Role of STIM1- and Orai1-mediated Ca^{2+} entry in Ca^{2+}-induced epidermal keratinocyte differentiation

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Summary

The uppermost thin layer on the surface of the skin, called the epidermis, is responsible for the barrier function of the skin. The epidermis has a multilayered structure in which each layer consists of keratinocytes (KCs) of different differentiation status. The integrity of KC differentiation is crucial for the function of skin and its loss causes or is accompanied by skin diseases. Intracellular and extracellular Ca^{2+} is known to play important roles in KC differentiation. However, the molecular mechanisms underlying Ca^{2+} regulation of KC differentiation are still largely unknown. Store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} influx pathway in most non-excitable cells. SOCE is evoked in response to a fall in Ca^{2+} concentration in the endoplasmic reticulum. Two proteins have been identified as essential components of SOCE: STIM1, a Ca^{2+} sensor in the ER, and Orai1, a subunit of Ca^{2+} channels in the plasma membrane. In this study, we analyzed the contribution of SOCE to KC growth and differentiation using RNAi knockdown of STIM1 and Orai1 in the human keratinocyte cell line, HaCaT. KC differentiation was induced by a switch in extracellular Ca^{2+} concentration from low (0.03 mM; undifferentiated KCs) to high (1.8 mM; differentiated KCs). This Ca^{2+} switch triggers phospholipase-C-mediated intracellular Ca^{2+} signals (Ca^{2+}-switch-induced Ca^{2+} response), which would probably involve the activation of SOCE. Knockdown of either STIM1 or Orai1 strongly suppressed SOCE and almost completely abolished the Ca^{2+}-switch-induced Ca^{2+} responses, resulting in impaired expression of keratin1, an early KC differentiation marker. Furthermore, loss of either STIM1 or Orai1 suppressed normal growth of HaCaT cells in low Ca^{2+} and inhibited the growth arrest in response to a Ca^{2+} switch. These results demonstrate that SOCE plays multiple crucial roles in KC differentiation and function.

Key words: Store-operated calcium entry, Calcium channels, Orai channels, Epidermal keratinocyte

Introduction

The skin functions as physical protection for the body from harmful pathogens, hazardous chemicals or dehydration. This barrier function of the skin is carried out by the outermost thin layer called epidermis. The epidermis is a multilayered structure composed primarily of cells termed keratinocytes. Each layer of the epidermis consists of keratinocytes in different differentiation status. While undifferentiated keratinocytes in the basal layer are highly proliferative, they exit the cell cycle and undergo differentiation upon migrating to upper layers. Therefore, the integrity of differentiation process of keratinocytes is critical for skin barrier function. Defects in this barrier function are known to cause many skin diseases (Proksch et al., 2008).

Many factors interact to induce KC differentiation including calcium (Ca^{2+}), vitamin D, and cell to cell contact (Bikle, 2010; Dotto, 1999; Yuspa et al., 1988). It has been reported that there is a gradient of extracellular Ca^{2+} concentration in the epidermis from low in the basal layer to high in the stratum granulosum, the uppermost layer of epidermis (Pillai et al., 1993). Indeed, increases of extracellular Ca^{2+} concentration (Ca^{2+} switch) can induce KCs to exit the cell cycle and express several differentiation markers in isolated primary KCs *in vitro* (Pillai et al., 1990). Furthermore, low extracellular Ca^{2+} concentration is critical to maintain the highly proliferative nature of undifferentiated KCs. It has previously been shown that the Ca^{2+} switch is sensed by a Ca^{2+}-sensing receptor (CaR) in the plasma membrane of KCs (Tu et al., 2004). CaR is a G-protein-coupled receptor coupled to G_q type alpha subunits, and thus activation of CaR leads to activation of the phospholipase C pathway (Hofer and Brown, 2003). CaR-mediated PLC signaling is initially mediated by PLCβ and subsequently by PLCγ (Xie and Bikle, 1999). Suppression of the intracellular Ca^{2+} increase with chelators, or suppression of PLCγ activity attenuate KC differentiation, suggesting that Ca^{2+} signaling is a key signaling pathway for Ca^{2+}-switch-induced KC differentiation (Li et al., 1995). However, the exact molecular mechanism underlying Ca^{2+}-switch-induced Ca^{2+} mobilization is largely unknown. Several Ca^{2+}-permeable channels are suggested to be involved in Ca^{2+} signaling in Ca^{2+}-switch-induced KC differentiation including transient receptor potential family channels (Beck et al., 2008; Cai et al., 2006; Müller et al., 2008).

Store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} influx pathway in most non-excitable cells (Parekh and Putney, 2005). As its name suggests, SOCE is activated by depletion of Ca^{2+} stores in the endoplasmic reticulum (ER). SOCE is known to be involved in cell proliferation and differentiation processes (Darbellay et al., 2009; Hwang and Putney, 2012; Johnstone et al., 2010). SOCE is mediated essentially by two classes of proteins, the STIM and Orai proteins (Feske et al., 2006; Liou et al., 2005; Roos et al., 2005; Vig et al., 2006; Zhang

et al., 2006). STIM proteins (STIM1 and STIM2) are single transmembrane proteins expressed in ER membrane with an EF-hand motif in the N-terminus facing the ER lumen. This EF-hand motif functions as a sensor for stored Ca^{2+} content (Liou et al., 2005). Reduction of ER luminal Ca^{2+} induces STIM1 to oligomerize and translocate to ER–plasma membrane junction termed puncta in which Orai1, a pore-forming subunit of SOC channels, is activated apparently by direct interaction with STIM1 (Liou et al., 2007; Park et al., 2009). Although translocation and puncta formation of ectopically expressed STIM1 has been demonstrated in the HaCaT keratinocyte cell line (Ross et al., 2007), the role of endogenous STIM1 and Orai1 proteins in SOCE in KCs has not yet been investigated.

