Published in final edited form as:
Exp Dermatol. 2015 January ; 24(1): 55–57. doi:10.1111/exd.12568.

Calmodulin 4 is dispensable for epidermal barrier formation and wound healing in mice

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Abstract

Calcium-mediated signals play important roles in epidermal barrier formation, skin homeostasis, and wound repair. Calmodulin 4 (Calm4) is a small, Ca^{2+} binding protein with strong expression in suprabasal keratinocytes. In mice, Calm4 first appears in the skin at the time of barrier formation and its expression increases in response to epidermal barrier challenges. In this study, we report the generation of Calm4 knockout mice and provide evidence that Calm4 is dispensable for epidermal barrier formation, maintenance, and repair.

Keywords

Epidermis; Calcium-binding Protein; Skin Barrier; Wound Repair

Background

Calcium (Ca^{2+}) plays a critical role in mammalian skin homeostasis and is a key signaling molecule during epidermal wound repair (1). Keratinocytes in particular rely on Ca^{2+}-mediated cues for proliferation, differentiation, and migration processes, as well as for the formation and maintenance of an intact epidermal barrier (2, 3). A large number of Ca^{2+}-binding proteins facilitate Ca^{2+} signaling and intracellular trafficking in the skin, including Calmodulins and Calmodulin-like (Calml) proteins 3 and 5 (1, 4, 5). We previously identified the mouse homolog of Calml5, Calmodulin 4 (Calm4), which is present in stratified epithelia, vibrissae hair follicles, and perichondral osteoblasts (6). In the skin, Calm4 is specifically expressed in suprabasal keratinocytes beginning at E15.5, coinciding with the onset of epidermal stratification and barrier formation (6, 7). Calm4 interacts in a Ca^{2+}-dependent manner with several other proteins, including calreticulin, stratifin, and annexin V, and shows enhanced protein expression after acute disruption of the epidermal Ca^{2+} gradient, suggesting a role for Calm4 in the late stages of wound repair (8). Here we

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Author Contributions

JCL, AK, and MIM designed the study; JCL and AK performed the research; JCL, AK, PB, and MIM analyzed the data; JCL and MIM wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

report that the genetic ablation of Calm4 in mice does not affect epidermal barrier formation, skin homeostasis, or the dynamics of wound healing *in vivo*.

Questions Addressed

In this study, we investigated the consequences of Calm4 deletion in mouse skin.

Experimental Design

See Supporting Information (Appendix S1).

Results

Loss of Calm4 does not affect epidermal barrier formation or keratinocyte differentiation

Calm4 was deleted from the mouse genome by replacing its open reading frame with a lacZ reporter cassette (Figure 1 A, B). Mice were generated in a CD-1 and C57 Bl/6 background, with neither background presenting any overt gross phenotype. In mice heterozygous at the Calm4 locus, the lacZ pattern faithfully recapitulated Calm4 expression in stratified epithelia, hair follicles, the nail bed, and limb bones starting at embryonic day (E) 15.5 (Figure 1 C). Cross-sections of E16.5 skin showed the specific localization of Calm4-lacZ in suprabasal keratinocytes (Figure 1 C). $Calm4^{-/-}$ mice were phenotypically indistinguishable from wild-type controls from birth into adulthood (data not shown). Dye exclusion assays of E15.5 to E17.5 embryos, transepidermal water loss (TEWL) measurements, and cornified envelope preparations from newborn mice indicated proper formation of the epidermal barrier in $Calm4^{-/-}$ mice (Figure 1 D, E, Figure S1). Late embryonic and early postnatal $Calm4^{-/-}$ skin revealed no defects in epidermal stratification or differentiation (Figure 1 F, G, Supp. Figure S1) beyond a slightly more granular appearance of filaggrin staining at E17.5 (Figure 1 F). Of note, Calm5, which shares 83% nucleotide sequence homology with its genomic neighbor Calm4 and displays a very similar expression pattern in skin (7), is not upregulated in $Calm4^{-/-}$ skin (Figure 1 G).

Calm4 deletion does not significantly alter the skin transcriptome

To assess the global effect of Calm4 deletion on skin homeostasis, we performed RNAseq on whole back skin isolated from newborn (postnatal day 1) and young adult mice (postnatal day 21). As expected, the P1 and P21 samples clustered together based on age, but the overall gene expression profiles were so similar between samples of the same group that they did not cluster together by genotype (Figure 2 A). After correcting for a false discovery rate of 0.05, none of the already limited number of differentially expressed mRNAs passed the significance threshold (Table S1). However, we were able to verify several gene expression changes by quantitative PCR, including Calm4 and Calm5, which were both among the top transcripts reduced in $Calm4^{-/-}$ mice (Table S1, Figure S2). This supports our earlier results suggesting that a functional compensation by Calm5 in $Calm4^{-/-}$ skin is unlikely.

