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Serum Response Factor is Essential for the Proper Development of Skin Epithelium

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Abstract

Mammalian epidermis is a stratified epithelium that serves as a barrier protecting the organism from mechanical stress and dehydration. Previous studies have demonstrated the importance of the actin cytoskeleton in the establishment of a functional skin epithelium. Despite what is known about the actin cytoskeleton in epithelial sheet formation, the molecules important for controlling the actin cytoskeleton during epidermal development have not been determined. Serum response factor (SRF) is a transcription factor that is considered to be an important regulator of the actin cytoskeleton. To examine the role of SRF in the developing mouse epidermis, we have employed gene targeting to ablate *Srf* in keratinocytes. Conditional inactivation of *Srf* during the embryonic timepoint leads to a defect in the organization of the epidermis. Immunohistochemical analyses demonstrated a marked loss of the filamentous actin cytoskeleton and E-cadherin localization in epidermis, as well as an aberration in the localization of tight junction proteins. Moreover, impairment of the "inside out" epidermal barrier was shown. *Srf* conditional knock out keratinocytes are unable to establish proper intercellular connections or form an epithelial sheet as shown by histological examination and induced keratinocyte differentiation experiments. Our results demonstrate that *Srf* is essential for the actin mediated sealing of epithelial cell-cell contacts and the development of functional stratified skin epithelium *in vivo*.

Introduction

Mammalian epidermis is a stratified epithelium that serves as a barrier protecting the organism from mechanical stress, dehydration, and microbial insults. The efficient barrier function provided by the skin is established during embryogenesis (reviewed in (Koster and Roop 2007)), where coordinated cell movements (Lechler and Fuchs 2005) and newly established and maintained adhesive connections (Brandner 2009; Fuchs and Raghavan 2002) permit the formation of a self-renewing, stratified tissue. This complex and precisely coordinated stratification program would require the ability of the cell cytoskeleton to undergo rapid and repeated modifications to facilitate cell migration (Disanza et al. 2005; Small et al. 2002) as well the formation of cell-cell adhesive connections (Vaezi et al. 2002; Vasioukhin et al. 2000), both of which are important for the proper development of skin epithelium.

Previous studies have demonstrated the importance of the actin cytoskeleton in the establishment of a functional skin epithelium. A study performed in epidermal keratinocytes of transgenic mice expressing EGFP fused with Beta actin demonstrated that keratinocytes undergo intricate remodeling of their actin cytoskeleton during epithelial sheet formation and subsequent stratification (Vaezi et al. 2002). The actin cytoskeleton was also shown to be necessary for the formation of adhesion zippers, which are intermediates in interepidermal membrane sealing that are important for the formation of an epithelial sheet (Vaezi et al.

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2002; Vasioukhin et al. 2000). In addition, the actin cytoskeleton serves as an anchoring point for multiple molecules important for cell adhesion in epidermis (reviewed in (Fuchs and Raghavan 2002; Schneeberger and Lynch 2004)). Despite what is known about the role of the actin cytoskeleton in the establishment of an epithelial sheet, the molecules controlling the actin cytoskeleton during the development of stratified skin epithelium have yet to be determined.

Serum response factor (SRF) is a highly conserved and widely expressed transcription factor that is considered to be an essential regulator of the actin cytoskeleton, and has been shown to be important for basic biological processes such as cell migration, adhesion, and proliferation (reviewed in (Miano et al. 2007)). SRF orchestrates a complex program of gene expression through its binding to a 10 base pair degenerate sequence known as the CArG box (consensus=CCW_6GG) (Johansen and Prywes 1995; Miano 2003; Treisman et al. 1998). Based upon this sequence, over 400 genes in the mouse genome are considered to be potential SRF targets, of which over 200 have been confirmed (Miano et al. 2007). In relation to the primary function of SRF dependent transcription, more than half of the confirmed targets encode proteins with functions related to actin dynamics, the formation of lamellipodia and filopodia, integrin-cytoskeletal interactions, and muscle contraction (reviewed in (Miano et al. 2007)). Previous studies using conditional knock out mice have demonstrated a role for SRF in processes such as the proper assembly and function of cardiomyocytes and vascular smooth muscle cells (Miano et al. 2004), vascular endothelial cell remodeling (Holtz and Misra 2008), visceral smooth muscle contractile function (Mericskay et al. 2007), and the extension and arborization of axonal projections (Wickramasinghe et al. 2008). A recent study examining the role of SRF in mouse skin demonstrated that postnatal ablation of SRF leads to hyperproliferative skin disease in mice (Koegel et al. 2009). Disruption of the actin cytoskeleton and reduced cell-cell contacts were observed in these mice (Koegel et al. 2009). While this showed that SRF is important in the postnatal maintenance of skin epithelial homeostasis, a role for SRF in the development of stratified skin epithelium has yet to be investigated. Additionally, the mechanism underlying the generation of intercellular contacts that is mediated by SRF is currently unknown.

In this study, conditional inactivation of *Srf* during the embryonic timepoint demonstrates a novel role for SRF in epidermal development. We show that SRF is critical for establishment of the epidermal barrier, possibly through regulating expression and localization of cell adhesion molecules. Further study to investigate the role of SRF during the process of epithelial sheet formation demonstrated a defect in the actin-mediated sealing of intercellular contacts, providing a potential mechanism for the intercellular adhesion defect observed throughout *Srf* ablated epidermis.

Materials and Methods

Mice

B6.129-*Srf*^{tm1Rmn}/J (Miano et al. 2004) and Tg(KRT14-cre)1Amc/J (mixed background of 129/Sv; C57BL6/J; CBA/J; Hsd:ND4 Swiss Webster) (Dassule et al. 2000) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at the University of Wisconsin-Madison. All mouse procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee at the University of Wisconsin-Madison. Mice heterozygous for a loxP flanked *Srf* allele (Miano et al. 2004) and Krt14 cre allele (Dassule et al. 2000) were generated by mating, and then backcrossed to mice homozygous for the floxed *Srf* allele. In all experiments, littermates heterozygous or homozygous for the loxP flanked *Srf* allele, but lacking the Krt14 cre allele, were used as controls. At least three mice were examined per mutant and control group for each experiment.

Histology

Animals were euthanized by decapitation, and tissues were immediately removed and immersion fixed in Bouin's fixative overnight at 4°C. Tissues were then rinsed, dehydrated and embedded in paraffin. Paraffin blocks were sectioned 6 μm thick on an RM 2135 microtome (Leica Microsystems; Wetzlar, Germany) and mounted on glass slides. Following Hematoxylin and Eosin (H&E) staining, slides were imaged on an Eclipse E600 microscope (Nikon; Tokyo, Japan) using a SPOT camera (Spot Diagnostics; Sterling Heights, MI).

