, Lu KH, Wuttke W, Welsch CW, Nagasawa H, Quadri SK. Recent studies on functions and control of prolactin secretion in rats. Recent Prog Horm Res 1972;28:471–526. [PubMed: 4569231]

Mendelsohn M, Haddad JG. Postnatal fall and rise of circulating 25-hydroxyvitamin D3 in the rat*. J Lab Clin Med 1975;86:32–7. [PubMed: 1151140]

Muller-Rover S, Handjiski B, van der Veen C, Eichmuller S, Foitzik K, McKay IA, et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol 2001;117:3–15. [PubMed: 11442744]

Oda Y, Hu L, Bul V, Elalieh H, Reddy JK, Bikle DD. Coactivator MED1 ablation in keratinocytes results in hair-cycling defects and epidermal alterations. J Invest Dermatol 2012;132:1075–83. [PubMed: 22189783]

Oda Y, Tu CL, Menendez A, Nguyen T, Bikle DD. Vitamin D and calcium regulation of epidermal wound healing [e-pub ahead of print]. J Steroid Biochem Mol Biol 2015; 10.1016/j.jsbmb.2015.08.011.

Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, et al. Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci USA 2001;98:7498–503. [PubMed: 11416220]

Paus R Principles of hair cycle control. J Dermatol 1998;25:793–802. [PubMed: 9990771]

Roth W, Deussing J, Botchkarev VA, Pauly-Evers M, Saftig P, Hafner A, et al. Cathepsin L deficiency as molecular defect of furless: hyperproliferation of keratinocytes and pertubation of hair follicle cycling. FASEB J 2000;14:2075–86. [PubMed: 11023992]

Sakai Y, Kishimoto J, Demay MB. Metabolic and cellular analysis of alopecia in vitamin D receptor knockout mice. J Clin Invest 2001;107:961–6. [PubMed: 11306599]

Schelling CP, Didierjean L, Rizk M, Pavlovitch JH, Takagi T, Heizmann CW. Calcium-binding proteins in rat skin. FEBS Lett 1987;214:21–7. [PubMed: 3569514]

Slominski AT, Kim TK, Li W, Yi AK, Postlethwaite A, Tuckey RC. The role of CYP11A1 in the production of vitamin D metabolites and their role in the regulation of epidermal functions. J Steroid Biochem Mol Biol 2014a;14(Pt A):28–39.

Slominski AT, Kim TK, Takeda Y, Janjetovic Z, Brozyna AA, Skobowiat C, et al. RORalpha and ROR gamma are expressed in human skin and serve as receptors for endogenously produced noncalcemic 20-hydroxy- and 20, 23-dihydroxyvitamin D. FASEB J 2014b;28:2775–89. [PubMed: 24668754]

Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J. Key role of CRF in the skin stress response system. Endocrine Rev 2013;34:827–84. [PubMed: 23939821]

Sooy K, Kohut J, Christakos S. The role of calbindin and 1,25dihydroxyvitamin D3 in the kidney. Curr Opin Nephrol Hyptertens 2000;9:341–7.

Stenn KS, Paus R. What controls hair follicle cycling? Exp Dermatol 1999;8:229–33. [PubMed: 10439219]

Stenn KS, Paus R. Controls of hair follicle cycling. Physiol Rev 2001;81:449–94. [PubMed: 11152763]

Thomasset M, Parkes CO, Cuisinier-Gleizes P. Rat calcium-binding proteins: distribution, development, and vitamin D dependence. Am J Physiol 1982;243:E483–8. [PubMed: 7149019]

Tu CL, Crumrine DA, Man MQ, Chang W, Elalieh H, You M, et al. Ablation of the calcium-sensing receptor in keratinocytes impairs epidermal differentiation and barrier function. J Invest Dermatol 2012;132:2350–9. [PubMed: 22622426]

Van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, Dijcks FA, Ederveen AG, et al. Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptorindependent mechanisms. J Bone Miner Res 2003;18:1725–36. [PubMed: 14584880]

Wan Y, Saghatelian A, Chong LW, Zhang CL, Cravatt BF, Evans RM. Maternal PPAR gamma protects nursing neonates by suppressing the production of inflammatory milk. Genes Dev 2007;21:1895–908. [PubMed: 17652179]

