HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2013 April 01.

Published in final edited form as:

J Invest Dermatol. 2012 October ; 132(10): 2350–2359. doi:10.1038/jid.2012.159.

Ablation of the calcium-sensing receptor in keratinocytes impairs epidermal differentiation and barrier function

Chia-Ling Tu[1], **Debra Crumrine**[2], **Mao-Qiang Man**[2], **Wenhan Chang**[1], **Hashem Elalieh**[1], **Michael You**[1], **Peter M. Elias**[2], and **Daniel D. Bikle**[1]

[1]Endocrine Unit, Veteran Affairs Medical Center and University of California, San Francisco, California, USA

[2]Dermatology Service, Veteran Affairs Medical Center and University of California, San Francisco, California, USA

Abstract

The calcium-sensing receptor (CaR) plays an essential role in mediating Ca^{2+}-induced keratinocyte differentiation *in vitro*. In this study, we generated keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice to investigate the function of the CaR in epidermal development *in vivo*. ^{Epid}CaR-/- mice exhibited a delay in permeability barrier formation during embryonic development. Ion capture cytochemistry detected the loss of the epidermal Ca^{2+} gradient in the ^{Epid}CaR-/- mice. The expression of terminal differentiation markers and key enzymes mediating epidermal sphingolipid transport and processing in the ^{Epid}CaR-/- epidermis was significantly reduced. The ^{Epid}CaR-/- epidermis displayed a marked decrease in the number of lamellar bodies and lamellar body secretion, thinner lipid-bound cornified envelopes and a defective permeability barrier. Consistent with *in vivo* results, epidermal keratinocytes cultured from ^{Epid}CaR-/- mice demonstrated abnormal $Ca^{2+}{}_I$ handling and diminished differentiation. The impairment in epidermal differentiation and permeability barrier in ^{Epid}CaR-/- mice maintained on a low calcium (0.02%) diet is more profound and persistent with age then in ^{Epid}CaR-/- mice maintained on a normal calcium (1.3%) diet.

Deleting CaR perturbs the epidermal Ca^{2+} gradient and impairs keratinocyte differentiation and permeability barrier homeostasis, indicating a key role for the CaR in normal epidermal development.

Address correspondence to Dr. Chia-Ling Tu, Endocrine Unit (111N), VA Medical Center, 4150 Clement Street, San Francisco, California 94121. Tel: (415) 7502089; Fax: (415) 7506929; chia-ling.tu@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 http://www.nature.com/jid

Introduction

The mammalian epidermis is a highly organized, stratified squamous epithelium consisting of basal, spinous, granular, and cornified keratinocyte layers. Each layer is defined by distinctive morphologic and biochemical features indicative of its state of differentiation (Fuchs, 1990). After mitosis in the basal layer, keratinocytes differentiate progressively across the epidermis toward the stratum corneum (SC). Keratinocytes at each differentiation stage express distinctive marker genes. Keratins are predominantly expressed by basal (K5, K14) and spinous (K1, K10) keratinocytes (Eichner *et al.*, 1986). Whereas spinous keratinocytes start to produce transglutaminase and involucrin, granular keratinocytes generate loricrin and filaggrin (Hohl, 1990; Rice and Green, 1979). Keratinocytes in the stratum granulosum (SG) also synthesize sphingolipid precursors and store them in lamellar bodies (LB), lipid-filled membrane structures that eventually fuse with the plasma membrane to release their contents into the extracellular space at the SG/SC interface. These lipid precursors are then enzymatically processed and incorporated into the lipid lamellar membranes (Holleran *et al*., 2006). In corneocytes, CE protein precursors are extensively cross-linked to form the CE (Thacher and Rice, 1985). Subsequently, CE and lipid lamellar membranes encase the keratin-filled corneocytes to form the permeability barrier. Moreover, epidermal LBs deliver a family of lipids and peptides that display antimicrobial activity and deposit them in the extracellular matrix in the SC (Aberg *et al*., 2007), contributing to the antimicrobial defense of the epidermis (Elias, 2007).

Extracellular calcium (Ca^{2+}_o) plays a critical role in keratinocyte differentiation (Eckert, 1989; Menon *et al.*, 1992), as elevated $[Ca^{2+}]_o$ triggers an increase in the level of intracellular free Ca^{2+} (Ca^{2+}_i), which subsequently promotes cell differentiation (Bikle *et al.*, 1996; Sharpe *et al.*, 1989). The progressive increases in Ca^{2+}_i in response to Ca^{2+}_o during keratinocyte differentiation are physiologically relevant, as a steep Ca^{2+} gradient can be found in the epidermis increasing from the basal layer to the stratum granulosum (SG) (Menon *et al.*, 1985). Formation of the Ca^{2+} gradient coincides with key developmental milestones of barrier formation and development of SC (Elias *et al.*, 1998). Perturbation of this Ca^{2+} gradient by barrier disruption or iontophoresis results in increased proliferation and disarray of the differentiated cell layers (Lee *et al.*, 1994; Menon *et al.*, 1994). Conversely, changes in Ca^{2+}_o level in the outer epidermis *in vivo*, following barrier perturbation, directly regulate lamellar body secretion and the expression of differentiation markers (Lee *et al.*, 1994; Lee *et al.*, 1992). These data support a pivotal role for Ca^{2+} in epidermal differentiation and barrier homeostasis.

In keratinocytes, the Ca^{2+}_i response to Ca^{2+}_o resembles that in parathyroid cells, which sense Ca^{2+}_o via the Ca^{2+}-sensing receptor (CaR), a member of family C of the G-protein coupled receptor (GPCR) superfamily (Brown and MacLeod, 2001). CaR is expressed predominantly in the suprabasal keratinocyte layers than in basal layer (Komuves *et al.*, 2002). Previous studies on cultured human keratinocytes demonstrate that mobilization of intracellular Ca^{2+}_i and E-cadherin-mediated cell-cell adhesion are two pathways critical for promoting cell differentiation in response to extracellular Ca^{2+} (Ca^{2+}_o), and that the CaR plays an obligatory role in regulating these processes (Tu *et al.*, 2001; Tu *et al.*, 2008). CaR mediates the Ca^{2+}_o-activation of E-cadherin pathways by promoting the translocation of E-

cadherin and its upstream effector Rho A to the plasma membrane (Tu *et al.*, 2011). Inhibiting CaR expression *in vitro* blocks the $Ca^{2+}{}_o$-induced formation of the E-cadherin/catenin adhesion complex (Tu *et al.*, 2008).

To determine definitively the role of CaR in epidermal development, we have generated keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice by breeding floxed CaR (CaRfl/fl) mice (Chang *et al.*, 2008) with mice expressing Cre recombinase under the control of the human keratin 14 (K14) promoter that targets cells in the stratum basale (SB) of the epidermis (Vasioukhin *et al.*, 1999). In contrast to the early death (mostly 5-7 days after birth) in mice lacking the full-length CaR (Ho *et al.*, 1995) precluding detailed analysis of the skin phenotype, ^{Epid}CaR-/- mice have no growth impediment or metabolic derangement, allowing us to examine the role of the epidermal CaR in mediating $Ca^{2+}{}_o$-induced signaling responses, cell differentiation, and permeability barrier function during both development and adult life.

Results

Loss of epidermal Ca^{2+} gradient in keratinocyte-specific CaR knockout mice

Keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice were generated by breeding floxed CaR (CaRfl/fl) mice with transgenic mice expressing Cre recombinase under the control of the human keratin 14 (K14) promoter (Figure 1a). All mice were maintained on a regular (normal Ca^{2+}) diet containing 1.3% Ca^{2+} unless otherwise specified. By PCR analyses of genomic DNA from different tissues isolated from 3-day-old ^{Epid}CaR-/- mice, we confirmed that excision of exon 7 only occurred in the epidermis but not in other tissues surveyed or in the epidermis of control mice (CaRfl/fl littermates lacking the Cre recombinase) (Figure 1b). QPCR analysis of the epidermal RNA using primers targeting exon 7 showed that the level of CaR mRNA was reduced by 92% in the epidermis of ^{Epid}CaR-/- compared to controls (Figure 1c). Immunohistochemical staining using an antibody (Chang *et al.*, 1998) against an epitope (amino acids 1043-1057) in the C-terminal tail of CaR also demonstrated a considerable decrease in CaR protein throughout all cell layers in ^{Epid}CaR-/- epidermis as compared to controls (Figure 1d). ^{Epid}CaR-/- mice and control littermates displayed no significant difference in growth (Figure S7) and gross appearance over the first year of life.

