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Decreased Calcium-Sensing Receptor Expression Controls Calcium Signaling and Cell-To-Cell Adhesion Defects in Aged Skin

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The calcium-sensing receptor (CaSR) drives essential calcium ion (Ca^{2+}) and E-cadherin–mediated processes in the epidermis, including differentiation, cell-to-cell adhesion, and epidermal barrier homeostasis in cells and in young adult mice. We now report that decreased CaSR expression leads to impaired Ca^{2+} signal propagation in aged mouse (aged >22 months) epidermis and human (aged >79 years, donor age) keratinocytes. Baseline cytosolic Ca^{2+} concentrations were higher, and capacitive Ca^{2+} entry was lower in aged than in young keratinocytes. As in *Casr*-knockout mice ($^{Epid}CaSR^{-/-}$), decreased CaSR expression led to decreased E-cadherin and phospholipase C-γ expression and to a compensatory upregulation of STIM1. Pretreatment with the CaSR agonist N-(3-[2-chlorophenyl]propyl)-(R)-alpha-methyl-3-methoxybenzylamine normalized Ca^{2+} propagation and E-cadherin organization after experimental wounding. These results suggest that age-related defects in CaSR expression dysregulate normal keratinocyte and epidermal Ca^{2+} signaling, leading to impaired E-cadherin expression, organization, and function. These findings show an innovative mechanism whereby Ca^{2+}- and E-cadherin–dependent functions are impaired in aging epidermis and suggest a new therapeutic approach by restoring CaSR function.

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INTRODUCTION

In this study, we report that the loss of calcium-sensing receptor (CaSR) leads to impaired calcium ion (Ca^{2+}) and E-cadherin signaling in aged human epidermal keratinocytes (AHEKs) and epidermis. Ca^{2+} is essential for normal keratinocyte (KC) proliferation, differentiation, and migration and wound repair (Cordeiro and Jacinto, 2013). Raised extracellular Ca^{2+}, epidermal barrier perturbation, and mechanical or laser stimulation all act through intracellular Ca^{2+} release and through subsequent store-operated or voltage-sensitive Ca^{2+} entry (Numaga-Tomita and Putney, 2013; Tu et al., 2005) that propagates Ca^{2+} signaling to neighboring KCs both laterally and vertically (Kumamoto et al., 2017; Tsutsumi et al., 2013). Epidermal Ca^{2+} signaling is driven by a marked Ca^{2+} gradient (Forslind et al., 1999; Menon and Elias, 1991), with Ca^{2+} concentrations approximately fourfold higher in the uppermost viable KCs than in the basal cells (Elias et al., 1998; Mauro et al., 1998). Much of this Ca^{2+} gradient and the resulting Ca^{2+} signaling depend on Ca^{2+} sequestered within the endoplasmic reticulum (ER) by SERCA (Celli et al., 2016). Although raising extracellular Ca^{2+} levels increases KC differentiation, it also decreases lipid secretion and barrier repair in terminally differentiated stratum granulosum KCs (Lee and Lee, 2018). Thus, an approach that enhances KC sensitivity to Ca^{2+} could optimize differentiation, migration, and barrier homeostasis, especially in an aging epidermis.

The G-protein–associated CaSR senses Ca^{2+} concentrations in the micromolar to the millimolar range, making it particularly useful in sensing changes in extracellular or organelle Ca^{2+} concentrations. In KCs, CaSR signaling activates phospholipase C (PLC)-β by Gq and leads to inositol triphosphate–mediated acute release of calcium from intracellular calcium stores. CaSR expression is essential for epidermal differentiation and barrier function (Komuves et at., 2002, Tu et al., 2012), controlling both KCs' ability to take up Ca^{2+} and store it in the ER (Tu et al., 2007). CaSR mediates the formation and stabilization of the E-cadherin signaling complex, leading to E-cadherin–mediated adherens junction and cell-to-cell adhesion (Tu et al., 2008, Tunggal et al., 2005). Mice with conditional knockout of the *Casr* in the epidermis ($^{Epid}Casr^{-/-}$) display a loss of the epidermal Ca^{2+} gradient, impaired KC differentiation, and defective permeability barrier (Tu et al., 2012). Conversely, experimental CaSR overexpression accelerates epidermal differentiation and permeability barrier formation (Turksen and Troy, 2003). Combined vitamin D receptor and CaSR deletion delay wound re-epithelization (Oda et al., 2017), and deleting

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Abbreviations: AHEK, aged human epidermal keratinocyte; Ca^{2+}, calcium ion; CaSR, calcium-sensing receptor; ER, endoplasmic reticulum; HK, human keratinocyte; KC, keratinocyte; NHEK, neonatal human epidermal keratinocyte; NPS R-568, N-(3-[2-chlorophenyl]propyl)-(R)-alpha-methyl-3-methoxybenzylamine; PLC, phospholipase C; TG, thapsigargin

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CaSR from young adult mice epidermis decreases E-cadherin expression and impairs Ca^{2+} signal propagation (Tu et al., 2019). Aged human epidermis and human keratinocyte (HK) show similar defects in Ca^{2+} signaling, expression, and re-epithelialization as those seen in mice in which CaSR was experimentally ablated. These studies suggest that CaSR may be a relevant target for improving Ca^{2+}- and E-cadherin–mediated processes in an aged epidermis.

RESULTS

Epidermal Ca^{2+} signaling after laser stimulation is blunted in aged mouse epidermis and aged HK monolayers

Previous studies showed that aged HKs respond sluggishly to mechanical stimulation (Denda et al., 2017). To assess lateral calcium signaling in aged versus that in young epidermis, we used a multiphoton excitation microscopy–based laser stimulation assay previously developed for $^{Epid}Casr^{-/-}$ studies (Tu et al., 2019). This experimental approach selectively perturbs a selected area of 20 × 20 μm^2 (corresponding roughly to one or two cells) in the stratum basale of the epidermis of mice expressing the fluorescent Ca^{2+} reporter GCaMP3 under the keratin 14 promoter. After laser stimulation, we monitored Ca^{2+} propagation by tracking epidermal fluorescence using time-lapse imaging (Tu et al., 2019) (Figure 1a). Ca^{2+} propagation spread to a significantly smaller area in the aged (>22 months) than in the young (6–8 weeks) mice (Figure 1b and d and Supplementary Figure S1). The KC cytosolic Ca^{2+} response after perturbation also was lower in the aged than in the young mice (Figure 1c and e and Supplementary Figure S1).