In this study, we analyzed the involvement of STIM1 and Orai1 in SOCE in HaCaT KCs and their importance for Ca^{2+}-switch-induced KC differentiation. siRNA-mediated knockdown of STIM1 and Orai1 strongly suppressed SOCE in HaCaT cells. Interestingly, the suppression of SOCE impaired Ca^{2+} storage in undifferentiated cells. Ca^{2+}-switch-induced Ca^{2+} responses were also abolished by the defect of SOCE, leading to a failure in the induced expression of *keratin 1* mRNA, an early differentiation marker gene. Furthermore, STIM1 and Orai1 knockdown suppressed steady state proliferation of undifferentiated KCs and also inhibited Ca^{2+}-switch-induced cell growth arrest. These results establish an important contribution of STIM1- and Orai1-mediated SOCE to Ca^{2+} homeostasis and commitment of KC differentiation.

Results

Induction of keratinocyte differentiation changes neither expression levels nor post-translational modification of STIM and Orai proteins

To analyze the involvement of SOCE in KC differentiation, we transfected HaCaT cells with siRNAs against either STIM1 or Orai1. KC differentiation was induced by Ca^{2+} switch. Cells were cultured routinely in 0.03 mM Ca^{2+}-containing keratinocyte growth medium (KGM) to maintain undifferentiated status. Then the Ca^{2+} concentration was increased to 1.8 mM (Ca^{2+} switch) and cells were cultured up to 72 hours. Western blotting of both proteins was carried out to verify the efficiency of siRNA-mediated knockdown of STIM1 and Orai1 and to analyze whether their expression levels or posttranslational modification are changed during KC differentiation (Fig. 1). Both proteins were substantially reduced by siRNA, although some small amounts remained that could be seen on longer exposures of the gels (not shown). The mean level of STIM1 increased somewhat

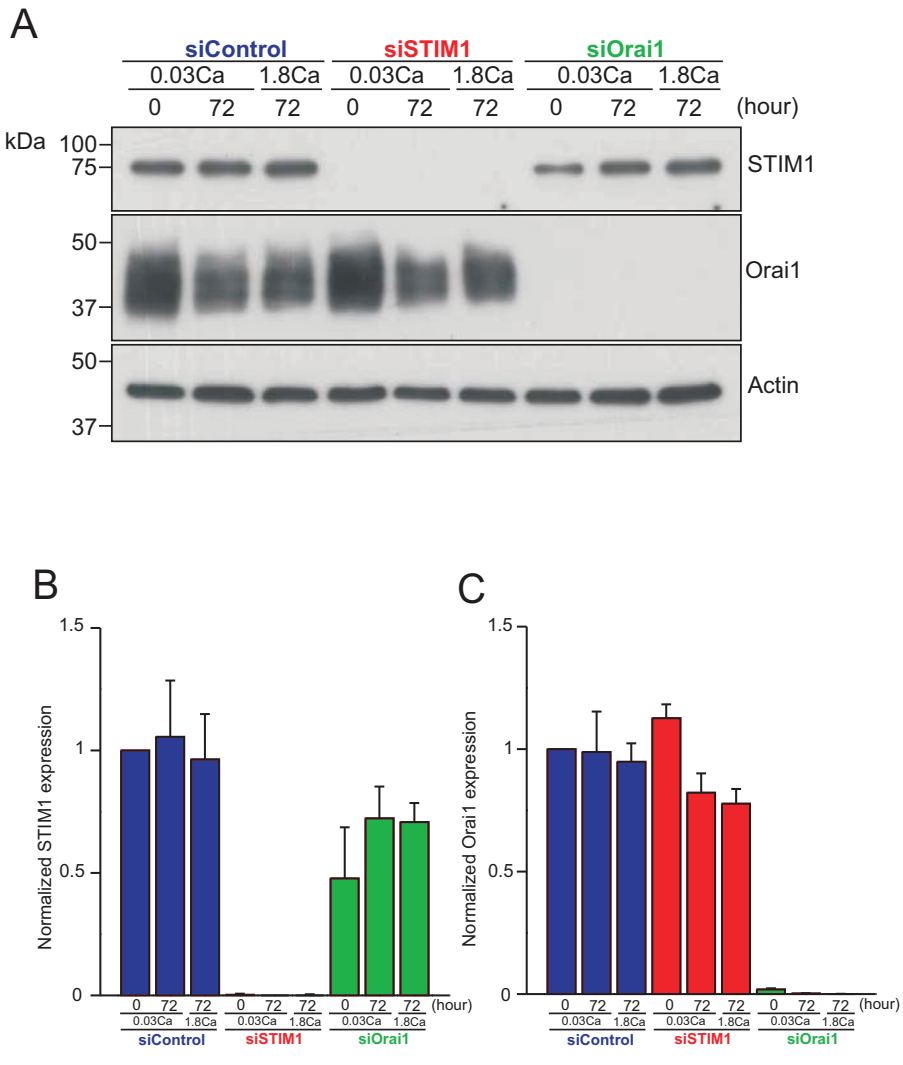

Fig. 1. STIM1 and Orai1 protein expression during keratinocyte differentiation. (**A**) Western blotting showing STIM1 and Orai1 protein expression in siRNA-transfected HaCaT cells before and after Ca^{2+} switch. Note the broadening of the Orai1 band due to glycosylation, as shown by Fukushima et al. (Fukushima et al., 2012). The full lane westerns for both STIM1 and Orai1 are shown in supplementary material Fig. S1. (**B**,**C**) Densitometric analysis of STIM1 (B) or Orai1 (C) expression. Intensities of bands of STIM1 or Orai1 were normalized by those of actin. Relative expression to the sample at time 0 from siControl-transfected cells are shown. Data are means ± s.e.m. from three independent experiments.

after 72 hours in culture. However, neither STIM1 nor Orai1 showed any difference in terms of their expression level and posttranslational modification during KC differentiation, that is when the 72 hour levels in 0.03 mM Ca^{2+} are compared to those in 1.8 mM Ca^{2+}.

