Reversal of Murine Epidermal Atrophy by Topical Modulation of Calcium Signaling

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Cytosolic Ca^{2+} signals are performed by Ca^{2+} releases from the endoplasmic reticulum and Ca^{2+} influx from the extracellular medium. Releases rely on the refilling of the intracellular Ca^{2+} stores by the Ca^{2+} influx "Store-Operated Calcium Entry" (SOCE) via the channel Orai1. Here we show that Orai1 expression, SOCE amplitude, and epidermal proliferation are decreased in the epidermis of patients with skin fragility when compared with aged nonatrophic skin. Epidermal atrophy was induced in mice by the inhibition of Orai1 with small interfering RNA and the topical application of a SOCE blocker BTP2. The inhibition of Orai1 impaired the heparin-binding epidermal growth factor (HB-EGF)-induced Ca^{2+} influxes and fully prevented the mitogen effect of HB-EGF in primary human keratinocytes. Importantly, epidermal proliferation correlated with Orai1 expression in mice. Conversely, the topical application of an Orai1 activator, the benzohydroquinone (BHQ), increased the epidermal thickness and proliferation, whereas the pro-proliferative effect of BHQ was prevented by the inhibition of Orai1. Finally, the topical application of BHQ reversed the epidermal atrophy induced by corticosteroids in mice. The topical modulation of Ca^{2+} signals may thus be a promising therapeutic strategy in dermatology.

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INTRODUCTION

Human epidermal atrophy is observed in chronic cutaneous insufficiency/fragility, a clinical syndrome characterized by the dermo–epidermal atrophy of the skin caused by aging, chronic UV exposure, or long-term corticosteroid use (Kaya and Saurat, 2007; Kaya *et al.*, 2008). The prevalence of skin fragility is around 32% in individuals aged over 60 years (Mengeaud *et al.*, 2012).

Epidermal homeostasis is regulated by the balance between the proliferation of basal keratinocytes and the differentiation of suprabasal keratinocytes. In a murine model of epidermal atrophy, it has been shown that proliferation is impaired, whereas differentiation occurs normally (Kaya *et al.*, 2006; Pastore *et al.*, 2008). Ca^{2+} signals are known to control keratinocyte differentiation, but it is poorly understood whether Ca^{2+} signals also control keratinocyte proliferation (Hennings *et al.*, 1980; Sharpe *et al.*, 1989; Yuspa *et al.*, 1989; Tang and Ziboh, 1991; Tu *et al.*, 1999; Numaga-Tomita and Putney, 2012).

It has been shown that the stimulation of EGFR activates the phosphoinositol 3-kinase pathways (Rodrigues *et al.*, 2000; Li *et al.*, 2001) and triggers cytosolic Ca^{2+} signals that control cell proliferation (Moolenaar *et al.*, 1986; Cheyette and Gross, 1991; Sanchez-Gonzalez *et al.*, 2010; Tajeddine and Gailly, 2012; Leroy *et al.*, 2013). Accordingly, several epidermal mitogens, including epidermal growth factor (EGF), elicit Ca^{2+} signals, which are fully inhibited in keratinocytes with depleted intracellular Ca^{2+} stores (Watt *et al.*, 1991; Sharpe *et al.*, 1993; McGovern *et al.*, 1995; Hoq *et al.*, 2011). These results strongly suggest that Ca^{2+} releases from the endoplasmic reticulum, the principal intracellular Ca^{2+} store, are involved in the control of keratinocyte proliferation.

As the Ca^{2+} content of the endoplasmic reticulum is limited, Ca^{2+} releases rely on the refilling of the stores through a Ca^{2+} influx from the extracellular medium, which is named Store-Operated Calcium Entry (SOCE) (Putney, 1986; Li *et al.*, 1997; Hofer *et al.*, 1998a, 1998b; Parekh and Putney, 2005). SOCE silently refills the Ca^{2+} stores and is inactivated when stores are full (Jousset *et al.*, 2007; Darbellay *et al.*, 2009, 2010, 2011; Shen *et al.*, 2011). The plasma membrane Ca^{2+} channel Orai1 is the main molecular partner of SOCE (Feske *et al.*, 2006; Vig *et al.*, 2006; Zhang *et al.*, 2006). Orai1 is activated by STIM1, the Ca^{2+} sensor of the endoplasmic reticulum (Liou *et al.*, 2005; Roos *et al.*, 2005; Wang *et al.*, 2009; Zhou *et al.*, 2009). The inhibition of the sarco/endoplasmic Ca^{2+} ATPase with thapsigargin (Tg) or benzohydroquinone (BHQ) activates Orai1. As Orai1-knockout mice present with epidermal atrophy (Gwack

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Abbreviations: BHQ, benzohydroquinone; CP, clobetasol propionate; EGF, epidermal growth factor; HB, heparin binding; PHK, primary human keratinocyte; siRNA, small interfering RNA; SOCE, Store-Operated Calcium Entry; Tg, thapsigargin