We next examined Ca^{2+} signaling in response to laser stimulation in the aged (>79 years) HKs (AHEKs) versus that in neonatal KC human (neonatal human epidermal KCs [NHEKs]) monolayers using the cell-permeant, calcium-sensitive fluorescent probe Calcium Green 1-AM (Thermo Fisher Scientific, Waltham, MA). In three separate experiments conducted on cells from three separate pairs of donors, we found that aged HKs monolayers responded with blunted calcium propagation (Figure 1f and g) and a lower average increase in the aged single cells' cytosolic calcium concentration (Figure 1h).

Ca^{2+} signaling in the aged versus that in the young KCs

We next compared the response to the extracellular Ca^{2+} and the intracellular Ca^{2+} stores and the capacitive cytosolic Ca^{2+} response in neonatal KCs with those in KCs obtained from aged (>79 years) humans (Figure 2a and b, respectively). Fura2-loaded KCs were exposed to 1.2 mM extracellular calcium. Traces representative of six (AHEK) to seven (NHEK) experiments on cells from three separate donors are shown in Figure 2a top panel (NHEK) and bottom panel (AHEK). Whereas NHEKs responded to raised extracellular calcium with a robust and rapid increase in cytosolic Ca^{2+} concentration, most AHEKs had a much more limited and slower response during the experiment time frame. Overall, AHEKs cytosolic Ca^{2+} response to increased extracellular calcium was significantly less pronounced than the response in NHEKs (Figure 2b).

Next, we compared intracellular Ca^{2+} stores and cytosolic Ca^{2+} capacitive influx in NHEKs (Figure 2c and d) with those in AHEKs (Figure 2e and f) in KCs monolayers cultured in low (0.07 mM) and high (1.2 mM) calcium-containing medium for 24 hours (Figure 2c–f). KCs were first placed in 0 mM extracellular Ca^{2+}. After a brief period of equilibration, 1 μM thapsigargin (TG), a concentration that releases both the ER and Golgi Ca^{2+} stores, was added to the medium to assess intracellular Ca^{2+} stores. Extracellular Ca^{2+} then was raised to a final concentration of 1.2 mM to quantify capacitive Ca^{2+} entry (Figure 2 and Table 1).

First, we found that baseline cytosolic Ca^{2+} concentration was markedly elevated and heterogeneous in the aged KCs. Responses of aged KCs to both TG and calcium switch were notably more variable than those in young KCs. Whereas 82% of all NHEK cells cultured in low calcium and 93% of NHEK cells cultured in high calcium responded to both TG and calcium switch, only 44% and 53% of AHEK cells cultured in low and high calcium, respectively, responded to TG or raised extracellular Ca^{2+} concentration.

CaSR and E-cadherin protein expression is downregulated in aged human epidermis

In nonexcitable cells such as KCs, Ca^{2+} influx is often regulated by store-operated Ca^{2+} entry (Numaga-Tomita and Putney, 2013; Tu et al., 2005; Vandenberghe et al., 2013), which requires PLC-mediated release of Ca^{2+} from intracellular stores such as the ER or Golgi and refill through STIM1 migration to the plasma membrane (Numaga-Tomita and Putney, 2013). These processes lead to adherens junction and desmosome reorganization, mediated by E-cadherin.

To define the changes in the CaSR-dependent signaling pathway, we first compared CaSR, E-cadherin, and STIM1 protein expression in total epidermal lysate from aged mice with those from young mice (Figure 3a). We found that both CaSR and E-cadherin protein expression was consistently decreased, whereas STIM1 levels were elevated in the aged mice. A similar pattern of E-cadherin downregulation was seen in young mice in which CaSR was experimentally ablated (Tu et al., 2019).

We then compared the expression levels of CaSR, E-cadherin, STIM1, PLCγ1, and PLCβ1 in NHEKs with the expression levels of those in AHEKs from three to four separate donors per group. We found that CaSR and E-cadherin levels were consistently downregulated in AHEKs from four separate donors (Figure 3b and Supplementary Figure S2a) compared with those in NHEKs from four separate donors. PLCγ1 and PLCβ1 levels were also consistently decreased, whereas STIM1 levels were increased in AHEKs compared with the levels in NHEKs. Exposure to high calcium appeared to reduce the difference in STIM1 level between NHEKs and AHEKs, but it did not normalize the expression levels of the other proteins. We observed a similar pattern in the $^{Epid}Casr^{-/-}$ mouse, where PLCγ1 levels were downregulated and STIM1 levels upregulated (Supplementary Figure S2b) compared with the pattern in wild-type mice. PLCβ1 was upregulated in $^{Epid}Casr^{-/-}$ mice.

Aged KCs display defective E-cadherin staining, slower migration, and impaired cell-to-cell adhesion

Previous studies (Tu et al., 2019) showed that decreased or absent epithelial CaSR levels lead to delayed re-epithelialization both in mice and in scratch assays using HKs through defective E-cadherin reorganization or CaSR-mediated cytosolic concentration increases. Passage 2 KCs