Knockdown of either STIM1 or Orai1 suppressed SOCE and Ca^{2+} release-activated Ca^{2+} current in HaCaT cells

Next, we analyzed the functional suppression of STIM1 and Orai1 by siRNAs using Ca^{2+} imaging (Fig. 2). After the depletion of intracellular ER stores by thapsigargin (TG), restoration of extracellular Ca^{2+} evoked large store-operated Ca^{2+} entry in HaCaT cells. To avoid saturation of the ratiometric fluorescent Ca^{2+} indicator Fura-5F, we utilized 0.5 mM Ca^{2+}-containing HBSS instead of 1.8 mM. Both STIM1 and Orai1 siRNAs strongly diminished TG-induced SOCE. STIM1 siRNA almost completely suppressed SOCE, while there was residual SOCE in Orai1 siRNA-transfected cells. The residual influx seen in the Orai1-knockdown cells likely results from the small remaining amount of Orai1. This influx was completely blocked by 2-aminoethyl borate (2APB) and by Synta 66 (supplementary material Fig. S2). Synta 66 has high specificity for Orai channels (Beech, 2012), and 2APB, at the concentration used here, does not block Orai2 or Orai3 (DeHaven et al., 2008). These results indicate that STIM1 and Orai1 constitute the major Ca^{2+} sensor and SOC channel in HaCaT cells.

STIM1 and Orai1 constitute the prototypical SOC channel that gives rise to a Ca^{2+}-selective Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). We next analyzed the effect of STIM1 and Orai1 knockdown on I_{CRAC} in HaCaT cells. Cells were dialyzed with 10 mM BAPTA and 20 µM inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] through the patch pipette to induce I_{CRAC}. As observed in other many other cell types, endogenous I_{CRAC} was too small to detect when using 1.8 mM Ca^{2+}-containing extracellular solution (Fig. 3A). Therefore, we used a technique whereby a rapid change to a divalent-cation-free (DVF) extracellular solution transiently increases the size of an inward sodium I_{CRAC}, due to removal of Ca^{2+} block (DeHaven et al., 2007). With this technique, we could readily record inwardly rectifying currents in siControl-transfected cells (Fig. 3A,C). Both STIM1 and Orai1 knockdown significantly reduced I_{CRAC} in HaCaT KCs (Fig. 3B). These results indicated that STIM1 and Orai1 are the major constituents of endogenous CRAC channels which underlie the SOCE pathway in HaCaT KCs.

Knockdown of either STIM1 or Orai1 reduces intracellular Ca^{2+} store content in HaCaT cells cultured in low Ca^{2+} medium

Strikingly, in STIM1 siRNA-transfected cells the passive Ca^{2+} leak when TG was first added was significantly suppressed (Fig. 2A). This reduction of Ca^{2+} release from TG-sensitive stores was completely restored by an additional 24-hour culture in 1.8 mM Ca^{2+}-containing medium (Fig. 2B). Note in Fig. 2B the similar kinetics of release and decline in the $[Ca^{2+}]_i$ signal upon thapsigargin addition. This indicates that the knockdown of STIM1 and Orai1 did not affect other aspects of cellular Ca^{2+} metabolism such as mechanisms of intracellular buffering or plasma membrane extrusion. Ca^{2+} release by TG was slightly lower in cells cultured with 0.03 mM Ca^{2+} than that in cells cultured with 1.8 mM Ca^{2+}, suggesting that stores are partially depleted in the low extracellular Ca^{2+} concentration of 0.03 mM. A more reliable method to assess intracellular Ca^{2+} store content

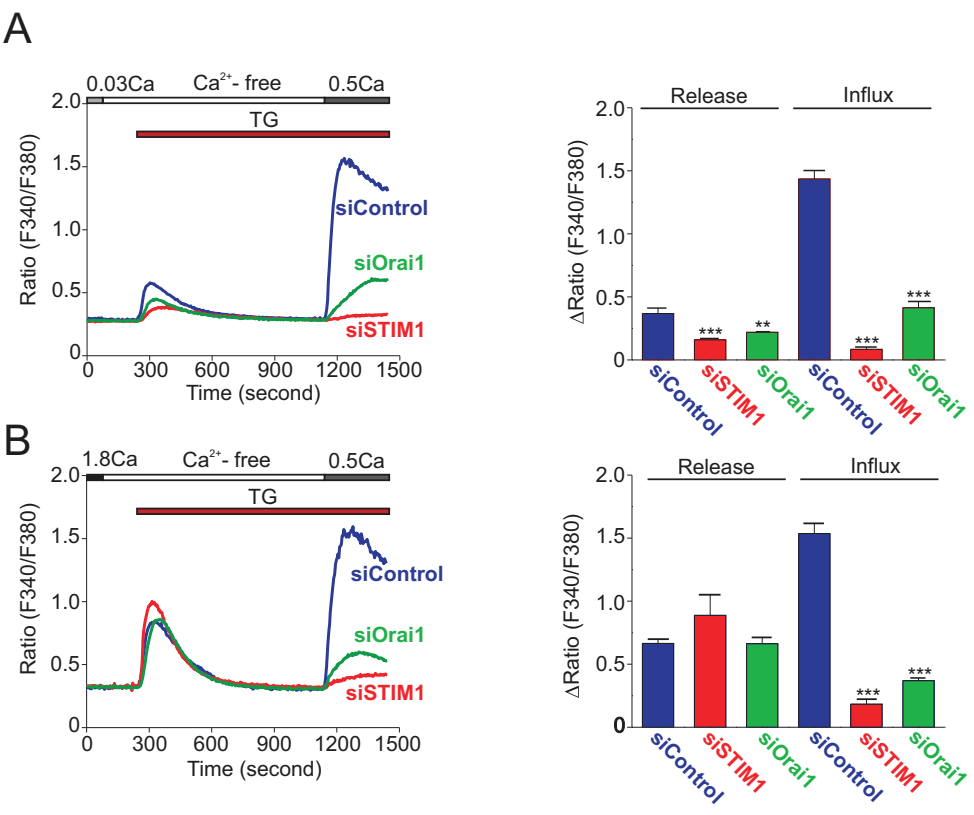

Fig. 2. Both STIM1 and Orai1 are indispensable for TG-induced SOCE in HaCaT cells. (**A**,**B**) Average time courses of TG-induced Ca^{2+} responses in siRNA-transfected HaCaT cells cultured in KGM-2 containing 0.03 mM Ca^{2+} (A) or 1.8 mM Ca^{2+} (B). Right panels showed peak $[Ca^{2+}]$ rises attributable to Ca^{2+} release and influx evoked by TG. Data are means ± s.e.m. from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ (one-way ANOVA followed by Tukey's test).

