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THE ROLE OF THE CALCIUM-SENSING RECEPTOR IN EPIDERMAL DIFFERENTIATION

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ABSTRACT

Calcium regulates the proliferation and differentiation of keratinocytes both *in vivo* and *in vitro*. Elevated extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) raises the intracellular free calcium ($[Ca^{2+}]_i$) and activates differentiation-related genes. Cells lacking the calcium sensing receptor (CaR) fail to respond to $[Ca^{2+}]_o$ and to differentiate, indicating a role for CaR in keratinocyte differentiation. These concepts derived from *in vitro* experiments have been tested and confirmed in two mouse models.

CALCIUM IS A KEY REGULATOR FOR EPIDERMAL DIFFERENTIATION

The mammalian epidermis is a highly specialized, highly organized, stratified squamous epithelium consisting of basal, spinous, granular and cornified cell layers. Each layer is defined by distinguishing morphological and biochemical features and state of differentiation of the keratinocytes (1). While cell proliferation occurs only in basal layer, post-mitotic keratinocytes undergo progressive differentiation as they travel across the epidermis towards the stratum corneum. Keratinocytes in each epidermal layer express distinct biochemical markers. Keratin 5 (K5) and 14 (K14) are predominantly expressed in basal keratinocytes (2). In spinous cells, synthesis of two early differentiation markers, keratins K1 and K10, is initiated (2, 3). In granular keratinocytes, proteins associated with the later stages of differentiation are expressed. These late differentiated markers include: profilaggrin (4), the precursor of the keratin cross-linking protein, filaggrin, transglutaminase I (5), and cornified envelope precursors such as involucrin (6) and loricrin (7). In corneocytes, loricrin and other structure proteins are extensively cross-linked by transglutaminase I to form the cornified envelope.

Calcium-induced Keratinocyte differentiation

Primary keratinocytes provide an excellent *in vitro* model for studying epidermal differentiation (8). Changes in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) play a crucial regulatory role in keratinocyte differentiation (8, 9, 10). When isolated epidermal keratinocytes are cultured at a $[Ca^{2+}]_o$ below 0.07 mM, they proliferate rapidly, express a basal cell phenotype, but fail to develop intercellular contacts and to form cornified envelopes. The differentiation of

keratinocytes is triggered by increasing the $[Ca^{2+}]_o$ above 0.1 mM (8, 9, 11). Elevation of $[Ca^{2+}]_o$ leads to characteristic morphological changes such as desmosome formation, stratification and cornification (12-14). Calcium also induces the redistribution of protein kinase Cα (PKCα) (15, 16) and calmodulin to the plasma membrane where they augment the calcium signaling process. These events are accompanied by a rearrangement of actin filaments from a perinuclear to a radial pattern (12). The expression of biochemical markers in cultured keratinocytes is tightly regulated by $[Ca^{2+}]_o$ in the medium and follows a definite sequence, first the keratins K1 and K10, followed by involucrin, then transglutaminase I, and finally profilaggrin and loricrin (17). Most of these differentiation-specific proteins are regulated at a transcriptional and, in some cases, at a post-translational level (e.g., transglutaminase I and profilaggrin) by $[Ca^{2+}]_o$. These temporal changes in gene expression observed *in vitro* closely reflect the spatial changes in gene expression in the epidermis.

Calcium gradient in epidermis

Ion capture cytochemistry and proton-induced X-ray emission (PIXE) analyses have demonstrated the existence of a steep gradient of Ca^{2+} in mouse and human epidermis (18-20): Ca^{2+} content is low in basal and spinous layers, whereas calcium levels increase progressively towards the outer stratum granulosum, and decreases again in the stratum corneum. Several studies indicated the importance of the calcium gradient for regulating epidermal function. First, perturbation in permeability barrier function leads to a decline in calcium levels in the outer epidermis and disruption of the epidermal calcium gradient (21-23), which subsequently stimulates the secretion of preformed lamellar bodies from the outmost stratum granulosum cells

(24). Normal calcium gradient is restored in 6-24 hours after barrier disruption in parallel with barrier recovery (21-24). Second, the formation of the Ca^{2+} gradient coincides with key developmental milestones of barrier formation and stratum corneum development (20). Finally, skin diseases characterized by an abnormal barrier, such as essential fatty acid deficiency and psoriasis, are accompanied by a loss of the calcium gradient (23, 25, 26). Though the factors that form and maintain the Ca^{2+} gradient *in vivo* are unknown; nevertheless, the pivotal role of calcium in epidermal differentiation is further supported by the observations that changes of Ca^{2+} level in the outer epidermis, independent of barrier perturbation, directly regulate lamellar body secretion and the expression of differentiation–specific markers *in vivo* (27, 28).