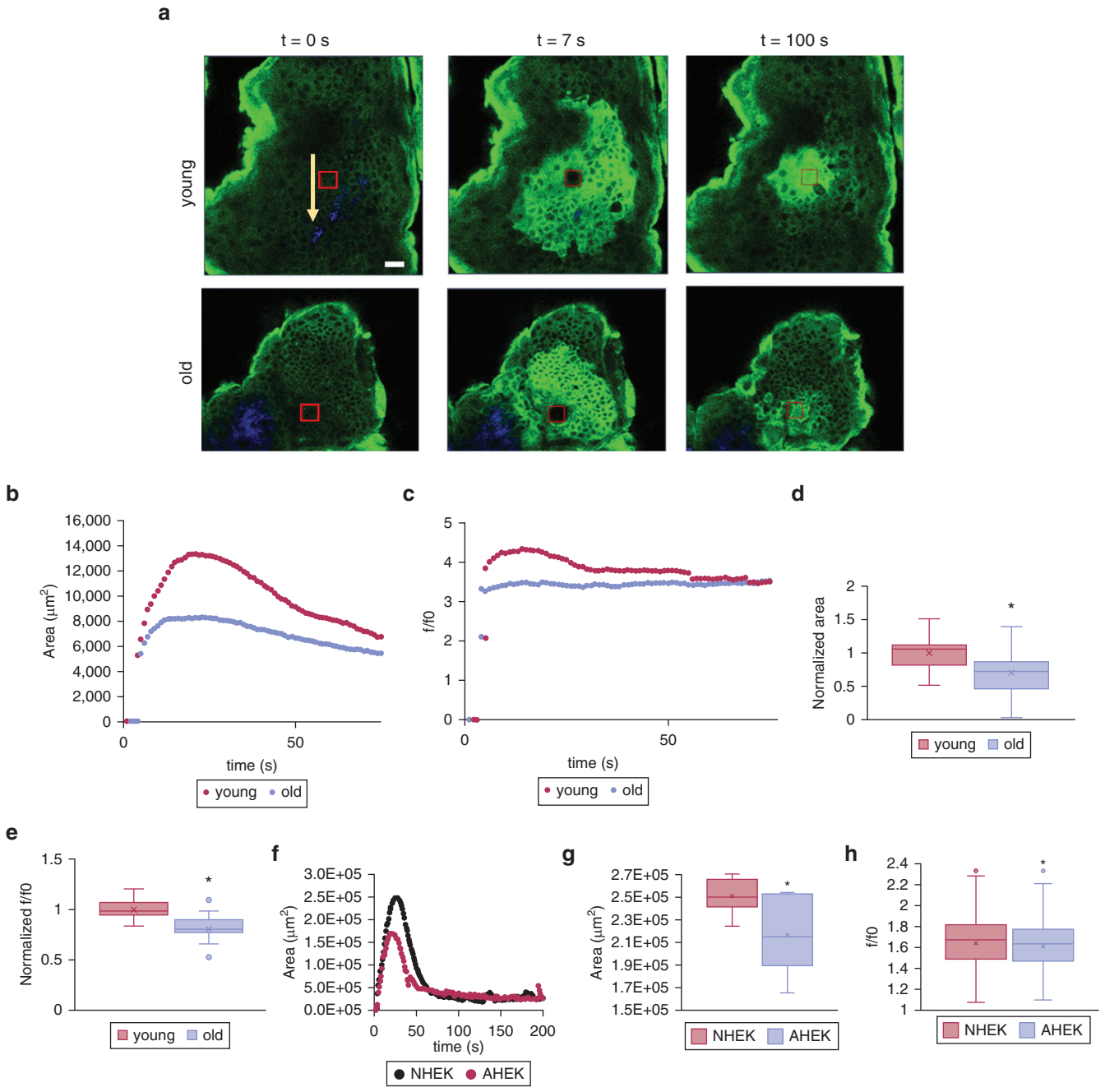

Figure 1. Calcium response to laser perturbation in aged compared with that in young ^{Epid}GCaMP mice and human keratinocytes monolayers. (**a**) Calcium response to laser perturbation to a 20 × 20 μm^2 SB region (red box) of young and old ^{Epid}GCaMP mouse epidermis. Arrows indicate dermal collagen (blue). (**b**) Time traces of response area and (**c**) Cytosolic Ca^{2+} concentration increase in young (red) and aged (blue) mice. (**d**) Distribution of maximum response area and (**e**) maximum cytosolic Ca^{2+} concentration increase over baseline for young and aged mice. Data were normalized to young mice mean value. n = 18 (aged mice) and 19 (young mice) traces from two biopsies per mouse and from three separate mouse pairs. (**f**) Time traces of calcium response area after laser perturbation in NHEK (black) compared with that in AHEK (red). (**g**) Distribution of maximum calcium response area (n = 15 traces from three separate experiments) and (**h**) single-cell maximum cytosolic Ca^{2+} concentration increase (n = 1,370–1,552 from three experiments). NHEK is indicated in red, and AHEK is indicated in blue. f/f0 represents baseline fluorescence. Asterisks indicate $P < 0.05$ by a two-tailed *t*-test. AHEK, aged human epidermal keratinocyte; NHEK, neonatal human epidermal keratinocyte; s, second; t, time; SB, stratum basale.

from aged or neonatal donors were plated in low calcium on plastic dishes for time-lapse imaging or multichambered glass slides for immunofluorescence microscopy until 80% confluent. Extracellular calcium levels were then raised to 1.2 mM to promote cell-to-cell adhesion and E-cadherin expression. After 24 hours in high calcium, the epithelial sheets were perturbed with a scratch assay and imaged at 100-minute intervals for 12–24 hours (Figure 4a). Time-lapse images revealed that epithelial sheets from aged KCs were slower on average than those from NHEKs at closing the defect (Figure 4b) owing to an initial delay at 100 minutes (asterisk, Figure 4b). Moreover, whereas NHEKs migrated as a sheet, aged cells appeared not to migrate in unison, but instead lost cell-to-cell adhesion and developed gaps as the