Ion capture cytochemistry and electron microscopic studies were performed to examine the impact of CaR knockout on the epidermal Ca^{2+} gradient. There was a steep Ca^{2+} gradient (Figure 2a), increasing from SB to SG in the epidermis of 10-week-old control mice, reaching the highest level in the uppermost SG. In the ^{Epid}CaR-/- epidermis, Ca^{2+} in the outer epidermis was reduced and the Ca^{2+} gradient was lost (Figure 2a). Measurement of cytosolic Ca^{2+} of epidermal keratinocytes derived from 5-day-old mice revealed a blunted $Ca^{2+}{}_i$ response to $Ca^{2+}{}_o$ in ^{Epid}CaR-/- cells. As shown in Figure 2b, raising $[Ca^{2+}]_o$ from 0.03 to 2 mM elicited a robust increase in $[Ca^{2+}]_i$ in control keratinocytes from 174 ± 10 to a peak of 833 ± 32 nM (mean ± SD; n=50), whereas ^{Epid}CaR-/- keratinocytes displayed a reduced $Ca^{2+}{}_i$ response to elevated $[Ca^{2+}]_o$ (from 75 ± 8 to 195 ± 23 nM; n=44). The blunted $Ca^{2+}{}_i$ response was correlated with a decrease in the $Ca^{2+}{}_i$ pool, as revealed by ionomycin administration in the absence of $Ca^{2+}{}_o$ (Figure 2c). Ionomycin (2 μM) induced Ca^{2+} release from internal stores and, as a result, a rise in $Ca^{2+}{}_i$ (increased from 201 ± 22 to

347 ± 34 nM, n=24) in control keratinocytes. ^{Epid}CaR-/- keratinocytes exhibited a substantial reduction of the rise in Ca^{2+}_i (from 155 ± 20 to 203 ± 26 nM, n=25) (Figure 2c). These results indicated that the loss of the epidermal Ca^{2+} gradient in ^{Epid}CaR-/- mice might be attributed to the defect in Ca^{2+}_i homeostasis in keratinocytes.

Increased cell proliferation and decreased differentiation gene expression in ^{Epid}CaR-/- epidermis

To determine whether CaR knockout affects epidermal differentiation, we examined the expression of CE precursors involucrin, loricrin and filaggrin and the cross-linking enzyme transglutaminase (TG) 1 in the epidermis of 3-day-old ^{Epid}CaR-/- mice and their control littermates. QPCR demonstrated a 30-50% decrease in their mRNA levels in the ^{Epid}CaR-/- (KO) epidermis as compared to controls (Figure 3a), whereas the early differentiation marker keratin 1 and the basal cell marker keratin 5 were not affected. Similar results were observed in skin from 10-week-old mice (data not shown). No change in the expression of keratin 6, a hyperproliferation marker, was detected in epidermis of ^{Epid}CaR-/- mice as compared to control (data not shown). Similarly, immunohistochemical staining (Figure 3b), and immunoblotting (Figure 3c) analyses showed the decline in the protein level of these differentiation markers in ^{Epid}CaR-/- epidermis. To verify that the defect in differentiation was a direct consequence of CaR knockout in keratinocytes, epidermal keratinocytes were isolated from the back skin of 5-day-old ^{Epid}CaR-/- and control mice and cultured in medium containing 0.05 mM Ca^{2+}. $CaCl_2$ (0.15 or 1.05 mM) was added to the culture to induce differentiation. Consistent with the *in vivo* results, Ca^{2+}-induced expression of terminal differentiation markers in the ^{Epid}CaR-/- epidermal keratinocytes was considerably reduced as compared to control cells (Figure S1).

To investigate whether the CaR regulates keratinocyte proliferation, we compared the number of proliferating cells in the epidermis of 7-day-old ^{Epid}CaR-/- (CaR-KO) and control littermates by immunohistochemical staining for proliferating cell nuclear antigen (PCNA, Figure S2c). The quantified data shown in Figure S2a indicated a moderate (~25%), yet significant, increase in cell proliferation in ^{Epid}CaR-/- epidermis. Isolated ^{Epid}CaR-/- keratinocytes also demonstrated a significant increase in proliferation in the presence of 0.05 mM Ca^{2+} (Figure S2b). However, hematoxylin and eosin (H&E) staining of the skin of 3-day-old mice showed that the number and organization of nucleated keratinocyte layers in ^{Epid}CaR-/- epidermis were normal as compared to control littermates (Figure S2d).

Our earlier studies indicate that the CaR is a positive regulator of E-cadherin-mediated cell-cell adhesion that plays key roles in epidermal differentiation (Perez-Moreno *et al.*, 2003; Tu *et al.*, 2008). To investigate whether E-cadherin-mediated cell-cell adhesion is perturbed by CaR ablation, we isolated keratinocytes from 7-day-old ^{Epid}CaR-/- and control mice and cultured them in medium containing 0.05 mM Ca^{2+}. After the cultures reached ~90% confluence, 2 mM Ca^{2+} was added to induce intercellular adhesion. As shown in Figure S3, high $[Ca^{2+}]_o$ induced association of β-catenin and E-cadherin at cell-cell junctions in control keratinocytes but not in ^{Epid}CaR-/- cells. This was accompanied by ineffectual Ca^{2+}_o-induced recruitment of upstream effectors, GTPase Rho and tyrosine kinase Fyn, with E-cadherin to the cell-cell contacts in ^{Epid}CaR-/- keratinocytes (Figure S3).

Delayed establishment of the permeability barrier and altered epidermal sphingolipid metabolism in ^{Epid}CaR-/- mice

To determine whether CaR deletion affects epidermal sphingolipid homeostasis, which is critical for maintaining the permeability barrier, we first compared the abundance of the lamellar bodies (LB) in the epidermis of 10-week-old ^{Epid}CaR-/- and control littermates. The number of LB in the keratinocytes in the upper SG and their secretion at the SG/stratum corneum (SC) junction were noticeably reduced in ^{Epid}CaR-/- epidermis (Figure 4a). Furthermore, measurement of the thickness of the lipid-bound CE in the proximal corneocytes in ^{Epid}CaR-/- epidermis revealed a 35% reduction (9.5 ± 0.85 nm; n=30) as compared to the controls (14.8 ± 0.8 nm; n=30) (Figure 4b). These observations suggested an altered sphingolipid metabolism in ^{Epid}CaR-/- epidermis. We then compared the expression of several key enzymes that mediate sphingolipid biosynthesis, transport and processing in the epidermis of 3-day-old ^{Epid}CaR-/- mice and their control littermates by qPCR. Except for glucosylceramide synthase (UGCG), the expression of enzymes mediating sphingolipid biosynthesis (serine palmitoyl transferase SPT2, fatty acid elongase ELOVL4, C4-5 trans desaturases DES1 and DES2) was largely not affected by CaR knockout. On the contrary, the mRNA levels of the lipid transporter ABCA12 and sphingolipid processing enzymes β-glucocerebrosidase (GCB) and sphingomyelinase (SMase) in the epidermis of ^{Epid}CaR-/- (KO) mice were reduced 30-40% as compared to controls (Figure 4c), indicating a down-regulated sphingolipid transport and processing. Similar results were found in 10-week-old ^{Epid}CaR-/- mice (data not shown).

