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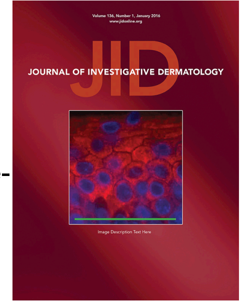

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The calcium-sensing receptor regulates epidermal intracellular Ca^{2+} signaling and re-epithelialization after wounding

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Running title: **Wound re-epithelialization requires the CaSR**

Abbreviations: CaSR, Ca^{2+}-sensing receptor; Ca^{2+}_i, intracellular Ca^{2+}; Ca^{2+}_o, extracellular Ca^{2+}; PLC, phospholipase C; AJ, adherens junctions; PI3K, phosphatidylinositol 3-kinase; PIP5K1α, phosphatidyl inositol 4-phosphate 5-kinase 1α; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; EGFR, epidermal growth factor receptor; Vdr, vitamin D receptor; α -SMA, smooth muscle actin-α

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ABSTRACT

Extracellular Ca^{2+} (Ca^{2+}_o) is a crucial regulator of epidermal homeostasis and its receptor, the Ca^{2+}-sensing receptor (CaSR), conveys the Ca^{2+}_o signals to promote keratinocyte adhesion, differentiation, and survival via activation of intracellular Ca^{2+} (Ca^{2+}_i) and E-cadherin-mediated signaling. Here, we took genetic loss-of-function approaches to delineate the functions of CaSR in wound re-epithelialization. Cutaneous injury triggered a robust CaSR expression and a surge of Ca^{2+}_i in epidermis. CaSR and E-cadherin were co-expressed at the cell-cell membrane between migratory keratinocytes in the nascent epithelial tongues. Blocking the expression of CaSR or E-cadherin in cultured keratinocytes markedly inhibited the wound-induced Ca^{2+}_i propagation and their ability to migrate collectively. Depleting CaSR also suppressed keratinocyte proliferation by down-regulating the E-cadherin/epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK) signaling axis. Blunted epidermal Ca^{2+}_i response to wounding and retarded wound healing were observed in the keratinocyte-specific CaSR knockout ($^{Epid}Casr^{-/-}$) mice, whose shortened neo-epithelia exhibited declined E-cadherin expression and diminished keratinocyte proliferation and differentiation. Conversely, stimulating endogenous CaSR with calcimimetic NPS-R568 accelerated wound re-epithelialization through enhancing the epidermal Ca^{2+}_i signals and E-cadherin membrane expression. These findings demonstrated a critical role for the CaSR in epidermal regeneration and its therapeutic potential for improving skin wound repair.

INTRODUCTION

Ca^{2+} maintains the normal homeostasis of mammalian epidermis by regulating keratinocyte adhesion, differentiation, and survival (Calautti et al., 2005; Yuspa et al., 1989), and growing

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evidence substantiate its involvement in wound repair. An elevation of Ca^{2+} concentration is detected in the wound bed and surrounding fluid within minutes after injury (Jungman et al., 2012; Lansdown et al., 1999), and elevated Ca^{2+} is the determining factor for wound fluid to stimulate cell motility in keratinocytes (Grzesiak and Pierschbacher, 1995). Increasing Ca^{2+} levels in the extracellular milieu of wound bed accelerates skin wound closure in mice (Kawai et al., 2011). Furthermore, mechanical or laser wounding triggers a rapid and transient increase in Ca^{2+}_i that spread from wound site to neighboring cell layers in epithelial sheets (Tsutsumi et al., 2013) and the epidermis of *C. elegans* (Xu and Chisholm, 2011) and embryos of *Drosophila* and *Xenopus* (Razzell et al., 2013; Soto et al., 2013), and blocking the Ca^{2+}_i propagation inhibits the ability of epithelial cells to close wound (Agle et al., 2010; Xu et al., 2012). These findings suggest that the surge of Ca^{2+}_i mobilization is one of the earliest signals produced at wound sites to trigger epithelial healing (Cordeiro and Jacinto, 2013; Wood, 2012).

The CaSR, a member of the family C G-protein coupled receptor, senses the changes in Ca^{2+}_o levels and initiates diverse cellular responses in epidermal keratinocytes. Activated CaSR instigates phospholipase C (PLC)-mediated Ca^{2+}_i accumulation and coordinates Ca^{2+}_i mobilizations from internal stores and across membrane channels through direct interactions of the receptor with various Ca^{2+}_i modulators, i.e. 1,4,5-trisphosphate receptor (IP_3R), Ca^{2+}-ATPase, and PLCγ1 (Tu et al., 2007). CaSR also activates the Rho GTPase-mediated signaling to facilitate the actin-cytoskeleton remodeling and the formation of E-cadherin/catenin adherens junction (AJ) (Tu et al., 2011; Tu and You, 2014), which play an obligatory role in transducing the outside-in signals by activating and integrating various intracellular signaling cascades. Assembly of AJs stimulates MAPK pathway through the recruitment and activation of EGFR to control cell proliferation and migration (Fedor-Chaiken et al., 2003; Pece and Gutkind, 2000)

and engages phosphatidylinositol 3-kinase (PI3K) to activate Akt pathway to promote keratinocyte survival and differentiation (Calautti et al., 2005; Pang et al., 2005; Pece et al., 1999). Moreover, the E-cadherin/PI3K/phosphatidyl inositol 4-phosphate 5-kinase 1α (PIP5K1α) signaling complex activates PLC-γ1 via phosphatidylinositol 3,4,5-triphosphate to sustain elevated Ca^{2+}_i levels as keratinocytes differentiate (Xie and Bikle, 2007; Xie et al., 2009). CaSR-deficient keratinocytes display blunted Ca^{2+}_i response to Ca^{2+}_o due to depleted internal stores and aberrant Ca^{2+}_i influx and severely impaired intercellular adhesion (Tu et al., 2007; Tu et al., 2008). $^{Epid}Casr^{-/-}$ mice, in which the Casr gene is deleted specifically in the keratinocytes, exhibit a delay in permeability barrier formation during embryonic development and the skins of adult mice manifest a loss of the epidermal Ca^{2+} gradient, impaired keratinocyte differentiation, abnormal sphingolipid metabolism, and defective permeability barrier (Tu et al., 2012).

Our previous studies show that restricting dietary calcium or deleting CaSR exacerbate the deficit in wound healing in mice caused by deletion of the vitamin D receptor (Vdr) from keratinocytes (Oda et al., 2016; Oda et al., 2017), implicating the interaction of calcium and vitamin D signaling in regulating repair processes. Wound re-epithelialization is mostly affected by the concurrent deletion of Vdr and Casr genes due to perturbance of a number of pathways relevant to wound healing, including β-catenin and AJ signaling (Oda et al., 2017). As recent studies show that VDR controls the renewal and activation of epidermal stem cells during wound repair via the β-catenin pathway (Oda et al., 2018), the role of calcium in wound healing remains indistinguishable in the models examined. To surely identify the mechanism underlying the calcium action in wound healing, we deleted CaSR in keratinocytes *in vivo* and *in vitro* in this study. Importantly, our results demonstrated the requirement for CaSR in the initiation of acute epidermal Ca^{2+}_i signaling and the formation of the E-cadherin-containing AJ involved in the

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proliferation and migration of the keratinocytes to re-epithelialize the wound and their subsequent differentiation to regenerate the epidermis.

RESULTS

Ablation of CaSR Delays Wound Repair

As a first step to define the role of CaSR in wound repair, we compared the CaSR expression in intact and puncture-wounded skin. Immunohistochemistry showed that the CaSR was present in all epidermal cell layers in the undamaged skin with predominant expression in the basal keratinocytes of the interfollicular epidermis (Figure 1a, upper panel). CaSR expression was significantly increased one day after injury in the thickened epidermis at the wound margins and in the nascent epithelial tongue above the wound bed (Figure 1a, lower panel). QPCR analyses of RNA extracted from the wounds confirmed an increase (12-fold) in CaSR message level (Figure 1b) compared to the intact skins. To examine the impact of CaSR ablation on wound repair, we compared the progression of wound healing in $^{Epid}Casr^{-/-}$ mice and their $Casr^{fl/fl}$ control littermates by measuring the wound size. Wound closure in $^{Epid}Casr^{-/-}$ mice was delayed as reflected by their significantly bigger wound sizes versus controls (Figure 1c and 1d). Measurements of the span of the neo-epithelia in wounds three days after injury showed that the distance of epidermal keratinocytes traveled across the wound beds was reduced by 37% in $^{Epid}Casr^{-/-}$ mice (Figure 1e and 1f), suggesting that CaSR ablation delayed wound closure by effecting on re-epithelialization.