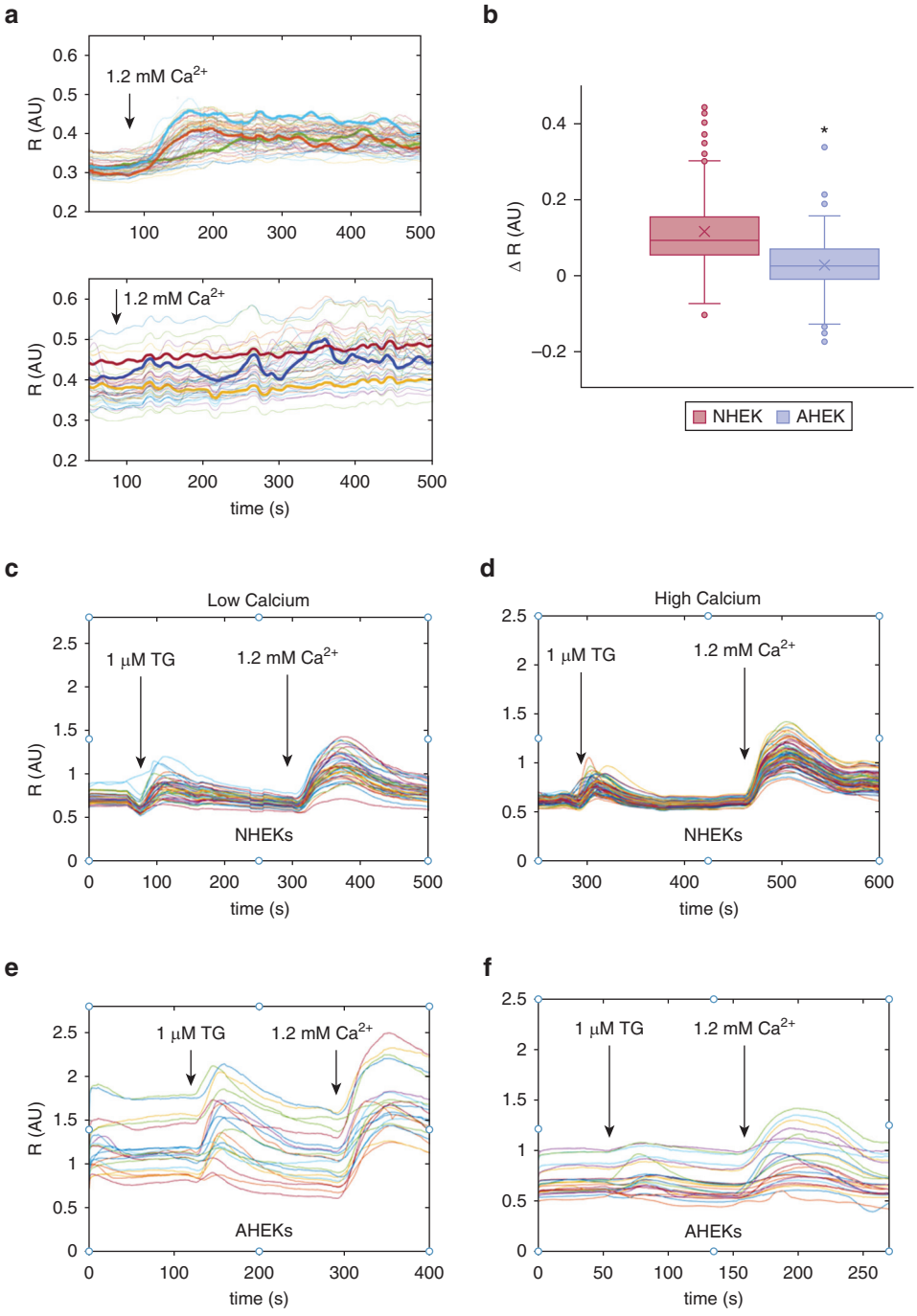

Figure 2. Calcium signaling is impaired in aged human keratinocytes. (**a**) Response to high extracellular calcium of FURA2-labeled NHEK (top panel) and AHEK (bottom panel) monolayers. Representative traces of six to seven separate experiments on cells cultured from three neonatal and three aged donors. (**b**) Distribution of single-cell cytosolic Ca^{2+} variation after calcium switch reported as ΔR. n = 220–410 cells per group from six (AHEK) to seven (NHEK) separate experiments on cells cultured from three neonatal and three aged donors. Asterisk denotes $P < 0.05$. (**c**–**f**) Representative traces of cytosolic Ca^{2+} concentrations in keratinocytes at baseline and in response to 1 μM TG followed by 1.2 mM [Ca^{2+}]. (**c**) NHEK and (**d**) AHEK in 0.07 mM Ca^{2+}. (**e**) NHEK versus (**f**) AHEK cultured in 1.2 mM [Ca^{2+}] for 24 hours. Data are reported as the ratio R of the fluorescence intensity at 340 nm excitation (f_{bound}) over the fluorescence intensity at 390 nm excitation (f_{free}). n = 102–380 cells per group from three to seven separate experiments on cells cultured from three neonatal and three aged donors. Results are summarized in Table 1. [Ca^{2+}], intracellular calcium concentration; AHEK, aged human epidermal keratinocyte; AU, arbitrary unit; Ca^{2+}, calcium ion; NHEK, neonatal human epidermal keratinocyte; s, second; TG, thapsigargin.

sheets migrated (Figure 4a). E-cadherin immunofluorescence staining was performed 6 hours after the scratch assay and revealed decreased and irregular E-cadherin plasma membrane staining in aged (Figure 4d) compared with that in young (Figure 4c) KC monolayers. Gaps in KC cell-to-cell adhesion were colocated with absent E-cadherin staining.

The CaSR agonist N-(3-[2-chlorophenyl]propyl)-(R)-alpha-methyl-3-methoxybenzylamine rescues Ca^{2+} wave propagation, intracellular calcium concentration, response to increased extracellular calcium, and E-cadherin translocation in AHEKs

N-(3-[2-chlorophenyl]propyl)-(R)-alpha-methyl-3-methoxybenzylamine (NPS R-568) selectively (Nemeth et al., 1998; Tang et al., 2018) binds to the transmembrane domain of the CaSR and increases its stability (Huang et al., 2011), thereby increasing its Ca^{2+} sensitivity and enhancing the effects of extracellular Ca^{2+} on CaSR (Fox et al., 1999). To test whether enhancing the CaSR response would also normalize Ca^{2+} wave propagation and signaling and E-cadherin translocation to the plasma membrane, we pretreated aged KCs with 0.5 or 1 μM NPS R-568 for 24 hours. Vehicle-treated aged KCs and neonatal foreskin KCs were used as controls.

We first found that similar to the epidermal response to laser stimulation, vehicle-treated aged KCs displayed markedly diminished propagation of calcium response to mechanical ablation (Figure 5a–c). However, pretreatment with

Table 1. Response to Thapsigargin and High Calcium in NHEKs and AHEKs

	Low Calcium		High Calcium	
Measured Quantities	**NHEK**	**AHEK**	**NHEK**	**AHEK**
Baseline	0.644 ± 0.005	1.06 ± 0.05[1]	0.704 ± 0.006	0.97 ± 0.05[1]
P1	0.33 ± 0.01	0.22 ± 0.04[1]	0.232 ± 0.007	0.25 ± 0.02
P2	0.45 ± 0.02	0.26 ± 0.02[1]	0.44 ± 0.01	0.34 ± 0.02[1]
Percentage of cells responding to 1μM thapsigargin	83 ± 8	44 ± 8[1]	92 ± 3	53 ± 12[1]

Abbreviations: $[Ca^{2+}]$, intracellular calcium concentration; AHEK, aged human epidermal keratinocyte; NHEK, neonatal human epidermal keratinocyte; P, peak.

Capacitive calcium entry traces (Figure 2) were analyzed to determine the baseline intracellular calcium levels (baseline), peak calcium release from stores after exposure to 1 μM thapsigargin (P1), and peak capacitive calcium entry after medium supplementation with 1.2 mM $[Ca^{2+}]$ (P2). Data are reported as the ratio R of the fluorescence intensity at 340 nm excitation (f_{bound}) over the fluorescence intensity at 390 nm excitation (f_{free}). The percentage of AHEKs responding to thapsigargin is reported in the last row of the table.

[1]Statistically significant difference between the NHEK and AHEK values determined by two-tailed *t*-test with $P < 0.05$. n = 102–380 single cell traces from three to seven experiments per group from three aged and three neonatal donors.