We next examined the integrity of the epidermal permeability barrier of the ^{Epid}CaR-/- mice and their control littermates by lanthanum perfusion assays, in which lanthanum was allowed to infiltrate from SB toward SG through the intercellular spaces of neighboring keratinocytes. In the control epidermis, an intact barrier stops the infiltration of lanthanum at the SG/SC interface (Figure 5a). In contrast, lanthanum diffused into the extracellular space between SG and SC in the ^{Epid}CaR-/- epidermis (Figure 5a), indicating a defective barrier.

To determine whether CaR ablation affects establishment of the permeability barrier during embryonic development, we stained E15.5, E16.5, E17.5 and E18.5 embryos of ^{Epid}CaR-/- mice and control littermates with 0.0125% toluidine blue. At E15.5, both control and ^{Epid}CaR-/- embryos were stained due to immature barrier function, although ^{Epid}CaR-/- embryos displayed slightly higher susceptibility to dye penetration than controls (Figure 5b). As embryos aged, specific skin sites acquire impermeable characteristics (Hardman *et al.*, 1998). At E16.5, control embryos showed large areas on the back skin resistant to stain, whereas ^{Epid}CaR-/- embryos remained almost completely stained (Figure 5c), indicating a delay in the formation of the permeability barrier in these embryos. Deferred barrier establishment were more apparent in the E17.5 ^{Epid}CaR-/- embryos. At this age, nearly the entire surface of control embryos became stain-resistant, while large areas of skin in ^{Epid}CaR-/- embryos retained permeability to dye penetration (Figure 5d). Nonetheless, permeability barriers were fully developed in the E18.5 ^{Epid}CaR-/- and control embryos (data not shown).

Impaired epidermal barrier functions in ^{Epid}CaR-/- mice under dietary Ca^{2+} restriction

To examine whether the CaR ablation associated-abnormalities in structure leads to aberrant permeability barrier function, we measured the trans-epidermal water loss (TEWL) in the skins of 4-month-old ^{Epid}CaR-/- and control mice before and after acute barrier disruption by sequential tape stripping. When the mice were maintained on a regular (normal Ca^{2+}) diet containing 1.3% Ca^{2+}, the basal TEWL rate (Figure S4a) tended to be higher and the kinetics of permeability barrier recovery after tape stripping tended to be slower (Figure S4b) in skins of ^{Epid}CaR-/- mice than controls, but the differences were not significant. However, as shown in Figure 6b, when these mice were maintained on a low Ca^{2+} (0.02%) diet, ^{Epid}CaR-/- mice manifested a significant delay in barrier recovery (33 and 47% recovery 3 and 6 hours, respectively, after barrier disruption versus 47 and 71% recovery in control skins), although their increased basal TEWL rate compared to controls did not attain significance (Figure 6a).

The expression patterns of terminal differentiation markers (Figure S5a) and enzymes mediating sphingolipid metabolism (Figure S5b) in 4-month-old mice raised on a low Ca^{2+} (0.02%) diet and a normal (1.3%) Ca^{2+} diet showed that dietary Ca^{2+} restriction further decreased the production of CE precursors and down-regulated sphingolipid metabolism in ^{Epid}CaR-/- mice (a 60-90% decrease in their mRNA levels as compared to controls), contributing to the defects in the barrier functions. On the contrary, when these mice were raised on the 1.3% Ca^{2+} diet, the gene expression was only slightly reduced in the ^{Epid}CaR-/- mice as compared to controls. This may explain why the basal barrier function and its recovery after tape stripping in these mice were not significantly different from control littermates when there is enough dietary Ca^{2+}.

Innate immune defense against microbial infection is another important epidermal function that is highly dependent on the integrity of SC and permeability barrier (Elias, 2007). Injury stimulates the production of soluble cytokines, such as IL-1 and IL-6, which mediate an inflammatory response, and activate the microbial pattern recognition receptors Toll-like receptor 2 (TLR2) and its co-receptor CD14 to increase the production of antimicrobial peptides (Schauber *et al.*, 2007). To evaluate whether loss of CaR affects the epidermal innate immune response, we compared the expression of TLR2, CD14, IL-1b, IL-6 and the anti-microbial peptide cathelicidin (camp) in the wounds and uninjured skin of 4-month-old ^{Epid}CaR-/- mice and their control littermates maintained on a low (0.02%) Ca^{2+} diet. As shown in Figure 6c, the injury-induced expression of these innate immune recognition genes and cytokines was significantly reduced (45-60% of the control) in ^{Epid}CaR-/- skin. Previous studies have implicated 1,25-dihydroxyvitamin D3 ($1,25D_3$) as a positive regulator of the innate immune response via increasing the expression of TLR2 and CD14 and the production of anti-microbial peptides (Schauber *et al.*, 2007; Weber *et al.*, 2005). We next assessed the levels of 25-hydroxyvitamin D3 ($25D_3$) 1-α-hydroxylase (CYP27B1), the enzyme responsible for converting $25D_3$ to active $1,25D_3$, and the $1,25D_3$ receptor (VDR) by qPCR. Figure 6d demonstrated that the expression of these modulators of $1,25D_3$ production and action were also markedly diminished in ^{Epid}CaR-/- skin. Therefore, the reduced innate immune response in ^{Epid}CaR-/- mice may be attributed to the down-regulation of $1,25D_3$/VDR-dependent signaling. Similar to the lack of effect of the CaR

ablation on the permeability barrier in mice raised on a normal Ca^{2+} diet, however, the changes in the innate immune response and expression of CYP27B1 and VDR in ^{Epid}CaR-/- mice under dietary Ca^{2+} restriction were not found in mice on the 1.3% Ca^{2+} diet (Figure S6).

Discussion

The CaR has emerged as a pivotal mediator by which keratinocytes respond to changes in Ca^{2+} levels. In the present study, we generated keratinocyte-specific CaR knockout mice to investigate the role of CaR in epidermal development and barrier function. Prominent epidermal characteristics of ^{Epid}CaR-/- mice include the loss of the Ca^{2+} gradient, inhibited expression of differentiation-related genes and impaired barrier function. ^{Epid}CaR-/- mice differ in their epidermal phenotype compared to mice lacking the full-length CaR (Ho *et al.*, 1995; Oda *et al.*, 2000), which also display abnormal differentiation of the epidermis. The epidermis of these global "CaR knockout" mice exhibits a steeper Ca^{2+} gradient, excess and premature LB secretion (Komuves *et al.*, 2002) and reduced nucleated keratinocyte layers (Oda *et al.*, 2000), as opposed to the loss of Ca^{2+} gradient, diminished LB secretion, but relatively organized cellular layers in ^{Epid}CaR-/- mice. These differences may be due to incomplete CaR deletion in the homozygous global CaR knockout in that these mice continue to express an alternatively spliced variant of CaR (Oda *et al.*, 2000; Rodriguez *et al.*, 2005), which may compensate for the loss of the full-length CaR. Additionally, they suffer severe hyperparathyroidism, hypophosphatemia and hypercalcemia. Since LB secretion and barrier homeostasis are closely regulated by Ca^{2+} (Elias and Feingold, 2001; Elias *et al.*, 1998), the hypercalcemia resulting from the uncontrolled PTH secretion may alter the Ca^{2+} gradient and cause the excess LB secretion. The accentuated epidermal Ca^{2+} gradient may also change the cellular morphology and organization of the nucleated keratinocytes in the epidermis of these mice.

