Transient Receptor Potential Vanilloid 4 (TRPV4) Is Downregulated in Keratinocytes in Human Non-Melanoma Skin Cancer

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A subgroup of the transient receptor potential (TRP) channels, including vanilloid 1 (TRPV1), TRPV2, TRPV3, TRPV4, and TRP ankyrin 1 (TRPA1), is expressed in cutaneous peptidergic somatosensory neurons, and has been found in skin non-neuronal cells, such as keratinocytes. Different cancer cells express TRPs, where they may exert either pro- or antitumorigenic roles. Expression and function of TRPs in skin cancers have been, however, poorly investigated. Here, we have studied the distribution and expression of TRPs by immunohistochemistry and messenger RNA (mRNA) in human healthy skin and human keratinocytic tumors, including intraepidermal proliferative disorders (solar keratosis (SK) and Bowen's disease), and non-melanoma skin cancer (NMSC; basal cell and squamous cell carcinomas). Similar TRPV1, TRPV2, and TRPV3 staining was found in keratinocytes from healthy and tumor tissues. TRPA1 staining was increased solely in SK samples. However, the marked TRPV4 staining and TRPV4 mRNA expression, observed in healthy or inflamed skin, was abrogated both in premalignant lesions and NMSC. In a human keratinocyte cell line (HaCaT), TRPV4 stimulation released IL-8, which in turn downregulated TRPV4 expression. Selective reduction in TRPV4 expression could represent an early biomarker of skin carcinogenesis. Whether the cytokine-dependent, autocrine pathway that results in TRPV4 downregulation contributes to NMSC mechanism remains to be determined.

Journal of Investigative Dermatology advance online publication, 17 April 2014; doi:10.1038/jid.2014.145

INTRODUCTION

The superfamily of transient receptor potential (TRP) channels encompasses six subfamilies, which are widely and differently expressed in various tissues and organs where they mediate pleiotropic functions in health and disease (Nilius *et al.*, 2007). A subgroup of channels defined as thermo-TRPs because of their ability to sense changes in temperature are localized to a subpopulation of peptidergic primary sensory neurons where they transduce nociceptive signals by a heterogenous variety of physicochemical stimuli and drive neurogenic inflammatory responses (Geppetti and Holzer, 1996; Nilius *et al.*, 2007). This subgroup of channels includes vanilloid 1 (TRPV1, the capsaicin receptor), TRPV2, TRPV3, and TRPV4, and TRP ankyrin 1 (TRPA1, the mustard oil receptor; Nilius *et al.*, 2007). Activators of thermo-TRP may be summarized as follows: TRPV1 is activated by vanilloid compounds (capsaicin, resiniferatoxin), noxious heat ($\geqslant$43 °C), low pH, and certain eicosanoids (Caterina *et al.*, 1997; Tominaga *et al.*, 1998; Ho *et al.*, 2008); TRPV3 and TRPV2 are activated by innocuous warm temperatures (32–39 °C) and noxious high temperature (>52 °C), respectively; TRPV4 is activated by mild high temperature (>24 °C) and by hypoosmotic stimuli (Liedtke *et al.*, 2000; Cheng *et al.*, 2010); and TRPA1 is activated by moderate low temperatures and a large series of environmental irritants and reactive molecules, including reactive oxygen species and their by-products (Bautista *et al.*, 2006; Eid *et al.*, 2008; Materazzi *et al.*, 2008; Biro and Kovacs, 2009).

Apart from their more prominent expression in nociceptors, thermo-TRPs may be expressed in non-nociceptive neurons (Chen *et al.*, 2013). More importantly, for the present study, some thermo-TRPs are found in non-neuronal cells (Earley *et al.*, 2009; Nozawa *et al.*, 2009; Nassini *et al.*, 2012) including skin cells (Atoyan *et al.*, 2009; Sokabe *et al.*, 2010; Radtke *et al.*, 2011). TRPV1 expression has been

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Abbreviations: BCC, basal cell carcinoma; BD, Bowen's disease; GSK, GSK1016790A; HaCaT, human keratinocyte cell line; mRNA, messenger RNA; NMSC, non-melanoma skin cancer; PGE_2, prostaglandin E2; SCC, squamous cell carcinoma; SK, solar keratosis; TNF-α, tumor necrosis factor-α; TRP, transient receptor potential; TRPA, TRP ankyrin; TRPV, TRP vanilloid; 4αPDD, 4α-phorbol-12,13-didecanoate

