SUPPLEMENTAL MATERIALS AND METHODS

Laser irradiation and Epidermal Ca^{2+}_i Imaging

To investigate the spatial and temporal dynamics of epidermal Ca^{2+}_i in response to wounding, skins of GCaMP3-expressing mice were wounded by laser irradiation and GCaMP fluorescence was imaged with two-photon excitation confocal microscopy. Dorsal skin biopsies were placed on an agar plate secured to a heated stage of an upright Zeiss LSM 780 coupled to a Ti:Saph laser (Chameleon Ultra II, Coherent) and imaged within 1 hour after excision. The excitation wavelength was tuned at 900nm (~15mW) and two spectral windows at 550/50nm and 445/25nm were used to visualize GCaMP fluorescence in epidermis and to detect dermal collagen as spatial reference, respectively. Laser wavelength was set at 800 nm (~140mW) to irradiate cells in an area of 20x20 μm^2 in specified epidermal cell layer. Irradiation parameters were optimized to consistently elicit a Ca^{2+}_i response without permanent cell damage. The change in GCaMP fluorescence ΔF was expressed as the ratio of change with respect to the baseline fluorescence $[(F_t-F_0)/F_0]$. Three planes Z-stacks were acquired to monitor the vertical Ca^{2+}_i propagation throughout the SG, SS, and SB. Representative data shown were the average recordings of 35-60 individual cells. Statistical significance was evaluated using recordings from 8 mice of each genotype.

Ca^{2+}_i Imaging in Cultured Keratinocyte Sheets

Keratinocytes were cultured to confluence on glass-bottomed chambers in medium containing 0.03 mM $CaCl_2$. Cultures were incubated with 1.2 mM $CaCl_2$ in medium for 24h and loaded with 10μM Calcium Green-1AM (Life Technologies) for 45 min prior to imaging by a Zeiss Axio Imager 2 inverted fluorescence microscope. Scratches were made to keratinocyte

sheets with a 23-gauge needle in bath solution containing 1.2 mM $CaCl_2$. Changes in Ca^{2+}_i levels in cells neighboring the scratched area were imaged before and for 20 min after wounding or until baseline levels had been resumed. The data were expressed as $\Delta F/F_0$ $[(F_t-F_0)/F_0]$. To compare the effects of gene manipulation or drugs, we followed $\Delta F/F_0$ over time by imaging with 10X objective and quantitated the intensity ($\Delta F/F_0$) in multiple 50 x50 μm^2 regions at different distances (at intervals of 200 μm) from the wound site. Data shown represent the average $\Delta F/F_0$ of six separate recordings from six independent batches of keratinocytes.

Cell Proliferation

To assess cell proliferation in neo-epithelia, wound sections were stained with a proliferating cell nuclear antigen (PCNA) staining kit (Invitrogen) according to manufacturer's instruction. The PCNA(+) cells within 600μm of the wound edges were quantitated using BIOQUANT image analysis program (Nashville, TN). Statistical significance was evaluated by examining three sections from each of six mice per genotype. Cell proliferation in cultured keratinocytes was assessed by measuring the rate of DNA synthesis. Pre-confluent keratinocytes were infected with Ad-ASCaR or Ad-control adenovirus in medium containing 0.03 mM $CaCl_2$ for 3 days. Cultures were then incubated in EGF-free medium containing 0.03 or 1.2 mM $CaCl_2$ for 24 h, with the last 4 h in 2μCi/mL [^{3}H]thymidine (77 Ci/mmol [methy-^{3}H]thymidine; ICN Pharmaceuticals, Inc., Costa Mesa, CA). Finally, the cells were washed and solubilized in 1 N NaOH, and the radioactivity in the washed acid precipitate was measured in a scintillation counter. Each experiment was done in triplicate and repeated four times.

Quantitative Real-Time PCR (q-PCR)

RNA was isolated from full-thickness skin wounds including 1-mm-wide rims using RNA Stat-60 RNA extraction reagent (Tel-Test) and purified with RNeasy RNA purification kit (Qiagen, Chatsworth, CA). Equal amounts of total RNA samples were reverse transcribed by M-MLV reverse transcriptase, and qPCR was performed as previously described (Tu *et al.*, 2011). Levels of mRNA were normalized to mitochondrial ribosomal protein L19.