The effect of CaR depletion on differentiation is due, at least in part, to the inability of $Ca^{2+}{}_i$ stores to properly handle $Ca^{2+}{}_i$ mobilization as seen in keratinocytes of Hailey-Hailey disease patients, which are deficient in the Golgi Ca^{2+}-ATPase, SPCA1, have depleted $Ca^{2+}{}_i$ stores, and do not respond to elevated $[Ca^{2+}]_o$ (Behne *et al.*, 2003; Hu *et al.*, 2000). The CaR localization in keratinocytes is predominantly intracellular and to a lesser extent in the plasma membrane (Tu *et al.*, 2007). It is likely that the CaR on the cell surface could readily sense changes in $[Ca^{2+}]_o$, but the function of the CaR in the intracellular compartments is unclear. The shared characteristics of CaR-deficient and SPCA1-deficient keratinocytes in $Ca^{2+}{}_i$ handling suggest that the CaR and SPCA1 are parts of a mechanism that transduces $Ca^{2+}{}_o$ signals into cellular responses in keratinocytes. We found that the CaR forms protein complexes with several modulators of the $Ca^{2+}{}_i$ stores and Ca^{2+} channels, i.e. PLCγ1, IP_3R and SPCA1, in the *trans*-Golgi (Tu *et al.*, 2007). The CaR could readily participate and serve as a coordinator of signaling events regulating $Ca^{2+}{}_i$ mobilization due to its intrinsic ability to sense $Ca^{2+}{}_o$ and possibly $Ca^{2+}{}_i$.

Besides modulating Ca^{2+} signaling, the CaR regulates critical steps in E-cadherin-mediated cell-cell adhesion in keratinocytes (Tu *et al.*, 2008). Knockout of CaR *in vivo* down-regulated E-cadherin-mediated signaling as indicated by the greatly reduced E-cadherin/

catenin adhesion complex at cell-cell contacts and decreased membrane-association of E-cadherin with its upstream regulators Rho A and tyrosine kinase Fyn in Ca^{2+}-treated ^{Epid}CaR-/- keratinocytes (Figure S3). The E-cadherin-dependent signaling cascade also functions as a mechanism for sustaining an elevated $Ca^{2+}{}_i$ level, which is critical for keratinocyte differentiation (Xie and Bikle, 2007). As a result of compromised $Ca^{2+}{}_i$ handling and E-cadherin-mediated cell-cell adhesion, loss of CaR in keratinocytes impeded differentiation.

The loss of CaR also affected the establishment and integrity of the permeability barrier (Figures 4 and 5). The thinner lipid-bound CE in corneocytes in ^{Epid}CaR-/- mice was attributed to both the reduced production and secretion of lamellar bodies, owing to down-regulated sphingolipid transport and processing, and decreased levels of CE proteins. Although TEWL in the resting state was comparable in the ^{Epid}CaR-/- mice and their control littermates, the permeability barrier defects in ^{Epid}CaR-/- epidermis were exacerbated when these animals were subjected to dietary Ca^{2+} restriction. Under such a condition, ^{Epid}CaR-/- mice showed a marked retardation in the recovery of the barrier following its disruption (Figure 6b). As the epidermal antimicrobial defense is highly dependent on the permeability barrier status, it was not surprising that the expression of TLR2, CD14 and cathelicidin in response to wounding in ^{Epid}CaR-/- mice on a 0.02% Ca^{2+} diet was attenuated (Figure 6c). VDR null and CYP27B1-deficient mice share similar epidermal phenotypes as ^{Epid}CaR-/- mice: abnormal epidermal Ca^{2+} gradient, decreased LB numbers and secretion, delayed recovery of permeability barrier function, and defective differentiation (Bikle *et al.*, 2004; Oda *et al.*, 2009), indicative of important roles for $1,25D_3$/VDR as well as calcium and CaR in permeability barrier homeostasis. It is known that $1,25D_3$ promotes keratinocyte differentiation via many of the same pathways as Ca^{2+} (Bikle and Pillai, 1993), such as by increasing $Ca^{2+}{}_i$ levels and activating protein kinase C and phospholipase C signaling. $1,25D_3$ augments the sensitivity to the prodifferentiating actions of Ca^{2+} in keratinocytes by increasing the expression of CaR (Ratnam *et al.*, 1999). Conversely, dietary Ca^{2+} supplements up-regulate VDR expression in the epidermis of the vitamin D-deficient rat (Zineb *et al.*, 1998). It is conceivable that $1,25D_3$/VDR-mediated pathways are targets of CaR action for regulating the permeability barrier homeostasis and the innate immune response, and that dietary Ca^{2+} restriction suppressed $1,25D_3$/VDR signaling (Figure 6d) in ^{Epid}CaR-/- epidermis and further impeded the function of the permeability barrier.

It is unlikely that the effect of dietary Ca^{2+} restriction on the barrier function and innate immune response in ^{Epid}CaR-/- mice was due to metabolic derangement because their serum calcium and phosphate levels were comparable to mice on a normal Ca^{2+} diet (Figure S7), although mice on a Ca^{2+}-restricted diet had slightly reduced serum calcium levels ($P<0.02$ in controls and $P=0.08$ in ^{Epid}CaR-/- mice). Likewise, the body size and weight gain of ^{Epid}CaR-/- mice were not significantly different from their control littermates during this period (Figure S8). By comparing the expression pattern of terminal differentiation markers (Figure 3) and enzymes mediating sphingolipid metabolism in 3-day-old (Figure 4) and 4-month-old mice (Figure S5) raised on a normal (1.3%) Ca^{2+} diet, we found that the impairment in gene expression in the ^{Epid}CaR-/- neonates was partially restored in the adult mice (Figure S5), suggesting adaptation when there is enough dietary Ca^{2+}. Conversely,

when these mice were raised on a low (0.02%) Ca^{2+} diet, the expression of differentiation marker genes (Figure S5a) and sphingolipid mediators (Figure S5b) in ^{Epid}CaR-/- mice was further suppressed, contributing to the defects in the barrier functions observed in these mice, indicative of a failure of compensatory mechanisms.

In summary, selective knockout of CaR in keratinocytes resulted in a loss of the epidermal Ca^{2+} gradient, impaired epidermal differentiation and defective permeability barrier. These phenomena are found in other diseases of the skin in which calcium regulation is altered (Hu *et al.*, 2000; Savignac *et al*., 2011). Complementary to the phenotypes of ^{Epid}CaR-/- mice, mice with CaR over-expression in the epidermis display accelerated epidermal differentiation and permeability barrier formation (Turksen and Troy, 2003). These observations clearly support a role for the CaR in regulating epidermal differentiation and barrier homeostasis.

Materials and Methods

Generation of keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice and PCR analysis for Casr gene excision

Generation of floxed CaR (CaR fl/fl) mice was described previously (Chang *et al.*, 2008). Keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice and littermate controls were produced by cross breeding K14Cre+/ CaR fl/fl and CaR fl/fl mice. All mice were maintained on a regular (normal Ca^{2+}) diet containing 1.3% Ca^{2+}, unless specified otherwise, according to protocols approved by the Animal Care Subcommittee, San Francisco Veterans Affairs Medical Center. Genomic DNA from different tissues of ^{Epid}CaR-/- mice and their control littermates were analyzed by PCR using 3 primers (primer 1, 5'-CCTCGAACATGAACAACTTAATTCGG-3'; primer 2, 5'-TGTGACGGAAAACATACTGC-3'; primer 3, 5'-CGAGTACAGGCTTTGATGC-3') targeting *Casr* gene exon 7 (Figure 1a). Whereas primers 2 and 3 detected floxed exon 7; the presence of the exon7-deleted *Casr* allele can only be detected by primers 1 and 3, as the product derived from the floxed *Casr* allele was much larger and amplified poorly under the condition used.

Descriptions of other methods used in this study are available in the Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Michelle Hamilton and Dia Bou Dargham for providing technical support. This work was supported by grants RO1 AR056256 (C L.T), RO1 AG21353 (W.C) and PO1 AR39448 (D.B) from the National Institutes of Health and a Merit Review Award from the Department of Veterans Affairs (D.B).