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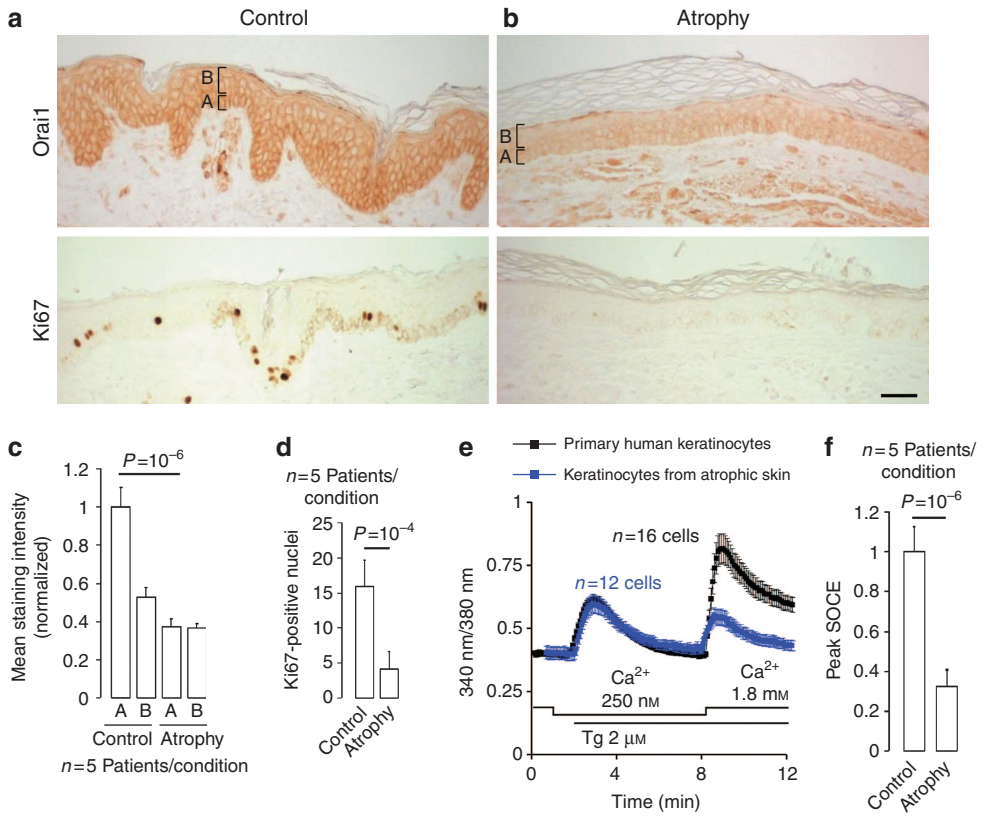

Figure 1. Store-Operated Calcium Entry (SOCE) is compromised in epidermal atrophy. Histological sections of healthy human skin (**a**) and atrophic skin (**b**) stained for Orai1 and Ki67; A = basal layer, B = suprabasal layers. (**c**) Quantification of Orai1 expression determined by the intensity of staining in **a** and **b** (mean ± SD, see Materials and Methods). (**d**) Quantification of the Ki67-positive nuclei per 2 mm of the dermo–epidermal junction (mean ± SD, see Materials and Methods). (**e**) Cytoplasmic Ca^{2+} assessed by Fura-2 probe 24 hours after isolation of primary keratinocytes. Intracellular Ca^{2+} stores were depleted using 2 µM thapsigargin (Tg) in a medium containing 250 nM Ca^{2+}, and, subsequently, 1.8 mM Ca^{2+} was added to the external medium to reveal SOCE (one representative experiment). (**f**) Peak Tg-induced SOCE amplitude measured in five patients/condition (mean ± SD). Bar = 150 µm.

et al., 2008) and the cutaneous phenotype of Orai1-deficient patients has not been studied yet (Byun *et al.*, 2010; Feske *et al.*, 2010; Chang *et al.*, 2012), the role of Orai1 in the epidermis and epidermal atrophy has to be clarified. Interestingly, Orai1 polymorphism may be associated with susceptibility to atopic dermatitis (Chang *et al.*, 2012).

RESULTS

Orai1 expression and SOCE amplitude are decreased in the epidermis of patients with epidermal atrophy

In healthy human skin, Orai1 was detected throughout the epidermis, and the level of expression of Orai1 was higher in the basal layer than in the suprabasal layers (Figure 1a, upper panel, a = basal layer, b = suprabasal layers). The skin of patients with skin fragility is characterized by epidermal thinning and linearization, which is due to the loss of rete ridges (Figure 1b, upper panel; Kaya and Saurat, 2007). Interestingly, the gradient of Orai1 expression that is observed in healthy aged epidermis was lost in atrophic skin, and the expression of Orai1 was markedly reduced (Figure 1a and b, upper panel). In control epidermis, measurements of the intensity of Orai1 staining revealed that Orai1 expression is 48 ± 7% lower in the suprabasal layers than in the basal layer (Figure 1c). In epidermal atrophy, signal quantification showed a decreased Orai1 expression of 62 ± 5% in the basal layer and 29 ± 3% in suprabasal layers, when compared with healthy aged skin of the same average age (Figure 1c and Supplementary Data S1 online). Thus, Orai1 expression is markedly decreased in the basal layer of atrophic epidermis.

Epidermal proliferation has been shown to be slightly decreased in healthy aged skin compared with young healthy skin (Giangreco *et al.*, 2010). In epidermal atrophy, the number of cells positive for the proliferation marker Ki67 was markedly decreased when compared with aged healthy skin (Figure 1a and b, lower panel). The determined average frequency of Ki67-positive nuclei was 4 ± 3 nuclei per 2 mm and 16 ± 4 nuclei per 2 mm in atrophic and control skin, respectively (Figure 1d). Thus, epidermal proliferation is impaired in epidermal atrophy, whereas epidermal differentiation occurs normally (Supplementary Data S2 online).

As Orai1 is known to control SOCE, we then explored the amplitude of Tg-induced SOCE in keratinocytes isolated from healthy and atrophic skin by using the fluorescent cytosolic Ca^{2+} indicator Fura-2. For this purpose, we isolated primary human keratinocytes (PHKs) from unused surgical specimens. As shown in Figure 1e, the resting cytosolic Ca^{2+} levels and the amount of Ca^{2+} released from intracellular stores by Tg