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

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Abstract

The calcium-sensing receptor (CaR) plays an essential role in mediating Ca²⁺-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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ gradient and impairs keratinocyte differentiation and permeability barrier homeostasis, indicating a key role for the CaR in normal epidermal development.
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\textsuperscript{2+}) plays a critical role in keratinocyte differentiation (Eckert, 1989; Menon et al., 1992), as elevated [Ca\textsuperscript{2+}]o triggers an increase in the level of intracellular free Ca\textsuperscript{2+} (Ca\textsuperscript{2+}i), which subsequently promotes cell differentiation (Bikle et al., 1996; Sharpe et al., 1989). The progressive increases in Ca\textsuperscript{2+}i in response to Ca\textsuperscript{2+}o during keratinocyte differentiation are physiologically relevant, as a steep Ca\textsuperscript{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\textsuperscript{2+} gradient coincides with key developmental milestones of barrier formation and development of SC (Elias et al., 1998). Perturbation of this Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} in epidermal differentiation and barrier homeostasis.

In keratinocytes, the Ca\textsuperscript{2+}i response to Ca\textsuperscript{2+}o resembles that in parathyroid cells, which sense Ca\textsuperscript{2+} via the Ca\textsuperscript{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\textsuperscript{2+}i and E-cadherin-mediated cell-cell adhesion are two pathways critical for promoting cell differentiation in response to extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}\textsubscript{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\textsuperscript{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\textsuperscript{CaR-/-} mice have no growth impediment or metabolic derangement, allowing us to examine the role of the epidermal CaR in mediating Ca\textsuperscript{2+}\textsubscript{o}-induced signaling responses, cell differentiation, and permeability barrier function during both development and adult life.

**Results**

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

Keratinocyte-specific CaR knockout (Epid\textsuperscript{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\textsuperscript{2+}) diet containing 1.3% Ca\textsuperscript{2+} unless otherwise specified. By PCR analyses of genomic DNA from different tissues isolated from 3-day-old Epid\textsuperscript{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\textsuperscript{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\textsuperscript{CaR-/-} epidermis as compared to controls (Figure 1d). Epid\textsuperscript{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\textsuperscript{2+} gradient. There was a steep Ca\textsuperscript{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\textsuperscript{CaR-/-} epidermis, Ca\textsuperscript{2+} in the outer epidermis was reduced and the Ca\textsuperscript{2+} gradient was lost (Figure 2a). Measurement of cytosolic Ca\textsuperscript{2+} of epidermal keratinocytes derived from 5-day-old mice revealed a blunted Ca\textsuperscript{2+}\textsubscript{i} response to Ca\textsuperscript{2+}\textsubscript{o} in Epid\textsuperscript{CaR-/-} cells. As shown in Figure 2b, raising [Ca\textsuperscript{2+}]\textsubscript{o} from 0.03 to 2 mM elicited a robust increase in [Ca\textsuperscript{2+}]\textsubscript{i} in control keratinocytes from 174 ± 10 to a peak of 833 ± 32 nM (mean ± SD; n=50), whereas Epid\textsuperscript{CaR-/-} keratinocytes displayed a reduced Ca\textsuperscript{2+}\textsubscript{i} response to elevated [Ca\textsuperscript{2+}]\textsubscript{o} (from 75 ± 8 to 195 ± 23 nM; n=44). The blunted Ca\textsuperscript{2+}\textsubscript{i} response was correlated with a decrease in the Ca\textsuperscript{2+}\textsubscript{i} pool, as revealed by ionomycin administration in the absence of Ca\textsuperscript{2+}\textsubscript{o} (Figure 2c). Ionomycin (2 μM) induced Ca\textsuperscript{2+} release from internal stores and, as a result, a rise in Ca\textsuperscript{2+}\textsubscript{i} (increased from 201 ± 22 to
347 ± 34 nM, n=24) in control keratinocytes. EpidCaR-/- keratinocytes exhibited a substantial reduction of the rise in Ca^{2+}\textsubscript{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 EpidCaR-/- mice might be attributed to the defect in Ca^{2+}\textsubscript{i} homeostasis in keratinocytes.