Immunohistochemical Staining

Wounded mouse skins were fixed with 4% formaldehyde, embedded in paraffin, and sectioned across the center of the wound. Sections were deparaffinized, rehydrated, and washed followed by incubation with primary antibodies to the proteins of interest and then with appropriate biotinylated secondary antibodies, followed by ABC-peroxidase (Vector, Burlingame, CA) and a DAB substrate. Secondary antibody alone was used as a control to establish the specificity of immunoreactivity (data not shown).

Immunofluorescence Staining

Keratinocytes were cultured on coverslips in medium containing 0.03 mM $CaCl_2$ until confluent. Cultures were then exposed to 50 ng/ml EGF for 5 min or 2mM $CaCl_2$ for 10 min. Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.5% NP-40 in PBS. After incubation with 10 μg/mL of primary antibodies and, subsequently, with 10 μg/mL of the appropriate fluorescein- or Texas Red-conjugated secondary antibody (Invitrogen, Carlsbad, CA), coverslips were mounted and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

Protein lysate Preparation and Immunoblotting

Conditions for preparing total keratinocyte lysates and membrane proteins, immunoblotting, and immunoprecipitation were described previously (Tu et al., 2008). A volume of 100 μg protein samples was used in immunoblotting analyses. Cell lysate containing 200 μg membrane protein were immunoprecipitated by 3 μg of designated antibodies, followed by Sepharose-conjugated protein G (Thermo Fisher Scientific, Rockford, IL).

Antibodies

The following antibodies (Abs) were used in this study: monoclonal Abs for α2 integrin (BD Biosciences, San Jose, CA), β-actin (Sigma-Aldrich, St Louis, MO), Rho A, and β-catenin, EGFR, actin (Santa Cruz Biotechnology, Santa Cruz, CA), ERK, and phosphor-ERK (Millipore, Temecula, CA); polyclonal Abs against E-cadherin, desmoglyein 1, phospho-EGFR (Tyr845) (Santa Cruz Biotechnology), keratin 1, loricrin, and filaggrin (Covance, Berkeley, CA), and CaSR (RRID AB2636871) (Tu et al., 2011).

Statistical Analysis

Quantitated data of various assays were analyzed with Student's t-Test (for comparing two sample groups) and one-way ANOVA (for comparing more than two groups) and the statistical difference is determined significant if P-value is <0.05.