Immunohistochemistry on frozen sections

Immunohistochemistry was performed as described in (Verdoni et al. 2008). Primary antibodies and dilutions used were Ki67 (1:100; Thermo Scientific, Fremont, CA), KRT14 (1:1,000; Covance, Emeryville, CA), KRT1 (1:500; Covance, Emeryville, CA), FLG (1:1,000; Covance, Emeryville, CA), E-cadherin (1:2,000; Sigma-Aldrich, Inc., St. Louis, MO), OCLN (1:100; Invitrogen, Camarillo, CA), CLDN1 (1:400; Invitrogen, Camarillo, CA) and ZO1 (1:100; Zymed, San Francisco, CA). Sections were rinsed in PBS, and incubated with Alexa Fluor 488 conjugated secondary antibody (1:400; Molecular Probes, Eugene, OR) and Alexa Fluor 568 conjugated phalloidin (1:50; Molecular Probes, Eugene, OR) for 45 minutes at room temperature. Slides were counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1:200; Sigma-Aldrich, Inc., St. Louis, MO). Images were captured on a Zeiss 510 Confocal Laser Scanning System and Axio Imager Microscope using LSM 510 Software (release 4.2) (Carl Zeiss MicroImaging, Inc., Thornwood, NY) or a Spot Image Analysis system (Spot Diagnostics; Sterling Heights, MI).

Western Blotting

Skin was incubated in a 5 mM EDTA/1X PBS solution for 1 hour at 37°C. Whole epidermis was isolated using forceps and homogenized in RIPA Buffer (1XPBS with 1% NP-40 and 0.1% SDS) containing a protease inhibitor cocktail. Protein concentrations were determined using the Precision Red Protein Assay Reagent (Cytoskeleton Inc., Denver, CO) according to the manufacturer's instructions. Equal amounts of protein were subjected to immunoblot analyses using antibodies against SRF (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (1:1,000; Abcam Inc, Cambridge, MA). Horse-radish peroxidase conjugated secondary antibodies were used (1:2,000; Jackson Immunoresearch, West Grove, Pa) prior to detection with a chemiluminescent reagent (Amersham ECL Plus Western Blotting Detection System, General Electric, Buckinghamshire, UK) and exposure to X-ray film (Thermo Scientific, Rockford, IL).

Outside-in barrier assays

For Lucifer Yellow penetration, newborn mice were euthanized by a lethal injection of ketamine and xylazine (200 mg/kg, 20 mg/kg bodyweight respectively), and immersed for 1 hour in 1 mM Lucifer Yellow (Sigma-Aldrich, Inc., St. Louis, MO) dissolved in PBS. Back skin was used for frozen sectioning and analysis of dye penetration. For Toluidine Blue staining, newborn mice were euthanized by a lethal injection of a ketamine and xylazine, immersed in methanol for 5 minutes, washed briefly with PBS, and incubated for 15 minutes in .1% Toludine Blue (Ricca Chemical Company, Arlington, TX). After a brief rinse in PBS, mice were immediately photographed.

Water Loss

Live newborn mice were separated from their parents, incubated in a chamber at 30°C, and weighed at 30 minute intervals for a 4 hour period. Pups were not fed during this period and urination was not observed in the mice used for the analysis. Weight loss was expressed as the percentage of initial individual weight that was lost during the course of the experiment.

Keratinocyte Culture

Primary epidermal keratinocytes were isolated from the skin of newborn mice using an overnight dispase treatment (5 mg/ml) in CnT-57 media (CellnTec, Bern, Switzerland). Keratinocytes were isolated from the epidermis using a 30 minute treatment with TrypLE select (Invitrogen, Carlsbad, CA) and plated on Collagen coated coverslips (BD Biosciences, San Jose, CA) at 50–70% confluency in CnT-57 media. Keratinocytes were allowed to attach for 36 hours, upon which the media was switched to CnT-02 media for 24 hours. Keratinocyte differentiation was accomplished by increasing the basal calcium concentration in the media from .07 mM to 2 mM for the times indicated.

Immunohistochemistry on cultured cells

Cells were briefly rinsed in PBS 3 times, and fixed for 10 minutes with 4% paraformaldehyde. After 2 rinses in PBS, culture plates were placed on ice for 2 minutes while cells were in the 3rd rinse of PBS. Cells were then permeabilized with ice cold methanol for 1 minute while on ice. Cells were washed in PBS 3 times, and E-cadherin (1:2,000, Sigma-Aldrich, Inc., St. Louis, MO) or SRF antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) was applied for 1 hour. Following 3 rinses in PBS, the cells were incubated with Alexa Fluor 488 conjugated secondary antibody (1:400; Molecular Probes, Eugene, OR) and Alexa Fluor 568 conjugated phalloidin (1:50; Molecular Probes, Eugene, OR) for 1.5 hours. Cells were rinsed 3 times in PBS, and counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1:200; Sigma-Aldrich, Inc., St. Louis, MO). Unless otherwise stated, all steps were performed at room temperature. Images were captured on a Zeiss 510 Confocal Laser Scanning System and Axio Imager Microscope using LSM 510 Software (release 4.2) (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Cell Proliferation Quantification

Frozen sections of back skin were stained for Ki67 and DAPI. Overlapping images of skin were photographed using the Spot Image Analysis system (Spot Diagnostics; Sterling Heights, MI). Cells were counted and length and area measurements were taken on the digital images using ImageJ software (http://rsb.info.nih.gov/ij). Cell numbers for each section were determined for a minimal epidermal length of 2000 μm. Two separate sections were analyzed for each animal.

Image Processing

Images were processed and adjustments to contrast, brightness, and color balance were made using Adobe Photoshop CS Software (Adobe Systems Inc., San Jose, CA).

Statistical Analyses

A two-tailed, unpaired *t*-test was performed for statistical comparison of all numerical values. GraphPad Prism software (GraphPad software, Inc., San Diego, CA) was used for statistical analysis and to create all graphs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Conditional ablation of *Srf* using Krt14 cre recombinase leads to failure of eyelid closure, skin abnormalities, and perinatal lethality

To determine if SRF may play a potential role in the proper development of skin epithelium, we generated mice with an embryonic ablation of *Srf* specifically in the epidermis (hereby referred to as conditional knock out mice, or cKO) using cre recombinase under the control of the Keratin (Krt) 14 promoter. The Krt14 promoter is strongly induced at embryonic day 13.5–14.5, and by birth, most keratinocytes in the skin are positive for the cre mediated

recombination event (Vasioukhin et al. 1999). The use of Krt14 cre mice allowed examination of the role of SRF in the development and stratification of skin epithelium because the majority of these processes occur following the timepoint of Krt14 cre induction (reviewed in (Koster and Roop 2007)). *Srf* cKO mice are born at the expected Mendelian ratios and show obvious abnormalities upon birth. cKO mice display an open eye phenotype indicating a failure of eyelid closure (Fig. 1A,B). The skin appears to be smoother in cKO mice than controls (Fig. 1A,B). Pups do not survive beyond postnatal day 0 (P0).