To determine whether injury alters the Ca^{2+}_i levels, we compared epidermal Ca^{2+}_i dynamics in response to wounding in transgenic mice expressing the fluorescent Ca^{2+} sensor protein GCaMP3 (Tian et al., 2009) under the control of keratin14 promoter. We performed focal laser

wounding on mouse dorsal skins and imaged GCaMP signals in different epidermal cell layers using a confocal microscopy. Laser irradiation in the upper stratum granulosum (SG) immediately triggered a surge of Ca^{2+}_i, as indicated by the intensified GCaMP fluorescence, which radiated from wound site to the neighboring cell layers (Figure 2a). The Ca^{2+}_i signals were spread crosswise in SG and downward through the stratum spinosum (SS) to the stratum basale (SB) where Ca^{2+}_i propagated with a peak intensity >3 fold stronger than the SG (Figure 2b). These Ca^{2+}_i propagation events typically persisted for several minutes before dissipated. We subsequently employed an *in vitro* scratch wound model to test whether the transient Ca^{2+}_i surges are required for wound restitution. Scratching confluent keratinocyte sheets immediately induced Ca^{2+}_i waves spreading from the wound site to distant undamaged areas (Supplementary Figure S1a and S1b). We treated keratinocytes with BAPTA, an intracellular Ca^{2+} chelator, or 2-APB, an IP_3R and TRP channel blocker, for 15 min prior to wounding to prevent Ca^{2+}_i propagation (Supplementary Figure S1c) and examined its impact on wound closure 24 hours later. Pretreatment with BAPTA or 2-APB markedly inhibited the ability of keratinocyte to migrate collectively to close wound (Figure 2c and 2d), demonstrating the prerequisite of Ca^{2+}_i signals in re-epithelialization.

Wound-induced Epidermal Ca^{2+}_i Responses and Keratinocyte Migration Require CaSR and E-cadherin

The robust Ca^{2+}_i propagation in SB keratinocytes (Figure 2b) coincided with the predominant CaSR expression, suggesting a role for CaSR in controlling Ca^{2+}_i mobilization responding to wounding. Indeed, as shown in Figure 3a, the epidermal Ca^{2+}_i wave triggered by laser wounding in the basal cell layer in the skins of CaSR knockout ($^{Epid}Casr^{-/-}$//$GCaMP^{+/+}$) mice was reduced to 20-40% of the Ca^{2+}_i response in the control ($^{Epid}Casr^{+/+}$//$GCaMP^{+/+}$) skins (Figure 3b, 3c, and 3d).

Our previous studies unveiled that E-cadherin is a critical downstream effector for CaSR in regulating keratinocyte survival, adhesion, and differentiation (Tu et al., 2008; Tu et al., 2011). Immunohistochemistry of 3-day-old wounds showed that CaSR and E-cadherin were co-expressed in all keratinocyte layers in the nascent epithelia including the migratory front (Supplementary Figure S2a, S2a', S2b, and S2b') where the expression of desmoglein1, a desmosome component, was excluded (Supplementary Figure S2c and S2c'). The intense presence of CaSR and E-cadherin in the cell-cell membrane between migratory keratinocytes located at the tip of the epithelial tongues (Supplementary Figure S2a'' and S2b'') suggested their involvement in mediating collective keratinocyte migration during re-epithelialization. To test this idea, we examined the effects of gene silencing of CaSR and E-cadherin on wound restitution. Blocking CaSR expression inhibited the translocation of E-cadherin from cytoplasmic compartment to cell membrane (Supplementary Figure S3a). Consistent with the role of E-cadherin-dependent AJ in the CaSR-mediated Ca^{2+}_i accrual (Tu et al., 2011; Xie et al., 2009), inhibiting the expression of either CaSR or E-cadherin with CaSR- (siCaSR) or E-cadherin-targeting siRNA (siEcad) (Supplementary Figure S3a and S3b) profoundly diminished the wound-induced Ca^{2+}_i propagation (Figure 3e and 3f) and, the collective directional keratinocyte migration after wounding (Figure 3g and 3h). These data indicate that CaSR and E-cadherin modulate the epithelial sheet migration, at least partly, via Ca^{2+}_i signals.

CaSR Ablation Impairs Keratinocyte Proliferation and Differentiation During Wound Re-epithelialization

To ascertain that wound re-epithelialization was impacted by CaSR ablation, we compared the morphology and gene expression of neo-epithelia in 5-day-old skin wounds from $^{Epid}Casr^{-/-}$ mice and control littermates. The neo-epithelia in $^{Epid}Casr^{-/-}$ mice (Figure 4b) appeared thinner

than those in the controls (Figure 4a and 4c) with reduced numbers of cell layers comprised of flattened nucleated keratinocytes. Measurement of neo-epithelial areas showed a 35% decrease in $^{Epid}Casr^{-/-}$ mice (Figure 4d). Immunohistochemical staining of wound sections for proliferating cell nuclear antigen (PCNA) showed decreased numbers of proliferating keratinocytes in the neo-epithelia in $^{Epid}Casr^{-/-}$ mice (Figure 4f and 4g), correlated with a declined cyclin D1 (Ccnd1) expression (Figure 4h).

Immunohistochemistry detected strong E-cadherin localizations in the cell-cell membrane in the migratory keratinocytes at the tip of the epithelial tongues and in the suprabasal keratinocytes adjacent to the wound margins in control mice (Figure 4a and 4k), while the E-cadherin expression was significantly diminished in the neo-epithelia of $^{Epid}Casr^{-/-}$ mice (Figure 4b and 4l). QPCR assays confirmed a 45% reduction in E-cadherin expression in wounds from $^{Epid}Casr^{-/-}$ mice (Figure 4e) versus controls. Notably, epidermal differentiation in the neo-epithelia started in the suprabasal cell layers near the wound margins (Figure 4j), where intense cell-cell membrane localizations of E-cadherin were first noticed (Figure 4a and Supplementary Figure S2b), corroborating a role for E-cadherin-mediated cell-cell adhesion in promoting keratinocyte differentiation. Corresponding to a reduction of membrane-localized E-cadherin, qPCR analyses (Figure 4i) and immunohistochemical staining (Figure 4j) showed that the levels of keratinocyte differentiation markers keratin (K) 1, transglutaminase (TGM) 1, loricrin, and filaggrin (FLG) in the neo-epithelia of $^{Epid}Casr^{-/-}$ mice were reduced by 32-47%.