Calcium signaling in the keratinocytes

Acute elevation of extracellular calcium induces a rapid biphasic increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) in keratinocytes; the initial spike of increased $[Ca^{2+}]_i$ followed by a prolonged plateau (29-31). In addition, levels of inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) also increase rapidly in response to elevated $[Ca^{2+}]_o$ (32, 33), implicating activation of the phospholipase C (PLC) pathway. The rise in IP_3 is believed to account for the initial rise in $[Ca^{2+}]_i$ by releasing calcium from intracellular stores. The sustained increase in $[Ca^{2+}]_o$ likely results from a stimulated transmembrane calcium influx (34), which presumably occurs as a consequence of activation of both calcium-sensitive chloride channels, leading to hyperpolarization of the membrane (35), and voltage-independent nonspecific cation channels permeable to calcium (36). Previous studies have shown that the $[Ca^{2+}]_i$ response to $[Ca^{2+}]_o$ changes with differentiation. The acute rise in $[Ca^{2+}]_i$ in response to increased $[Ca^{2+}]_o$ is greater in

undifferentiated normal keratinocytes (37, 38) and in transformed keratinocytes that fail to differentiate than in differentiated keratinocytes (39). Nonetheless, the basal $[Ca^{2+}]_i$ level increases as cells differentiate (31, 39). It is well established that this increase in $[Ca^{2+}]_i$ is essential for the differentiation response (29, 31, 40). Blocking the rise in $[Ca^{2+}]_i$ with intracellular calcium chelators (BAPTA) (41) or blocking membrane calcium flux with lanthanum (42) prevents calcium-induced keratinocyte differentiation. Furthermore, it has been demonstrated that PLCγ1 plays a key role in sustaining the increased $[Ca^{2+}]_i$ (43) since blocking calcium stimulation of PLCγ1 impedes the ability of $[Ca^{2+}]_o$ to increase $[Ca^{2+}]_i$ and to induce differentiation in keratinocytes. Also, raised $[Ca^{2+}]_o$ stimulates PKC by increasing the amount of DAG resultant from the activated PLC pathway (44). Among the various PKC isoenzymes in epidermis, PKCα is associated with the latter stages of calcium-induced differentiation (15). Down-regulation of PKCα (15) or blocking its production (16, 45) subsequently reduces the calcium-stimulated expression of late differentiation markers.

Calcium-sensing receptor in the keratinocytes

The mechanism by which the keratinocyte responds to the change in $[Ca^{2+}]_o$ is not fully understood. Yet, the response to $[Ca^{2+}]_o$ in keratinocytes resembles to that in the parathyroid cells (46), which is now known to sense $[Ca^{2+}]_o$ via an extracellular calcium-sensing receptor (CaR). Besides in the parathyroid gland, this receptor has been found expressed in various tissues that do not directly involve in calcium homeostasis (47-53). We have identified the same receptor in the keratinocytes (37, 38). The ability of a selective activator of the CaR, NPS R-467, to increase

the $[Ca^{2+}]_i$ response to $[Ca^{2+}]_o$ and to activate the differentiation marker genes in keratinocytes supports the notion that CaR modulates the response to $[Ca^{2+}]_o$ (53).