Calm4 is dispensable for keratinocyte migration and wound healing

Calm4 expression increased after challenging the epidermal barrier by tape stripping in mice and full-thickness wounding of human skin *ex vivo* (8), indicating a possible role for Calm4 in epidermal barrier restoration and re-epithelialization. To further test this possibility, we created 8mm full-thickness wounds on the backs of 7-week-old mice and monitored the rates of wound closure for 13 days. There was no difference in the rate or amount of wound closure between Calm4$^{-/-}$ and WT mice (Figure 2 B). Furthermore, there was no detectable change in keratinocyte migration between WT, Calm4$^{+/-}$, and Calm4$^{-/-}$ in an *ex vivo* outgrowth assay (Figure 2 C). Calm4$^{-/-}$ mice also did not show any significant alterations in TEWL measurements of tape-stripped adult back skin (Figure S2).

Conclusions

In summary, our results indicate that Calm4 is dispensable for epidermal barrier formation, skin homeostasis, keratinocyte migration, and wound healing. Calm4$^{-/-}$ mice displayed no overt phenotypes and accordingly, their skin transcriptomes were very similar to wild-type controls. While we found no evidence of functional compensation by the highly homologous Calm5 or other Calmodulin isoforms, it is possible that other Ca^{2+}-binding proteins can functionally compensate for Calm4 in epidermal barrier formation and/or restoration, e.g. Calml3, which is highly expressed during the wound re-epithelialization stage in human skin (4). We also cannot rule out that Calm4 plays a non-redundant role in certain specialized responses to epidermal challenges that we did not address, e.g. pathogen exposure. However, the lack of an *in vivo* phenotype in mice genetically null for a small epidermal Ca^{2+}-binding protein is not unique to Calm4. S100A9$^{-/-}$ mice also appear grossly normal, even in several *in vivo* inflammatory assays, where S100A9 is normally expressed at high levels by both keratinocytes and skin-infiltrating leukocytes (9). Our findings therefore reinforce the notion that the establishment, maintenance, and repair of the epidermal barrier are tightly regulated events involving a large and sometimes redundant assortment of signaling molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Gustavo Gutierrez-Cruz and Dr. Hong-Wei Sun for help with the RNAseq experiments, Kimberly Hilsen for assistance with mouse breeding, and Dr. Mary Ann Stepp for input relating to the wound healing studies. This research was supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the NIH.

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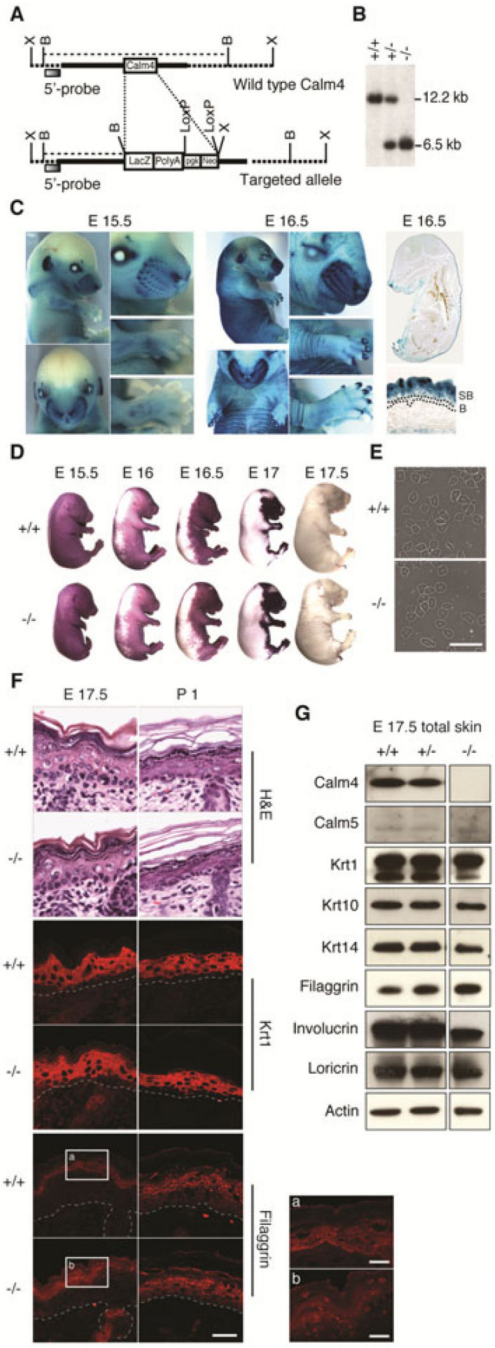

Figure 1. Normal epidermal barrier formation and keratinocyte differentiation in Calm4$^{-/-}$ mice