Abbreviations

Ca^{2+}_o	extracellular Ca^{2+}
Ca^{2+}_i	intracellular Ca^{2+}
CaR	calcium-sensing receptor
TG	transglutaminase
LB	lamellar bodies
CE	cornified envelope
SB	stratum basale
SG	stratum granulosum
SC	stratum corneum
TEWL	trans-epidermal water loss
qPCR	quantitative polymerase chain reaction
$1,25D_3$	1,25-dihydroxyvitamin D3
$25D_3$	25-hydroxyvitamin D3
VDR	$1,25D_3$ receptor

References

Aberg KM, Man MQ, Gallo RL, et al. Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. J Invest Dermatol. 2007; 128:917–25. [PubMed: 17943185]

Behne MJ, Tu CL, Aronchik I, et al. Human keratinocyte ATP2C1 localizes to the Golgi and controls Golgi Ca2+ stores. J Invest Dermatol. 2003; 121:688–94. [PubMed: 14632183]

Bikle DD, Chang S, Crumrine D, et al. 25 Hydroxyvitamin D 1 -Hydroxylase Is Required for Optimal Epidermal Differentiation and Permeability Barrier Homeostasis. J Invest Dermatol. 2004; 122:884–92.

Bikle DD, Pillai S. Vitamin D, calcium, and epidermal differentiation. Endocr Rev. 1993; 14:3–19. [PubMed: 8491153]

Bikle DD, Ratnam A, Mauro T, et al. Changes in calcium responsiveness and handling during keratinocyte differentiation. Potential role of the calcium receptor. J Clin Invest. 1996; 97:1085–93. [PubMed: 8613532]

Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. Physiol Rev. 2001; 81:239–97. [PubMed: 11152759]

Chang W, Pratt S, Chen TH, et al. Coupling of calcium receptors to inositol phosphate and cyclic AMP generation in mammalian cells and Xenopus laevis oocytes and immunodetection of receptor protein by region-specific antipeptide antisera. J Bone Miner Res. 1998; 13:570–80. [PubMed: 9556057]

Chang W, Tu C, Chen TH, et al. The extracellular calcium-sensing receptor (CaSR) is a critical modulator of skeletal development. Sci Signal. 2008; 1:ra1. [PubMed: 18765830]

Eckert RL. Structure, function, and differentiation of the keratinocyte. Physiol Rev. 1989; 69:1316–46. [PubMed: 2678169]

Eichner R, Sun TT, Aebi U. The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. J Cell Biol. 1986; 102:1767–77. [PubMed: 2422179]

Elias PM. The skin barrier as an innate immune element. Semin Immunopathol. 2007; 29:3–14. [PubMed: 17621950]

Elias PM, Feingold KR. Coordinate regulation of epidermal differentiation and barrier homeostasis. Skin Pharmacol Appl Skin Physiol. 2001; 14(Suppl 1):28–34. [PubMed: 11509904]

Elias PM, Nau P, Hanley K, et al. Formation of the epidermal calcium gradient coincides with key milestones of barrier ontogenesis in the rodent. J Invest Dermatol. 1998; 110:399–404. [PubMed: 9540982]

Fuchs E. Epidermal differentiation: the bare essentials. J Cell Biol. 1990; 111:2807–14. [PubMed: 2269655]

Hardman MJ, Sisi P, Banbury DN, et al. Patterned acquisition of skin barrier function during development. Development. 1998; 125:1541–52. [PubMed: 9502735]

Ho C, Conner DA, Pollak MR, et al. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Nat Genet. 1995; 11:389–94. [PubMed: 7493018]

Hohl D. Cornified cell envelope. Dermatologica. 1990; 180:201–11. [PubMed: 2192924]

Holleran WM, Takagi Y, Uchida Y. Epidermal sphingolipids: Metabolism, function, and roles in skin disorders. FEBS Lett. 2006; 580:5456–66. [PubMed: 16962101]

Hu Z, Bonifas JM, Beech J, et al. Mutations in ATP2C1, encoding a calcium pump, cause Hailey-Hailey disease. Nat Genet. 2000; 24:61–5. [PubMed: 10615129]

Koch PJ, de Viragh PA, Scharer E, et al. Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. J Cell Biol. 2000; 151:389–400. [PubMed: 11038185]

Komuves L, Oda Y, Tu CL, et al. Epidermal expression of the full-length extracellular calcium-sensing receptor is required for normal keratinocyte differentiation. J Cell Physiol. 2002; 192:45–54. [PubMed: 12115735]

Lee SH, Elias PM, Feingold KR, et al. A role for ions in barrier recovery after acute perturbation. J Invest Dermatol. 1994; 102:976–9. [PubMed: 8006464]

Lee SH, Elias PM, Proksch E, et al. Calcium and potassium are important regulators of barrier homeostasis in murine epidermis. J Clin Invest. 1992; 89:530–8. [PubMed: 1737844]

Menon GK, Elias PM, Feingold KR. Integrity of the permeability barrier is crucial for maintenance of the epidermal calcium gradient. Br J Dermatol. 1994; 130:139–47. [PubMed: 8123567]

Menon GK, Elias PM, Lee SH, et al. Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. Cell Tissue Res. 1992; 270:503–12. [PubMed: 1486603]

Menon GK, Grayson S, Elias PM. Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. J Invest Dermatol. 1985; 84:508–12. [PubMed: 3998499]

Oda Y, Tu CL, Chang W, et al. The calcium sensing receptor and its alternatively spliced form in murine epidermal differentiation. J Biol Chem. 2000; 275:1183–90. [PubMed: 10625662]

Oda Y, Uchida Y, Moradian S, et al. Vitamin D receptor and coactivators SRC2 and 3 regulate epidermis-specific sphingolipid production and permeability barrier formation. J Invest Dermatol. 2009; 129:1367–78. [PubMed: 19052561]

Ratnam AV, Bikle DD, Cho JK. 1,25 dihydroxyvitamin D3 enhances the calcium response of keratinocytes. J Cell Physiol. 1999; 178:188–96. [PubMed: 10048583]

Rice RH, Green H. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. Cell. 1979; 18:681–94. [PubMed: 42494]

Rodriguez L, Tu C, Cheng Z, et al. Expression and functional assessment of an alternatively spliced extracellular Ca2+-sensing receptor in growth plate chondrocytes. Endocrinology. 2005; 146:5294–303. [PubMed: 16166224]

Savignac M, Edir A, Simon M, et al. Darier disease: a disease model of impaired calcium homeostasis in the skin. Biochim Biophys Acta. 2011; 1813:1111–7. [PubMed: 21167218]

Schauber J, Dorschner RA, Coda AB, et al. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. J Clin Invest. 2007; 117:803–11. [PubMed: 17290304]

Sharpe GR, Gillespie JI, Greenwell JR. An increase in intracellular free calcium is an early event during differentiation of cultured human keratinocytes. FEBS Lett. 1989; 254:25–8. [PubMed: 2776884]

Thacher SM, Rice RH. Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. Cell. 1985; 40:685–95. [PubMed: 2578891]

Tu CL, Chang W, Bikle DD. The extracellular calcium-sensing receptor is required for calcium-induced differentiation in human keratinocytes. J Biol Chem. 2001; 276:41079–85. [PubMed: 11500521]

Tu CL, Chang W, Bikle DD. The role of the calcium sensing receptor in regulating intracellular calcium handling in human epidermal keratinocytes. J Invest Dermatol. 2007; 127:1074–83. [PubMed: 17124506]

Tu CL, Chang W, Bikle DD. The calcium-sensing receptor-dependent regulation of cell-cell adhesion and keratinocyte differentiation requires Rho and filamin A. J Invest Dermatol. 2011; 131:1119–28. [PubMed: 21209619]

Tu CL, Chang W, Xie Z, et al. Inactivation of the calcium sensing receptor inhibits E-cadherin-mediated cell-cell adhesion and calcium-induced differentiation in human epidermal keratinocytes. J Biol Chem. 2008; 283:3519–28. [PubMed: 18065418]

Turksen K, Troy TC. Overexpression of the calcium sensing receptor accelerates epidermal differentiation and permeability barrier formation in vivo. Mech Dev. 2003; 120:733–44. [PubMed: 12834872]

Vasioukhin V, Degenstein L, Wise B, et al. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc Natl Acad Sci U S A. 1999; 96:8551–6. [PubMed: 10411913]

Weber G, Heilborn JD, Chamorro Jimenez CI, et al. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J Invest Dermatol. 2005; 124:1080–2. [PubMed: 15854055]