Cell proliferation during wound re-epithelialization is critically regulated by growth factors and their downstream signaling cascades, including the EGFR/MAPK signaling axis (Muller et al., 2012; Repertinger et al., 2004; Seeger and Paller, 2015). As shown in Figure 5a, blocking the activity of EGFR and MEK with specific inhibitors, AG1478 and U0126, respectively, inhibited

cell proliferation in cultured keratinocytes. Growing evidence indicate that clustering membrane-localized E-cadherin is required to activate EGFR and stimulate MAPK in epithelial cells (Fedor-Chaiken et al., 2003; Pece and Gutkind, 2000). In epidermal keratinocytes, CaSR stabilizes AJ through recruitment and activation of Rho-family GTPase and induction of cytoskeletal reorganization (Tu et al., 2011; Tu and You, 2014). Fluorescence immunostaining (Supplementary Figure S4a, S4b, S4c), and co-immunoprecipitation (Supplementary Figure S4d) demonstrated that blocking CaSR expression with adenoviruses carrying a full-length CaSR antisense cDNA (Ad-ASCaSR; Supplementary Figure S3c) suppressed the Ca^{2+}-induced trans-localization of RhoA and E-cadherin to the cell membrane, hence inhibited the formation of E-cadherin/β-catenin adhesion complex and the insertion of cytoskeletal actin into AJ at cell-cell contact. We then tested whether inhibition of CaSR expression affected the E-cadherin/EGFR interaction and MAPK activation. Fluorescence immunostaining (Figure 5b) demonstrated that inducing the formation of cell-cell contacts by raising Ca^{2+}_o concentration from 0.03 to 2 mM substantially increased the colocalization of EGFR with E-cadherin at the cell membrane in the control keratinocytes, whereas inhibiting CaSR expression by Ad-ASCaSR reduced the association of EGFR with E-cadherin (Figure 5b). The E-cadherin/EGFR interaction was verified by co-immunoprecipitation of plasma membrane protein using antibodies for either protein (Supplementary Figure S5a). Additionally, Ad-ASCaSR infection markedly suppressed the ability of Ca^{2+} and EGF to activate EGFR and ERK, reflected by reduced levels of phosphorylated EGFR and phosphorylated ERK1/2, respectively, (Figure 5c, Ad-ASCaSR vs. Ad-control). The impact of CaSR depletion on ERK activation was specific for EGFR/MAPK signaling, since Ad-ASCaSR infection had no effect on the IGF-induced ERK phosphorylation, which is mediated via PI3K pathway (Haase et al., 2003). Conversely, EGFR inhibitor AG1478

inhibited Ca^{2+}-stimulated phosphorylation of EGFR and ERK (Supplementary Figure S5b), substantiating the crosstalk between CaSR and EGFR pathways. As a consequence of the diminished E-cadherin/EGFR/ERK signaling, CaSR depletion greatly inhibited keratinocyte proliferation (Figure 5d).

Activating Endogenous CaSR Facilitates Wound Healing

To test whether manipulating endogenous CaSR activity affects wound repair, we topically treated wounds on the dorsal skins of wild-type C57Bl/6J mice with allosteric CaSR agonist (calcimimetic) or antagonist (calcilytic) and examined their effects on wound closure. As shown in Figure 6a, the calcimimetic NPS-R568 accelerated, whereas the calcilytic NPS-2143 delayed, wound closure. Measurements of the length of the neo-epithelia in 3-day-old wounds demonstrated that NPS-R568 increased wound re-epithelialization by 56% (Figure 6b and 6c), correlated with the ability of NPS-R568 to augment the intensity and expanse of the wound-induced Ca^{2+}_i waves (Figure 6d and 6e). QPCR analyses of wounded skins showed that topical NPS-R568 treatment up-regulated E-cadherin expression (Figure 6f) and increased the levels (30-73%) of differentiation markers in the neo-epithelia (Figure 6g). Also, as shown in Supplementary Figure S6, pre-treating keratinocyte sheets with NPS-R568 increased the total and cell membrane-localized E-cadherin after wounding. These data demonstrated that activating endogenous epidermal CaSR effectively facilitated epidermal wound repair by enhancing the Ca^{2+}_i and E-cadherin signaling.

CaSR Ablation in Keratinocytes Impacts on Acute immune Response and Dermal Repair

To examine whether deleting CaSR from keratinocytes influences other wound repair processes, we evaluated inflammatory cell infiltration and the distribution of the myofibroblasts in the dermal granulation tissue. While fluorescence immunostaining of 1-day-old skin wounds

using CD45 antibody (Supplementary Figure S7a) showed that $^{Epid}Casr^{-/-}$ mice and control mice exhibited comparable leukocytes infiltration near the wound margins; however, qPCR analyses of skin wounds (Supplementary Figure S7b) revealed a 39-58% reduction in the expressions of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) and regulators for immune response (cathelicidin, TLR2, MCP1, MIP1α, and Cox2) in $^{Epid}Casr^{-/-}$. Furthermore, as the message levels of various dermal extracellular matrix (ECM) were equivalent in $^{Epid}Casr^{-/-}$ and control wounds five days after injury (Supplementary Figure S7d), the number of α–smooth muscle actin (αSMA)-positive myofibroblasts (Supplementary Figure S7c) and the expression of several ECM-remodeling enzymes (MMP3, 9, and 10) were decreased by 33-53% in $^{Epid}Casr^{-/-}$ (Supplementary Figure S7d). It was conceivable that these changes in acute immune responses and dermal remodeling also contributed to the delayed wound healing in $^{Epid}Casr^{-/-}$ mice.

DISCUSSION

Ca^{2+} is a key regulator of keratinocyte differentiation, but its actions and underlying mechanisms in wound healing remain unclear. In the present study, we discovered that epidermis responds to injury with a profound increase in CaSR expression and our results demonstrated pivotal roles played by the epidermal CaSR in initiating early Ca^{2+}_i responses and E-cadherin signaling to stimulate keratinocyte proliferation, migration, and differentiation for wound re-epithelialization.

The rapid induction of the Ca^{2+}_i propagation at a wound site signifies Ca^{2+}_i as a transcription- and translation-independent damage signal to initiate epithelial healing (Cordeiro and Jacinto, 2013). Wound-elicited Ca^{2+}_i waves protect corneal endothelium from excess apoptosis (Justet et al., 2016). In *Drosophila* embryos epidermal Ca^{2+}_i surge instigates H_2O_2 release via activation of a NAPDH oxidase to recruit inflammatory cells at the wound site (Razzell et al., 2013). The

epidermal $Ca^{2+}{}_i$ signal acts upstream of Rho-GTPases to modulate actin polymerization and actin-myosin network constriction to close wound in nematodes and Xenopus embryos, respectively (Soto et al., 2013; Xu and Chisholm, 2011). In this study we showed that wound-induced $Ca^{2+}{}_i$ propagation is required for effective epithelial sheet migration (Figure 2c). Interestingly, we uncovered differential $Ca^{2+}{}_i$ responses to wounding in separate keratinocyte layers of mammalian stratified epidermis (Figure 2a), with the strongest response in the basal layer where CaSR was highly expressed and cells were activated to proliferate and migrate after wounding. In line with the obligatory role of CaSR in controlling $Ca^{2+}{}_i$ mobilization in keratinocytes (Tu et al., 2007), inhibiting CaSR expression abolished wound-induced epidermal $Ca^{2+}{}_i$ response, suppressed collective directional keratinocyte migration (Figure 3), and impeded wound restitution *in vivo* (Figure 1).

Despite conventional views linking the down-regulation of E-cadherin with increased cell motility, E-cadherin appears to promote epithelial migration under several circumstances (Kardash et al., 2010; Rodriguez et al., 2012). In *Drosophila*, E-cadherin functions as an integrator of mechanical signals and is necessary for collective directional migration of border cell clusters (Cai et al., 2014). E-cadherin-dependent traction forces of the leading-edge cells coordinate migration of renal epithelial sheets (Li et al., 2012). E-cadherin acts downstream to CaSR to mediate keratinocyte sheet migration likely through regulating intercellular adhesion and $Ca^{2+}{}_i$ accrual (Xie et al., 2009). Membrane expression of E-cadherin was reduced in the leading edges of the shortened epithelial tongues in $^{Epid}Casr^{-/-}$ wounds (Figure 4b), consistent with the decreased migration rate of CaSR- or E-cadherin-deficient keratinocytes *in vitro* (Figure 3g).