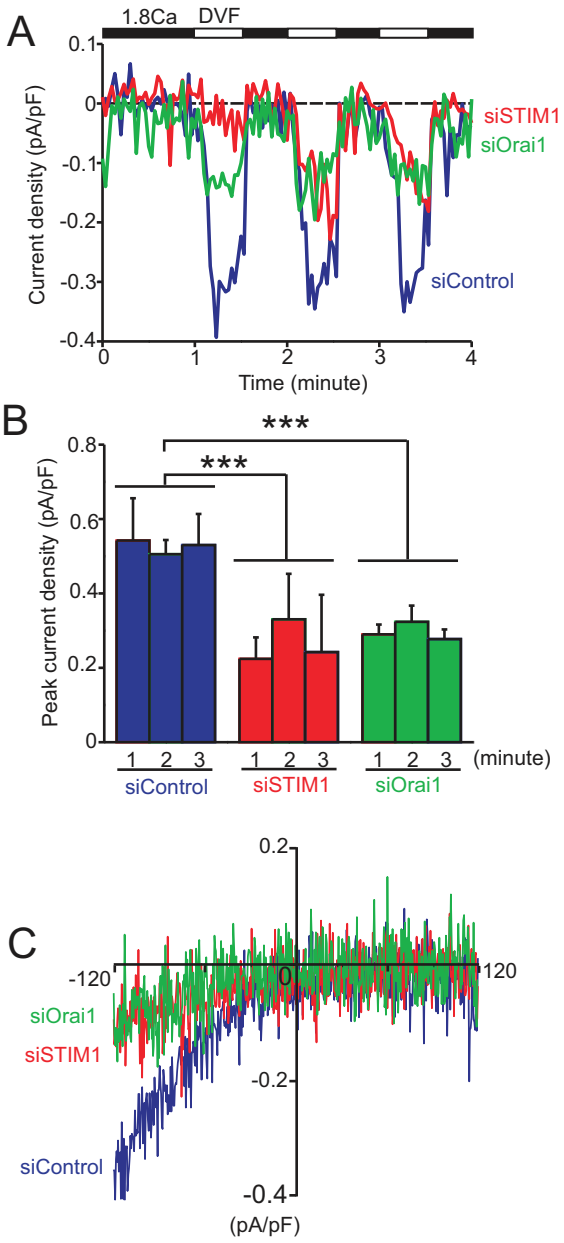

Fig. 3. Both STIM1 and Orai1 are important for I_{CRAC} in HaCaT. (**A**) Average time courses of I_{CRAC} induced by intracellular dialysis of 20 μM Ins(1,4,5)P_3 and 10 mM BAPTA. Switches of extracellular solution are indicated in the horizontal bar above the traces. (**B**) Peak inward current recorded at the holding of −120 mV. Data are means ± s.e.m.; siControl, n=4; siSTIM1 and siOrai1, n=3. ***P<0.001 (ANOVA followed by Tukey's test). (**C**) Representative current–voltage relationship of I_{CRAC} in divalent-cation-free solution.

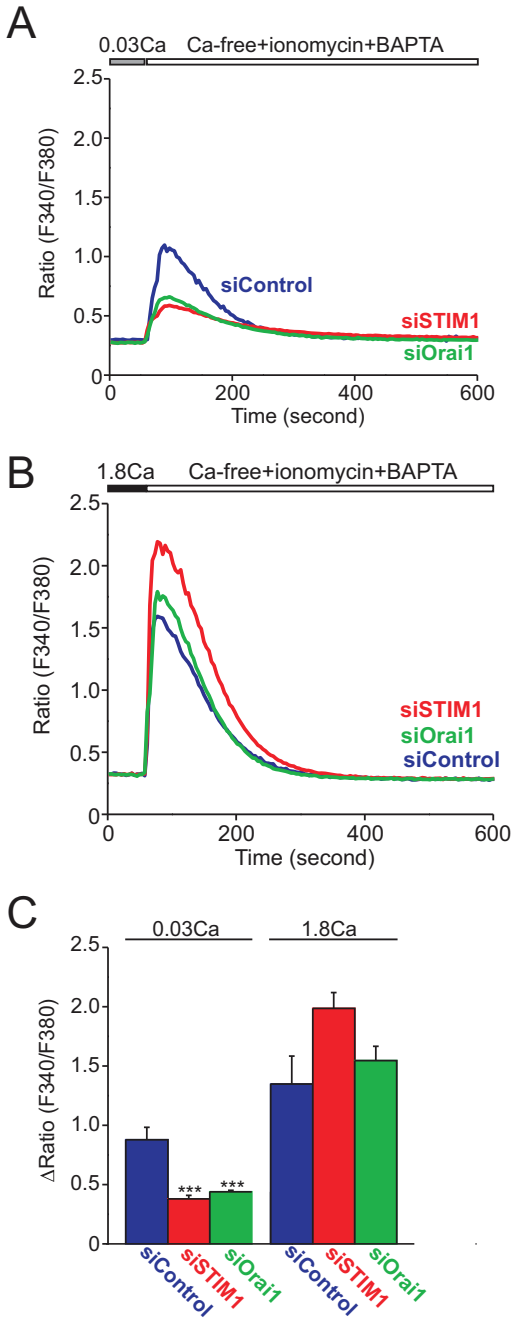

Fig. 4. Both STIM1 and Orai1 are important for maintenance of intracellular stored Ca^{2+} in HaCaT cells cultured in low Ca^{2+} medium. Average time courses of Ca^{2+} release from whole intracellular Ca^{2+} stores by ionomycin in siRNA-transfected HaCaT cells cultured in low (**A**) or high (**B**) Ca^{2+} medium. (**C**) Peak [Ca^{2+}] rises after whole intracellular store depletion by 20 μM ionomycin. Data are means ± s.e.m. from three independent experiments. ***P<0.001 (one-way ANOVA followed by Tukey's test).