NPS R-568 restored the Ca^{2+} wave propagation in a dose-dependent fashion (Figure 5a–c). Pretreatment with NPS R-568 for 24 hours also restored the AHEKs cytosolic calcium response to increased extracellular calcium in a dose-dependent manner (Figure 5d and Supplementary Figure 3). CaSR stimulation with NPS R-568 also partially rescued E-cadherin plasma membrane translocation (Figure 5e and f). These findings show that enhancing CaSR

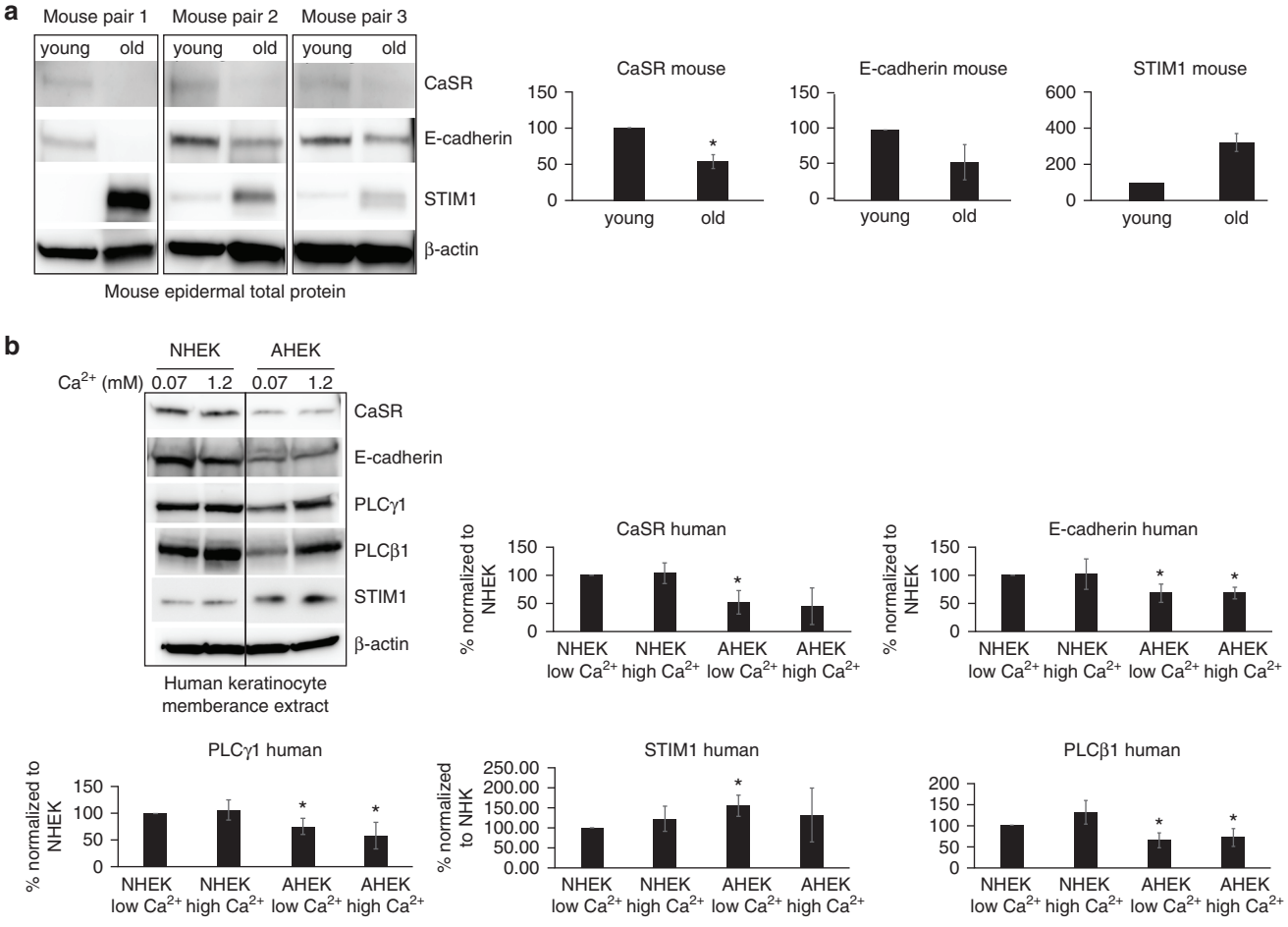

Figure 3. Calcium-signaling molecules expression in aged and young mice and neonatal and aged human keratinocytes. (**a**) Epidermal lysate from three aged and three young mice was probed for levels of CaSR, E-cadherin, and STIM1 using western blotting, and the differences in expression levels were quantified (bar graphs). (**b**) Crude membrane extracts of NHEK and AHEK cultured in low or high calcium for 24 hours were probed for CaSR, E-cadherin, PLCγ1, PLCβ1, and STIM1 using western blotting, and the expression levels were quantified (bar graphs). Data are representative of three to four different sets of aged and neonatal cells. Asterisks denote $P < 0.05$ by a two-tailed *t*-test. AHEK, aged human epidermal keratinocyte; Ca^{2+}, calcium ion; CaSR, calcium-sensing receptor; NHEK, neonatal human epidermal keratinocyte; PLC, phospholipase C.