Members of the EGF family induce the rapid proliferation and migration of keratinocytes at the wound edge through the EGFR/MAPK signaling cascade to promote re-epithelialization (Haase et al., 2003; Loo et al., 2011; Repertinger et al., 2004; Shirakata et al., 2005). The engagement of E-cadherin in newly formed cell contacts is critical for recruitment and sensitization of EGFR to allow responses to low levels of EGF and activation of downstream MAPK and Rac1 signaling cascades (Betson et al., 2002; Fedor-Chaiken et al., 2003; Pece and Gutkind, 2000). The findings that depleting CaSR inhibited EGF-stimulated phosphorylation of EGFR and ERK (Figure 5c) while EGF inhibitor blocked Ca^{2+}-induced activation of ERK (Supplementary Figure S5b) support a signaling scheme in which the E-cadherin/EGFR interface couples the CaSR to MAPK activation. Depleting CaSR reduced the interaction between E-cadherin and EGFR (Figure 5b and Supplementary Figure S5a) and diminished MAPK signaling (Figure 5c), leading to decreased cell proliferation *in vitro* (Figure 5d) and *in vivo* (Figure 4f). Furthermore, in the neo-epithelia of $^{Epid}Casr^{-/-}$ mice the membrane-localized E-cadherin in suprabasal keratinocyte layers was markedly reduced (Figure 4b), similar to the lack of E-cadherin adhesion complex formation in CaSR-depleted keratinocytes *in vitro* (Supplementary Figure S4), rendering the delay of epidermal differentiation during wound repair (Figure 4j).

In contrast to the negative impacts of genetic knockdown or pharmacological inhibition of CaSR on re-epithelialization, stimulating endogenous CaSR with an allosteric activator, NPS-R568, amplified the magnitude and duration of injury-induced Ca^{2+}_i propagation and accelerated wound closure (Figure 6). During cytokine-guided intestinal restitution, elevated Rho signaling stimulates the membrane localizations of E-cadherin and F-actin to reduce the paracellular space and enhance the epithelium barrier integrity (Hwang et al., 2012). Likewise, NPS-R568 increased the localization of E-cadherin to the cell-cell junctions between keratinocytes during

wound restitution (Supplementary Figure S6c). Our studies support that Ca^{2+}/CaSR signal is essential for cutaneous wound repair and convey clinical implications for the use of calcimimetics in improving outcomes of wound healing.

In summary, our previous and current studies support a working model for the actions of the CaSR in wound re-epithelialization (Figure 6h). Injury increases CaSR expression in keratinocytes at the wound margins and raises the Ca^{2+} levels in the extracellular milieu of those cells, activating the CaSR and downstream pathways: (1) CaSR couples to the Gαq and activates PLC to generate IP_3, which triggers calcium release from internal stores and subsequent Ca^{2+} influx through membrane channels, resulting in a transient increase in Ca^{2+}_i. The Ca^{2+}_i signals promote the reorganization of actin cytoskeleton and changes of cell adhesion likely through Rho-mediated signaling, enabling keratinocyte to migrate collectively. (2) CaSR forms a signaling complex with Rho and GEF to stabilize the E-cadherin/catenin adhesion complexes at the cell-cell contacts, which in turn activate EGFR and downstream MAPK pathway to support keratinocyte proliferation. (3) E-cadherin-mediated AJs engage PIP5K1α and PI3K and stimulate their effectors, PLCγ 1 and Akt, to promote Ca^{2+}_i accumulation, cell survival, and differentiation. CaSR integrates extracellular cues (wounding, Ca^{2+}_o) and intracellular signals (Ca^{2+}_i, E-cadherin, MAPK) to coordinate keratinocyte proliferation, migration, and differentiation to re-epithelialize the wounds.

Unexpectedly, our results indicate that the loss of keratinocyte CaSR had broader effects on wound repair besides re-epithelialization. It was uncertain whether the altered acute immune response and dermal matrix remodeling in $^{Epid}Casr^{-/-}$ mice skin (Supplementary Figure S7) was a direct consequence of impaired functions of keratinocytes, which have the ability to produce pro-inflammatory factors and ECM modifying enzymes, or an indirect outcome due to the impacts

on immune cells and fibroblasts. Further studies are needed in the future to address these questions and decipher the underlying mechanism.

MATERIALS AND METHODS

Mice and *In Vivo* Wounding

Generation of Casr$^{fl/fl}$ control and ^{Epid}Casr$^{-/-}$ mice, in which the entire transmembrane domain and intracellular portion of the CaSR is deleted in Keratin 14 (Krt14)-expressing keratinocytes, and the verification of gene ablation were described previously (Tu et al., 2012). ^{Epid}Casr$^{-/-}$ and K14-cre were bred with ROSA-GCaMP3 mice (Ai38; Jackson Laboratory, Sacramento, CA) to create ^{Epid}Casr$^{-/-}$//GCaMP$^{+/+}$ and ^{Epid}Casr$^{+/+}$//GCaMP$^{+/+}$, respectively. All mice were bred into the C57Bl/6J background and maintained on a normal calcium (1.3%) diet. Mice at 8 weeks of age were used in wound repair assessment. Four-mm full-thickness skin wounds were made on the backs of mice with a biopsy punch after hair depilation. To calculate the wound closure rate the wounds were photographed daily during a 7 to 10-day recovery period, and areas of the wounds were quantitated using ImageJ software (NIH). Wounds from at least 8 mice per genotype were evaluated. Wounds and their 2 mm-wide peripheral areas were excised for immunohistochemical or RNA extraction for gene expression analyses at the specified time points after wounding. The Institutional Animal Care and Use Committee at the San Francisco Veterans Affairs Medical Center approved all protocols.

Human Keratinocytes and Gene Silencing

Human neonatal foreskin keratinocytes (NHKs) were cultured in serum-free growth medium 154CF (Thermo Fisher Scientific) containing Human Keratinocyte Growth Supplement (Thermo

Fisher Scientific) and 0.03 mM $CaCl_2$. Pre-confluent keratinocytes were transfected with 20 nM siRNA targeting *CASR* and *CDH1* (E-cadherin) (siGENOME SMART pool; Dharmacon Inc., Lafayette, CO) using PepMute siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD), or infected with the Ad-ASCaR adenovirus (60 pfu/cell) in growth medium containing 0.03 mM $CaCl_2$ and cultured for 3 days prior to growth factor treatments or calcium (2 mM) exposure for AJ induction. The committee for Human Research at the San Francisco Veteran Affair Medical Center and the University of California San Francisco approved the use of human keratinocytes.

Keratinocyte Migration Assay (Scratch Wounding Model)

Confluent keratinocyte cultures were exposed to 7.5 µg/mL mitomycin for 4 hours to stop cell proliferation. The cultures were switched to medium containing 1.2 mM $CaCl_2$ for 1h before scratched with a sterile 20 µL-pipette tip (Mettler Toledo Rainin, LLC, Oakland, CA). In some experiments, keratinocyte cultures were pre-treated with vehicle (0.1% DMSO), 1,2-bis(2-aminophenoxy)ethane-N ,N ,N’ ,N’-tetraacetic acid-AM (BAPTA-AM, 10µM) or 2-aminoethoxydiphenyl borate (2-APB, 75µM) (Sigma-Aldrich, St Louis, MO) for 15 min prior to wounding. Cell migration was performed in the presence of 0.6 mM $CaCl_2$ and monitored by taking phase-contrast photographs on the same field before and after wounding. Migration efficiency was determined by quantitating the scratched areas that were re-occupied by migrating keratinocytes 24h after wounding using ImageJ. Statistical significance was evaluated using at least twelve different fields for four independent batches of keratinocytes.

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

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Delayed wound closure in the $^{Epid}Casr^{-/-}$ mice. Four-mm full thickness skin wounds were made on the back of (**a, b**) C57Bl/6J and (**c-f**) $^{Epid}Casr^{-/-}$ mice and their control littermates. (**a**) Sections of intact and wounded skin were stained with an antibody against CaSR. Red arrow and black racket indicate the wound margin and nascent epithelial tongue, respectively. CaSR(+) basal keratinocytes (arrowheads) are shown in insets. (**b**) QPCR assessment of CaSR message level in wounded and intact skins. Data were presented as mean+/-SE (n=6), * P<0.01. (**c**) Representative images and (**d**) closure rate of skin wounds in $^{Epid}Casr^{-/-}$ (KO) and control (cont) mice. Areas of the wounds were measured at specified time points after injury and normalized to the wound area at day 0. (**e**) H & E staining of wounds three days after injury. Epithelial tongues are outlined with dotted blue lines. Scale bar = 50 (**e**), 100 (**a**), or 800 µm (**c**). (**f**) The distances traveled by migratory keratinocytes are defined as the span between the wound margins and the tip of the epithelial tongues. Mean+/-SE (n=16-20). * P<0.01.