Wasserman RH, Corradino RA, Taylor AN. Vitamin D-dependent calcium-binding protein. Purification and some properties. J Biol Chem 1968;243:3978–86. [PubMed: 4298516]

Wernyj RP, Mattson MP, Christakos S. Expression of calbindin-D28k in C6 glial cells stabilizes intracellular calcium levels and protects against apoptosis induced by calcium ionophore and amyloid beta-peptide. Brain Res Mol Brain Res 1999;64:69–79. [PubMed: 9889325]

Xie Z, Komuves L, Yu QC, Elalieh H, Ng DC, Leary C, et al. Lack of the vitamin D receptor is associated with reduced epidermal differentiation and hair follicle growth. J Invest Dermatol 2002;118:11–6. [PubMed: 11851870]

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 1997;16:391–6. [PubMed: 9241280]

Zhu Y, Goff JP, Reinhardt TA, Horst RL. Pregnancy and lactation increase vitamin D-dependent intestinal membrane calcium adenosine triphosphatase and calcium binding protein messenger ribonucleic acid expression. Endocrinology 1998;139:3520–4. [PubMed: 9681503]

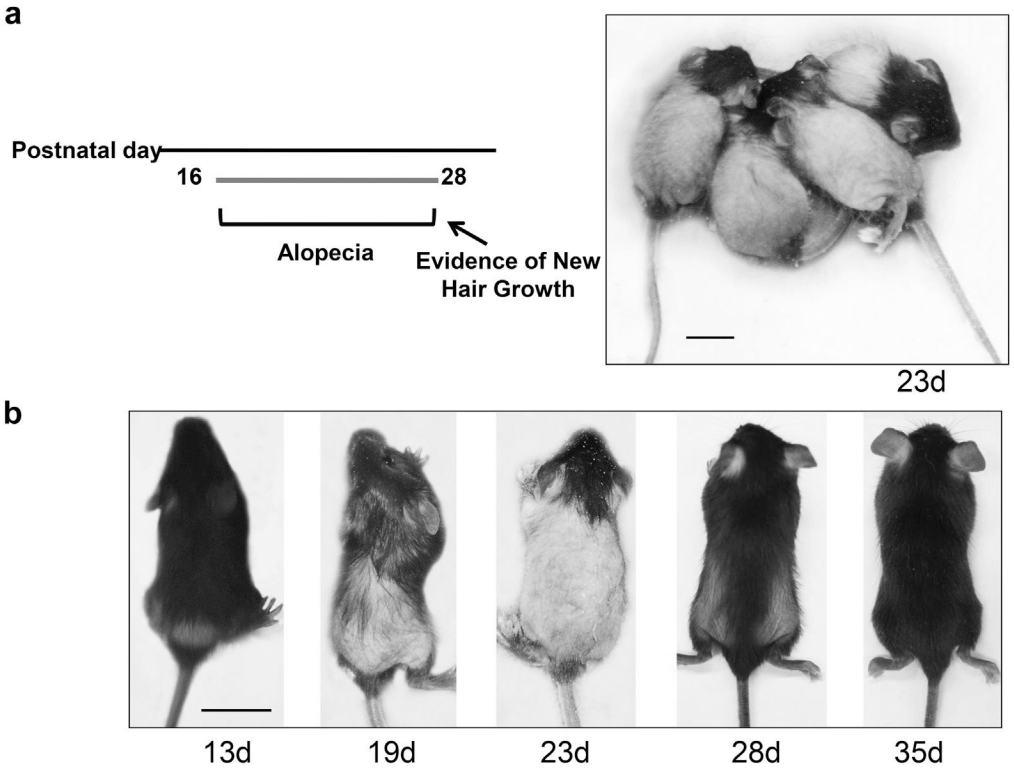

Figure 1. Development of alopecia.