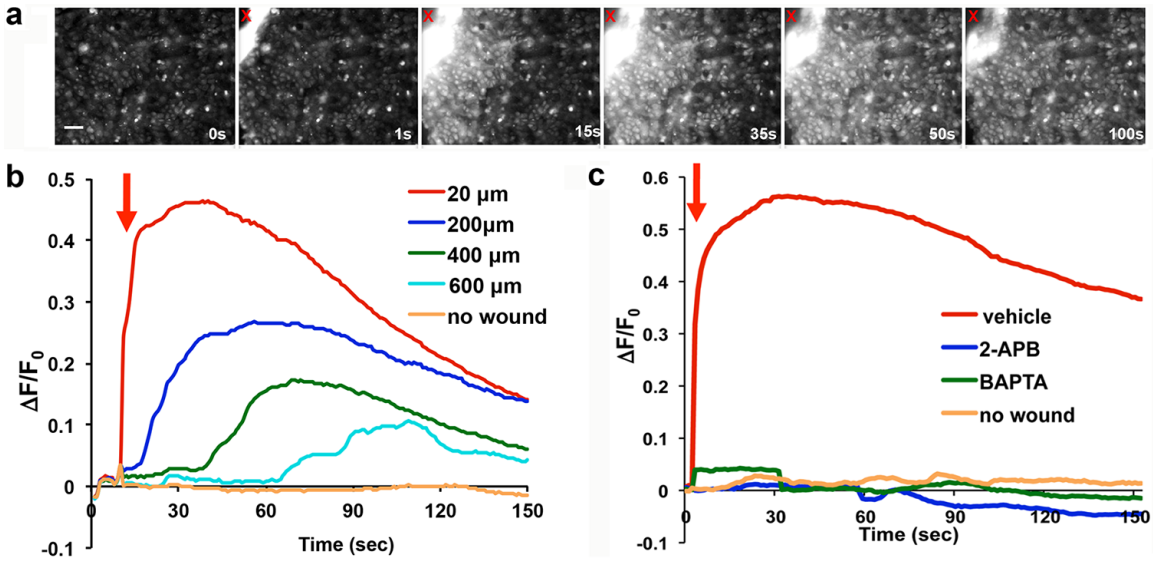

Figure S1. Scratch wounding triggered Ca^{2+}_i wave in keratinocyte sheet. Confluent human keratinocyte cultures were loaded with 5 µM calcium green-1 AM before Ca^{2+}_i measurement. (**a**) Calcium green fluorescence before and after scratch wounding. Bar = 100 µm. Red Xs indicate the wounded areas. (**b**) Representative trace of $\Delta F/F_0$ showed that the epidermal Ca^{2+}_i spread from the wound sites toward neighboring undamaged cell layers in a wave pattern. (**c**) Ca^{2+}_i measurement of cells pre-treated with vehicle (0.1% DMSO), 10µM BAPTA-AM or 75µM 2-APB for 15 min before imaging. Arrow indicates the time when scratch wounding was applied. Ca^{2+}_i propagation was detected immediately after wounding in the vehicle-treated cells, but not in cells pre-treated with BAPTA or 2-APB.