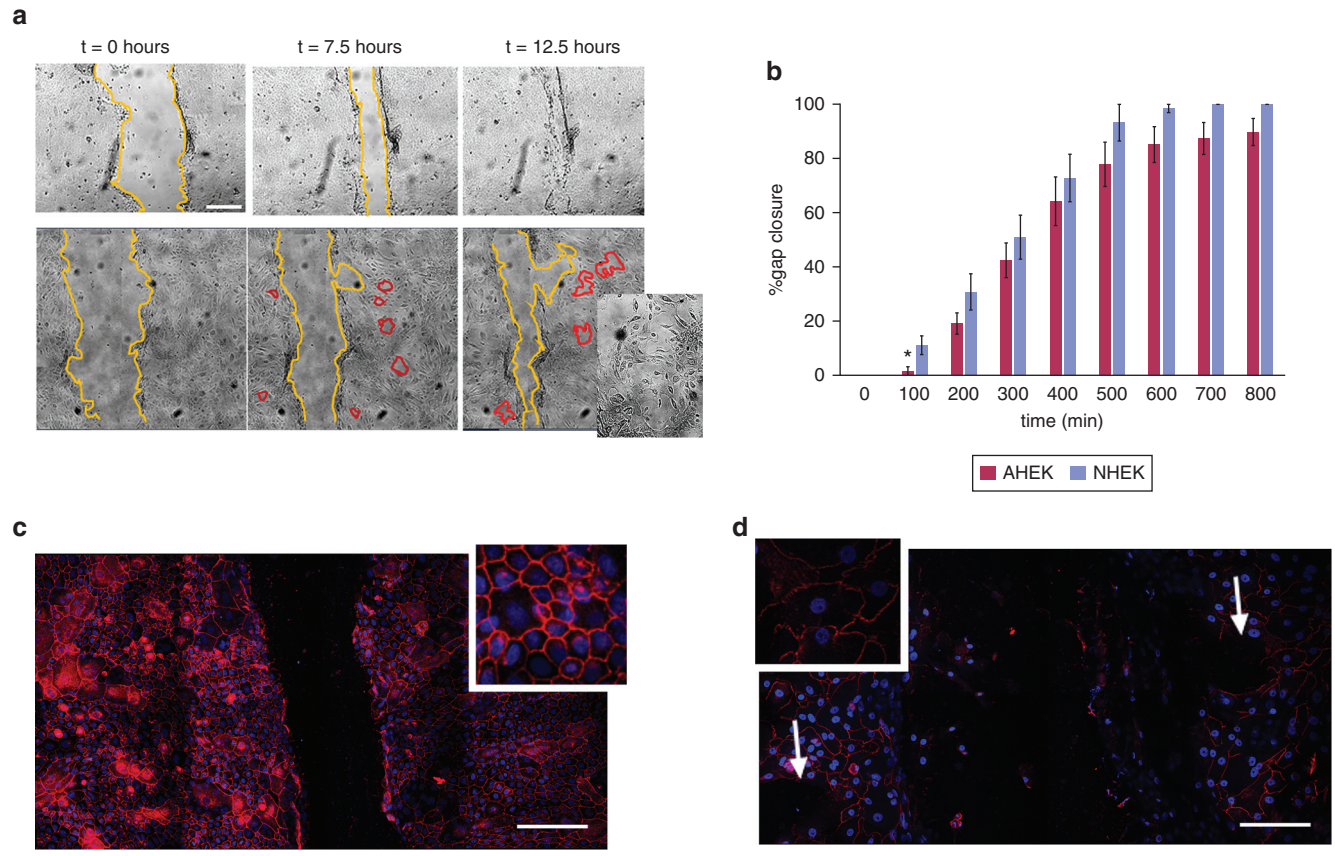

Figure 4. Impaired cell-to-cell adhesion in aged keratinocytes monolayers. (**a**) Brightfield time-lapse images of scratch assay of second passage keratinocytes monolayers from neonatal (top row) and aged (bottom row) donors in high calcium. The yellow lines highlight the gap area at different time points, whereas the red lines highlight the gaps occurring in the AHEK monolayers during sheet migration. Inset shows the gaps in the aged epidermal keratinocytes sheet. (**b**) Mean percentage gap closure as a function of time. AHEK is indicated with red bars, and NHEK is indicated with blue bars. Error bars represent the SEM. n = 4–8 wells from two experiments on cells from three to four donors per condition. (**c, d**) E-cadherin staining (red) of (**c**) NHEK and (**d**) AHEK monolayers 6 hours after scratching. Nuclear DAPI was used for counterstain (blue). Insets show higher detail of E-cadherin staining. White arrows in (**d**) show the gaps in cell-to-cell adhesion. AHEK, aged human epidermal keratinocyte; min, minute; NHEK, neonatal human epidermal keratinocyte.

activity through a pharmacological activator such as NPS R-568 can rescue the abnormal Ca^{2+} signaling and E-cadherin organization seen in aged KCs.

DISCUSSION

These results show that normal Ca^{2+} signaling and Ca^{2+}-signaling protein expression are impaired in aged epidermis and KCs. Similar to *Casr*-knockout cells and epidermis (Tu et al., 2019), Ca^{2+} propagation after perturbation in murine epidermis and cell monolayers from aged donors was significantly reduced compared with that from young controls. Moreover, CaSR expression was consistently downregulated in aged KCs. CaSR acts to release Ca^{2+} from the ER and Golgi through PLC-generated inositol triphosphate, which then binds to the inositol trisphosphate receptor on the ER (Tu et al., 2008). Knockdown of *CaSR* in human cells causes a reduction in both Gq-mediated activation of PLCβ and E-cadherin–mediated activation of PLCγ (Tu et al., 2005), which are in turn necessary for the acute and sustained KCs response to elevated extracellular calcium levels. Both PLCβ and PLCγ expression levels were consistently reduced in KCs from aged donors.

CaSR expression also stabilizes the E-cadherin complex, which in turn regulates cell-to-cell adhesion and cell migration and enables the sustained intracellular calcium level increase needed for KC differentiation through the recruitment of PLCγ. Decreased E-cadherin expression levels and translocation to the plasma membrane were associated with decreased CaSR expression in aged HKs, consistent with previous reports in mice (Tu et al., 2008) and human cells (Tu et al., 2011). Our data also suggest that decreased E-cadherin levels result in loss of cell-to-cell junction stability and concerted epithelial sheet migration during a scratch assay. More differentiated KCs tend to have a blunted Ca^{2+} response, and if aged KCs are more differentiated than young KCs, this might furnish an alternative mechanism to explain our findings. However, past reports show that aged KCs and skin are actually less differentiated both in mice (Bourguignon et al., 2013) and in humans (Berge et al., 2008; Diekmann et al., 2016; Dos Santos et al., 2015).

Taken together, these results show that decreased CaSR expression and function contribute significantly to the impaired Ca^{2+} signaling response seen in aging. Although changes in each of the Ca^{2+}-signaling components could also be expected to modify Ca^{2+} signaling, our finding that treatment with the CaSR agonist NPS-R568 restored normal Ca^{2+} signaling and E-cadherin distribution strongly suggests that decreased CaSR expression in aging drives both impaired