**Increased cell proliferation and decreased differentiation gene expression in EpidCaR-/ epidermis**

To determine whether CaR knockout affects epidermal differentiation, we examined the expression of CE precursors involucrin, loricin and filaggrin and the cross-linking enzyme transglutaminase (TG) 1 in the epidermis of 3-day-old EpidCaR-/- mice and their control littermates. QPCR demonstrated a 30-50% decrease in their mRNA levels in the EpidCaR/- (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 EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- and control mice and cultured in medium containing 0.05 mM Ca^{2+}. CaCl\textsubscript{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 EpidCaR-/- 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 EpidCaR-/- (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 EpidCaR-/- epidermis. Isolated EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- 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+}]\textsubscript{o} induced association of β-catenin and E-cadherin at cell-cell junctions in control keratinocytes but not in EpidCaR/- cells. This was accompanied by ineffectual Ca^{2+}\textsubscript{o} induced recruitment of upstream effectors, GTPase Rho and tyrosine kinase Fyn, with E-cadherin to the cell-cell contacts in EpidCaR-/- keratinocytes (Figure S3).
Delayed establishment of the permeability barrier and altered epidermal sphingolipid metabolism in EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- epidermis (Figure 4a). Furthermore, measurement of the thickness of the lipid-bound CE in the proximal corneocytes in EpidCaR-/- 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 EpidCaR-/- epidermis. We then compared the expression of several key enzymes that mediate sphingolipid biosynthesis, transport and processing in the epidermis of 3-day-old EpidCaR-/- 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 EpidCaR-/- (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 EpidCaR-/- mice (data not shown).

We next examined the integrity of the epidermal permeability barrier of the EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- mice and control littermates with 0.0125% toluidine blue. At E15.5, both control and EpidCaR-/- embryos were stained due to immature barrier function, although EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- embryos. At this age, nearly the entire surface of control embryos became stain-resistant, while large areas of skin in EpidCaR-/- embryos retained permeability to dye penetration (Figure 5d). Nonetheless, permeability barriers were fully developed in the E18.5 EpidCaR-/- and control embryos (data not shown).
Impaired epidermal barrier functions in EpidCaR-/- mice under dietary Ca\textsuperscript{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 EpidCaR-/- and control mice before and after acute barrier disruption by sequential tape stripping. When the mice were maintained on a regular (normal Ca\textsuperscript{2+}) diet containing 1.3% Ca\textsuperscript{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 EpidCaR-/- mice than controls, but the differences were not significant. However, as shown in Figure 6b, when these mice were maintained on a low Ca\textsuperscript{2+} (0.02%) diet, EpidCaR-/- 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\textsuperscript{2+} (0.02%) diet and a normal (1.3%) Ca\textsuperscript{2+} diet showed that dietary Ca\textsuperscript{2+} restriction further decreased the production of CE precursors and down-regulated sphingolipid metabolism in EpidCaR-/- 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\textsuperscript{2+} diet, the gene expression was only slightly reduced in the EpidCaR-/- 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\textsuperscript{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 EpidCaR-/- mice and their control littermates maintained on a low (0.02%) Ca\textsuperscript{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 EpidCaR-/- skin. Previous studies have implicated 1,25-dihydroxyvitamin D3 (1,25D\textsubscript{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\textsubscript{3}) 1-\alpha-hydroxylase (CYP27B1), the enzyme responsible for converting 25D\textsubscript{3} to active 1,25D\textsubscript{3}, and the 1,25D\textsubscript{3} receptor (VDR) by qPCR. Figure 6d demonstrated that the expression of these modulators of 1,25D\textsubscript{3} production and action were also markedly diminished in EpidCaR-/- skin. Therefore, the reduced innate immune response in EpidCaR-/- mice may be attributed to the down-regulation of 1,25D\textsubscript{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\textsuperscript{2+} diet, however, the changes in the innate immune response and expression of CYP27B1 and VDR in EpidCaR-/—mice under dietary Ca\textsuperscript{2+} restriction were not found in mice on the 1.3% Ca\textsuperscript{2+} diet (Figure S6).