Histological analysis of head sections at P0 demonstrates a severe defect in eyelid migration and fusion, where the tips fail to fuse at the proper point (Fig. 1C, arrowheads). Thickening and disorganization of the skin epithelium in the periocular area are obvious at the histological level (Fig. 1C).

To confirm the absence of SRF expression in epidermal keratinocytes, we performed western blot analyses for SRF in wild type (WT) and cKO epidermis. A complete ablation of SRF protein in cKO epidermis at P0 is shown (Fig. 1D). Immunohistochemical analysis for SRF in cultured keratinocytes showed that SRF is no longer expressed in the nuclei of cKO keratinocytes, while there is abundant nuclear expression in WT cells (Fig. 1E).

Keratinocyte-specific ablation of *Srf* leads to defects in the structural organization of skin epithelium

In order to closely examine the skin phenotype of cKO mice, we performed histological analyses. Hematoxylin and eosin staining revealed a thick and disorganized epithelium in P0 cKO mice, particularly within the more basal layers, where it was difficult to identify a clear epidermal-dermal boundary (Fig. 2A). Intercellular gaps were present in cKO mice, which suggested a loss of proper cell adhesion (Fig. 2A, arrowheads). Despite these abnormalities, morphological signs of complete epidermal development persisted as the stratum corneum was fully developed (Fig. 2A). Due to the abnormal thickening of the skin epithelium occurring in cKO mice, we hypothesized that the epithelium may be undergoing hyperproliferation, as was found to be a consequence of postnatal ablation of SRF in epidermis in a previous study (Koegel et al. 2009). An antibody against the proliferative marker Ki67 labeled cells in the basal, suprabasal, and apical layers of cKO epidermis. In contrast, WT epidermis showed labeling that was restricted to the innermost basal and suprabasal layers (Fig. 2B). A quantification of Ki67 positive cells in WT and cKO epidermis demonstrated that the number of proliferating cells per unit length was similar (Fig. 2C). The total number of cells per unit length in WT and cKO epidermis was also not significantly different (Fig. 2C). These data demonstrate that cKO epidermis is not undergoing hyperproliferation. However, the total number of cells per unit area was significantly less in mutant epithelium, showing that cells are less densely packed (Fig. 2C). In further support of a defect in adhesion, the area of the epidermis per unit length is greater in cKO epithelium (Fig. 2C), which quantifies the area for the section of epidermis apical to equally measured lengths of the basement membrane. An adhesion defect causing the expansion (i.e greater area/unit length) of the epidermis can explain why total cell number is not significantly different but the number of cells per unit area was significantly less in mutant epithelium. These results show that *Srf* plays a role in the development of the normal architecture of skin epithelium and is important for the spatial organization of cells in this tissue.

Epidermal differentiation is abnormal in *Srf* cKO mice

To determine if normal epidermal stratification is maintained in cKO epithelium, we examined the localization of differentiation markers in the skin. In normal epidermis, keratin 14 (KRT14) begins to be expressed upon epidermal commitment from the dermal layer and is expressed in the basal layer of epidermis (Byrne et al. 1994). In cKO mice, this localization is highly

abnormal, where KRT14 is expressed in all layers of the epidermis at very high levels as compared to WT (Fig. 3). Expression of keratin 1 (KRT1), which is normally localized to the suprabasal layers (Bickenbach et al. 1995; Byrne et al. 1994; Roop et al. 1988), is found in a scattered pattern in cKO epithelium, where many cells in these layers do not express KRT1 (Fig. 3). The expression of the terminal differentiation marker filaggrin (FLG) is also abnormal, where expression is no longer continuous in the most apical layers (Arbeit et al. 1994). Some cells overexpress this protein while others do not express the protein or express it at lower levels than WT (Fig. 3). While these results demonstrate that a subpopulation of cells are undergoing differentiation, they likely indicate that the majority of cells are remaining in an undifferentiated stage just following epidermal specification.

Srf cKO mice display defects in barrier function

The cKO pups are born at the expected Mendelian ratio but die within a day of birth. Since intercellular gaps at the histological level indicated abnormal cell-cell adhesion in the cKO epidermis, we hypothesized that the cause of death may be dehydration, and investigated the barrier function of the skin in *Srf* cKO mice. WT and *Srf* cKO mice have similar body weights just following birth (1.3851g ± .054 and 1.2916g ± .041, respectively). Subsequent body weight measurements at 30 minute intervals demonstrated that cKO mice lost as much as 6% of their body weight over a period of 4 hours, while control animals only lost as much as 1% during the same time period (Fig. 4A). This marked weight loss is strongly indicative of transepidermal water loss (Segre et al. 1999) (Furuse et al. 2002;Tunggal et al. 2005), and suggests a dysfunction in the "inside out" permeability barrier (Furuse et al. 2002;Tunggal et al. 2005). Assessment of the "outside in" permeability barrier with Toluidine Blue staining showed that the interdigit areas of the hands and feet of cKO mice have a significantly compromised permeability (Fig. 4B, arrowheads). Microscopic observation of back skin with a Lucifer Yellow penetration assay did not show any infiltration of the dye into the epidermal or dermal layers (Fig. 4C), which suggested that any impairment of the "outside in" barrier occurs only in the extremities.