In the absence of calbindin-D_{9k}, maternal deprivation of vitamin D combined with low dietary calcium (0.47%) affects postnatal hair follicle development. **(a)** At postnatal day 23, pups nursed by calbindin-D_{9k}–null mothers fed a D−/low-Ca diet have nearly complete hair loss over the trunk with sparing of the head and the base of the tail. **(b)** The progression of the hair cycling defect during lactation (days 13–28) and complete recovery by day 35. Scale bar = 1 cm. Ca, calcium; d, day; D−, vitamin D deficient.

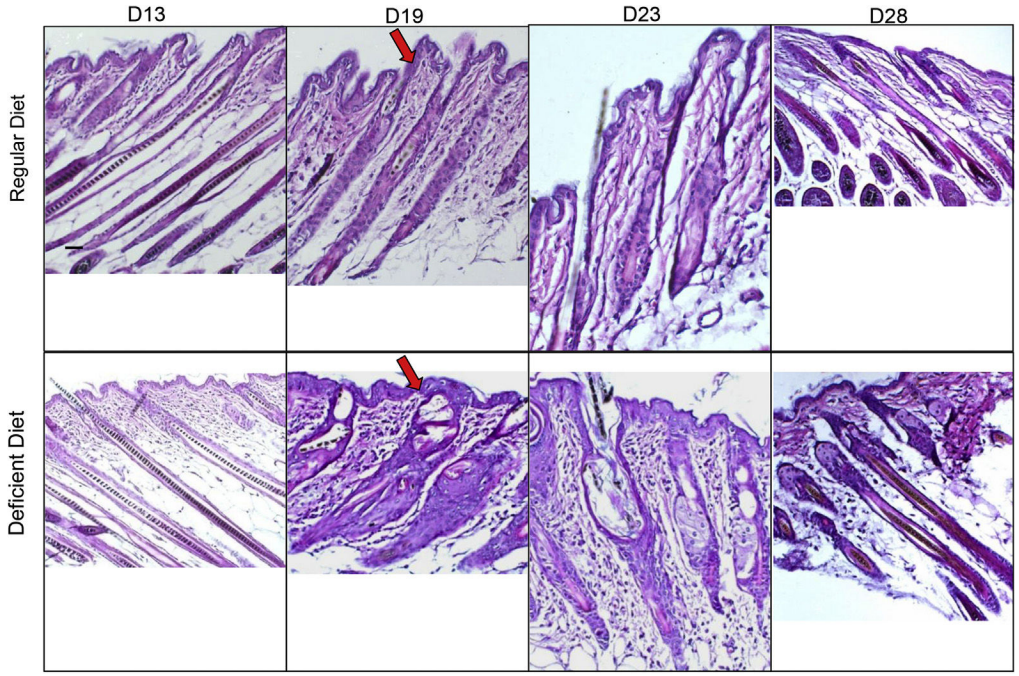

Figure 2. Epidermal morphology.

Hematoxylin and eosin-stained sections of the epidermis collected from pups (postnatal days 13–28) nursed by calbindin-D_{9k}–knockout mothers fed a diet containing vitamin D and calcium (1%) (upper panels) or fed the De−/low-Ca diet (lower panels). By day 19 the follicles become markedly dystrophic, and the hair shafts are lost. This is associated with marked thickening and hyperplasia of the epidermis (red arrow). Scale bar = 20 μm. Ca, calcium; D, day; D−, vitamin D deficient.