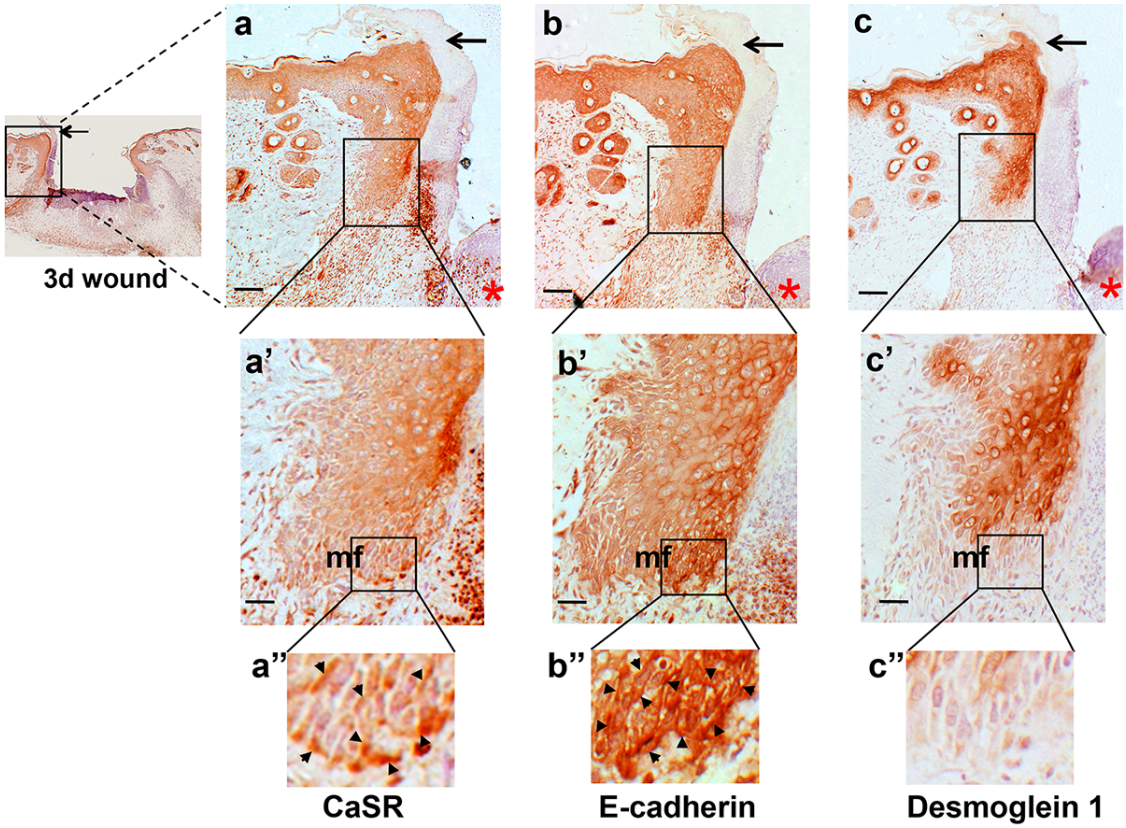

Figure S2. Expression of CaSR and E-cadherin in the neo-epithelia. Skin wounds were excised 3 days after injury, sectioned, and stained with antibodies against (**a**) CaSR, (**b**) E-cadherin, and (**c**) desmoglein 1 and followed by HRP-conjugated secondary antibody. Boxed areas in **a, b, c** were enlarged and shown in **a', b', and c'**, respectively. Red asterisks mark the wound beds and black arrows point to the wound margins. Bar = 200 (**a, b, c**) or 50 μm (**a', b', c'**). CaSR and E-cadherin were expressed in all cell layers including the migratory keratinocytes at the front (mf) of the neo-epithelia. In contrast, desmoglein1 was mostly absent in the migratory keratinocytes. (**a''**, **b''**, **c''**) Enlarged images of mf areas where strong membrane staining of (**a''**) CaSR and (**b''**) E-cadherin were detected. Arrowheads point the localization of CaSR and E-cadherin at the cell-cell membrane between migratory keratinocytes.

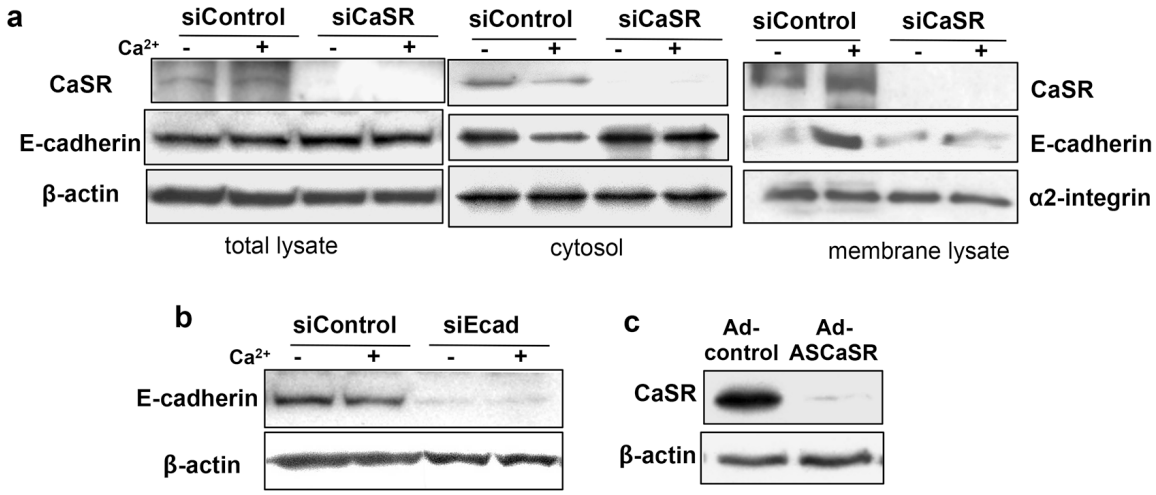

Figure S3. Suppression of CaSR and E-cadherin expression by gene silencing. (**a, b**) Human keratinocytes were transfected with a scrambled siRNA (siControl) or specific siRNAs targeting (**a**) CaSR (siCaSR) or (**b**) E-cadherin (siEcad) in medium containing 0.03 mM $CaCl_2$. Cells were then exposed to 2 mM $CaCl_2$ for 15 min to induce formation of cell-cell junctions. (**c**) Keratinocytes were infected by an adenovirus carrying a full-length CaSR antisense cDNA (Ad-ASCaSR) or an empty viral vector (Ad-control). Levels of CaSR and E-cadherin protein in total lysates, and in extracts derived from cytosol and membrane fractions were assessed by immunoblotting. β-actin was used as loading control for total and cytosol lysates, whereas α2-integrin was for plasma membrane protein. Blocking CaSR expression by siCaSR (**a**) or Ad-ASCaSR (see Figure S4) effectively inhibited the Ca^{2+}-stimulated membrane localization of E-cadherin.