The keratinocyte CaR sequence is identical to that of the parathyroid CaR. Besides the full-length CaR, keratinocytes produce an alternatively spliced variant of CaR. This spliced variant lacks exon 5, resulting in the loss of residues 460 to 536 in the extracellular domain (38). When cDNAs of full length CaR or its spliced variant were transfected into HEK 293 cells, the full length CaR was expressed as two major proteins of 140-kDa and 160-kDa, whereas the spliced variant of CaR was expressed as a single protein of 130-kDa (38, and Fig. 3). Deglycosylation analyses revealed that the full length CaR and its spliced variant have different N-linked glycosylation patterns (38). The spliced variant protein appears to have only high mannose type oligosaccharide chains, whereas the full length CaR has two different N-glycosylated oligosaccharide chains. The deletion of the 2 N-glycosylation sites at Asn468 and Asn488 within exon 5 may account for the change of glycosylation pattern in the spliced variant of CaR. When expressed in HEK293 cells, only the full-length CaR was able to mediate calcium-stimulated inositol phosphate (IP) production (38). Besides, this spliced variant moderately reduced the IP response of the full length CaR to $[Ca^{2+}]_o$ when they were coexpressed in HEK293 cells (38), suggesting that the ratio of the two forms of CaR could be a factor regulating the calcium responsiveness in cells. Also, when both forms of CaR were co-transfected into keratinocytes with an involucrin promoter/luciferase gene construct, only the full-length CaR enhanced the calcium-stimulated induction of the involucrin gene (Fig. 1), and this enhancement was blocked by an antisense CaR construct, indicating an important role for CaR in calcium-induced keratinocyte differentiation. Fluorescence immunostaining revealed that

unlike the full-length CaR (Fig. 2A) which displays distinct plasma membrane localization, the distribution of spliced variant (Fig. 2B) is strictly confined in the cytoplasm with a perinuclear pattern. Failure to generate a protein capable of being transferred to the cell surface due to a change in glycosylation is a likely basis for the failure of the CaR spliced variant to mediate cellular response to $[Ca]_o$.

The full-length CaR and its spliced variant are expressed differentially during keratinocyte differentiation. In Western analyses of keratinocyte membrane proteins (Fig. 3A) the endogenous CaR was detected by an antibody (ADDR) that reacts with both forms of CaR as three major bands of 120-kDa, 160-kDa and 185-kDa and a minor band of 130-kDa (which was best seen in longer exposed film). Whereas the 120-kDa band corresponds to the non-glycosylated full-length CaR, the 160-kDa band corresponds to one of the two major glycosylated forms (the 140-kDa and 160-kDa proteins) of full-length CaR (38). An antibody (hCaR4/6) that specifically reacts with the human CaR splice variant confirmed the 130-kDa protein as the spliced variant (Fig. 3B). The level of full-length CaR was higher in the less differentiated keratinocytes (5 days in culture) and decreased as the cells differentiated (Fig. 3A), which is consistent with the larger $[Ca]_i$ and IP responses in undifferentiated cells. On the other hand, the level of the spliced variant remained relatively unaffected during keratinocyte differentiation (Fig. 3B). The expression pattern of CaR message is similar to that of protein (38).

In the parathyroid cells and in HEK293 cells (Fig. 2A) that were transfected with cDNA for the full-length CaR, CaR was extensively expressed on the plasma membrane, though substantial intracellular distribution was detected. On the other hand, in keratinocytes only a low

level of the endogenous CaR protein is present on the plasma membrane, whereas the majority of the CaR protein is distributed within cytoplasm, especially in a perinuclear area. We have detected a considerable amount of CaR protein co-localized with the trans-golgi marker TGN38 (Fig. 4). Substantial perinuclear cytoplasmic localization of CaR is commonly observed in other cell types (52, 54-56). Presently it is unclear whether intracellular CaR simply represents nascent receptor protein halfway through the biosynthetic processing or whether it has distinct biological functions. Our preliminary studies have shown that CaR co-localizes and forms a signaling complex with other calcium-modulating proteins such as PLCγ1, IP3R and the Ca^{2+}-ATPase ATP2C1 in Golgi (57), an important intracellular calcium store in keratinocytes. These data raise the possibility that CaR mediates calcium sensing not only on the plasma membrane but also within the internal calcium stores as suggested previously (58).

The role of CaR in calcium-induced differentiation was elucidated by blocking the expression of CaR through transfection of a CaR antisense construct into keratinocytes (59). These cells have a decreased CaR protein level and a clearly reduced $[Ca^{2+}]_i$ response to $[Ca^{2+}]_o$ compared to the vector-transfected cells (59). Transfection of the anti-CaR construct also blocked the calcium-induced inhibition of cell proliferation and the calcium-stimulated expression of late differentiation markers (59). These results demonstrated that CaR mediated calcium signaling is essential for keratinocyte differentiation. While our observation that the antisense CaR reduced the $[Ca^{2+}]_i$ response to $[Ca^{2+}]_o$ can be interpreted as a consequence of hindered calcium sensing of extracellular calcium by the CaR, it is possible that antisense CaR transfection could disrupt a CaR mediated release of calcium from intracellular stores and/or CaR mediated increase in calcium influx through calcium channels. In either case the $[Ca^{2+}]_o$ mediated increase in $[Ca^{2+}]_i$

and subsequent expression of differentiation-related genes would be abolished by inhibition of CaR production.