involves the use of the Ca^{2+} ionophore ionomycin together with the fast Ca^{2+} chelator BAPTA, thereby reducing the likelihood that the peak release of Ca^{2+} will be blunted by uptake into other intracellular organelles (e.g. mitochondria) (Bird et al., 2008). Consistent with the data of TG-induced Ca^{2+} leak (Fig. 2), siRNAs against STIM1 and Orai1 similarly suppressed the ionomycin peaks (Fig. 4). The reduction in response to ionomycin was clearly due to differences in ER Ca^{2+} stores because when ER stores were depleted by thapsigargin prior to ionomycin addition, there was no difference in the remaining release due to ionomycin (supplementary material Fig. S3). Increasing extracellular Ca^{2+} to 1.8 mM again restored the Ca^{2+} contents to levels comparable to those in control siRNA

transfected cells. These results suggest that STIM1 and Orai1-mediated Ca^{2+} entry is constitutively active in KCs and plays an important role in maintaining intracellular Ca^{2+} stores when cells are exposed to low Ca^{2+} environments.

Ca^{2+}-switch-induced Ca^{2+} responses were abolished by knockdown of either STIM1 or Orai1 in HaCaT cells

KCs are thought to sense an increase in extracellular Ca^{2+} concentration through Ca^{2+}-sensing receptors (CaRs) on their cell surface and respond by undergoing differentiation (Tu et al., 2004). CaR is a G-protein-coupled receptor known to be coupled to $G\alpha_q$ protein which leads to induction of PLC-mediated Ca^{2+} responses (Hofer and Brown, 2003). Therefore, we next investigated how suppression of SOCE affects Ca^{2+}-switch-induced Ca^{2+} responses. Cells were initially kept in 0.03 mM Ca^{2+}-containing HBSS. In this cell line, raising extracellular Ca^{2+} to 1.8 mM Ca^{2+} only evoked minor Ca^{2+} responses in siControl-transfected cells possibly due to efficient global buffering of the Ca^{2+} influx within the cytoplasm (data not shown). However, increasing extracellular Ca^{2+} to 10 mM in control siRNA-transfected cells evoked an initial large Ca^{2+} increase followed by a lower but sustained increase (Fig. 5). These Ca^{2+}-switch-induced Ca^{2+} responses were substantially reduced in either STIM1 or Orai1 siRNA-transfected cells. Similar to the responses to thapsigargin, the residual response in the Orai-knockdown cells was further reduced by either 2APB or Synta 66 (supplementary material Fig. S2).

STIM1 and Orai1 are required for either normal proliferation or extracellular Ca^{2+}-dependent proliferation arrest

Induction of KC differentiation is associated with proliferation arrest (Fuchs, 1993; Micallef et al., 2009). We and others have reported that STIM1 and Orai1 have critical roles in proliferation and cell cycle regulation (Abdullaev et al., 2008; Chen et al., 2011; Smyth et al., 2009). Therefore, next we tested the effect of STIM1 and Orai1 knockdown on proliferation of HaCaT cells by using an XTT cell proliferation assay (see Materials and Methods). As shown in Fig. 6, STIM1 knockdown strongly, and Orai1 knockdown to a lesser extent, diminished proliferation in 0.03 mM Ca^{2+}-containing medium. These data suggest that STIM1 and Orai1 are important for growth of KCs in the undifferentiated condition. Strikingly, Ca^{2+} switch suppressed the proliferation of control cells by about half of that in 0.03 mM Ca^{2+} medium. However, Ca^{2+}-switch-induced suppression of cell growth was not seen or reduced in STIM1- or Orai1-knockdown cells, respectively. These results suggest that STIM1 and Orai1-dependent signaling is important in maintaining proliferation of undifferentiated KCs and that Ca^{2+} switch triggers cell growth arrest by a STIM1- and Orai1-dependent mechanism.

Ca^{2+}-switch-induced expression of an early differentiation marker gene *keratin 1* is diminished by either STIM1 or Orai1 knockdown

Intracellular Ca^{2+} increases upon Ca^{2+} switch are critical for the induction of keratinocyte differentiation (Li et al., 1995). To analyze the physiological effect of the defect of SOCE, we evaluated the expression of *keratin 1* (*KRT1*) mRNA using qRT-PCR (Fig. 7). *KRT1* is known to be an early differentiation marker of keratinocytes. *KRT1* expression can be observed in the suprabasal layer of epidermis (Yuspa et al., 1988). In 0.03 mM Ca^{2+}-containing medium, *KRT1* expression remained almost unchanged over 72 hours in all siRNA-transfected cells. In contrast, following Ca^{2+} switch, an increase in *KRT1* expression was first detected at 48 hours, and to a larger and statistically significant level at 72 hours in siControl-transfected cells. Strikingly, either STIM1- or Orai1-knockdown cells showed significant reduction of *KRT1* mRNA expression, indicating that both proteins are important for the induction of *KRT1* expression upon Ca^{2+} switch in HaCaT cells. These results indicate that Ca^{2+}-switch-induced KC differentiation requires STIM1- and Orai1-mediated SOCE.

Discussion

SOCE is the major Ca^{2+} influx pathway in most non-excitable cells and plays a critical role in cell fate decision (Parekh and Putney, 2005). Although the presence of SOCE has been reported in KCs (Korkiamäki et al., 2003; Tu et al., 2005), its importance in KC differentiation has not been assessed likely because the molecular players underlying SOCE, STIM1 and Orai1, have only recently been identified. In this study, we have discovered a number of important functions of both proteins with regard to Ca^{2+} in KCs, and in the physiologically important process of terminal differentiation. First, we demonstrated that STIM1 and Orai1 are responsible for SOCE in KCs. Second, we show that STIM1 and Orai1-mediated Ca^{2+} entry is also important for maintenance of intracellular Ca^{2+} stores when KCs are exposed