Figure 2. Epidermal Ca^{2+}_i propagation is required for wound closure. (**a, b**) Dorsal skins excised from $^{Epid}Casr^{+/+}$//$GCaMP^{+/+}$ mice were subjected to focal laser irradiation in a 20x20 μm^2 area (red box) in the upper stratum granulosum (SG). (**a**) Time-lapse GCaMP fluorescent images in SG, stratum spinosum (SS), and stratum basale (SB) before and after laser wounding. (**b**) Temporal changes in the intensity of GCaMP signal ($\Delta F/F_0$). Red arrow indicates the laser irradiation. Mean+/-SE (n=20-65 cells), P<0.01. The results are representative of 6 separate experiments. (**c, d**) Confluent keratinocyte cultures were treated with 0.1% DMSO (vehicle), 10µM BAPTA-AM, or 75µM 2-APB for 15 min prior to scratch wounding. (**c**) Representative micrographs of keratinocyte sheets at 0 and 24hr after scratch wounding. Scale bar = 50 µm.

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Dashed black lines mark the boundaries of scratched areas. Migratory fronts of keratinocytes are outlined in blue. (**d**) Quantitation of scratched areas re-occupied by migrating keratinocytes were made 24hr after wounding and normalized to vehicle control. Mean+/-SE (n=12), * P<0.01.

Figure 3. Blocking CaSR or E-cadherin expression diminished wound-induced Ca^{2+}_i propagation and collective keratinocyte migration. (**a-d**) Dorsal skins excised from $^{Epid}Casr^{-/-}$//$GCaMP^{+/+}$ ($^{Epid}Casr^{-/-}$) and $^{Epid}Casr^{+/+}$//$GCaMP^{+/+}$ (control) mice were subjected to laser irradiation in the stratum basale (SB). (**a**) Time-lapse GCaMP fluorescent images of SB and the quantitative measurements of (**b**) magnitude, (**c**) peak intensity, and (**d**) duration of the Ca^{2+}_i propagation following wounding. Mean+/-SE (n=35-60 cells), * P<0.01, # P<0.05. (**e-h**) Human keratinocytes were transfected with scrambled siRNA (siControl) or specific siRNA targeting CaSR (siCaSR) or E-cadherin (siEcad) prior to scratch wounding. (**e, f**) Confluent cultures were loaded with calcium green before imaging. (**e**) Time-lapse fluorescent images and (**f**) quantitation of temporal changes in the intensity (peak $\Delta F/F_0$) of Ca^{2+}_i propagations at various distances from wound site. Red X indicates wounded sites. Data were presented as mean+/-SE of 6 separate experiments. *P<0.01, #P<0.05. (**g**) Representative micrographs of keratinocyte sheets at 0 and 24hr after scratch wounding. Bar = 50 (**a, g**) or 100 μm (**e**). (**h**) Scratched areas re-occupied by migrating keratinocytes were quantified 24hr after wounding and normalized to siControl, (n=14), # P<0.05.

Figure 4. CaSR ablation reduced E-cadherin expression, keratinocyte proliferation, and differentiation in the neo-epithelia. Skin wounds were excised from $^{Epid}Casr^{-/-}$ mice and control littermates (**a-h, k, l**) four or (**i-j**) five days after injury, sectioned, and stained with antibodies

against (**a, b**) E-cadherin, (**f**) PCNA, or (**j**) differentiation markers. Basement membranes are outlined with dotted blue lines. Red arrows denote the wound margins and black brackets mark the spans of neo-epithelia. Arrowheads indicate areas with the strongest staining of E-cadherin in the cell membrane. Boxed areas in **a** and **b** are enlarged and shown in panel **k** and **l**, respectively. Bar = 50 (**a, b, f, k, l**) or 100 μm (**j**). The (**c**) thickness and (**d**) tissue size of neo-epithelia and (**g**) the number of PCNA(+) cells in representative fields were quantified and presented as mean+/- SE (n=16). Expression of E-cadherin (**e**), cyclin D1 (**h**) and differentiation markers (**i**) in wounds were determined by qPCR and normalized to the levels in control mice (n=6-8); * $P<0.01$, # $P<0.05$.

Figure 5. Blocking CaSR expression abolished interactions between E-cadherin and EGFR and suppressed EGFR-mediated ERK activation and keratinocyte proliferation. (**a**) Keratinocytes were pre-treated with vehicle (0.1% DMSO), 5μM AG1478, or 5μM U0126 for 30 min and cell proliferation was assessed in EGF-free medium containing 0.03 or 1.2 mM $CaCl_2$ for 24 hrs in the presence of [^{3}H]thymidine. (**b, c, d**) Keratinocytes were infected with adenoviruses carrying antisense CaSR cDNA (Ad-ASCaR) or empty viral vector (Ad-control) in medium with 0.03 mM $CaCl_2$ prior to calcium or growth factor treatments. (**b**) Cells were exposed to 2 mM $CaCl_2$ for 15 min to induce formation of cell-cell junctions. Fluorescence immunostaining was performed using antibodies against E-cadherin (red) and EGFR (green). Overlapped staining sites were visualized as yellow. Arrowheads indicate the colocalization of EGFR with E-cadherin at the cell membrane. Bar = 20 μm. (**c**) Keratinocytes were treated with EGF (50 ng/ml) or IGF1 (50 ng/ml) for 5 min, or $CaCl_2$ (2mM) for 15 min. Protein levels of ERK, phosphorylated ERK (p-ERK), EGFR, and phosphorylated EGFR (p-EGFR) were

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assessed by immunoblotting analyses of cell lysates. β-actin was used as a loading control. (**d**) Cell proliferation was assessed by [^{3}H]thymidine incorporation in medium with or without supplementation of EGF (0.1 ng/ml) for 24 hrs.

Figure 6. Activating endogenous CaSR augmented wound-induced Ca^{2+}_i propagation and re-epithelialization. (**a**) Four-mm full thickness skin wounds on the back of C57Bl/6J mice were topically treated with vehicle (0.05% DMSO), 0.1 nmole NPS-R568, or 0.1nmole NPS-2143 daily. Sizes of the wounds were measured at times indicated and normalized to the control wounds treated with vehicle. (**b**) H & E staining of wounds treated with NPS-R568 or vehicle for three days. Epithelial tongues are outlined with dotted blue lines. (**c**) The distances traveled by migratory keratinocytes in neo-epithelium were measured and presented as mean+/-SE (n=8), *P<0.01. (**d, e**) Cultured keratinocyte sheets were loaded with calcium green and pretreated with 5μM NPS-R568 or vehicle for 15 minutes prior to scratch wounding. (**d**) Time-lapse fluorescent images. Red Xs indicate wounded sites. Bar = 50 (**b**) or 100 μm. (**e**) Quantitation of peak $\Delta F/F_0$ at various distances from wound site. Mean+/-SE of 6 separate experiments. *P<0.01, #P<0.05. Expressions of (**f**) E-cadherin and (**g**) epidermal differentiation markers in wounds treated with NPS-R568 or vehicle were assessed by qPCR. Mean+/-SE (n=8); * P<0.01, # P<0.05. (**h**) A model depicting CaSR-mediated regulation of wound re-epithelialization. Details were described in Discussion.