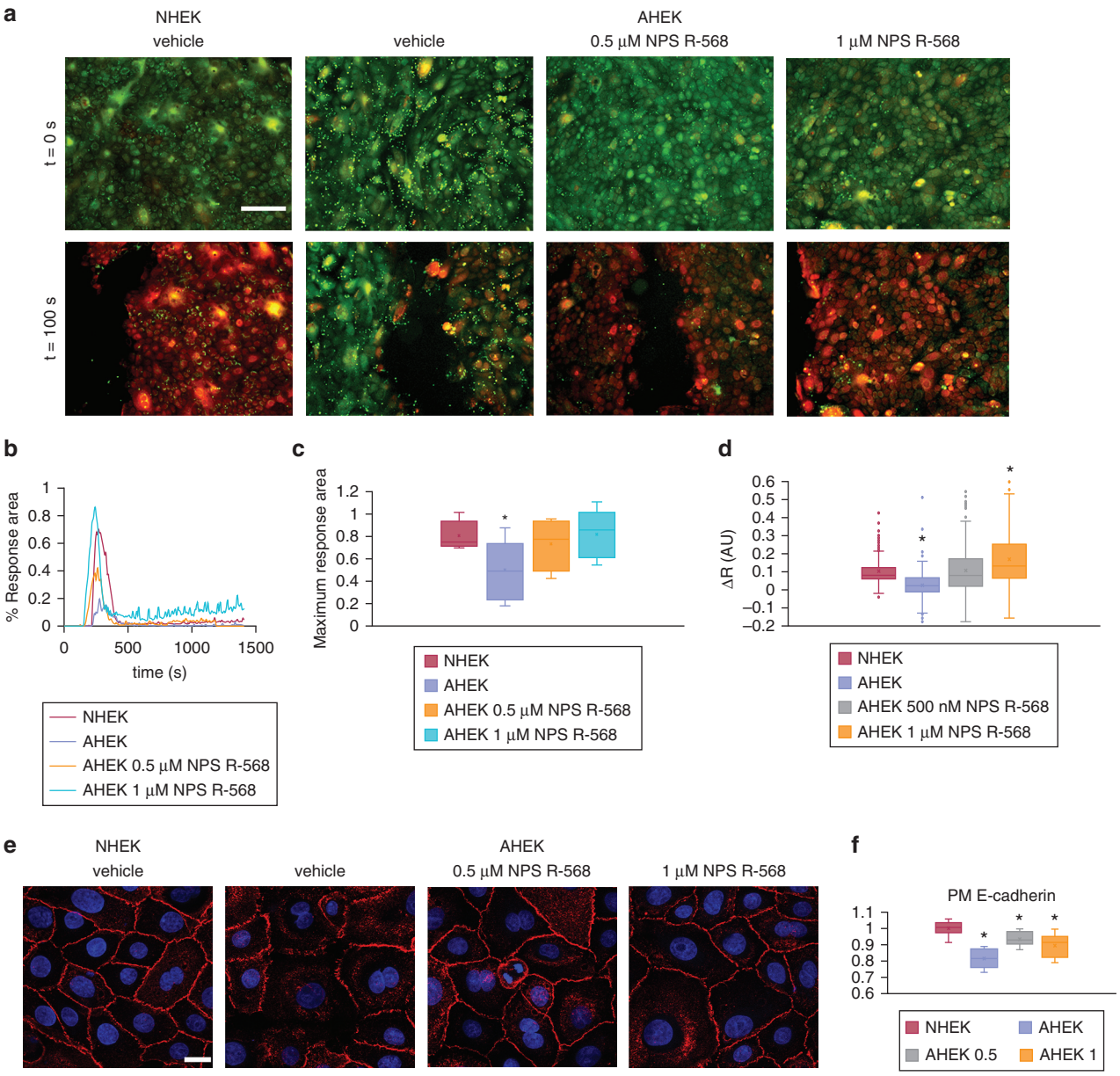

Figure 5. NPS R-568 normalizes E-cadherin and calcium response after scratch wounding. (**a**) NHEK and AHEKs before and 100 s after scratch. Red hue denotes higher cytosolic Ca^{2+}. Bar = 200 μm. (**b**) Time traces of percentage area with increased cytosolic Ca^{2+} level after scratch. Representative of five to eight experiments on cells from three neonatal and three aged donors per group. (**c**) Distribution of maximum calcium response area after scratch. NHEK (red) and AHEK (lilac) treated with vehicle, 0.5 μM NPS R-568 (orange), or 1 μM NPS R-568 (cyan). (**d**) Distribution of single-cell cytosolic Ca^{2+} response to increased extracellular calcium. Asterisks indicate statistical significance with $P < 0.05$. n = 192–409 cells per group from six to 10 experiments on cells from three neonatal and three aged donors. (**e**) Immunofluorescence E-cadherin staining (red) of NHEK and AHEK monolayers switched to 1.2 mM [Ca^{2+}] media containing 0 (DMSO vehicle), 0.5, or 1 μM NPS-R568 for 15 minutes. Images are representative of three separate experiments on cells from three neonatal and three aged donors. Bar = 20 μm. (**f**) Quantification of E-cadherin levels at the PM. Asterisks denote statistically significant difference from NHEKs levels determined by a two-tailed *t*-test with $P < 0.05$. [Ca^{2+}], intracellular calcium concentration; AHEK, aged human epidermal keratinocyte; AU, arbitrary unit; NHEK, neonatal human epidermal keratinocyte; NPS R-568, N-(3-[2-chlorophenyl]propyl)-(R)-alpha-methyl-3-methoxybenzylamine; PM, plasma membrane; s, second; t, time.

Ca^{2+} signaling and downstream changes in Ca^{2+}-signaling proteins. Although decreases in CaSR protein expression might be expected to impair NPS-R568 efficacy, this agent has been shown to increase CaSR function on mutant CaSR as well (Rus et al., 2008).

STIM1 expression also increased in both aged mouse epidermis and aged HKs, likely as a compensatory response. STIM1 expression was found to increase to a lesser extent in a previous report (Takei et al., 2016), although this report examined younger subjects (maximum age of 70 years) compared with our older subjects (aged >79 years).

Several questions remain regarding CaSR-mediated Ca^{2+} signaling in aged KCs. First, although raised cytosolic baseline Ca^{2+} is consistent with STIM1 upregulation, it also could be explained by impaired Ca^{2+} uptake or extrusion mechanisms, including functional defects in organelle and plasma membrane Ca^{2+} adenosine triphosphatase or defects in plasma membrane sodium ion and/or Ca^{2+} antiporters. We

do not see consistent differences in the expression of these proteins. However, more subtle differences in these proteins' functions, along with elevated STIM levels, may become apparent in subsequent investigations. Second, although increased STIM1 levels would suggest increased store-operated Ca^{2+} entry, similar to what was observed in *Casr*-knockout cells (Tu et al., 2008), we observe a significant defect in calcium entry after exposure to high (1.2 mM) extracellular calcium in aged compared with that in young cells in both proliferative and differentiative conditions. Further investigation into the calcium entry mechanisms, such as STIM1 translocation to the plasma membrane and Orai1 interactions, will be needed to address this defect. Moreover, KCs have also been shown to express molecules involved in noncapacitive calcium entry such as voltage-sensitive calcium channels (Denda et al., 2006; Lee et al., 1994), transient receptor potential channels (Peier et al., 2002), nonselective cation channels in undifferentiated KCs (Fatherazi et al., 2004), which could also play a role in the decreased response to raised extracellular calcium we observe in aged KCs. Finally, it is not clear what mechanisms underlie the increased variability seen in the aged KC response to extracellular Ca^{2+}, TG, or mechanical stimulation. Previous studies show that more substantial increases in cytosolic Ca^{2+} are seen in less differentiated KCs, both in response to extracellular Ca^{2+} (Kruszewski et al., 1991) and in response to mechanical stimulation (Dubé et al., 2012). In addition, basal cytosolic Ca^{2+} concentration is variable within the same colonies, depending on cell size (Pillai et al., 1993). Likewise, CaSR expression and function decrease as gingival KCs terminally differentiate (Fatherazi et al., 2004). Therefore, variations in aged KC response could be due to exaggerated intrinsic aging processes, mutations in response to environmental agents such as UV, or a combination of intrinsic and extrinsic processes.