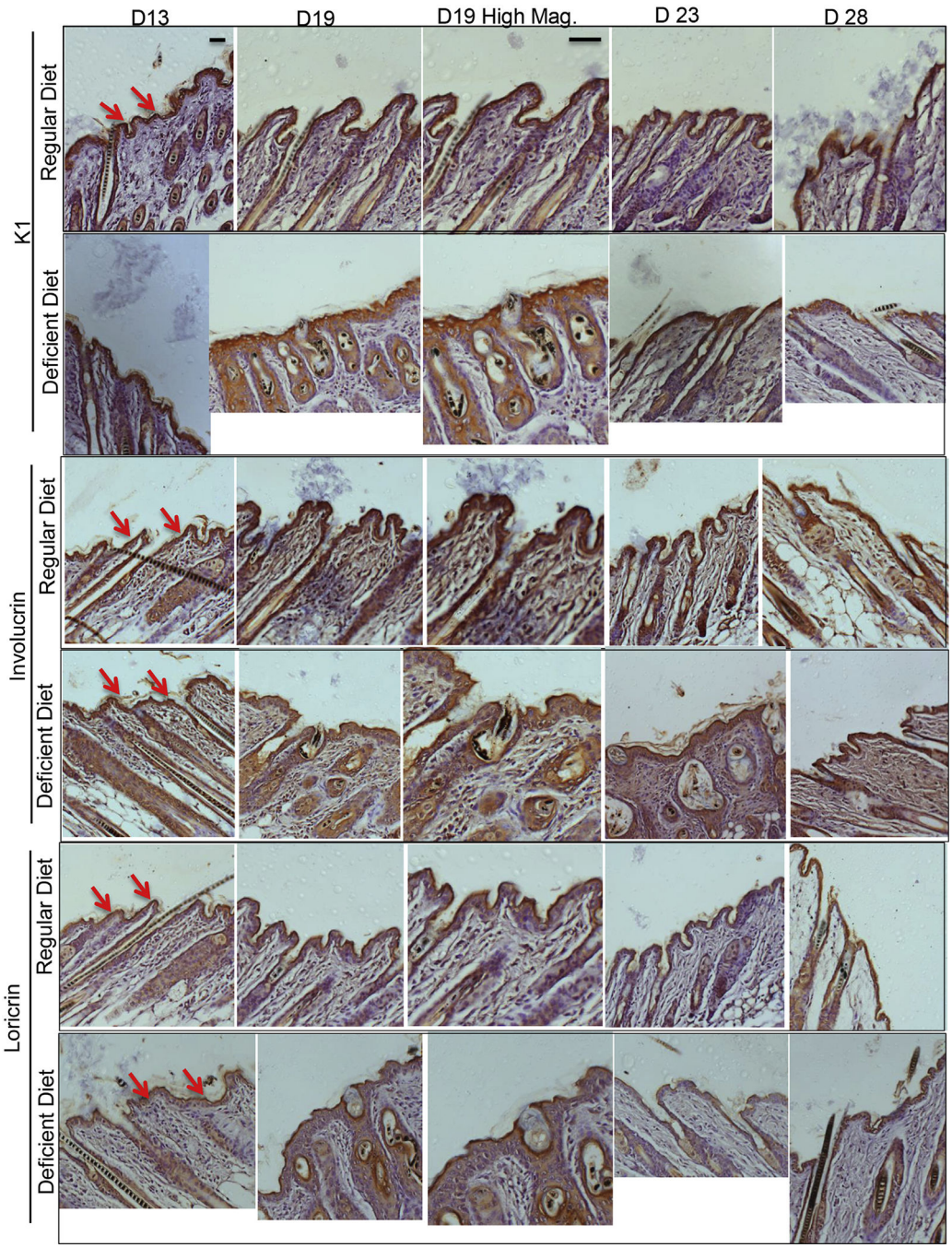

Figure 3. Immunohistochemistry for keratin 1, involucrin, and loricrin.

Representative samples of whole skin taken during days 13–28 from pups nursed by calbindin-D_{9k}–knockout mothers on the regular diet (upper panels) or D−/low-Ca diet (lower panels) (see legend to Figure 2) were immunoreacted with antibodies for keratin 1, involucrin, or loricrin. Higher magnifications of the immunohistochemistry results at 19 days are also shown. The red arrows point to the epidermis. Scale bars = 20 μm. Ca, calcium; D, day; D−, vitamin D deficient; K1, keratin 1.