Calcium receptor in mammalian epidermis

Immunocytochemistry and *in situ* hybridization confirmed the expression of CaR in mammalian skin (Fig. 5). An antibody recognizing both forms of CaR was used to detect the CaR protein in the human (Fig. 5A) and mouse (Fig. 5 B, C) epidermis. In both species the CaR protein is expressed in interfollicular suprabasal keratinocyte layers, whereas the keratinocytes in the basal layer contain little CaR. The strongest staining was observed in the cells of the upper stratum granulosum (Figs. 5A and B), whereas the corneocytes displayed weak staining. Noticeably, most of the CaR staining was intracellular, not on the plasma membrane, confirming the distribution of CaR in cultured keratinocytes. The specificity of the staining was established as the peptide-preabsorbed antibody failed to react with CaR protein in mouse skin (Fig. 5C). *In situ* hybridization using a DIG-labeled antisense RNA probe showed that the distribution of CaR message (Fig. 5D, E) is similar to that of CaR protein in human (Fig. 5D) and mouse (Fig. 5E) epidermis. While little or no CaR mRNA was seen in the basal layers of the interfollicular epidermis, the upper spinous and granular layers were labeled for CaR mRNA in both species. Hybridization using a sense probe showed no signal in mouse skin (Fig. 5F). These results demonstrated that the expression of CaR closely parallels the Ca^{2+} gradient in the epidermis, supporting the concept that CaR mediates keratinocyte differentiation *in vivo*. However, the importance of CaR in the epidermis is made clear in two animal models, CaR deficient (*Car-/-*) mice (60) and transgenic mice with epidermal overexpression of CaR (61).

Calcium receptor deficient mice and calcium receptor transgenic mice

In the *Car-/-* mice the CaR gene was disrupted by insertion of a neomycin resistance gene into exon 5 (60), which resulted in a complete loss of full-length CaR expression. However, immunocytochemistry, Western analyses, and *in situ* hybridization all detected the continuous expression of the alternatively spliced form of CaR in these animals (62, 63). Previous studies (60) showed that loss of calcium homeostasis results in hypercalcemia among other metabolic changes in these animals. By comparing the skin from 3-day-old *Car-/-* mice to their wild type (*Car+/+)* littermates, light microscopic studies revealed that *Car-/-* mice have a thinner epidermis with less organized differentiation sequence, demonstrated by abnormal flattening from the basal through suprabasal cells (62); however, this difference was less apparent after 7 days. In addition, PCNA staining indicated a modest increase in the proliferation of the basal layer in *Car-/-* epidermis (63). Electron microscopic examination (63) of the epidermis discovered various morphological abnormalities in the epidermis of *Car-/-* mice, including a looser and less organized stratum corneum, an expanded interface between stratum granulosum and stratum corneum, abnormal accumulation of large keratohyalin granules in the cells of lower granular layers, and premature secretion from lamellar bodies in the lower granular keratinocytes. Furthermore, keratinocyte differentiation in *Car-/-* mice (Fig. 6, right column) is altered in comparison with the *Car+/+* mice (Fig. 6, left column) . Although the basal keratin expression (judged by the immunolocalization of K14, data not shown) did not change, the mRNA (Fig. 6A, B, E, F) and protein (Fig. 6C, D, G, H) levels of the terminal differentiation genes loricrin (Fig. 6, A-D)and profilaggrin (Fig. 6, E-H) were greatly decreased in the epidermis

of *Car-/-* mice. In addition, keratinocytes from *Car-/-* mice failed to demonstrate a normal $[Ca^{2+}]_i$ response to $[Ca^{2+}]_o$ (62). These results indicate that the full-length CaR is required for normal epidermal differentiation and that this function cannot be replaced with the alternatively spliced CaR.