Xie Z, Bikle DD. The recruitment of phosphatidylinositol 3-kinase to the E-cadherin-catenin complex at the plasma membrane is required for calcium-induced phospholipase C-gamma1 activation and human keratinocyte differentiation. J Biol Chem. 2007; 282:8695–703. [PubMed: 17242406]

Zineb R, Zhor B, Odile W, et al. Distinct, tissue-specific regulation of vitamin D receptor in the intestine, kidney, and skin by dietary calcium and vitamin D. Endocrinology. 1998; 139:1844–52. [PubMed: 9528970]

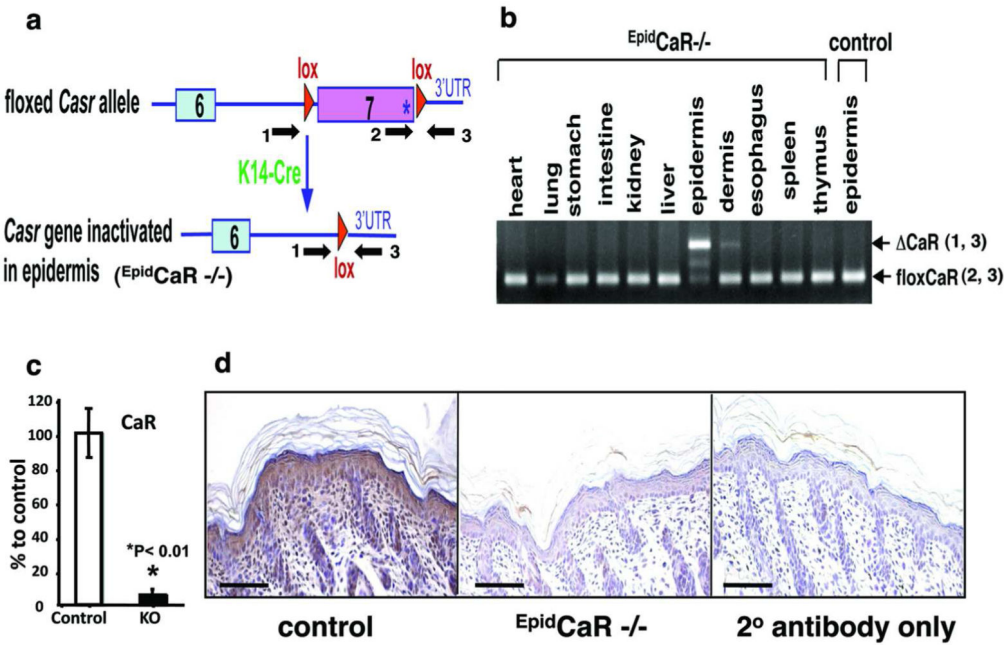

Figure 1. Ablation of the CaR gene, *Casr*, in mouse epidermis

(a) Generation of keratinocyte-specific CaR knockout (^{Epid}CaR-/-) mice. The floxed CaR mice were bred with Cre-expressing transgenic mice (K14-Cre) to delete exon 7 of the *Casr* gene in the epidermis. (b) PCR analyses of genomic DNA from different tissues of 3-day-old ^{Epid}CaR-/- mice using 3 primers (black arrows) targeting exon 7. The DNA fragment amplified from exon7-deleted allele by primers 1 and 3 was detected only in the epidermis. (c) qPCR analysis of epidermal RNA. The level of CaR mRNA in ^{Epid}CaR-/- (KO) epidermis was normalized to that in controls and presented as mean ± SD, n=4. *P<0.01. (d) Immunohistochemical staining of whole skin for CaR. Positive staining sites are visualized as brown. Bar = 25 μm.

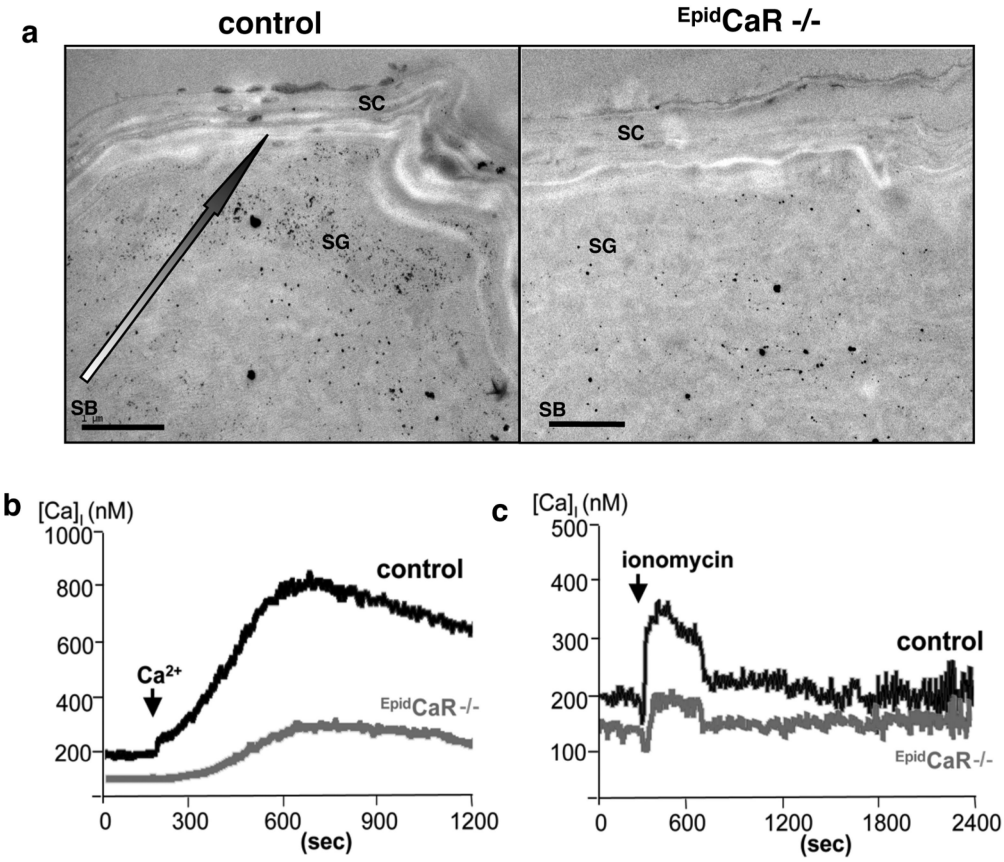

Figure 2. Loss of the epidermal Ca^{2+} gradient in ^{Epid}CaR-/- mice

(a) The epidermal Ca^{2+} level in 10-week-old mice was determined by the density of the electron-dense Ca^{2+} deposits. A steep Ca^{2+} gradient, indicated by a shaded arrow, starting from the stratum basale (SB) and reaching the highest level in the uppermost stratum granulosum (SG), was detected in the epidermis of control but not in ^{Epid}CaR-/- mice. Bar = 1 μm. (b, c) Keratinocytes from 5-day-old ^{Epid}CaR-/- and control mice were loaded with Fura-2. (b) $[Ca^{2+}]_i$ was initially measured in buffer containing 0.03 mM Ca^{2+} and then following the addition of 2 mM Ca^{2+}. (c) $[Ca^{2+}]_i$ was measured in buffer without $Ca^{2+}{}_o$ before and after ionomycin-induced store Ca^{2+} depletion. The data shown represent the average $[Ca^{2+}]_i$ of 25-50 individual keratinocytes during recording.