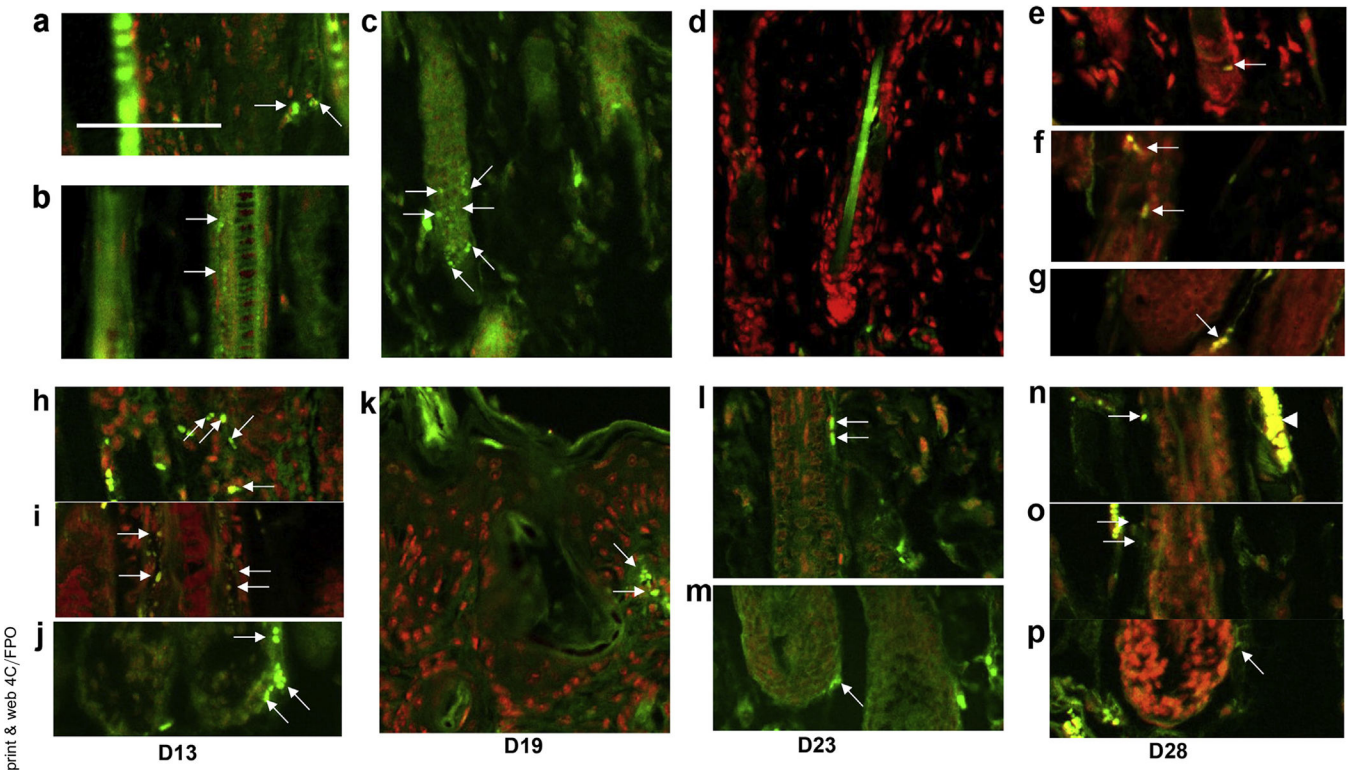

Figure 4. Impact of a D−/low-Ca diet on apoptosis during the first hair follicle cycle.

Apoptotic cells in the hair follicles are detected using TUNEL staining (green) in mice raised on (**a–g**) a normal diet (ND) and (**h–p**) a D−/low-Ca diet (DD) at the indicated times. Cell nuclei were stained with propidium iodide (red). The TUNEL-positive cells are indicated by arrows. Some TUNEL-positive cells (as in eeg) appear yellow in sections with a higher ratio of red/green fluorescence. The arrow head in **n** indicates red blood cells inside a blood vessel. (**a, b**) ND, day 13. (**h, j**) DD, day 13. (**a, h**) Bulge/isthmus region. (**b, i**) Central inner root sheath region. (**j**) DD, hair bulb region. (**c**) ND, epithelial strand region, day 19. (**k**) DD, dystrophic hair follicle, day 19. (**d**) ND, day 23. (**l–m**) DD, day 23. (**l**) Central inner root sheath region. (**m**) hair bulb region. (**e–g**) ND, day 28. (**n–p**) DD, day 28. (**e, n**) Bulge/isthmus region. (**f, o**) Central inner root sheath region. (**g, p**) Hair bulb region. Scale bar = 50 μm. Table 1 shows results from at least five sections of each region. Ca, calcium; D, day; D−, vitamin D deficient.