(**A**) Strategy to generate Calm4 knockout mice. B, BamHI; X, XbaI. (**B**) Southern Blot confirming successful targeting at the Calm4 locus. (**C**) Whole-mount lacZ staining of Calm4$^{+/-}$ embryos illustrates Calm4 expression in skin, vibrissae, eyelids, nail bed, and limb bones. Cross-sections at E 16.5 show Calm4/lacZ expression in nasal epithelium and confirm Calm4 specificity in suprabasal keratinocytes. B, basal; SB, suprabasal. (**D**) Dye exclusion assay reveals normal epidermal barrier formation in Calm4$^{-/-}$ embryos. (**E**)

Calm4$^{-/-}$ corneocytes are indistinguishable from wild-type. Scale bar, 50μm. (**F**) H&E histology and immunofluorescence stainings on E 17.5 and P 1 skin sections reveal no difference in epidermal stratification in Calm4$^{-/-}$ mice. Note the slightly more granular appearance of filaggrin staining at E 17.5 (insets). Dashed line demarcates epidermis/dermis junction. Scale bars, 30μm. (**G**) Western Blots of E 17.5 total skin extracts show no change in keratinocyte differentiation markers as well as Calm5 in the absence of Calm4.

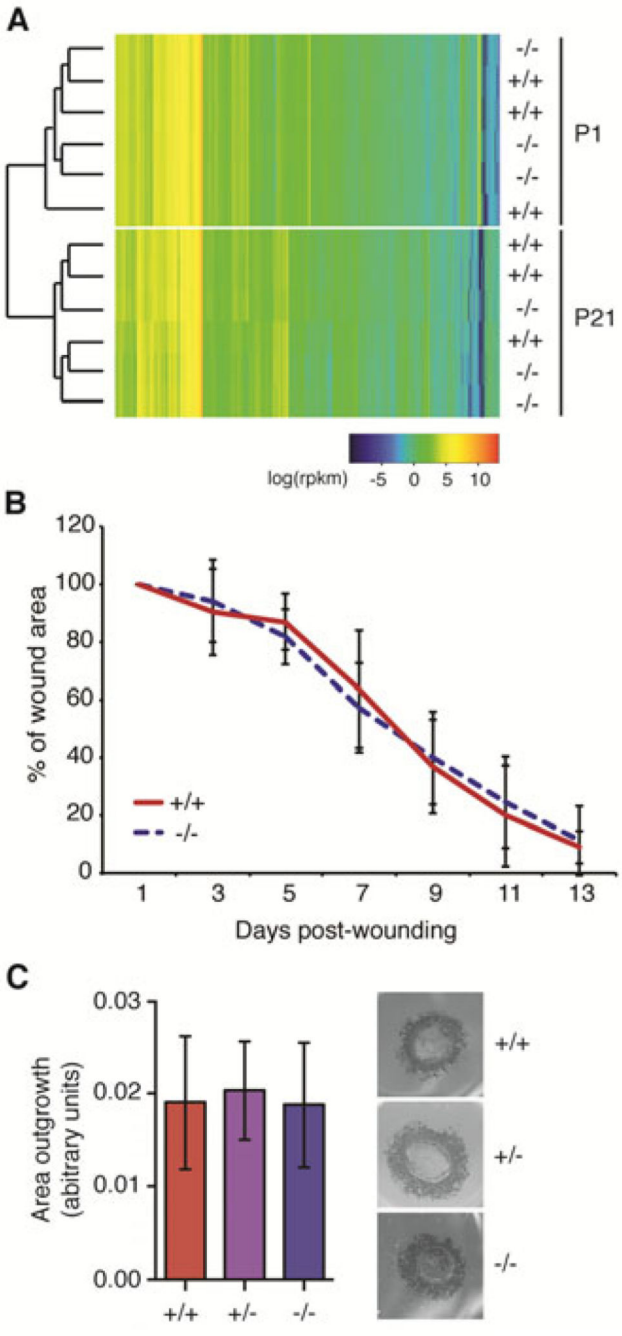

Figure 2. Calm4 is dispensable for epidermal homeostasis and wound healing

(**A**) Heatmap of mRNA expression levels in terms of log of rpkm (graph shows transcripts analyzed by RNAseq with rpkm values ≥ 1) collected from wild-type and Calm4$^{-/-}$ back skin (n=3 each) at P1 and P21. Note that hierarchical clustering groups the samples by age but not genotype. (**B**) 8mm full-thickness wounds heal normally in Calm4$^{-/-}$ mice over the course of 2 weeks. Percentages are relative to wound size on Day 1. Error bars = +/− SD. (**C**) Explant outgrowth assays reveal no difference in the migratory potential of Calm4$^{-/-}$

keratinocytes. Graph shows the mean outgrowth +/− SD of at least 11 individual mice per genotype. Images are representative outgrowths stained for Krt6 after 8 days in culture.