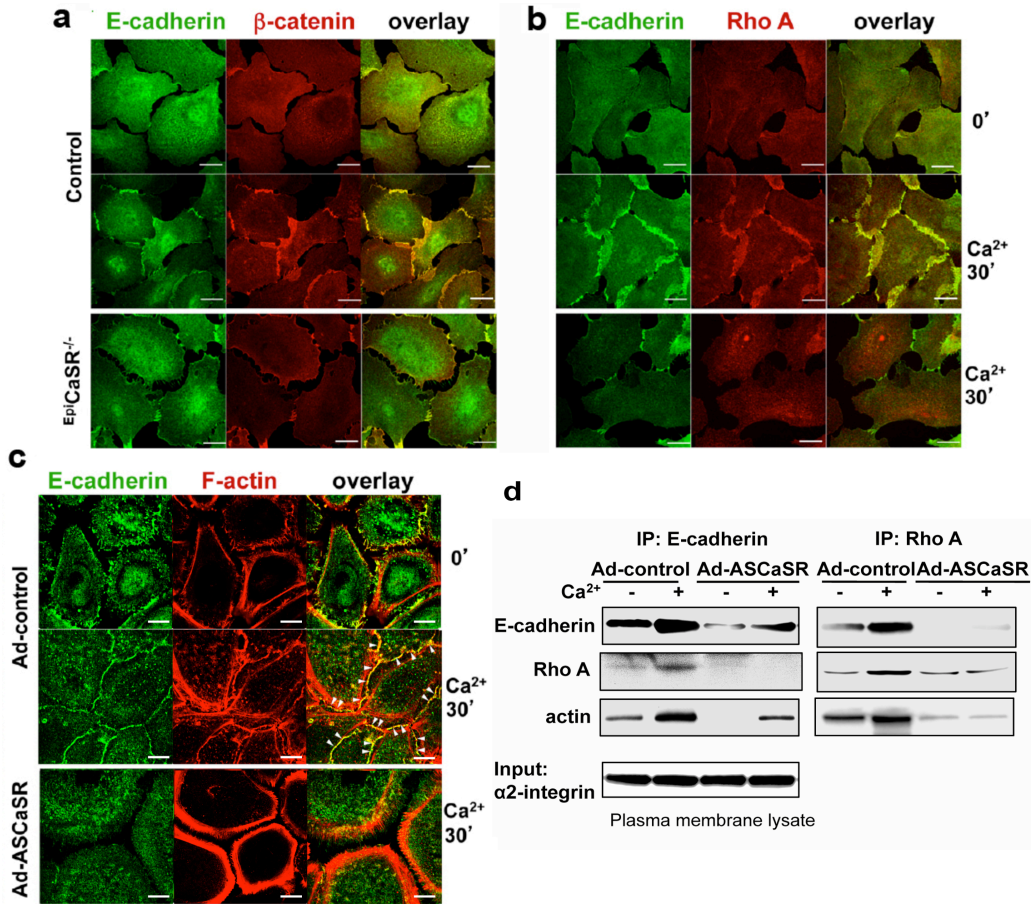

Figure S4. Blocking CaSR expression inhibited the formation of adherens junctions (AJ). (**a, b**) Epidermal keratinocytes isolated from newborn $^{Epid}Casr^{-/-}$ mice and their control littermates or (**c, d**) human keratinocytes infected with Ad-ASCaSR or control adenoviruses were exposed to 2 mM $CaCl_2$ for 30 min to induce the formation of cell-cell junctions. (**a-c**) Fluorescent immunostaining was performed using FITC-conjugated E-cadherin antibody (green), and Texas Red-conjugated antibodies (red) against (**a**) β-catenin and (**b**) Rho A, or (**c**) Texas Red-conjugated phalloidin (for F-actin). Overlapped staining sites are visualized as yellow. Arrowheads indicate the colocalization of F-actin with AJs. Bar = 20 (a, b) or 10 μm (c). (**d**) Plasma membrane lysates of adenovirus-infected keratinocytes were immunoprecipitated (IP) with antibodies against E-cadherin or Rho A. The immunoprecipitates were analyzed by immunoblotting for E-cadherin, Rho A, and actin. α2-integrin was used as a control for equal input of plasma membrane protein before precipitation. The Ca^{2+}-stimulated assembly of E-cadherin, β-catenin, Rho A, and F-actin into adhesion complexes at the cell-cell contract were evidently reduced in CaSR-deficient keratinocytes. Keratinocytes that lost the ability to form AJ appeared larger in size, with glossier surfaces and plumper shapes than control cells.