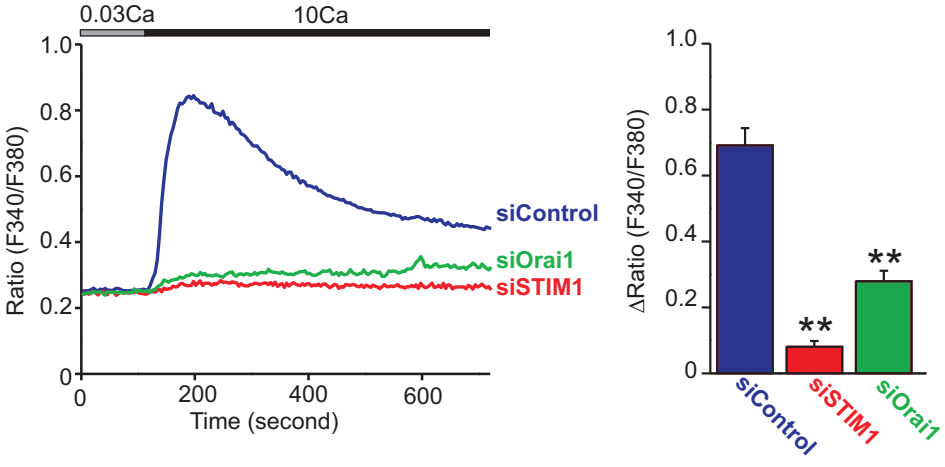

Fig. 5. Both STIM1 and Orai1 are required for Ca^{2+}-switch-induced Ca^{2+} responses in HaCaT cells. Average time courses of 10 mM extracellular Ca^{2+}-induced Ca^{2+} responses in siRNA-transfected HaCaT cells (left). Peak [Ca^{2+}] rises of Ca^{2+}-switch-induced Ca^{2+} responses (right). Data are means ± s.e.m. from three independent experiments. $^{**}P<0.01$ (one-way ANOVA followed by Tukey's test).

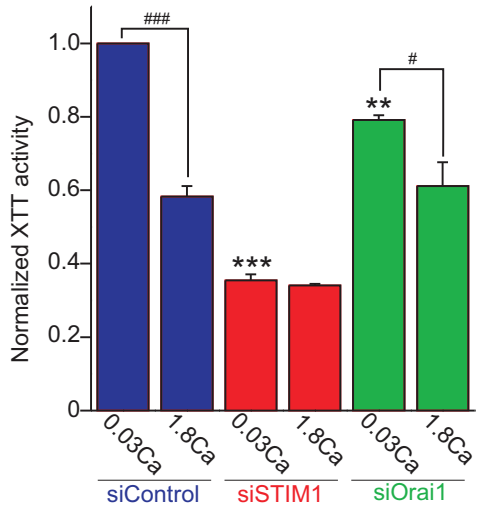

Fig. 6. STIM1 and Orai1 are important for normal cell growth and Ca^{2+}-switch-induced growth arrest. Cells were cultured for 72 hours in 0.03 mM or 1.8 mM Ca^{2+}-containing KGM-2. Cell growth was analyzed by the XTT cell proliferation assay. XTT activity in siSTIM1- and siOrai1-transfected cells is shown relative to that in siControl-transfected HaCaT cells in control low Ca^{2+} medium for 72 hours. Data are means ± s.e.m. from three independent experiments. $^{***}P<0.001$, $^{**}P<0.01$ versus siControl-transfected cells cultured in 0.03 mM Ca-KGM-2 for 72 hours. $^{###}P<0.001$, $^{#}P<0.05$ comparison between low and high Ca^{2+} condition in same siRNA-transfected cells (one-way ANOVA followed by Tukey's test).

to a low Ca^{2+} environment. Third, we have shown that the Ca^{2+} response underlying induction of differentiation by Ca^{2+} switch is also completely lost following knockdown of either STIM1 or Orai1. Fourth, STIM1 and Orai1 are required for normal KC proliferation in low Ca^{2+} culture condition. Finally, we have demonstrated that STIM1 and Orai1 deficiencies lead to suppression of growth arrest and the expression of the early differentiation marker *KRT1*. These results strongly suggest STIM1 and Orai1-mediated SOCE plays a critical role in cell fate decision of KCs.

STIM and Orai proteins have 2 (STIM1 and 2) and 3 (Orai1, 2 and 3) homologues in mammals, respectively. Since STIM2 has higher affinity compared to STIM1, STIM2 has been suggested to function to maintain resting levels of ER Ca^{2+} in non-stimulated cells (Brandman et al., 2007). In contrast, STIM1 functions as an ER Ca^{2+} sensor to more drastic reduction upon the stimulation of Ca^{2+} release and activates Orai channels in the plasma membrane. In this study, siRNA-mediated STIM1 knockdown almost completely suppressed TG-induced Ca^{2+} entry as well as I_{CRAC}, demonstrating that STIM1 is the major Ca^{2+} sensor to signal to Orai channels in response to store depletion in HaCaT KCs (Fig. 2). Unexpectedly, we found that the knockdown of STIM1 also attenuated TG-induced passive Ca^{2+} release from TG-sensitive Ca^{2+} stores in cells cultured in low Ca^{2+} medium, suggesting that STIM1 also functions to maintain basal ER Ca^{2+} levels in HaCaT cells cultured in low Ca^{2+} medium. Orai1 knockdown also caused strong suppression of TG-induced SOCE and diminished ER Ca^{2+} levels in cells cultured in low Ca^{2+} medium. However, in 1.8 mM Ca^{2+}-containing medium, there was no difference in the store Ca^{2+} contents in cells lacking either STIM1 or Orai1. Thus, STIM1 and Orai1, and by inference SOCE, appear to be required to maintain Ca^{2+} stores in undifferentiated KCs, either because of their constitutive activity or because of signaling from external humoral factors.