Keratinocytes from *Srf* cKO mice display an abnormal actin cytoskeleton and defective formation of cell-cell contacts

With recent genomic studies showing that nearly half of the confirmed SRF target genes are associated with functions related to actin treadmilling, contractility, and lamellipodial/filopodial formation (reviewed in (Miano et al. 2007)), *Srf* may play a direct role in the proper formation of the actin cytoskeleton in skin epithelium. Consistent with this notion, a postnatal ablation of *Srf* lead to a marked loss of filamentous actin localization to the cell periphery in epidermis (Koegel et al. 2009). To determine if *Srf* is also important for the establishment of filamentous actin localization, we stained P0 sections of WT and cKO skin with phalloidin, a molecule that binds to filamentous, or polymerized, actin (F-actin). In WT epithelium, F-actin accumulation is visible in a continuous fashion at the borders of each cell in the epithelium at P0, which represents the cortical actin cytoskeleton network assembled at cell-cell boundaries in mature epithelium (Fig. 5A) (Hodivala-Dilke et al. 1998). In stark contrast, the F-actin network has been severely ablated in cKO epithelium, where F-actin signal is visible at low levels only in the more basal layers of the epithelium (Fig. 5A). Due to the obvious disorganization of the epithelial layer in cKO skin and the appearance of areas devoid of cells, we suspected that adhesive connections between the epithelial cells may be affected. Since the adherens junction protein, E-cadherin, needs to be linked to the actin cytoskeleton for strong cell-cell adhesion (Adams et al. 1998; Angres et al. 1996; Vasioukhin et al. 2000), we hypothesized that the localization of E-cadherin may be affected in cKO mice. Immunohistochemical staining for E-cadherin in WT and cKO skin demonstrated that E-cadherin is expressed at markedly reduced levels in cKO epithelium, where membrane localization is limited to cells still displaying F-actin accumulation (Fig. 5A). Due to the

significant reduction of E-cadherin expression, it is likely that adherens junction formation is inefficient in SRF null epithelium and a loss of proper cell adhesion occurs.

Since the tight junction is another type of cell junction that physically associates with the actin cytoskeleton, we hypothesized that tight junctions may be affected as well in *Srf* cKO mice. Immunofluorescence showed that localization of the tight junction protein occludin is abnormal in *Srf* cKO epidermis, where it no longer localizes to the membranes in the stratum granulosum layer as seen in WT epidermis (Tunggal et al. 2005) (Fig. 5B). Claudin-1, which is proposed to be a central building block of tight junctions (Furuse et al. 1998), is almost completely ablated in *Srf* cKO epithelium, especially in the more apical layers where tight junctions are normally formed (Fig. 5B). Immunofluorescence for ZO-1, also a tight junction protein, showed membrane staining in WT epithelium, especially in the more apical layers. However, its localization was highly abnormal in mutant epithelium with membrane staining observable only in the more basal layers (Fig. 5B). These results show that the epidermal loss of SRF leads to a defect in epidermal tight junction assembly.

Epithelial sheet formation is defective in SrfcKO keratinocytes

To further examine the role of *Srf* in the establishment of epithelial cell-cell contacts, we studied WT and cKO keratinocytes during epithelial sheet formation *in vitro*. Keratinocytes were plated at relatively high density and epithelial sheet formation was induced by changing the calcium level in the media to stimulate their differentiation as described previously (Vaezi et al. 2002; Vasioukhin et al. 2000).

WT keratinocytes adhered to the collagen-coated surface upon plating, as shown by the formation of F-actin rich extensions in contact with the surface matrix (Fig. 6, 0 hr). At 3 hours past the switch to high calcium medium, two rows of punctated staining for E-cadherin appeared at the sites of cell-cell contact in WT keratinocytes. Extending radial actin fibers closely associated with rows of E-cadherin puncta were observed between the cells in contact (Fig. 6, inset). These intercellular structures with E-cadherin puncta and actin filaments are called “adhesion zippers”, which are intermediates in interepidermal membrane sealing (Vaezi et al. 2002;Vasioukhin et al. 2000). They are sites of active actin polymerization during the early phases of epithelial sheet formation (Vasioukhin et al. 2000). At 6 hours past the calcium switch, adhesion zippers have begun to disappear in WT keratinocytes as the adjacent cells make closer contacts with each other and E-cadherin expression is increased at the membranes. F-actin assembled into stress fibers within the cytoplasm of some adjacent cells as if it was a continuous network, which is also an intermediate step in the *in vitro* assembly of an epithelial sheet (Vaezi et al. 2002) (Fig. 6, 6 hr). At 24 hours past the calcium switch, filamentous actin networks were concentrated to cell-cell contacts and formed a honeycombed pattern along with E-cadherin localization, denoting sealed membranes (Fig. 6).

Srf cKO keratinocytes, which do not express SRF protein (Fig. 1), were irregularly shaped when plated on a collagen coated surface, with the cells extending their cytoplasm to large areas (Fig. 6, 0 hr). F-actin rich extensions were not observed contacting the coated surface matrix, which suggested that the formation of these extensions was defective in *Srf* cKO keratinocytes (Fig. 6, 0 hr). Consistent with this observation, *Srf* cKO keratinocytes did not form adhesion zippers at 3 hours past the calcium switch. While F-actin rich areas were observed at the edges of cells, the fiber-like protrusions contacting neighboring cells in WT keratinocytes were not observed in *Srf* cKO keratinocytes (Fig. 6, inset). E-cadherin expression was induced in *Srf* cKO keratinocytes and localized to some membranes at 3 hours past the calcium switch.

However, E-cadherin did not arrange in rows of puncta as observed in WT keratinocytes, which suggested that the arrangement of filamentous actin extensions is a prerequisite for proper E-

cadherin localization. It is also to note that E-cadherin expression was observed at variable levels in the *Srf* cKO culture, with some cells showing a relatively high level of expression in the membrane and others showing very little expression. *Srf* cKO keratinocytes did not change significantly in appearance after the 3 hour timepoint. There was no evidence of adhesion zipper formation or F-actin rich fiber like protrusions (Fig. 6, all timepoints). Although neighboring cells appeared to contact each other (Fig. 6, all timepoints), intercellular gaps were consistently present (Fig. 6, arrowheads). These results suggest that *Srf* cKO keratinocytes are defective in their ability to properly form an epithelial sheet.

Discussion

In this study, the role of SRF in the development of stratified skin epithelium was examined. Our results demonstrate that SRF is necessary for the proper organization and architecture of normal skin epithelium. Phenotypes associated with a loss of *Srf* at the embryonic timepoint suggest that SRF plays a role in the regulation of keratinocyte adhesion and differentiation. It was also shown that SRF is crucial for the development of "inside out" barrier function for body fluid retention as well as the "outside in" barrier in the extremities. Our results demonstrate that SRF is necessary for the proper development of a functional stratified skin epithelium *in vivo*.