Recently, Truksen and Troy (61) reported that in transgenic mice with CaR overexpressed in the basal cells in the epidermis, hair follicle differentiation and epidermal permeability barrier development were accelerated. Several morphological and biochemical changes were found in the epidermis from transgenic mice. Transgenic epidermis is thicker due to hypertrophic suprabasal keratinocytes, and the granular layer exhibits increased keratohyalin granules (61). In addition, the levels of early (keratin K1) and late (involucrin, loricrin and filaggrin) differentiation markers significantly increased in the transgenic epidermis, which could contribute to the accelerated barrier formation (61). Their observations complement the findings of increased proliferation and decreased expression of late differentiation markers in the epidermis of *Car-/-* mice (62, 63). Therefore, these results strongly support that CaR-mediated signaling is required for normal epidermal differentiation.

CONCLUSION

Calcium is a critical regulator of proliferation and differentiation in keratinocytes *in vivo* and *in vitro*. Epidermal keratinocytes express two forms of CaR, full-length CaR and its alternatively spliced variant lacking exon 5. Only the full-length CaR is able to mediate the calcium-stimulated IP synthesis. The expression of full-length CaR decreases during keratinocyte

differentiation, which may account for the loss of calcium responsiveness in differentiated cells. Down-regulation of CaR production prevents the calcium-induced $[Ca^{2+}]_i$ response and the calcium-stimulated expression of differentiation-related genes, demonstrating a role of CaR in mediating calcium signaling during keratinocyte differentiation. The intracellular localization of CaR and its associations with other calcium-modulating proteins in keratinocytes suggests the possibility that the role of CaR in keratinocytes differs from that in the parathyroid gland. The distribution of CaR in the epidermis parallels the calcium gradient that is vital for epidermis development and permeability barrier function. The epidermis lacking the full-length CaR exhibits various morphological abnormalities and displays decreased expression of differentiation markers. On the other hand, overexpression of CaR in the epidermis accelerates hair follicle formation, enhances permeability barrier development and stimulates expression of differentiation markers. These results indicate that CaR is required for normal epidermal differentiation *in vivo*, presumably by mediating the calcium signaling required for keratinocyte differentiation.

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Figure legends

Figure 1. The full-length CaR enhances calcium-stimulated transcriptional activation of involucrin. The cDNA for the full-length CaR (CaR), spliced variant (AltCaR), control vector or the combination of the full-length CaR and an CaR antisense construct (anti-CaR) were transiently transfected into keratinocytes along with a β-galactosidase expression vector and an involucrin promoter/luciferase reporter construct in KGM containing 0.03 mM Ca^{2+}. Cells were incubated in 1.2 mM Ca^{2+} for 48h before the luciferase and β-galactosidase activities were measured. The promoter activity is expressed as luciferase activity normalized to β-galactosidase activity. Calcium stimulated promoter activity is presented as the fold increase of the promoter activity of cells grown in 1.2 mM Ca^{2+} to the promoter activity of cells grown in 0.03 mM Ca^{2+}. The full-length CaR, but not the spliced variant enhanced the calcium-stimulated transcriptional activation of the involucrin promoter. However, this enhancement is suppressed by the CaR antisense construct. Each data point shows the mean ± S.D. (n=3).

Figure 2. Fluorescence immunostaining of the CaR protein exogenously expressed in HEK293 cells. HEK293 cells were transiently transfected with cDNAs for full-length CaR (A) or its spliced variant (B). The cells were stained with a polyclonal anti-CaR antibody that reacts with both forms of CaR, followed by fluorescein-conjugated anti-rabbit IgG. Fluorescent signals were detected with a confocal microscope. A clear plasma membrane distribution was detected in the cells expressing the full-length CaR, although considerable intracellular staining was noticed.

The alternatively spliced CaR localized strictly within cytoplasm in a perinuclear area. No staining was detected in the cells transfected with control vector (data not shown).