These results suggest that CaSR plays an essential role in mediating Ca^{2+} signaling and E-cadherin–mediated processes in the epidermis. Moreover, decreased CaSR expression and function contribute to impaired KC signaling and E-cadherin expression. These results also suggest that CaSR may be a different target in improving E-cadherin–mediated processes in aged epidermis.

MATERIALS AND METHODS

Please see Supplementary Materials and Methods for more information.

Laser perturbation assay

All animal procedures were approved by the Animal Studies Subcommittee (Institutional Animal Care and Use Committee) of the San Francisco Veterans Administration Medical Center (CA) and were performed in accordance with their guidelines. Live epidermal explants from GCaMP3$^{+/+}$-expressing young (aged 6–8 weeks) versus old (aged 22 months) mice placed dermis side down on a 3% agar gel were secured on the heated stage of an upright Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy, New York, NY) coupled to a Ti:Saph laser (Chameleon Ultra II, Coherent, Santa Clara, CA). Ca^{2+} signaling in the epidermis was stimulated by irradiating a spatially defined 20 × 20 μm^2 region on the basal layer of the epidermis with 800 nm (~140 mW). Irradiation parameters (laser intensity, scanning speed, number of iterations) were kept constant for all experiments and were optimized to consistently elicit a cytosolic Ca^{2+} response without permanent cell damage. The resulting GCaMP fluorescence was imaged with two-photon excitation microscopy. The excitation wavelength was 900 nm (~15 mW). Two spectral windows of 550 per 50 nm and 445 per 25 nm were used to visualize the GCaMP fluorescence in the epidermis and the second harmonic generation signal, respectively. Dermal collagen, identified with the second harmonic signal, was used as a spatial reference. Time series were analyzed in Fiji (Schindelin et al., 2012) and Matlab (MathWorks, Natick, MA). The change in GCaMP fluorescence was expressed as the ratio of the change with respect to the baseline fluorescence (f/f0), whereas the response area was measured as the area with significantly increased cytosolic calcium (f/f0 > 1.2), reported in μm^2. A total of three mice per age group was used for these experiments. Two separate skin biopsies (one per flank) per mouse were used to collect 18–19 times resolved cytosolic calcium traces per experimental group.

Cytosolic calcium imaging in KC monolayers and cultured KC sheets

Second to third passage KCs from newborns versus those from aged subjects (NHEK and AHEK, respectively) were cultured as described earlier to 70–90% confluence for single KC Ca^{2+} imaging and 100% confluence for scratch assays and laser perturbation. KCs were loaded with 10 μM Calcium Green-1AM (Life Technologies, Carlsbad, CA) for KC Ca^{2+} response to laser perturbation. Cells were placed on the heated stage of an upright Zeiss 780 two-photon confocal microscope, and calcium recordings before and after laser perturbation were acquired as described earlier using a dipping ×20 lens with numerical aperture = 1. For the response to calcium switch, scratch assays, and capacitive calcium entry after store depletion, KCs were loaded with 7.5 μM Fura-2 AM (Sigma-Aldrich, St. Louis, MO). Dyes were loaded for 45 minutes at 37 °C and washed three times with Hank's Balanced Salt Solution. Phenol red–free Hank's Balanced Salt Solution (Thermo Fisher) containing the appropriate extracellular Ca^{2+} (0, 0.07, or 1.2 mM) was used during imaging. Fura2-loaded cells were secured on a Zeiss Axio Imager 2 inverted fluorescence microscope and were alternately illuminated with 340 nm and 390 nm wavelengths. The fluorescence at emission wavelength 510 nm was recorded. Scratches were made to KC sheets with a 23-gauge needle. Changes in cytosolic Ca^{2+} levels in cells neighboring the scratched area were imaged before and for 50–200 seconds after wounding. For response to high extracellular calcium switch experiments, after a period of equilibration to establish a baseline of 60–120 seconds, a high calcium medium was added to the wells to a final concentration of 1.2 mM. Cells were imaged every second for additional 5–15 minutes Ca^{2+}. The response is expressed in μm^2 for area; R(arbitrary unit) = f_{390nm}/f_{340nm}, where f_{390nm} and f_{340nm} are the fluorescence intensities generated by excitation at 390nm and 340nm, respectively, corresponding to calcium-bound and -free FURA2, for single KC cytosolic Ca^{2+} responses; and ΔR((arbitrary unit) = $R_{hiCa} - R_{bl}$, where R_{hiCa} is the average R value between 2 and 4 minutes after calcium switch, and R_{bl} is the average baseline R before a switch to high calcium. For capacitive calcium entry after store depletion, 1μM TG (Sigma-Aldrich) was added to the culture well during ratiometric imaging. After the ratiometric signal returned to baseline, cells were exposed to 1.2 mM calcium-containing media. Cell migration was assessed using brightfield time-lapse imaging. Cells were plated on 24-well plates and switched to 1.2 mM extracellular Ca^{2+} for 24 hours before imaging on a Zeiss

Cell Observer (Carl Zeiss Microscopy) with full environmental control (37 °C and 5% carbon dioxide). A 10 μl pipette tip was used to scratch the cultures.

NPS R 568 treatment

For calcium imaging experiments, KCs monolayers were switched to 0.07 mM or 1.2 mM calcium and 0 (1:1,000 dilution of DMSO), 0.5, or 1 μM NPS R 568 (Sigma-Aldrich)–containing medium 24 hours before imaging. For E-cadherin immunofluorescence staining, cells were exposed to 1.2 mM calcium and NPS R 568 (0, 0.5,1 μM)–containing media 15 minutes before fixation.

Data availability statement

The data that support the findings of this study are available at https://doi.org/10.17632/s5mk3nd495.1 and from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: AC, TMM, CLT; Data Curation: AC, CLT; Formal Analysis: AC, CLT; Funding Acquisition: DDB, TMM; Investigation: AC, TMM, CLT; Methodology: AC, CLT; Project Administration: TMM; Resources: TMM, DDB; Software: AC; Supervision: TMM; Validation: AC, CLT; Visualization: AC; Writing — Original Draft Preparation: AC, TMM, CLT; Writing — Review and Editing: AC, TMM

Disclaimer

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.03.025.

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