Srf conditional knock out mice are born with their eyes open at birth, a defect caused by the improper migration and fusion of eyelid epithelium (reviewed in (Findlater et al. 1993)). Mutations in a number of genes have been shown to cause the open-eye phenotype in mice. Examination of the actin cytoskeleton in the keratinocytes of mutant mice born with open eyes demonstrated that proper actin filament organization is likely to play a central role in the normal migration of eyelid epithelium (Jin et al. 2008; Kato et al. 2007; Li et al. 2003; Shimizu et al. 2005; Tao et al. 2005; Thumkeo et al. 2005; Yu et al. 2008; Zenz et al. 2003; Zhang et al. 2003). In this study, it was shown that keratinocytes in the epidermis of *Srf* cKO mice have a loss of normal organization of actin filaments *in vivo*. Keratinocytes derived from *Srf* cKO mice are also defective in proper organization of actin filaments *in vitro*. Since cell migration requires the formation of actin projections (reviewed in (Disanza et al. 2005) (Small et al. 2002)) and these do not form in *Srf* null keratinocytes, this may lead to the failure of eyelid migration in *Srf* cKO mice.

In this study, it was shown that *Srf* conditional knock out mice have an abnormal epidermal barrier at birth. The "inside out" barrier is impaired, resulting in weight loss that occurs as a consequence of body fluid evaporation. The establishment of a watertight epidermal barrier for the inside out barrier depends upon the proper formation of the intercellular tight junctions between keratinocytes, as well as the stratum corneum (reviewed in (Proksch et al. 2008)). The intercellular tight junctions in the stratum granulosum layer are important for the escape of solutes from the skin, as shown by tracer experiments (Furuse et al. 2002). Consistent with this notion, gene knockout studies have demonstrated that loss of claudin 1, one of the major molecules comprising the tight junction (Furuse et al. 1998), results in leaky tight junctions and perinatal death due to transepidermal water loss (Furuse et al. 2002). A subsequent study also showed that mice epidermally deficient for E-cadherin display similar phenotypes as claudin 1 deficient mice in addition to aberrant localization of tight junction proteins, demonstrating that E-cadherin is essential for the inside out epidermal barrier through its regulation of tight junctions (Tunggal et al. 2005). E-cadherin expression, as well as localization of tight junction proteins occludin, claudin 1, and ZO1, is dramatically affected in *Srf* conditional knock out mice at birth. Based on the known function of SRF as a regulator of the actin cytoskeleton, SRF may function initially to establish localization of filamentous actin at cell borders, which could then serve as a scaffold whose assembly is a prerequisite for the localization of E-cadherin to cell membranes. E-cadherin may, in turn, regulate tight junction

formation as proposed previously (Tunggal et al. 2005). Alternatively, filamentous actin may directly influence tight junction formation through its association with tight junction proteins (reviewed in (Niessen 2007)). This study defines a possible role for SRF in the establishment of the epidermal barrier through the embryonic regulation of the actin cytoskeleton and subsequent E-cadherin localization and tight junction formation.

In a recent study examining the role of SRF in mouse skin, a postnatal loss of SRF (P2-P14) lead to an ablation of F-actin localization but not to a compromise in the E-cadherin localization (Koegel et al. 2009). Epidermal barrier functions were not examined in the study, but they were obviously not impaired to the extent that affects viability. The fact that E-cadherin localization was maintained in animals that did not die upon postnatal SRF ablation shows that the SRF function may be critical to the establishment of the epidermal barrier through regulating E-cadherin localization and subsequent tight junction formation.

A role for SRF in cell-cell adhesion through its regulation of the actin cytoskeleton is further supported by our results from an *in vitro* investigation of epithelial sheet formation. The formation of epithelial sheets is an essential feature of skin epidermis and requires the intricate remodeling of the actin cytoskeleton to establish sealed epithelial cell-cell contacts (Vaezi et al. 2002; Vasioukhin et al. 2000). Epidermal cells in mouse skin display abundant F-actin projectiles, which often pair in a manner resembling that in culture (Vasioukhin et al. 2000). *In vitro* investigation allows the study of the organization of actin filaments during the development of sealed epithelial cell-cell contacts in an epidermal layer. In this study, we showed that adhesion zipper formation is impaired in the early stages of forming an epithelial sheet in keratinocytes derived from *Srf* cKO mice. F-actin projections between neighboring cells were not present in *Srf* cKO keratinocytes. While E-cadherin was found localized to the membranes at some of the intercellular contacts in the *Srf* cKO culture, it did not arrange in rows of puncta associated with a radial actin fiber. This shows that SRF is essential for the initial stage of cell-cell adhesion and its loss results in failure of epithelial sheet formation. Aberrant epithelial sheet formation likely leads to weak intercellular adhesion, as evidenced by the presence of intercellular gaps in the cKO culture. It should be noted that a difference in the *in vitro* and *in vivo* localization of F-actin and E-cadherin in *Srf* cKO keratinocytes was observed. F-actin signal was lost *in vivo* but was detectable at cell edges *in vitro*. E-cadherin signal at cell borders was also lost *in vivo*, but was maintained *in vitro* (despite its abnormal localization pattern). These differences could be a reflection of cells in culture that contact surface matrix and neighboring cells versus those in an *in vivo* environment which contact neighboring cells and basement membrane. Moreover, the stage of differentiation between cultured cells and cells integrated into epidermis could vary and contribute to this change. Nevertheless, it is likely that the defects in cell-cell adhesion in *Srf* cKO keratinocytes are due primarily to defects in the actin cytoskeleton, which is abnormal in cKO epithelium as well as cultured keratinocytes. Our data show that defects in intercellular adhesion are an intrinsic property of *Srf* cKO keratinocytes due to their inability to establish or maintain epithelial cell-cell contacts *in vitro* and *in vivo*.

In this study, a loss of SRF expression in developing skin epithelium results in an epidermal differentiation defect. A differentiation defect was also observed in a previous study where SRF was ablated postnatally (P2-P14) (Koegel et al. 2009). The results of both studies show that SRF is necessary for the development and maintenance of a properly differentiating epithelium. The abnormal differentiation may be due to the adhesion defect observed in *Srf* cKO epithelium. It is known that the prevention of cell-cell contact in cultured keratinocytes leads to a delay in the expression of the differentiation markers KRT10 and involucrin (Charest et al. 2009). In addition, keratinocytes express higher levels of differentiation markers as cells reach confluency in culture and cell-cell contact increases (Kolly et al. 2005). Since *Srf* cKO

mice display an adhesion defect in epidermis, this may lead to an impairment of keratinocyte differentiation.

A loss of cell adhesion may also lead to the abnormal localization of proliferating cells in *Srf* cKO epithelium. In *Srf* cKO mice, proliferating cells were observed not only in the basal layer but also in the suprabasal layer, which may be due to a loss of adhesion to neighboring sites of contact during cell division and movement. The large intercellular gaps throughout cKO epithelium would allow the redistribution of cells in these areas, leading to the appearance of proliferating cells in the suprabasal layers. A defect in cell-cell adhesion in the suprabasal layers may be contributing to the maintenance of cycling cells in this area of the epithelium, allowing cells to remain in a proliferative state because of the absence of a contact dependent cue for cell quiescence.