Figure 3. Expression of the CaR protein during human keratinocyte differentiation. Human keratinocytes were grown in medium containing 0.03 mM or 1.2 mM Ca^{2+}. The membrane proteins were isolated from human foreskin keratinocytes (NHK) at the indicated time points (5, 7, 14 days in culture), and from HEK293 cells transfected with the cDNA for full-length human CaR (CaR), and the alternatively spliced CaR (AltCaR) or control vector. The CaR proteins were detected using an antibody (ADDR) recognizes both full-length human CaR and its alternatively spliced variant, or an antibody (hCaR4/6) that specifically reacts with the alternatively spliced CaR variant. In addition to the three bands (120-, 160- and 185-kDa), a 130-kDa band (observed on longer exposed film, data not shown) was specifically detected by ADDR in keratinocytes. Detection by hCaR4/6 confirmed that the 130-kDa protein present in keratinocyte membranes is the spliced variant of CaR. The specificity of the immunostaining of these bands was confirmed by incubation with the antibodies preabsorbed with the specific peptides against which they were raised. Adapted with permission from The Journal of Biological Chemistry (59).

Figure 4. Fluorescence immunostaining of the CaR protein endogenously expressed in human keratinocytes. Keratinocytes were stained with a polyclonal antibody recognizing both forms of CaR and a monoclonal antibody against a trans-golgi structural protein TGN38, followed by fluorescein-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG, respectively. Fluorescent signals were detected with a confocal microscope. A low level of CaR was detected

on the plasma membrane in some cells, whereas most of the CaR was detected within the cytoplasm with perinuclear distribution. Notice substantial co-localization of CaR and TGN38 in Golgi.

Figure 5. CaR expression in the mammalian epidermis. The expression of the CaR protein was detected by immunostaining using anti-CaR antibody (A, B, C), and the presence of CaR mRNA was detected by *in situ* hybridization using digoxygenin labeled antisense RNA probe (D, E, F) in human (A, D) and mouse (B, C, E, F) skin. The dark coloration in the basal cells of the human epidermis (A, D) is due to endogenous pigmentation. Incubation with peptide-preabsorbed antibody (C) or hybridization with a sense control probe (F) on mouse skin blocks staining for CaR, demonstrating the specificity of the detection of CaR expression. Magnification = 600x. Reprinted with permission from the Kluwer Academic Publishers (64).

Figure 6. Expression of late differentiation markers in the epidermis of wild-type (*Car*+/+) and CaR-deficient (*Car*-/-) mice. The expression of loricrin (A, B) and profilaggrin (E, F) mRNAs was detected by *in situ* hybridization in 3-day-old *Car*+/+ (left column) and *Car*-/- (right column). Loricrin (C, D) and profilaggrin/filaggrin (G, H) proteins were detected by immunohistochemistry. Bar=25 μm. Notice the decreased and non-uniform, localized mRNA and protein signals in *Car*-/- epidermis as opposed to wild type. Reprinted with permission from The Journal of Cellular Physiology (63).