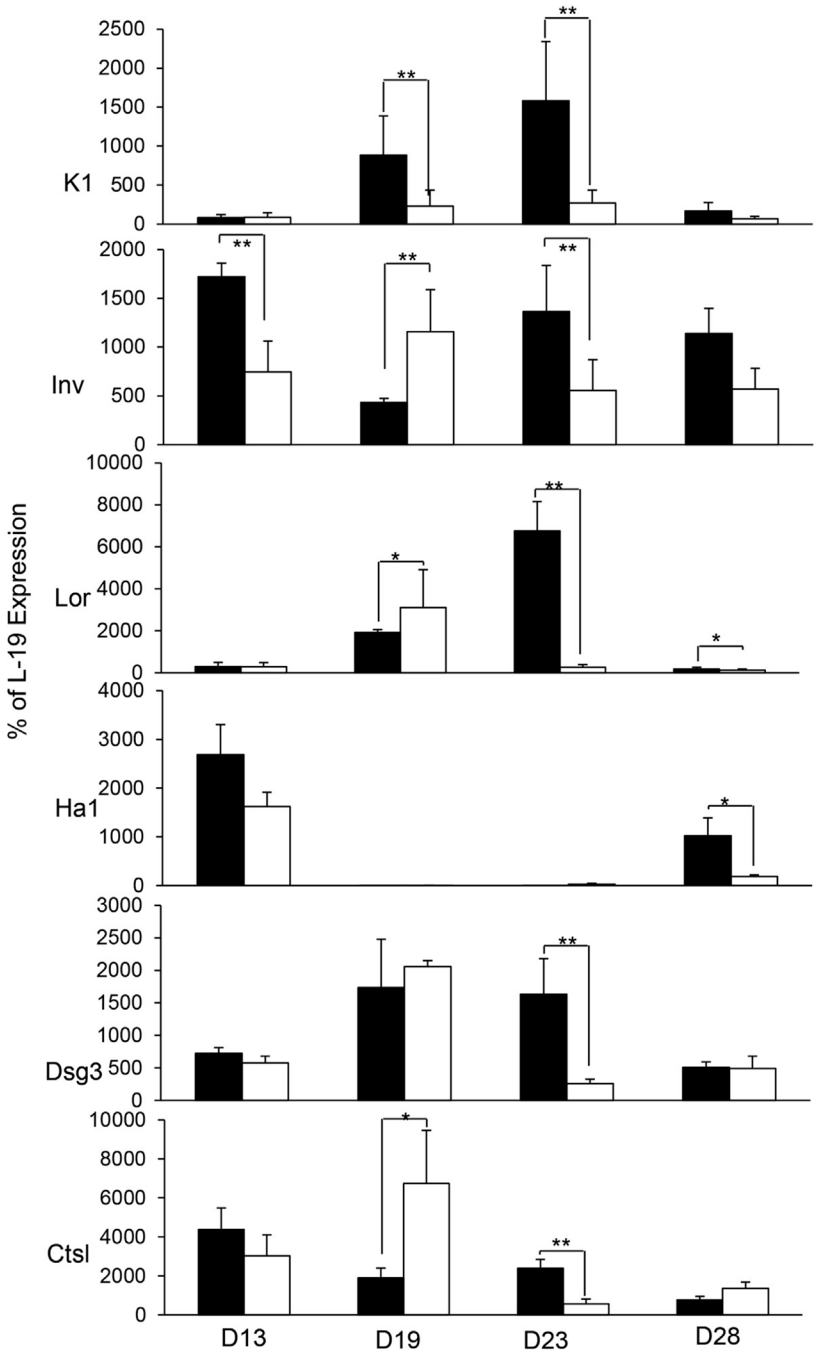

Figure 5. Messenger RNA levels of differentiation markers.