KC differentiation by Ca^{2+} switch is thought to result from activation of the G-protein- and PLC-coupled CaR expressed in the plasma membrane (Tu et al., 2004). CaR is one of the class of receptors preferentially coupled with Gq and Gi proteins (Hofer and Brown, 2003). In this study, we demonstrated that increasing extracellular Ca^{2+} induced large and sustained Ca^{2+} increases in control cells, which were almost completely abolished in either STIM1- or Orai1-knockdown cells. This is consistent with previous finding that Ca^{2+}-switch-induced Ca^{2+} responses were completely blocked by the treatment of 2-APB, a relatively specific inhibitor of STIM–Orai-mediated SOCE, in normal human keratinocytes (Tu et al., 2008). We did not observe any Ins(1,4,5)P_3-receptor-mediated Ca^{2+} release from ER in STIM1- or Orai1-knockdown cells. It is possible that STIM1- and Orai1-knockdown cells do not maintain sufficient ER Ca^{2+} content to give a measurable release as suggested by the data in Fig. 3. Alternatively, CaR-mediated Ca^{2+} release might be too small to be detected by our methods. Ca^{2+} switch with 1.8 mM Ca^{2+} which is sufficient to induce differentiation in HaCaT cells, could not evoke visible Ca^{2+} responses possibly because the responses are subtle and are sufficiently buffered to prevent large changes in global Ca^{2+} concentration. We considered the possibility that knockdown of STIM1 or Orai1 might affect the expression of CaR; we obtained a weak signal for CaR by qRT-PCR, although there was no visible change with knockdown of STIM1 or Orai1. We have not been able to successfully detect CaR by western blotting with the currently available antibodies.

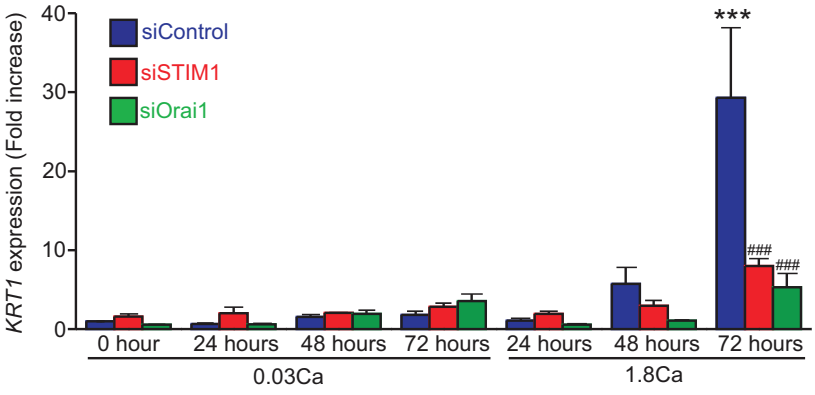

Fig. 7. Both STIM1 and Orai1 are required for Ca^{2+}-switch-induced differentiation marker expression in HaCaT cells. Ca^{2+}-switch-induced expression of an early differentiation marker gene *KRT1* in siRNA-transfected HaCaT cells. Fold increases of *KRT1* expression relative to the sample at time 0 from siControl-transfected cell are shown. Data are means ± s.e.m. from four independent experiments. $^{***}P<0.001$ versus siControl-transfected cells time 0; $^{###}P<0.001$ versus siControl-transfected cells cultured in high Ca^{2+} medium for 72 hours (one way ANOVA followed by Tukey's test).

KC differentiation is associated with cell cycle arrest at G0 (Fuchs, 1993). In our study, Ca^{2+} switch reduced cell proliferation by nearly half in control cells (Fig. 6). However, this Ca^{2+}-switch-induced suppression of proliferation was absent or reduced in STIM1- or Orai1-knockdown cells, respectively. Furthermore, proliferation of undifferentiated cells was diminished by either STIM1 or Orai1 knockdown. Thus, STIM1 and Orai1 are required for both steady state proliferation as well as Ca^{2+}-switch-induced suppression of proliferation, suggesting that SOCE may have at least two important roles in KC physiology. How STIM1 and Orai1 might contribute to a steady state proliferation in low Ca^{2+} medium is unclear. However, STIM1- and Orai1-mediated Ca^{2+} entry is apparently constitutively active in cells cultured in low Ca^{2+} medium, probably due to constitutive reduction of stored Ca^{2+} (Figs 2, 4), suggesting that this constitutive SOCE may be required for normal growth of KCs. With regard to cell growth arrest, it has been reported that the calcineurin/NFAT signaling pathway, which is known to require STIM1- and Orai1-mediated Ca^{2+} entry for its activation, regulates Ca^{2+}-switch-induced cell cycle arrest via expression of the cyclin-dependent kinase inhibitors p21 and p27 (Santini et al., 2001). Therefore, it is likely that STIM1 and Orai1-mediated Ca^{2+} entry plays an important role in KC growth arrest by activating NFAT signaling pathway.

The sequential expression of differentiation marker genes is one of the features of epidermal development (Yuspa et al., 1988). Since Ca^{2+} signaling is an early event after the stimulation of cells, we analyzed the contribution of STIM1 and Orai1 to the expression of an early differentiation marker of *KRT1* (Fig. 7). Expression of *KRT1* occurs mainly in the suprabasal layer and stratum spinosum in human epidermis (Yuspa et al., 1988). It has been reported that HaCaT cells differentiate in response to elevated Ca^{2+} in a manner similar to normal epidermal keratinocytes, but HaCaT cells require somewhat higher extracellular Ca^{2+} concentration and a longer time to launch the differentiation process (Micallef et al., 2009; Sakaguchi et al., 2003). As shown in Fig. 7, *KRT1* expression appeared 72 hours after Ca^{2+} switch in our HaCaT cells. We attempted to analyze KRT1 protein expression in the same time course, but the expression was not sufficient to reliably detect the protein by western blot. We could not extend the incubation times because the increasing cell density caused an increase in KRT1 expression, independently of Ca^{2+}. It is noteworthy that STIM1 and Orai1 knockdown did not affect the Ca^{2+}-independent, cell density-dependent *KRT1* expression (data not shown). This is consistent with the previous report that once keratinocyte cultures reach confluence, they start to express *KRT1* genes and are no longer affected by the extracellular Ca^{2+} concentration (Poumay and Pittelkow, 1995). In this study, the signaling mechanisms linking STIM1- and Orai1-mediated SOCE and *KRT1* expression remain elusive. Previous studies identified a regulatory element for Ca^{2+}-dependent *KRT1* expression in its 3′-flanking DNA sequence which is predicted to contain an activating protein (AP)-1 binding site (Huff et al., 1993; Rothnagel et al., 1993). It has been reported that c-Fos, one of the AP-1 transcription factors, expression is dependent on SOC channel activity in rat mast cells (Chang et al., 2006). Therefore, it is possible that STIM1 and Orai1 are required for the activation of AP-1 transcription factors in HaCaT cells. Alternatively, NFAT which is activated downstream of STIM1- and Orai1-mediated SOCE may regulate *KRT1* expression in an indirect manner, since an NFAT inhibitor cyclosporin A suppresses *KRT1* expression in murine KCs (Santini et al., 2001) and HaCaT cells (data not shown). It has been demonstrated that the expression of p21 depends on NFAT activity although the promoter of p21 has a DNA binding sequence for specificity protein (SP) 1/SP2 transcription factors but not that for NFAT. In fact, NFAT transactivates p21 gene promoter in synergism with Sp1/Sp2 (Santini et al., 2001). Therefore, it is possible that *KRT1* expression is regulated by similar cooperative mechanism between NFAT and AP-1.