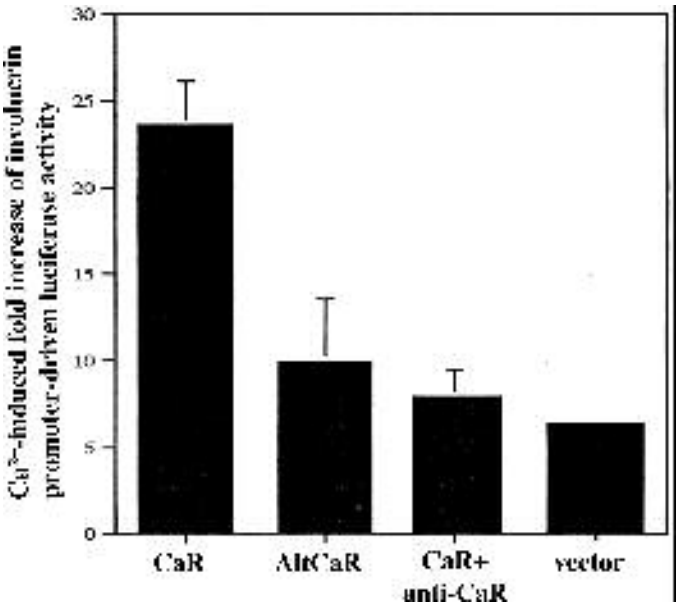

Figure 1

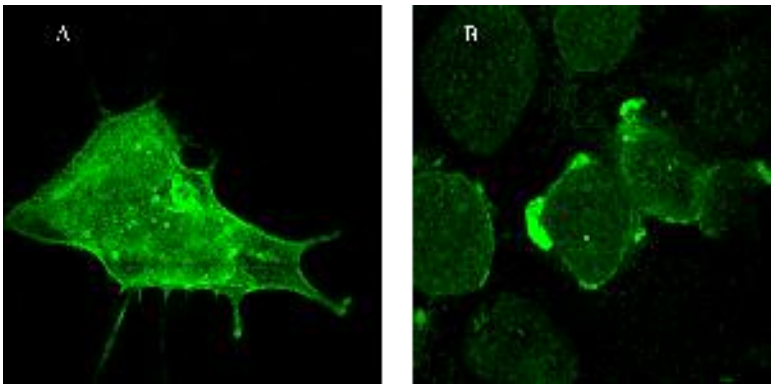

Figure 2

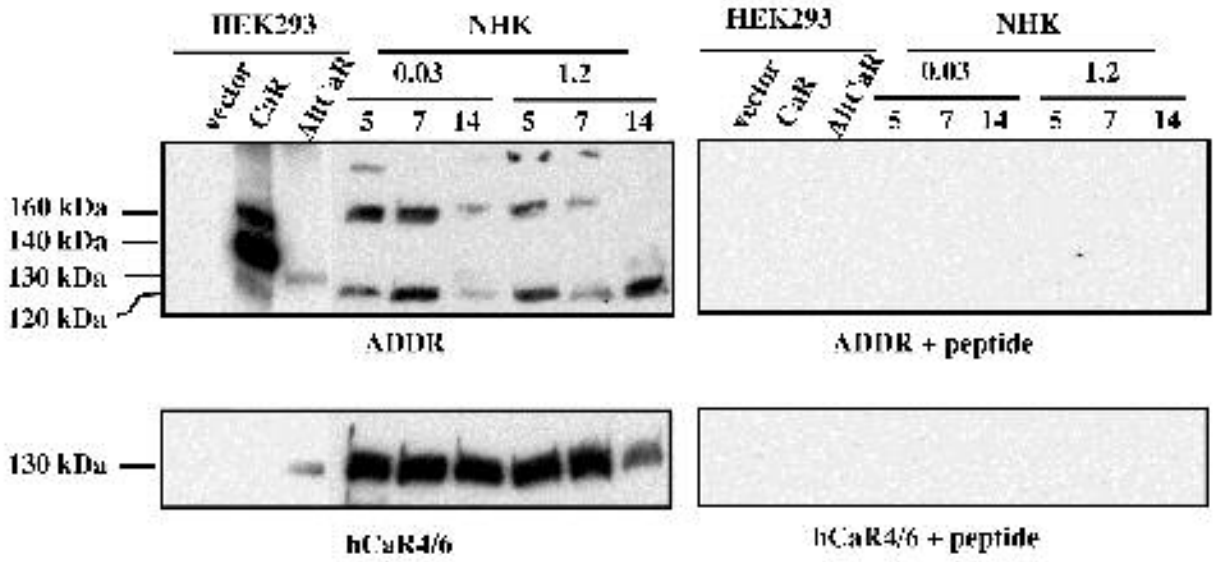
HEK293
NHK
HEK293
NHK
vector
CaR
ΔhCaR
0.03
1.2
0.03
1.2
5 7 14 5 7 14
5 7 14 5 7 14
160 kDa
140 kDa
130 kDa
120 kDa
ADDR
ADDR + peptide
130 kDa
hCaR4/6
hCaR4/6 + peptide