Our finding that epidermal SRF ablation during embryonic development does not result in an increased number of proliferating cells is in contrast to the result of postnatal ablation of SRF in epidermis. In that study, hyperproliferation of epidermal cells was observed (Koegel et al. 2009), which was hypothesized to be mediated by the mitogenic effects of inflammation. The absence of epithelial cell hyperproliferation in mice with an embryonic ablation of SRF could be due to an inefficient immune response, as newborn mice are generally less effective in eliciting responses to inflammatory stimuli.

In summary, this study defines a new role for SRF, which is to function in the development of a stratified epithelial sheet and the establishment of epithelial barrier in the skin. Our findings also suggest a possibility that SRF may play a general role in the establishment of a stratified epithelium (e.g. epithelial layers of the skin, esophagus, cervix, bladder, sweat glands, oral mucosa, and cornea) through its regulation of the actin cytoskeleton, which is important for epithelial sheet formation.

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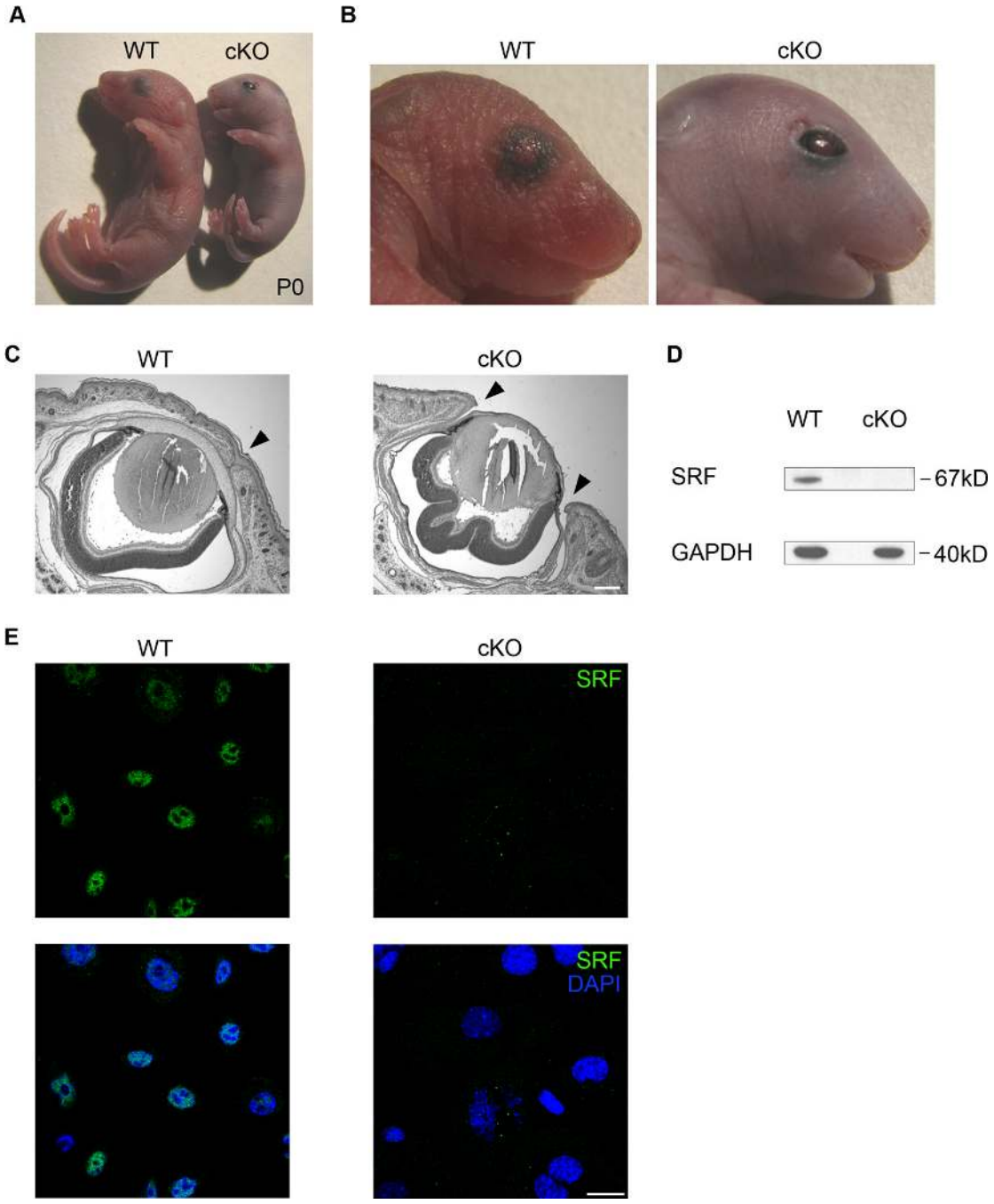

Fig. 1.

Loss of SRF expression in keratinocytes leads to failure of eyelid closure and hyperthickening of eyelid epithelium. *A and B*. Images of WT and *Srf* cKO mice at birth show smoothening of the skin in mutant mice and as well as a failure of eyelid formation. *C*. Hematoxylin and eosin staining shows that the leading edges of the eyelid epithelium fail to migrate and fuse at the proper point in *Srf* cKO mice (arrowheads). A hyperthickening of mutant epithelium is obvious as compared to WT. The scale bar represents 200 μm. *D*. Western blot analysis of whole epidermal lysate demonstrates a complete loss of SRF expression in cKO mice at birth. *E*. Immunohistochemical staining for SRF (green) and DAPI (blue) in cultured keratinocytes

demonstrates a complete ablation of nuclear SRF expression in cKO mice. The scale bar represents 20 μm.