In conclusion, we have shown that STIM1 and Orai1 constitute a major Ca^{2+} influx pathway in KCs. This Ca^{2+} entry is required for the maintenance of stored Ca^{2+} levels in intracellular organelles in the undifferentiated condition and for Ca^{2+} signaling in the induction of KC differentiation. Through these Ca^{2+}-influx-dependent processes, STIM1 and Orai1 play dual roles in KC physiology in both steady state proliferation and in signaling the onset of differentiation.

Materials and Methods

Cell culture and siRNA transfection

HaCaT cells were cultured in KGM-2 (Lonza) supplemented with 0.03 mM $CaCl_2$ and maintained at 37℃ in a humidifier incubator set at 5% CO_2. Culture medium was replaced every 2–3 days. To knockdown endogenous STIM1 and Orai1, cells were transfected with siRNAs by using Hiperfect (Qiagen) according to manufacturer's instruction. Briefly, 5 nM-siRNA–Hiperfect complex was added onto 1×10^6 cells in 100-mm tissue culture dish. The culture medium was exchanged 6 hours after transfection. Cells were incubated for 2–3 days and replated to 6-well plates at a cell density of $0.5–1.5\times10^5$ cells per well and transfected with siRNAs again. Cells were stimulated with 1.8 mM Ca^{2+}-containing KGM-2 2 days after the second siRNA transfection and used for experiments as indicated. Target sequences of siRNA against human STIM1 and Orai1 were as described previously (Mercer et al., 2006).

Cell lysis and western blotting

HaCaT cells were lysed in RIPA buffer [137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM EDTA, 5 mM sodium orthovanadate and protease inhibitor cocktail (Rosch)]. Cell lysates were resolved by a 4–20% gradient SDS-PAGE and subjected to immunoblot analysis with anti-human STIM1 (Prosci; 1:3000), anti-human Orai1 (Sigma; 1:5000), anti-actin (Sigma; 1:3000). The bands were scanned and the density of each band was determined using ImageJ software.

Ca^{2+} imaging

Measurement of changes in $[Ca^{2+}]_i$ was carried out as previously described (DeHaven et al., 2008). Cells were loaded with the fluorescent Ca^{2+} indicator Fura-5F. The fluorescence ratio images were recorded from cells incubated in HBSS (in mM): 145 NaCl, 3 KCl, 0.3 or 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4 adjusted with NaOH). $CaCl_2$ was omitted in Ca^{2+}-free solution.

Electrophysiology

Whole-cell currents were measured as described previously (DeHaven et al., 2007; DeHaven et al., 2008). The standard extracellular HBSS contained (in mM): 145 NaCl, 3 KCl, 10 CsCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (adjusted to pH 7.4 with NaOH). The standard divalent free solution (DVF) was prepared by removing the $CaCl_2$ and $MgCl_2$ from HBSS and adding 0.1 mM EGTA. The intracellular pipette solution contained: 145 mM cesium methanesulfonate, 10 mM BAPTA, 10 mM HEPES and 8 mM $MgCl_2$ (adjusted to pH 7.2 with CsOH) together with 20 μM Ins(1,4,5)P_3 (hexasodium salt). Currents were acquired with pCLAMP-10 (Axon Instruments) and analyzed with Clampfit (Axon Instruments).

We noted that knockdown of STIM1 caused morphological changes in the majority of cells, such that they were much more flattened and virtually impossible to patch. Thus, patch-clamp experiments were carried out on the minority of cells showing minimal morphological changes. It is possible that these cells had somewhat higher levels of residual STIM1, and this may explain why partial, albeit diminished I_{crac} was seen in these cells.

XTT cell proliferation assay

An XTT assay was carried out by following the manufacturer's instruction (ATCC). Briefly, siRNA-transfected HaCaT cells were seeded onto 96-well plates at a cell density of 3×10^3 cells/well. Cells were cultured in 0.03 mM or 1.8 mM Ca^{2+}-containing KGM-2 for 72 hours. After addition of XTT reagent, 96-well plates were incubated for 6 hours until an orange color developed. Absorbance

values at the wavelength of 475 nm and 660 nm were measured using a microtiter plate reader. Average of blank-subtracted absorbance was calculated from triplicate readings and normalized to that of siControl-transfected cells cultured 72 hours in 0.03 mM Ca^{2+}-containing KGM-2.

Quantitative RT-PCR

For quantitative RT-PCR, total RNAs were extracted from HaCaT cells with the RNeasy mini kit (Qiagen). cDNA synthesis was performed with Omniscript (Qiagen), using 0.5 μg total RNA, and quantitative RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) with the ABI Prism 7000 Instrument (Applied Biosystems), using specific oligonucleotides [human *KRT1*, forward 5′-ATTTCTGAGCTGAATCGTGTGATC-3′, reverse 5′-CTTGGCATC-CTTGAGGGCATT-3′ (Micallef et al., 2009); *GAPDH*, forward 5′-GAAGG-TGAAGGTCGGAGTC-3′, reverse 5′-GAAGATGGTGATGGGATTTC-3′].

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