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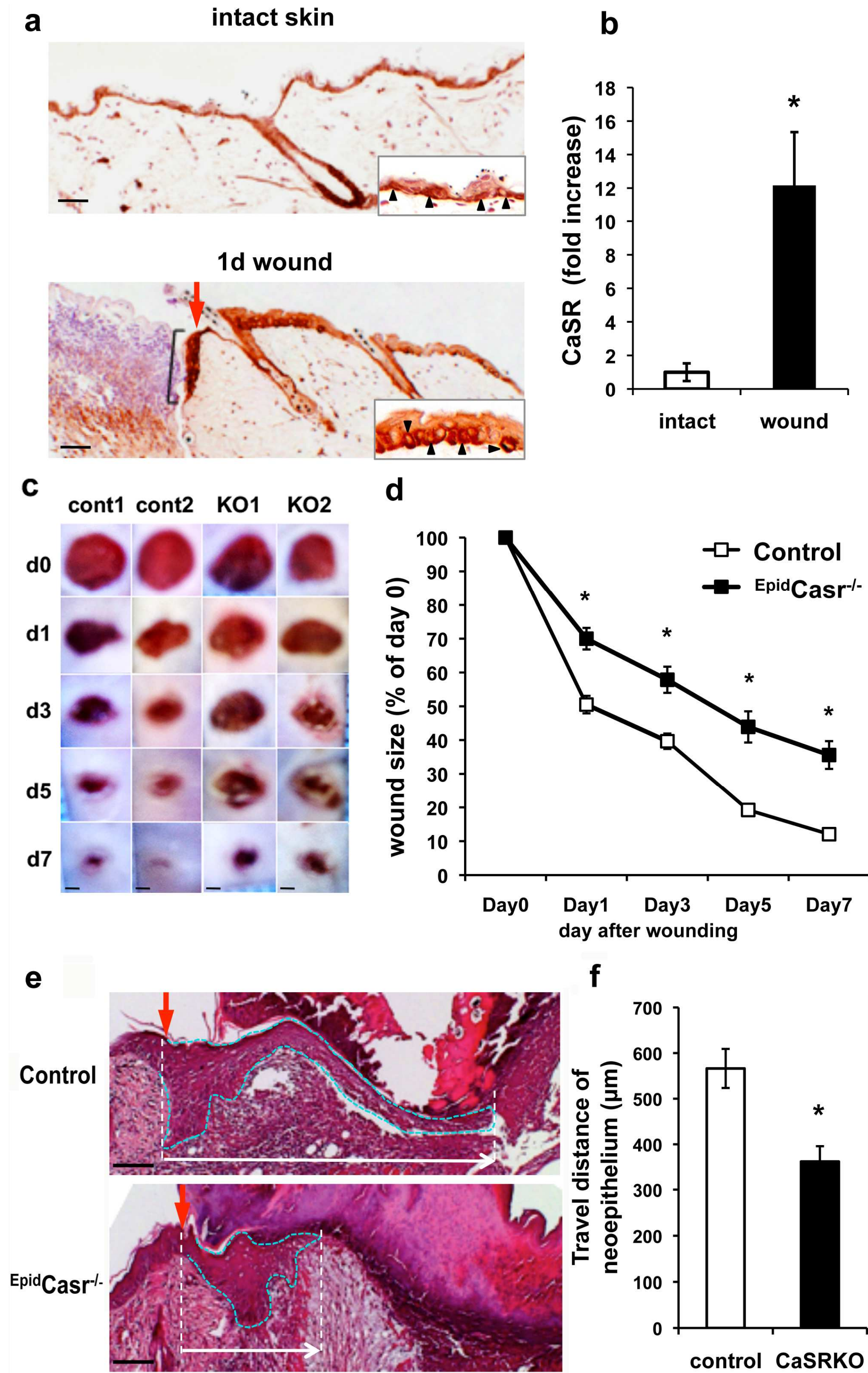
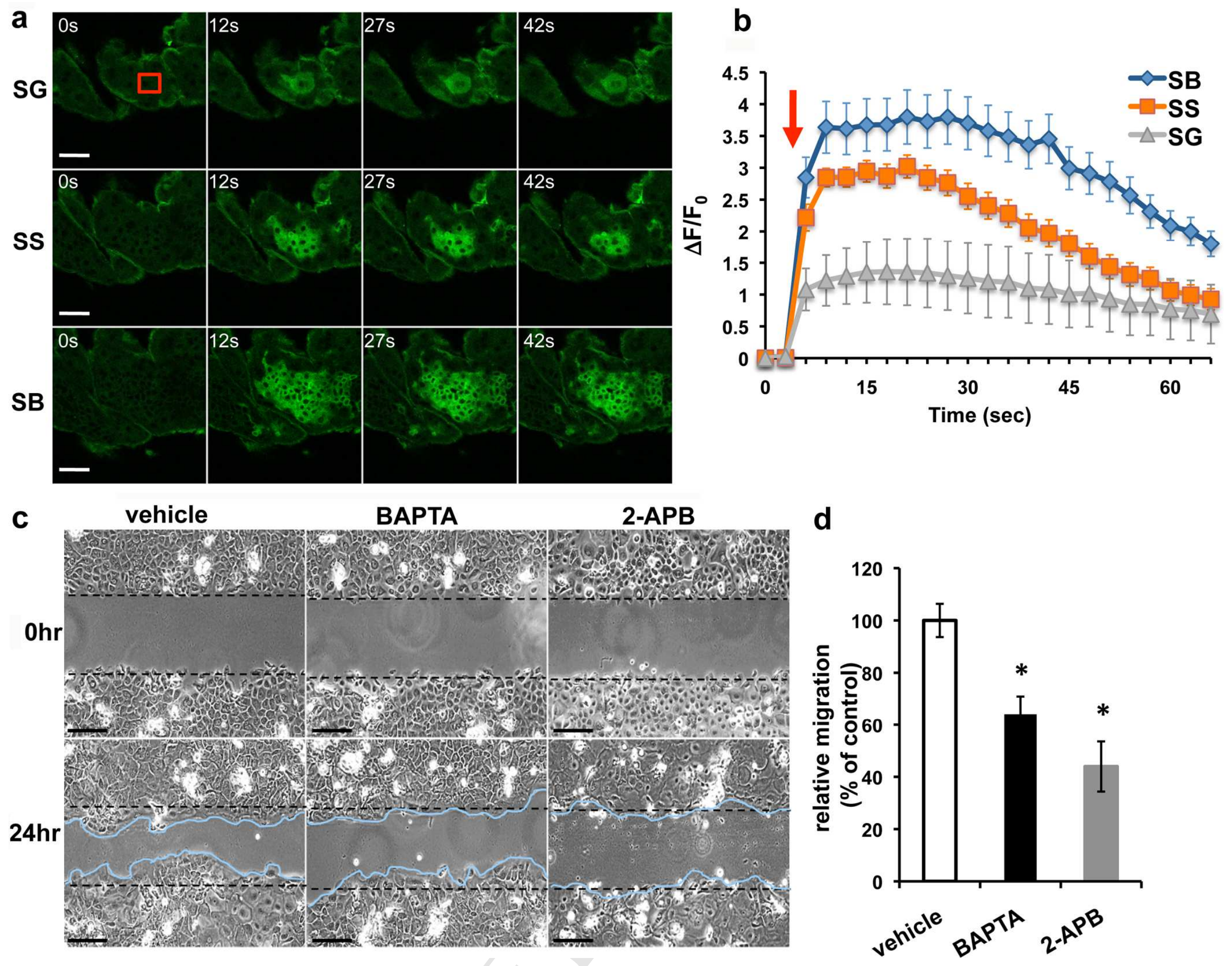


a
intact skin
1d wound
b
CaSR (fold increase)
18
16
14
12
10
8
6
4
2
0
*
intact
wound
c
cont1
cont2
KO1
KO2
d0
d1
d3
d5
d7
d
wound size (% of day 0)
100
90
80
70
60
50
40
30
20
10
0
Control
EpidCasr-/-
Day0
Day1
Day3
Day5
Day7
day after wounding
e
Control
EpidCasr-/-
f
Travel distance of neoepithelium (µm)
700
600
500
400
300
200
100
0
control
CaSRKO