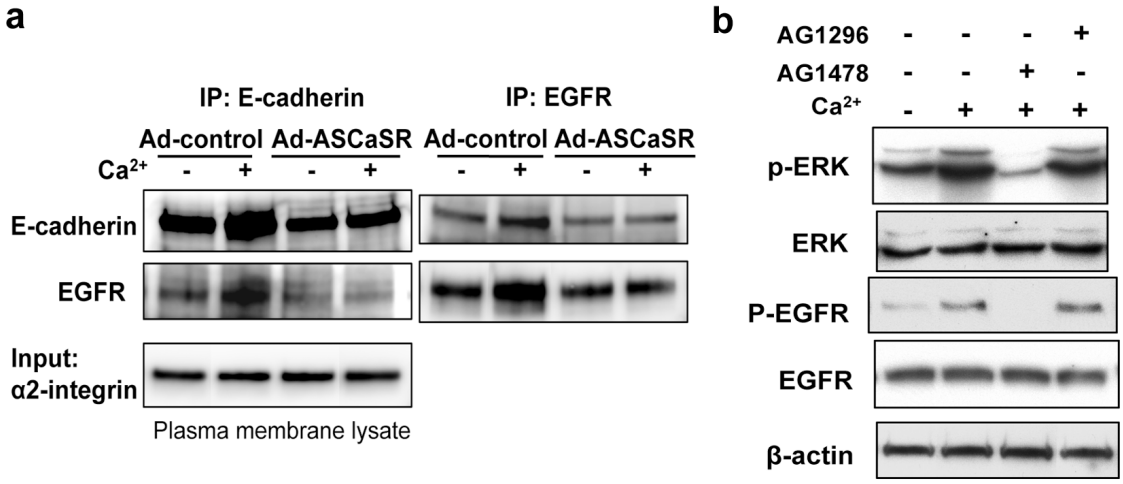

Figure S5. A cross talk between the CaSR and EGFR pathways. (**a**) Keratinocytes were infected by an adenovirus carrying a full-length CaSR antisense cDNA (Ad-ASCaSR) or an empty viral vector (Ad-control) in medium containing 0.03 mM $CaCl_2$. Cells were then exposed to 2 mM $CaCl_2$ for 15 min to induce formation of cell-cell junctions. Plasma membrane lysates of adenovirus-infected keratinocytes were immunoprecipitated (IP) with antibodies against E-cadherin or EGFR. The immunoprecipitates were analyzed by immunoblotting for E-cadherin and EGFR. α2-integrin was used as a control for equal input of plasma membrane protein before precipitation. The Ca^{2+}-stimulated association of E-cadherin and EGFR at the cell membrane was markedly reduced in CaSR-deficient keratinocytes. (b) Keratinocytes were pre-treated with vehicle (0.1% DMSO), 5μM AG1478, or 5μM AG1296 for 30 min before exposure to 2mM $CaCl_2$. Levels of ERK, phosphorylated ERK (p-ERK), EGFR, and phosphorylated EGFR (p-EGFR) were assessed by immunoblotting analyses of total lysates. β-actin was used as a loading control. Blocking EGFR signaling with AG1478 effectively inhibited the Ca^{2+}-stimulated activation of ERK, whereas PDGF inhibition by AG1296 had no effect.