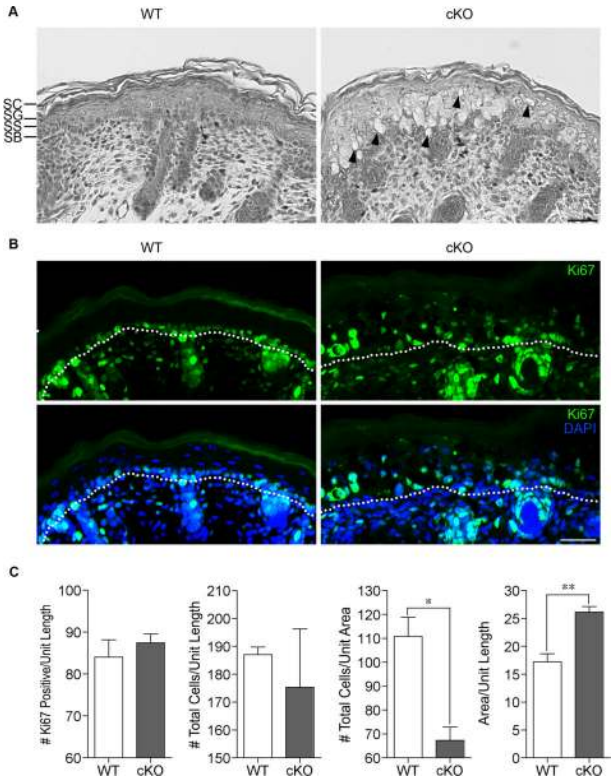

Fig. 2.

Hyperthickening of epidermis in *Srf* cKO mice is due to a loss of proper cell adhesion. *A*. Hematoxylin and eosin staining of back skin demonstrates thickening of the epidermal layer in cKO mice as compared to WT. Large areas devoid of cells are shown (arrowheads). SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale. The scale bar represents 20 μm. *B*. Immunohistochemical staining for the cell proliferation marker Ki67 (green) shows that cycling cells are restricted to the basal layer in WT epithelium while they are localized to the basal, suprabasal, and apical layers in cKO epithelium. Slides were counterstained with DAPI (blue). Dotted lines indicate the basement membrane. The scale bar represents 20 μm. *C*. Quantification of proliferating cells and total cell number shows that there are similar numbers of proliferating cells and total cells in WT and cKO epithelium. The area per unit length is significantly greater in cKO epidermis, and the density of cells per unit area is significantly less than WT, demonstrating that cells are less densely packed in cKO epithelium. Error bars represent s.e.m. * denotes statistical significance by *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

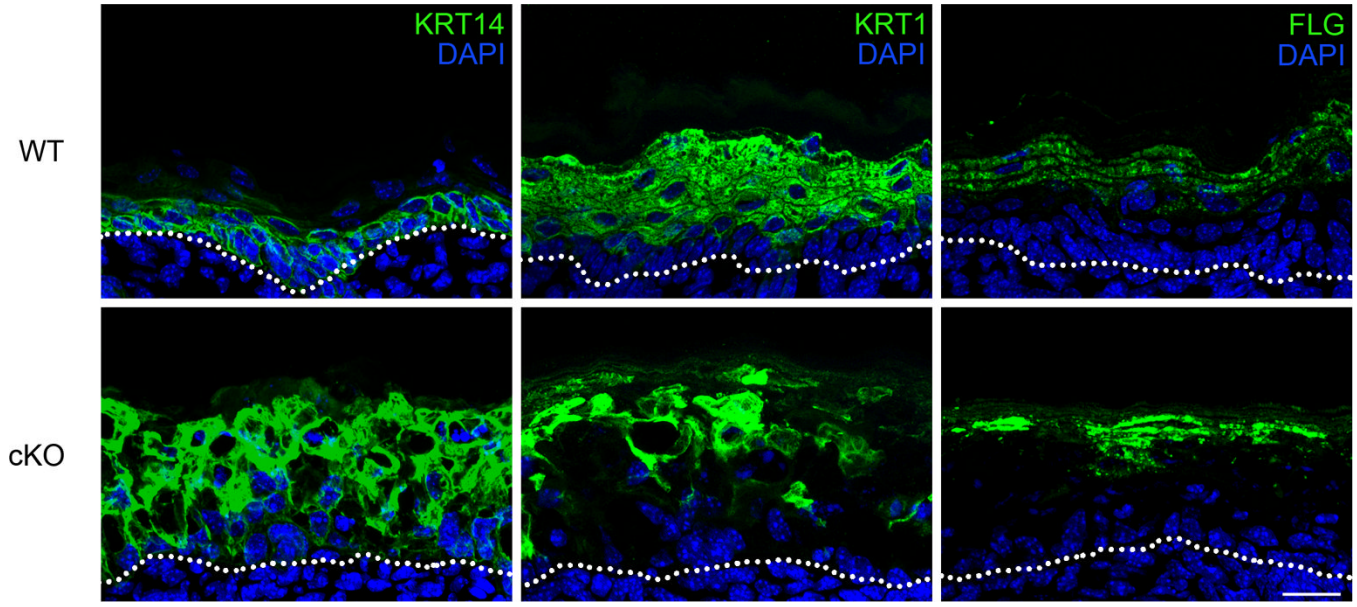

Fig. 3.

Srf cKO mice show a defect in epidermal differentiation. Immunohistochemical staining for the differentiation markers KRT14, KRT1, and FLG (green). KRT14 is ectopically expressed in cKO epithelium, showing localization to all layers. A patchy and overall reduced pattern of expression is shown for KRT1, an intermediate marker of differentiation, and FLG, a terminal differentiation marker. These staining patterns demonstrate that the majority of cells are remaining in an undifferentiated state in cKO epithelium. Slides were counterstained with DAPI (blue). Dotted lines indicate the basement membrane. The scale bar represents 20 μm.

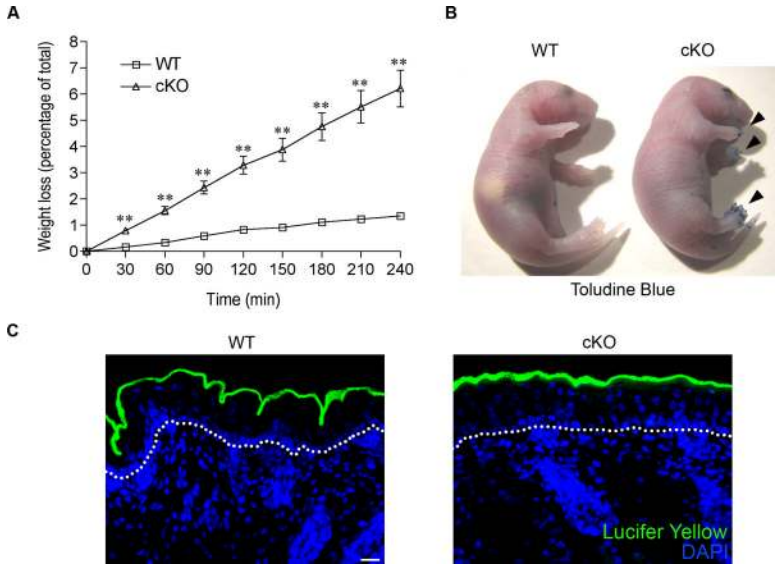

Fig. 4.