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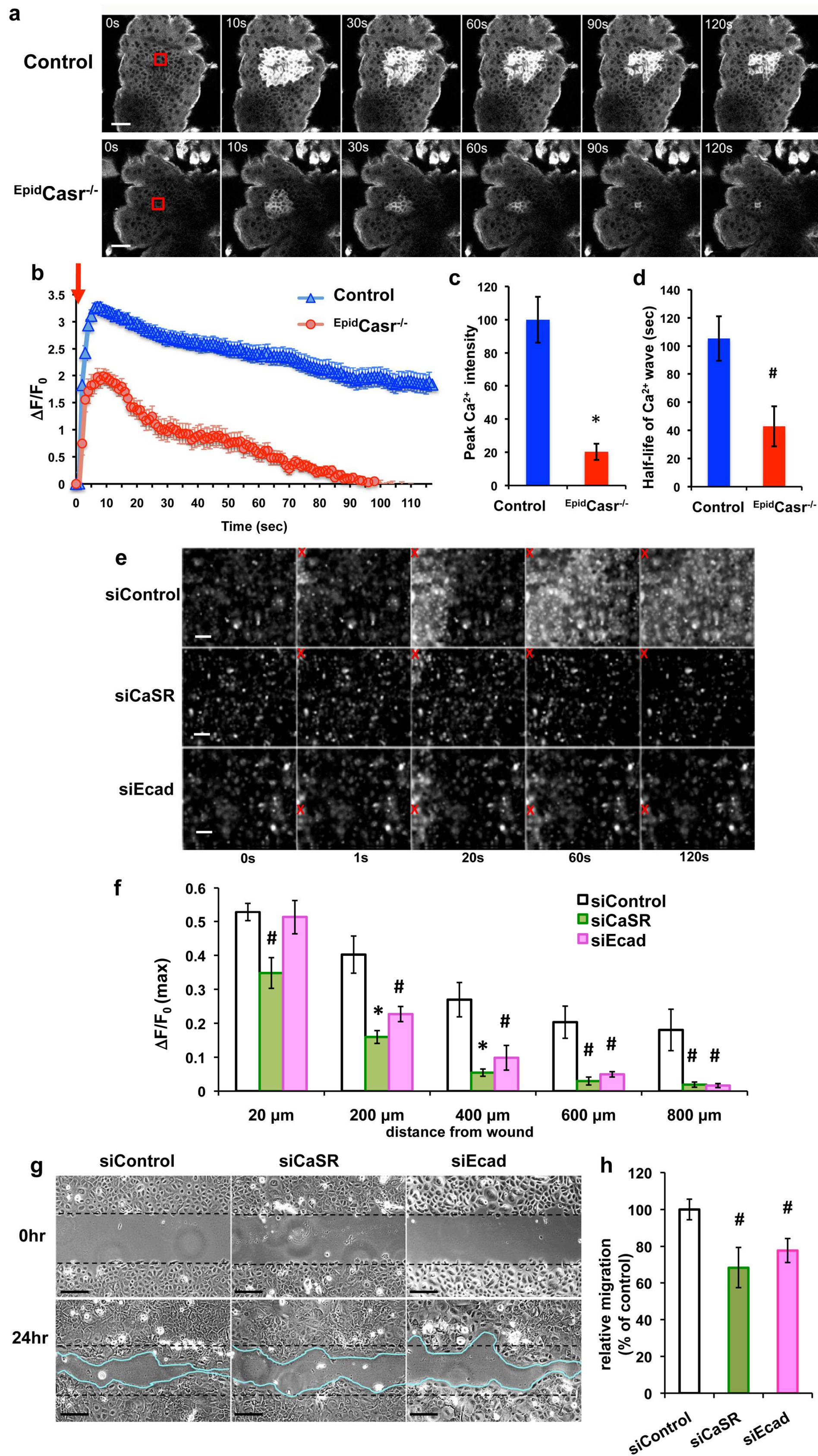
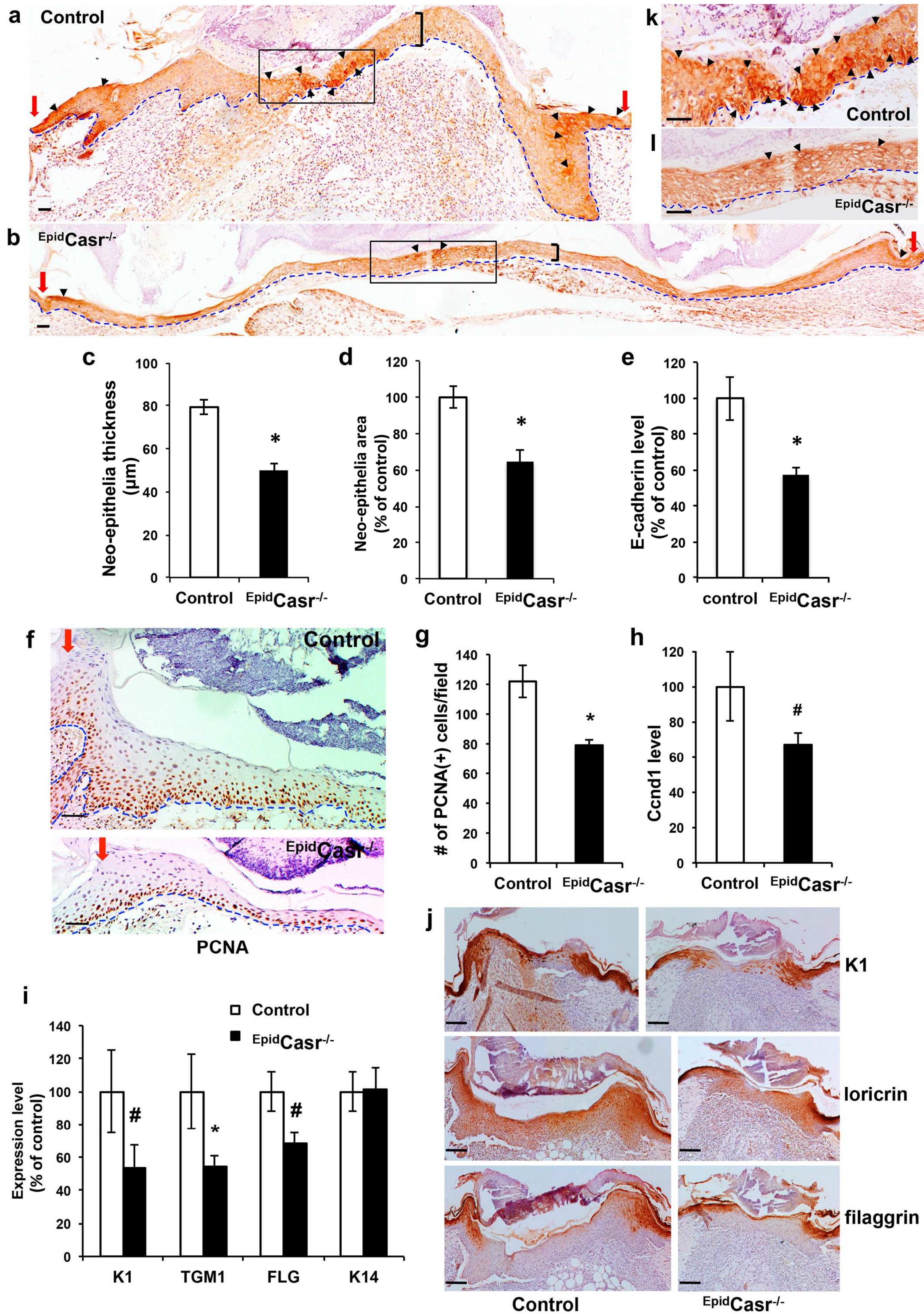
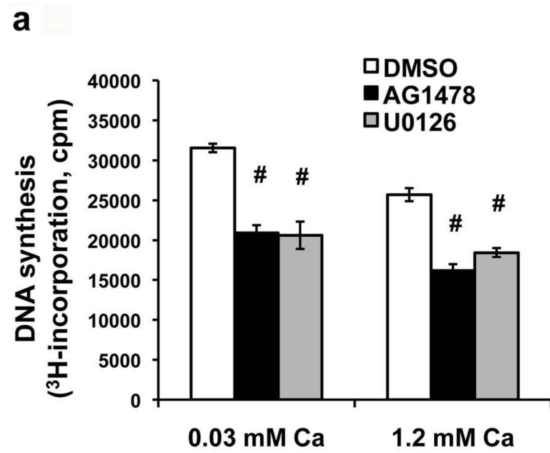
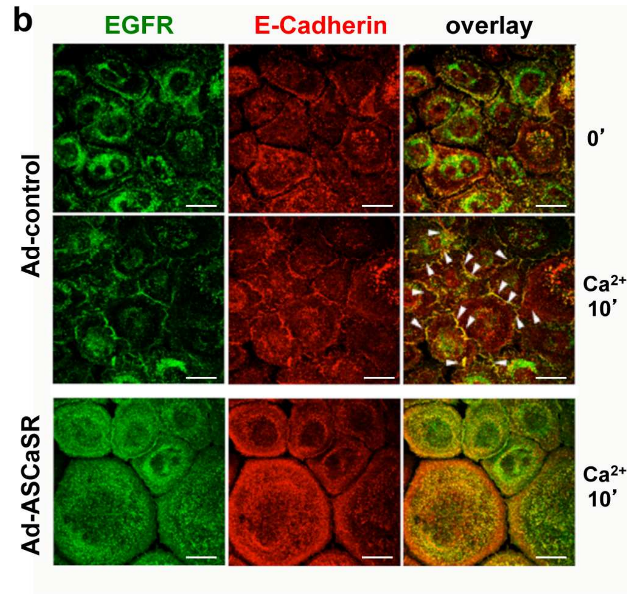