**Discussion**

The CaR has emerged as a pivotal mediator by which keratinocytes respond to changes in Ca\textsuperscript{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 EpidCaR-/— mice include the loss of the Ca\textsuperscript{2+} gradient, inhibited expression of differentiation-related genes and impaired barrier function. EpidCaR-/— 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\textsuperscript{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\textsuperscript{2+} gradient, diminished LB secretion, but relatively organized cellular layers in EpidCaR-/— 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\textsuperscript{2+} (Elias and Feingold, 2001; Elias et al., 1998), the hypercalcemia resulting from the uncontrolled PTH secretion may alter the Ca\textsuperscript{2+} gradient and cause the excess LB secretion. The accentuated epidermal Ca\textsuperscript{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\textsuperscript{2+}\textsubscript{i} stores to properly handle Ca\textsuperscript{2+}\textsubscript{i} mobilization as seen in keratinocytes of Hailey-Hailey disease patients, which are deficient in the Golgi Ca\textsuperscript{2+}-ATPase, SPCA1, have depleted Ca\textsuperscript{2+}\textsubscript{i} stores, and do not respond to elevated [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}\textsubscript{i} handling suggest that the CaR and SPCA1 are parts of a mechanism that transduces Ca\textsuperscript{2+}\textsubscript{o} signals into cellular responses in keratinocytes. We found that the CaR forms protein complexes with several modulators of the Ca\textsuperscript{2+}\textsubscript{i} stores and Ca\textsuperscript{2+} channels, i.e. PLC\textgamma1, IP\textgamma3R 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\textsuperscript{2+} mobilization due to its intrinsic ability to sense Ca\textsuperscript{2+}\textsubscript{o} and possibly Ca\textsuperscript{2+}\textsubscript{i}.

Besides modulating Ca\textsuperscript{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 EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- mice and their control littermates, the permeability barrier defects in EpidCaR-/- epidermis were exacerbated when these animals were subjected to dietary Ca\(^{2+}\) restriction. Under such a condition, EpidCaR-/- 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 EpidCaR-/- mice on a 0.02% Ca\(^{2+}\) diet was attenuated (Figure 6c). VDR null and CYP27B1-deficient mice share similar epidermal phenotypes as EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- 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 EpidCaR-/- mice). Likewise, the body size and weight gain of EpidCaR-/- 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 EpidCaR-/- 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\textsuperscript{2+} diet, the expression of differentiation marker genes (Figure S5a) and sphingolipid mediators (Figure S5b) in EpidCaR-/ 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\textsuperscript{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 EpidCaR-/ 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 (EpidCaR-/ 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 (EpidCaR-/ 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\textsuperscript{2+}) diet containing 1.3% Ca\textsuperscript{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 EpidCaR-/ 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.

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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

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Figure 1. Ablation of the CaR gene, Casr, in mouse epidermis
(a) Generation of keratinocyte-specific CaR knockout (EpidCasr-/-) 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 EpidCasr-/- 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 EpidCasr-/- (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 EpidCaR-/- 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 EpidCaR-/- mice. Bar = 1 μm. (b, c) Keratinocytes from 5-day-old EpidCaR-/- 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\(^{2+}\) 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 Epidermal 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 Epidermal 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 Epidermal CaR-/- epidermis. Bar = 50 μm.
Figure 4. Down-regulation of epidermal sphingolipid transport and processing in EpidCaR-/- 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 EpidCaR-/- mice. Bar = 0.5 μm. (b) The thickness of lipid-bound cornified envelope (CE) in the proximal corneocytes in EpidCaR-/- 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 transdesaturases (DES1 and DES2), glucosylceramide synthase (UGCG), lipid transporter ABCA12, sphingomyelinase (Smase) and β-glucocerebrosidase (GCB). The message levels in EpidCaR-/- (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 EpidCaR-/- 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 EpidCaR-/- epidermis. Bar = 0.5 μm. E15.5 (b), E16.5 (c) and E17.5 (d) embryos of EpidCaR-/- and control littermates were extracted with methanol and stained with toluidine blue. Staining indicated permeability for the dye and immature barrier function. Bar = 2 mm.
Figure 6. Delayed permeability barrier recovery and attenuated innate immune response in EpidCaR-/- skins

Four-month-old EpidCaR-/- mice and control littersmates 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, EpidCaR-/- 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 EpidCaR-/- skin were normalized to that in controls. Data were presented as mean ± SE, n=8 13. *P<0.01, #P<0.05, EpidCaR-/- vs. control.