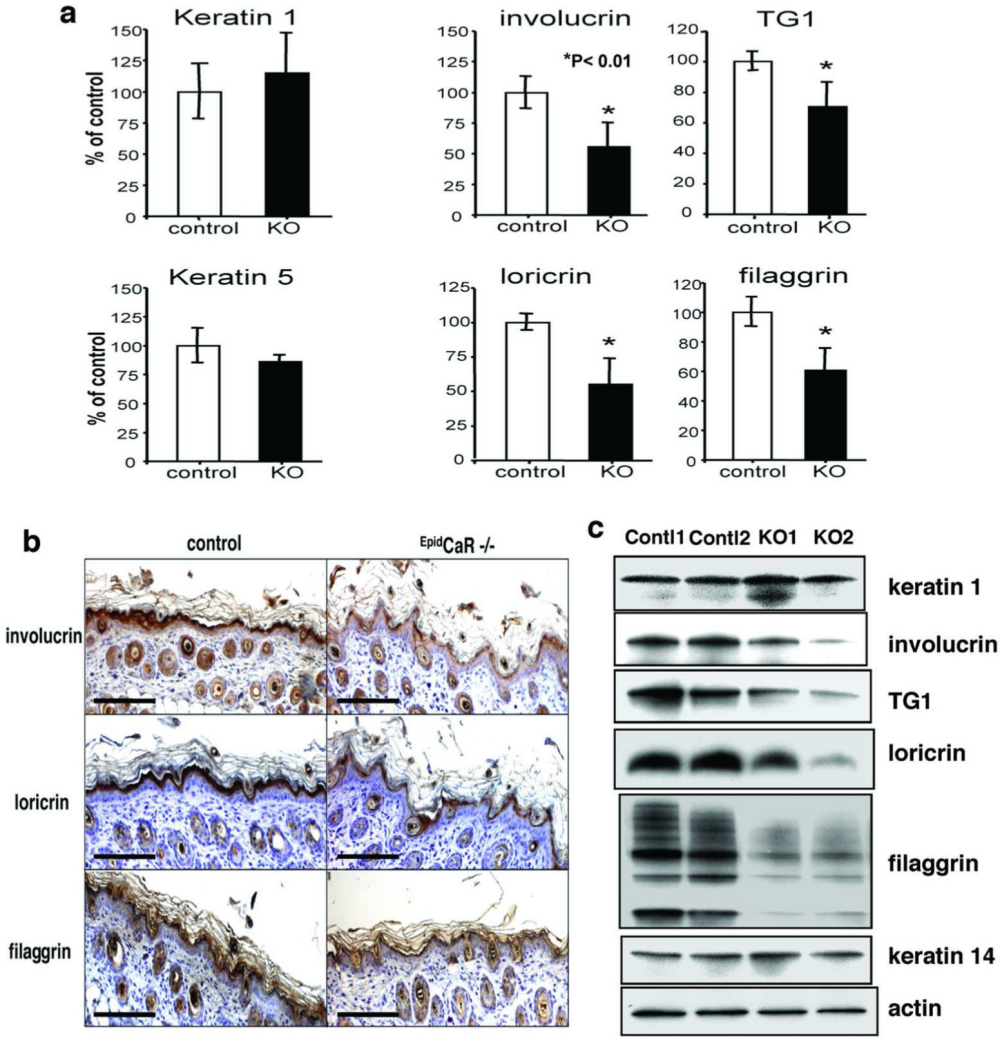

Figure 3. Reduced production of cornified envelope precursors in ^{Epid}CaR-/ neonates

(a) QPCR analyses of epidermal RNA for differentiation markers keratin 1, involucrin, transglutaminase (TG1), loricrin and filaggrin, and basal cell marker keratin 5. The message levels in ^{Epid}CaR-/- (KO) epidermis were normalized to that in controls and presented as mean ± SD, n=7-9. *P<0.01. Immunohistochemical staining of skin (b) and immunoblotting analyses of epidermal protein lysates (c) confirmed a decreased expression of involucrin, loricrin and filaggrin in ^{Epid}CaR-/- epidermis. Bar = 50 μm.

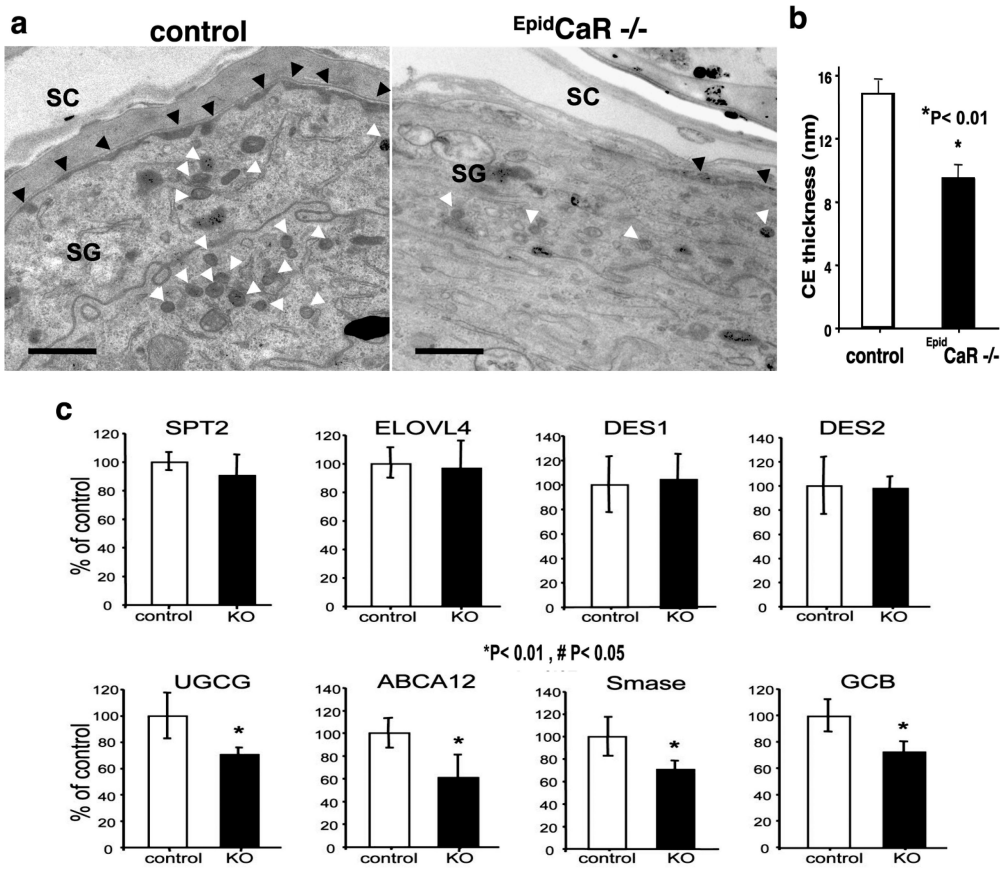

Figure 4. Down-regulation of epidermal sphingolipid transport and processing in ^{Epid}CaR-/- epidermis

(a) Fewer lamellar bodies (LB) (white arrowheads) in the upper SG keratinocytes and less LB secretion (black arrowheads) at the SG/SC junction in epidermis of 10-week-old ^{Epid}CaR-/- mice. Bar = 0.5 μm. (b) The thickness of lipid-bound cornified envelope (CE) in the proximal corneocytes in ^{Epid}CaR-/- epidermis was compared to controls. The data were presented as mean ± SD. *P<0.01. (c) QPCR analyses of epidermal RNAs of 3-day-old mice for serine palmitoyl transferase (SPT2), fatty acid elongase (ELOVL4), C4-5 trans desaturases (DES1 and DES2), glucosylceramide synthase (UGCG), lipid transporter ABCA12, sphingomyelinase (Smase) and β-glucocerebrosidase (GCB). The message levels in ^{Epid}CaR-/- (KO) epidermis were normalized to that of controls and presented as mean ± SD, n=7. *P<0.01, #P< 0.05.