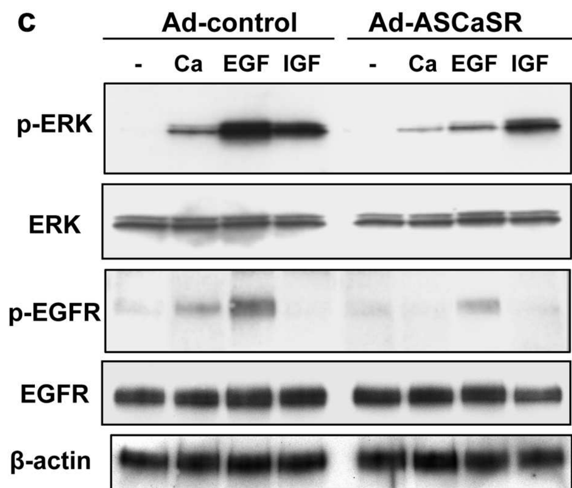
a
Control
EpidCasr-/-
0s
10s
30s
60s
90s
120s
b
Control
EpidCasr-/-
ΔF/F0
Time (sec)
c
Peak Ca2+ intensity
Control
EpidCasr-/-
d
Half-life of Ca2+ wave (sec)
Control
EpidCasr-/-
e
siControl
siCaSR
siEcad
0s
1s
20s
60s
120s
f
siControl
siCaSR
siEcad
ΔF/F0 (max)
20 µm
200 µm
400 µm
600 µm
800 µm
distance from wound
g
siControl
siCaSR
siEcad
0hr
24hr
h
relative migration (% of control)
siControl
siCaSR
siEcad

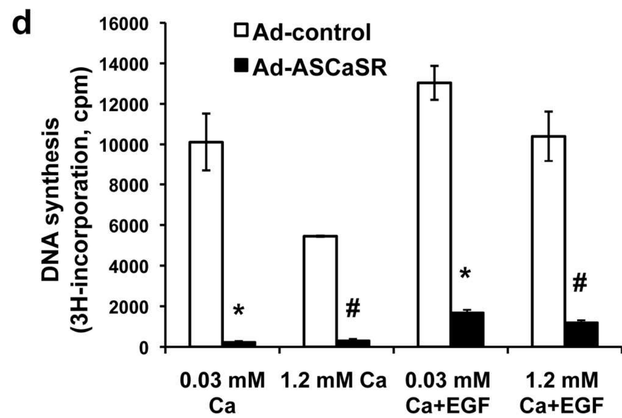


a Control
b EpidCasr-/-
k Control
l EpidCasr-/-
c
Neo-epithelia thickness (μm)
Control
EpidCasr-/-
d
Neo-epithelia area (% of control)
Control
EpidCasr-/-
e
E-cadherin level (% of control)
control
EpidCasr-/-
f Control
EpidCasr-/-
PCNA
g
# of PCNA(+) cells/field
Control
EpidCasr-/-
h
Ccnd1 level
Control
EpidCasr-/-
i
Control
EpidCasr-/-
Expression level (% of control)
K1
TGM1
FLG
K14
j
K1
loricrin
filaggrin
Control
EpidCasr-/-









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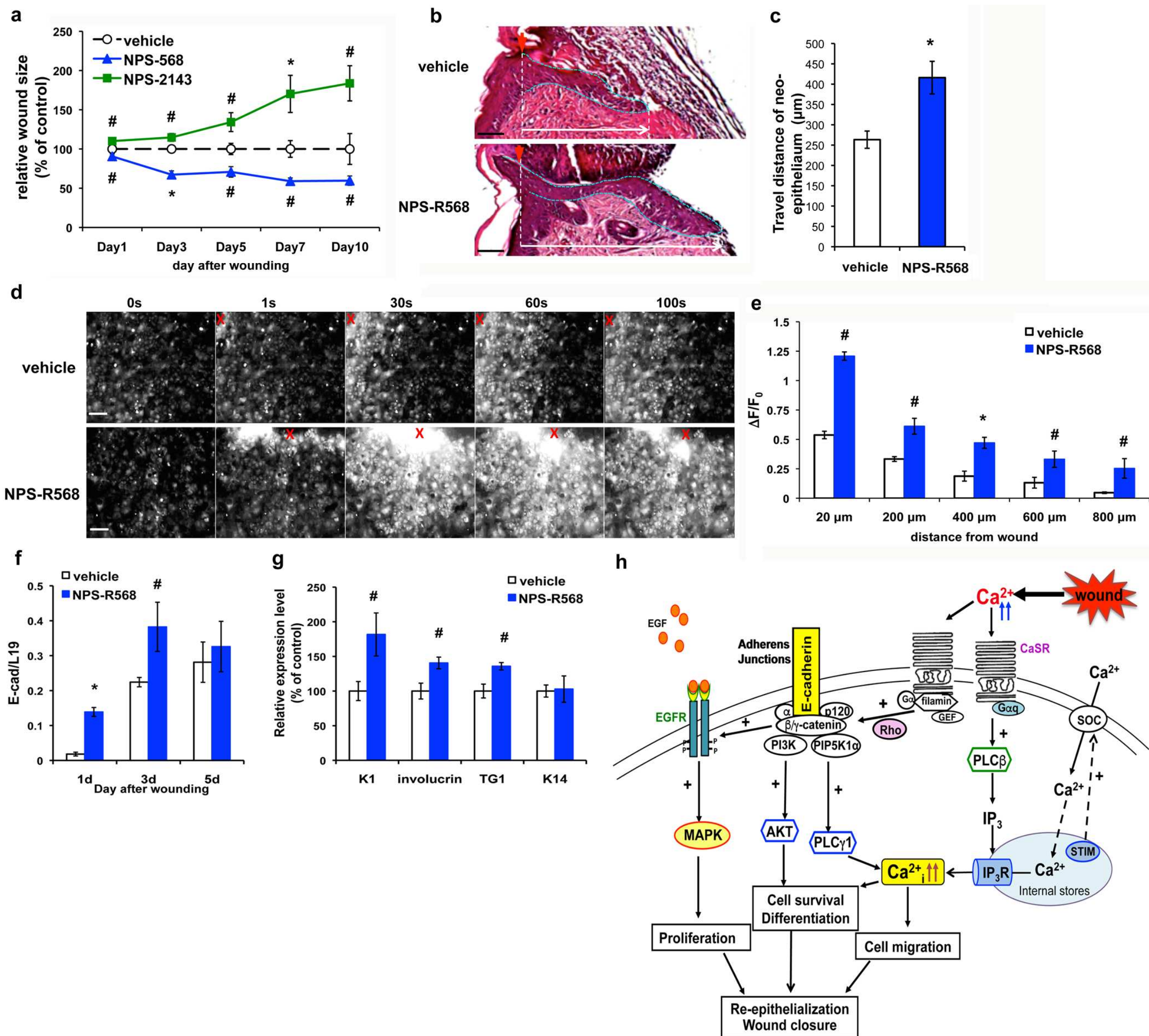

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