The messenger RNA levels for keratin 1, involucrin, loricrin, Ha1, cathepsin L, and desmoglein 3 were measured by quantitative real-time reverse transcriptase PCR in skin from pups nursed by calbindin-D_{9k}–knockout mothers on a D−/low-Ca diet (red bar) or D+/Ca+ diet (blue bar) during days 13–28 (see Figure 2 legend). (n = 3 mice per time point). The data are expressed as percentage of L19, the messenger RNA for a 60S ribosomal protein used as a loading marker. Error bars include standard deviation. *P < 0.05, **P <

0.01. Ca, calcium; Ctsl, cathepsin L; D−, vitamin D deficient; D+, vitamin D replete; Dsg3, desmoglein 3; Inv, involucrin; K1, keratin 1; Lor, loricrin.

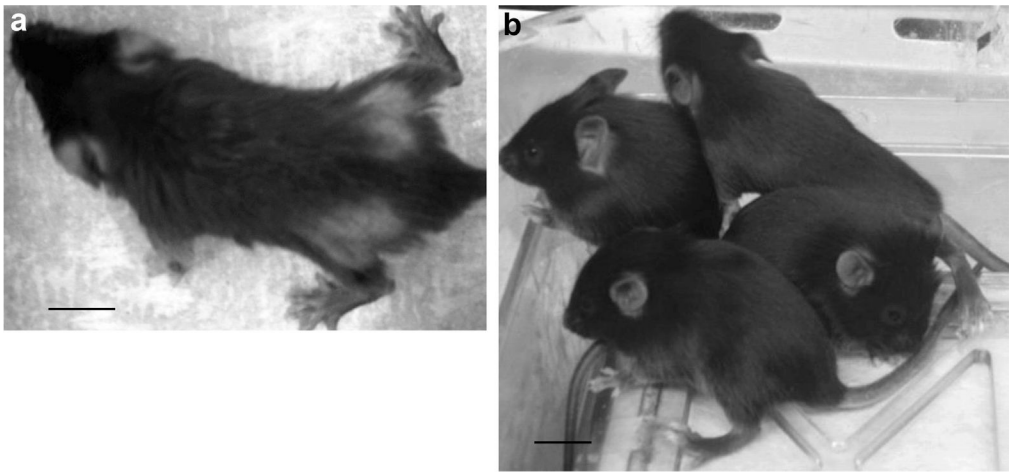

Figure 6. Rescue of the alopecia phenotype.

(a) Feeding the calbindin-D_{9k}–knockout mothers a vitamin D-deficient/high-calcium (2%), high-lactose (20%) diet during lactation (from birth to postnatal day 28) produced a less severe phenotype in the pups as shown by alopecia restricted to a patch on the back (pup at postnatal day 21). **(b)** Fostering pups to calbindin-D_{9k}–knockout mothers fed a diet containing vitamin D and calcium (1%) immediately after birth resulted in the complete rescue of the alopecia phenotype (pups at postnatal day 21). Scale bar = 1 cm.

Table 1.

Number of TUNEL-positive cells/hair follicle[1]

	Day 13		Day 19		Day 23		Day 28	
	Regular	Deficient	Regular	Deficient[2]	Regular	Deficient	Regular	Deficient
Bulge/isthmus	2.7 ± 0.5	3.5 ± 0.6	5.0 ± 0.8	—	< 1.0	1.3 ± 0.5	1.3 ± 0.3	1.3 ± 0.6
Central inner root sheath	4.7 ± 0.6	6.8 ± 1.7	< 1.0	—	< 1.0	2.8 ± 1.0	2.6 ± 0.5	< 1.0
Proximal bulb	< 1.0	4.0 ± 2.0	< 1.0	—	< 1.0	3.8 ± 0.9	< 1.0	< 1.0
Epithelial strand	NA	NA	10.8 ± 2.2	—	NA	NA	NA	NA
Total number	7.4 ± 1.1	14.3 ± 3.7	15.8 ± 3.3	6.5 ± 2.3		7.9 ± 2.4	3.9 ± 0.8	1.3 ± 0.6

Abbreviation: NA, not applicable.

[1]Results are assessed by the number of TUNEL-positive cells in the different regions of each hair follicle in the regular- and low-calcium diet (deficient-treated mice at the times evaluated histologically for loss and recovery of alopecia. Results are expressed as mean ± standard deviation per hair follicle.

[2]Day 19 deficient diet group had no definable hair follicle regions because of the dystrophic histology. Only total TUNEL-positive cell number is shown for this group.