Figure 3

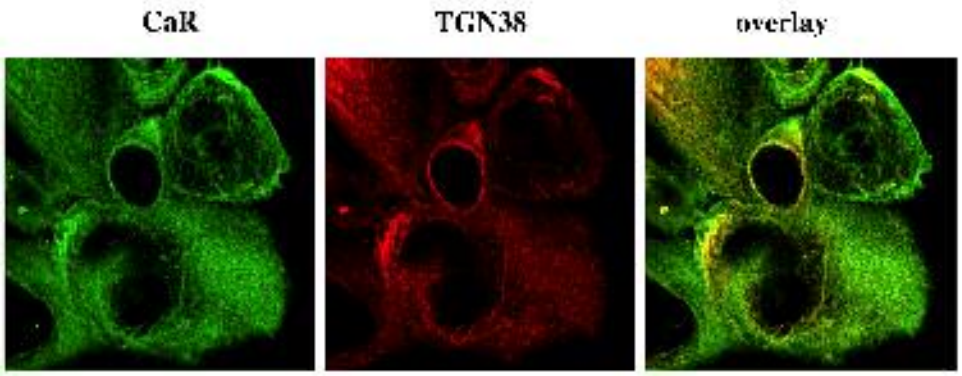

Figure 4

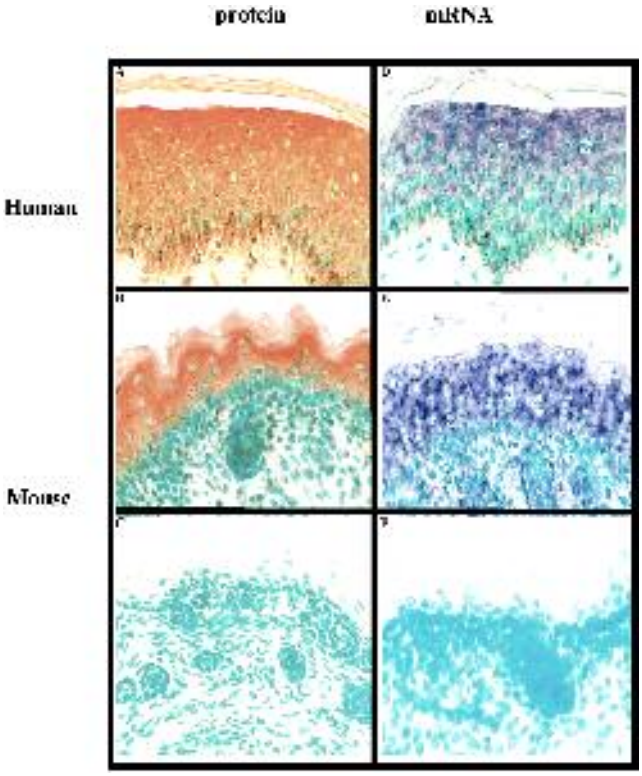

Figure 5

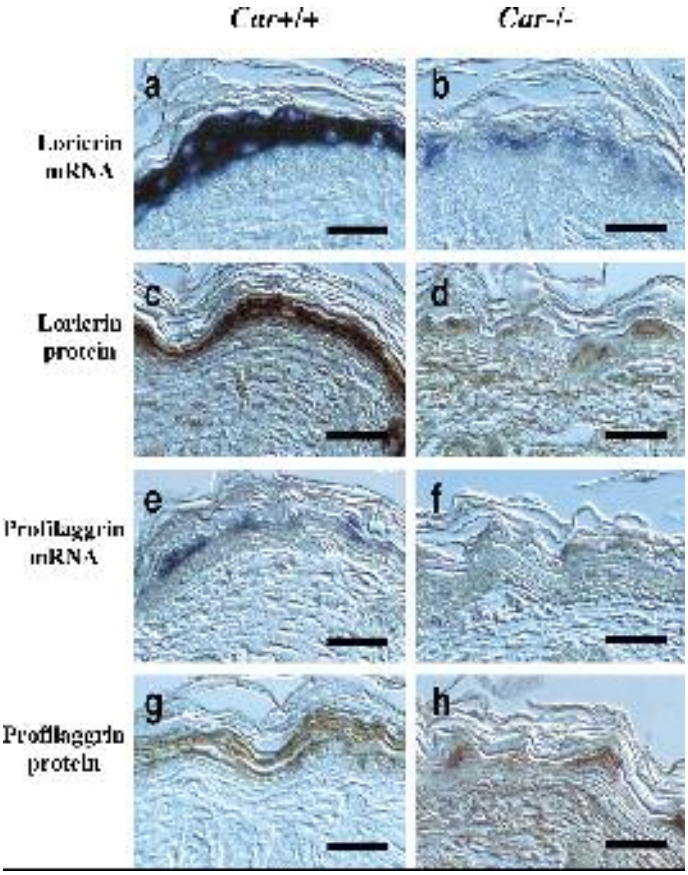
Car+/+
Car-/-
a
b
Loricrin mRNA
c
d
Loricrin protein
e
f
Profilaggrin mRNA
g
h
Profilaggrin protein

Figure 6