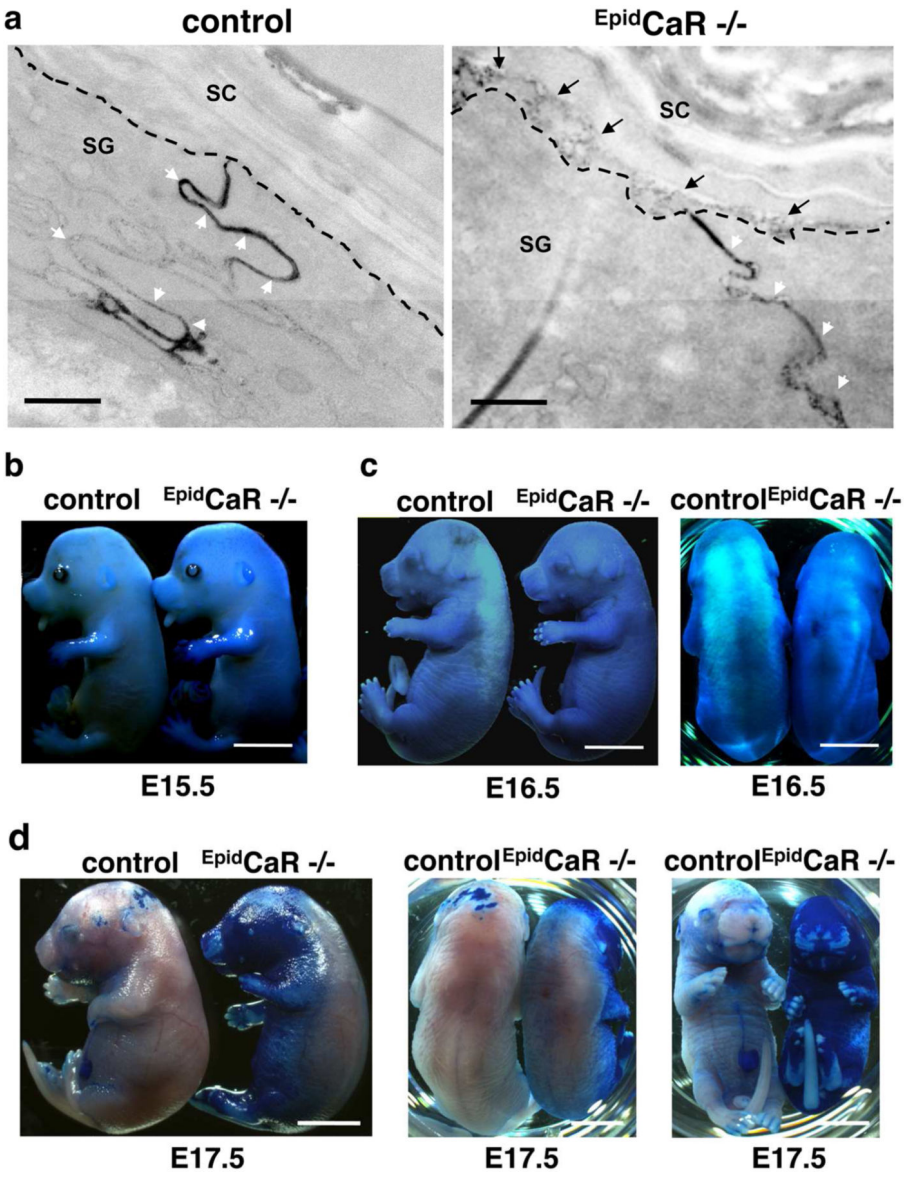

Figure 5. Defective permeability barrier and delayed permeability barrier development in ^{Epid}CaR-/- mice

(a) Permeability barrier integrity of 10-week-old mice was assessed by lanthanum perfusion. The movement of lanthanum (indicated by white arrows) stopped at the SG/SC interface (dashed line) in control epidermis, while lanthanum "leaked" into the extracellular space (black arrows) in ^{Epid}CaR-/- epidermis. Bar = 0.5 μm. E15.5 (b), E16.5 (c) and E17.5 (d) embryos of ^{Epid}CaR-/- and control littermates were extracted with methanol and stained with toluidine blue. Stainingindicated permeability for the dye and immature barrier function. Bar = 2 mm.

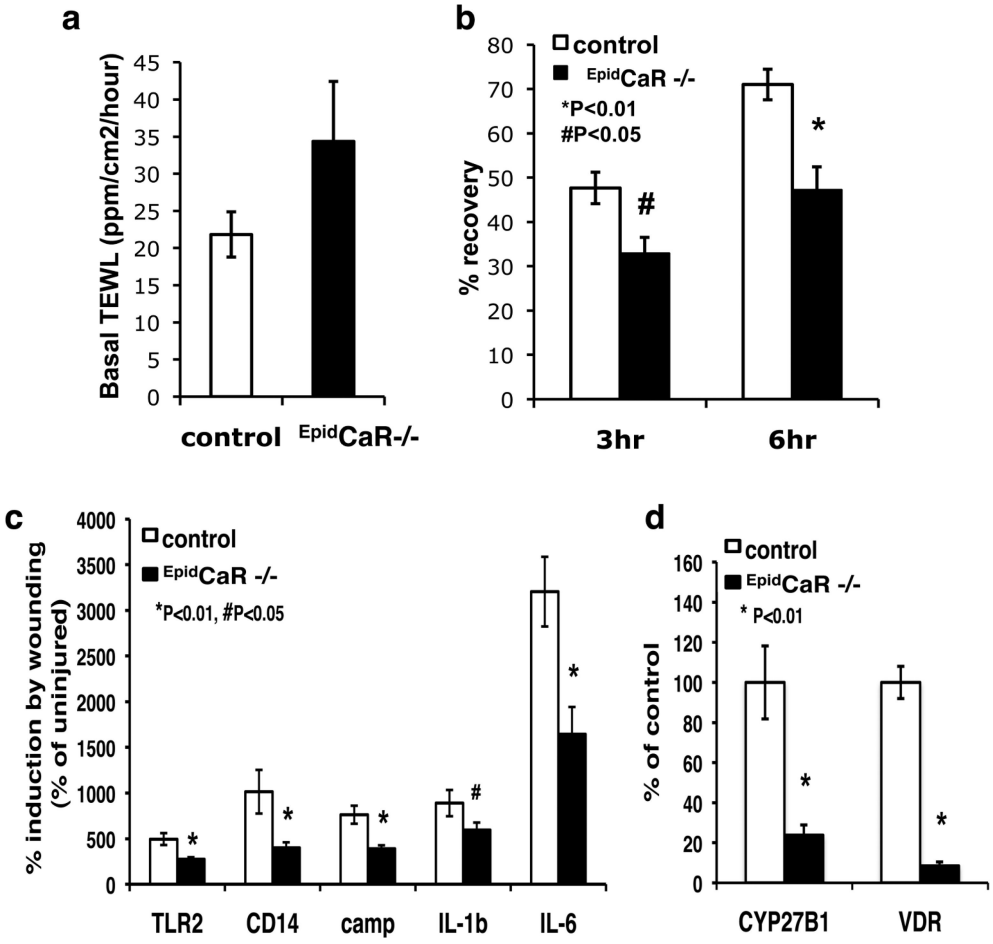

Figure 6. Delayed permeability barrier recovery and attenuated innate immune response in ^{Epid}CaR-/- skins

Four-month-old ^{Epid}CaR-/- mice and control littermates were maintained on a low calcium (0.02%) diet. (a, b) The permeability barrier was disrupted by tape stripping and trans-epidermal water loss (TEWL) was measured before (a) and 0, 3 and 6 hours after (b) tape stripping. Barrier recovery was expressed as % decrease of TEWL at 3 and 6 versus 0 hour after barrier disruption. Data were presented as mean ± SE, n=9-10. *P<0.01, #P<0.05, ^{Epid}CaR-/- vs. control. (c) Full-depth skin incisions were made on the back of the animals. Twenty-four hours later, total RNAs were collected from wounded and uninjured areas. Expressions of innate immune response genes and interleukins were analyzed by qPCR and their levels in the wound normalized to that in the uninjured area. (d) The message levels of CYP27B1 and VDR in ^{Epid}CaR-/- skin were normalized to that in controls. Data were presented as mean ± SE, n=8 13. *P<0.01, #P<0.05, ^{Epid}CaR-/- vs. control.