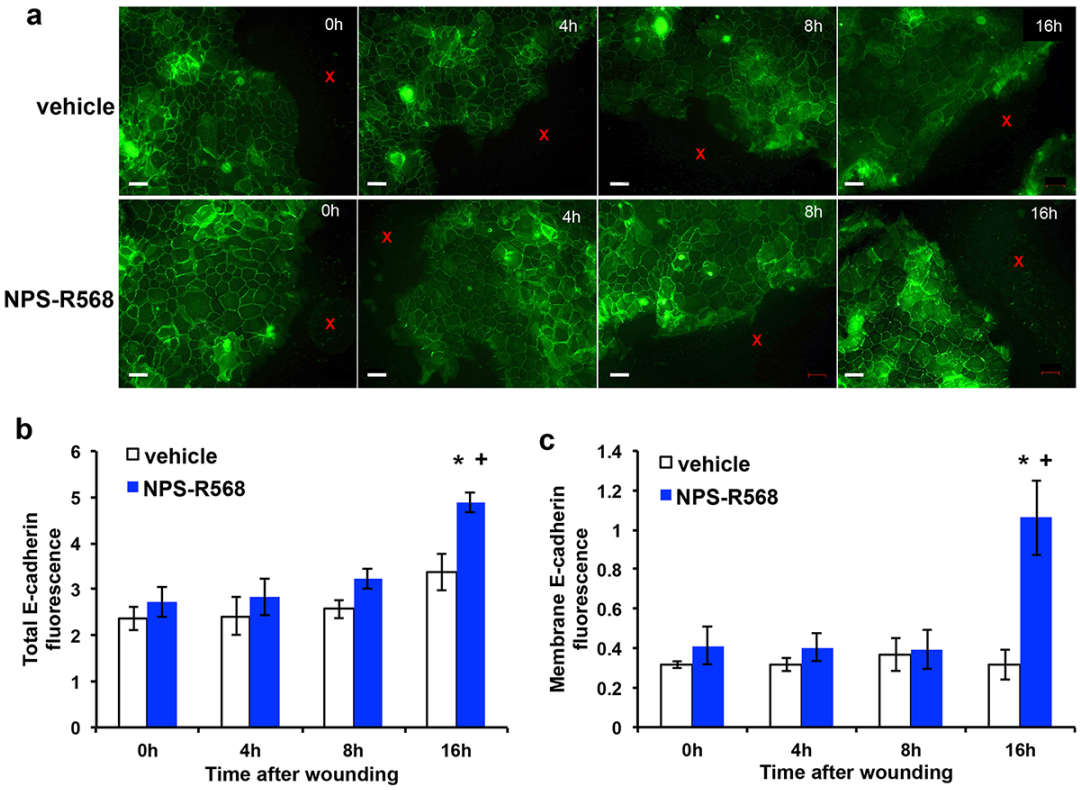

Figure S6. Activating endogenous CaSR by calcimimetic enhanced membrane localization of E-cadherin. Confluent keratinocyte cultures were pre-treated with vehicle (0.01% DMSO) or 5 μM NPS-R568 for 15 min prior to scratch wounding. (**a**) **I**mmunofluorescence staining for E-cadherin at 0, 4, 8, and 16hr after wounding. Bar = 20 μm. Red Xs indicate the denuded culture areas. Fluorescence of (**b**) total and (**c**) membrane localized E-cadherin within migrating keratinocyte sheets were quantitated and normalized to the number of nuclei visualized by DAPI (not shown). * $P<0.01$, compared to vehicle; + $P<0.05$, compared to 0h.