Srf cKO mice display defects in epidermal barrier formation. *A*. Body weight measurements in newborn mice over time show a significant weight loss in *Srf* cKO mice, which is indicative of dehydration and a defect in the "inside out" epidermal barrier. Error bars represent s.e.m. Error bars in WT are not observable due to extremely low variability. * denotes statistical significance by *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *B*. Toluidine Blue dye penetration assay demonstrates that the "outside in" barrier is compromised in the hands and feet of *Srf* cKO mice (arrowheads). *C*. Microscopic analysis of back skin sections derived from mice immersed in Lucifer Yellow dye further demonstrates that the "outside in" barrier is not disrupted in this tissue due to loss of SRF expression. Slides were counterstained with DAPI (blue). Dotted lines indicate the basement membrane. The scale bar represents 20 μm.

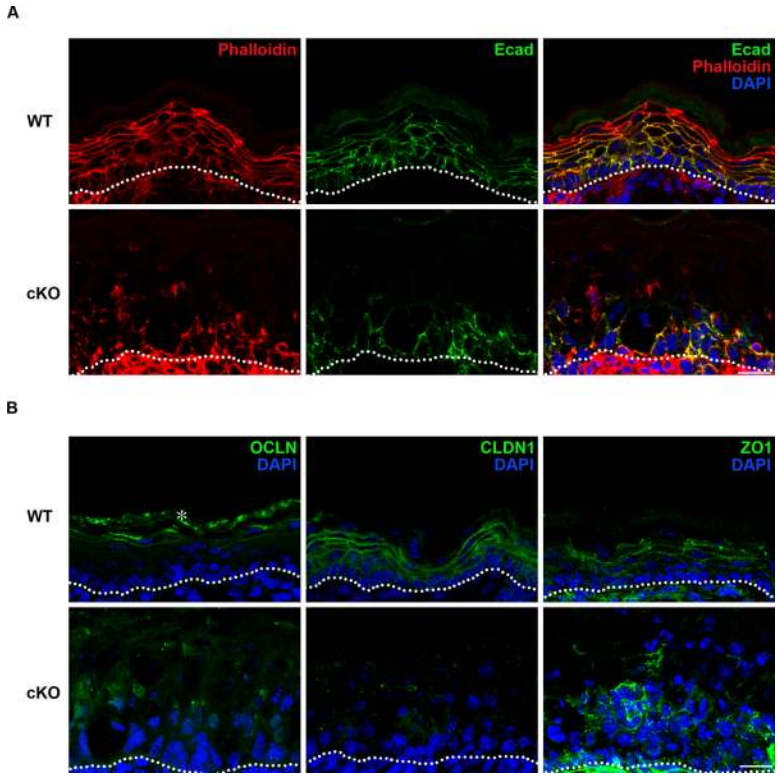

Fig. 5.

SRF expression is important for establishing the localization of F-actin, E-cadherin and tight junction proteins to cell borders in skin epithelium. *A*. Single slice confocal imaging for Phalloidin (red) and E-cadherin (Ecad, green) shows a loss of F-actin and E-cadherin localization to the majority of the epithelium in cKO mice, where both are maintained only in the more basal cells. *B*. Occludin (OCLN, green) localization is lost from the membranes in the stratum granulosum layer in *Srf* cKO mice. The asterisk denotes nonspecific binding to the stratum corneum. Claudin 1 (CLDN1, green) localization to cell borders has been almost completely lost throughout *Srf* cKO epithelium. ZO1 (green) localization is observable at cell borders only in the more basal layers in cKO epithelium. All slides were counterstained with DAPI (blue). Dotted lines indicate the basement membrane. All scale bars represent 20 μm.

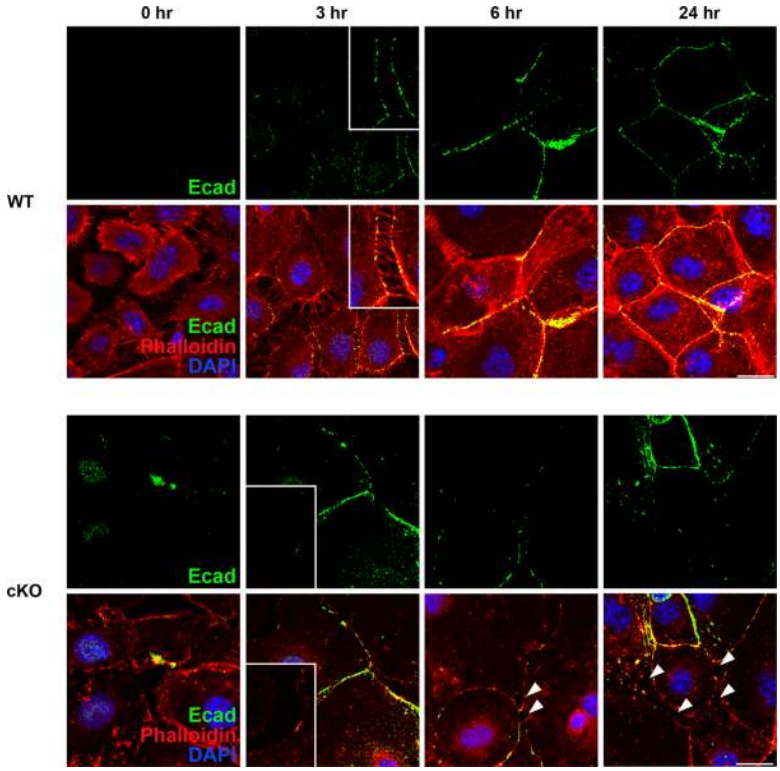

Fig. 6.

Epithelial sheet formation is impaired in *Srf* cKO keratinocytes. Keratinocytes plated at high density in low calcium medium (.07 mM) were switched to high calcium medium (2 mM) for the times indicated. Staining for Phalloidin (red) shows that F-actin projections are not formed in *Srf* cKO keratinocytes at any time point. E-cadherin (Ecad, green) is expressed in keratinocytes following the calcium switch in WT and *Srf* cKO keratinocytes, but its localization into puncta associated with F-actin projections is observable only in WT cells (inset, the adhesion zipper below magnified 2×). Adhesion zippers do not form in *Srf* cKO keratinocytes at any timepoint (inset, the intercellular space above magnified 2×), and intercellular gaps are consistently present (arrowheads). Cells were counterstained with DAPI (blue). The scale bar represents 20 μm.