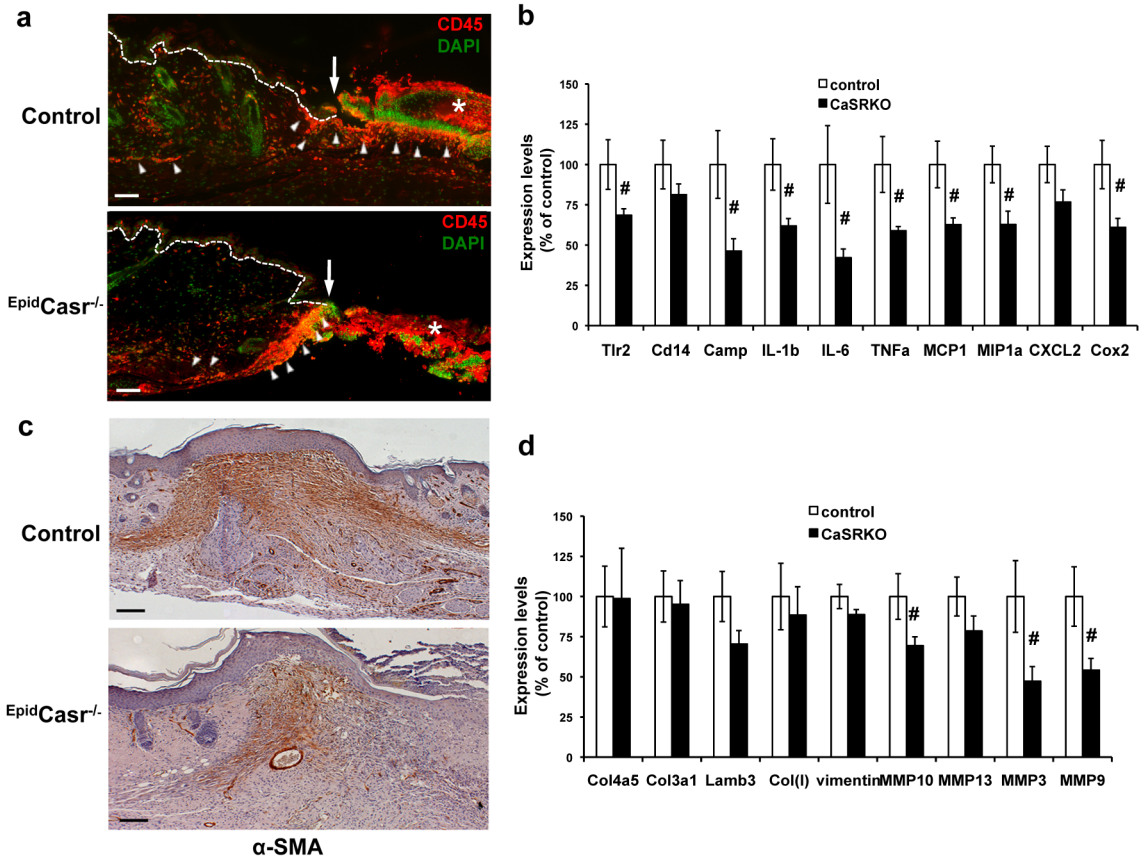

Figure S7. Comparisons of non-epithelial wound phenotypes in $^{Epid}Casr^{-/-}$ mice and control littermates. Skin wounds were excised one (a, b) or five (c, d) days after injury. (**a**) Wound sections were stained with Texas Red-conjugated antibody (red) for leukocyte antigen CD45, whereas nuclei were visualized by DAPI (green). Basement membranes of the epidermis adjoining the wound are outlined with dotted lines. Arrows point to the wound margins and arrowheads indicate areas with strong leukocyte infiltration. Asterisks mark the fibrin clots which nonspecifically retain fluorescence. (**c**) Wound sections were stained with α-smooth muscle actin (α-SMA) for myofibroblasts, followed by HRP-conjugated secondary antibody. Bar = 50 (**a**) or 100 μm (**c**). Expression of (**b**) inflammatory cytokines and regulators and (**d**) dermal extracellular matrix (ECM) proteins and ECM remodeling enzymes in wounds were determined by qPCR and normalized to the levels in control mice and presented as mean+/-SE (n=6); # P<0.05.