Received 3 September 2013; revised 29 January 2014; accepted 18 February 2014; accepted article preview online 18 March 2014

identified in epidermal and hair follicle keratinocytes, dermal mast cells, sebaceous gland–derived sebocytes, and dendritic cells (Stander *et al.*, 2004; Bodo *et al.*, 2005). TRPV2 has been found in keratinocytes (Axelsson *et al.*, 2009) and macrophages (Link *et al.*, 2010), and TRPV3 has been found in blood vessels (Earley *et al.*, 2010) and keratinocytes (Cheng *et al.*, 2010). The presence of TRPV4 has been reported in basal and suprabasal keratinocytes of healthy human skin (Chung *et al.*, 2003; Radtke *et al.*, 2011), where its function has been related to cell survival after skin exposure to noxious heat. Additional functions have been suggested for TRPV4 localized to skin cells. TRPV4 has been defined as osmo- and mechano-sensor for its contribution in responses to mechanical and osmotic stimuli (Liedtke and Kim, 2005). In particular, TRPV4 has been suggested to control the homeostasis of the skin permeability barrier, as a sort of osmotic pressure detector (Denda *et al.*, 2007). More recently, a highly relevant evolutionary function of TRPV4 has been proposed in mammalian skin both at the physiological and pathophysiological levels. Indeed, TRPV4 appears to contribute to UVB-induced damage and to UVB-evoked pain behavior by increasing the expression of the proalgesic/algogenic mediator endothelin-1 (Moore *et al.*, 2013). TRPA1 has been found in keratinocytes and in melanocytes, where it is activated by UVA irradiation, and *via* this mechanism it may contribute to the phototransduction process (Atoyan *et al.*, 2009; Bellono *et al.*, 2013). Thus, TRPA1 may contribute to an extraneural pathway of phototransduction, possibly contributing to a spectrum of UV-activated TRPs, whereby TRPV4 is sensing UVB and TRPA1 UVA (Bellono *et al.*, 2013; Moore *et al.*, 2013).

Non-melanoma skin cancer (NMSC) encompasses basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which account for ~80 and 20%, respectively, of the total tumor burden (Weinstock, 1994). Both cancer subtypes originate from the basal layer of the epidermis of the skin, but although BCC is characterized by a slow-growing rate and poor metastatic potential SCC shows opposite features, thus representing the major cause of the deaths attributable to NMSC (Weinstock, 1994). BCC and SCC occur primarily on sun-exposed areas of the body and have been associated with chronic sun and UV exposure (Kwa *et al.*, 1992; Bowden, 2004). Accumulating evidence suggests that oxidative stress and release of inflammatory mediators from epidermal cells contribute to tumor development (Marnett, 2000; Bachelor and Bowden, 2004; Perrotta *et al.*, 2011). Change in expression and identification of functions, relevant for tumor progression, have been reported for TRP channels in different types of cancer (Duncan *et al.*, 2001; Tsavaler *et al.*, 2001; Bode *et al.*, 2009; Oancea *et al.*, 2009; Lehen'kyi *et al.*, 2012; Santoni *et al.*, 2012). However, little information exists regarding TRP channel expression and function in skin tumors, and particularly in NMSC. Here, we show that TRPV4 is markedly downregulated in both the premalignant lesions of NMSC such as solar keratosis (SK) and Bowen's disease (BD), and in SCC and BCC. In addition, TRPV4 in cultured keratinocytes is downregulated by cell exposure to a variety of proinflammatory mediators, including IL-8, which is released from keratinocytes upon TRPV4 stimulation.

RESULTS

Localization of TRP in human healthy skin

We first investigated, by immunohistochemistry, the expression of TRP proteins in human healthy skin. Staining for TRPV1 was observed in the epidermis, basal, and suprabasal epidermal keratinocytes (Figure 1). TRPV2 and TRPV3 staining was detected in basal and suprabasal keratinocytes and endothelial cells (Axelsson *et al.*, 2009) (Figure 2a and b). TRPV4 protein expression was diffusely observed in basal and suprabasal epidermal keratinocytes, and it was also found in

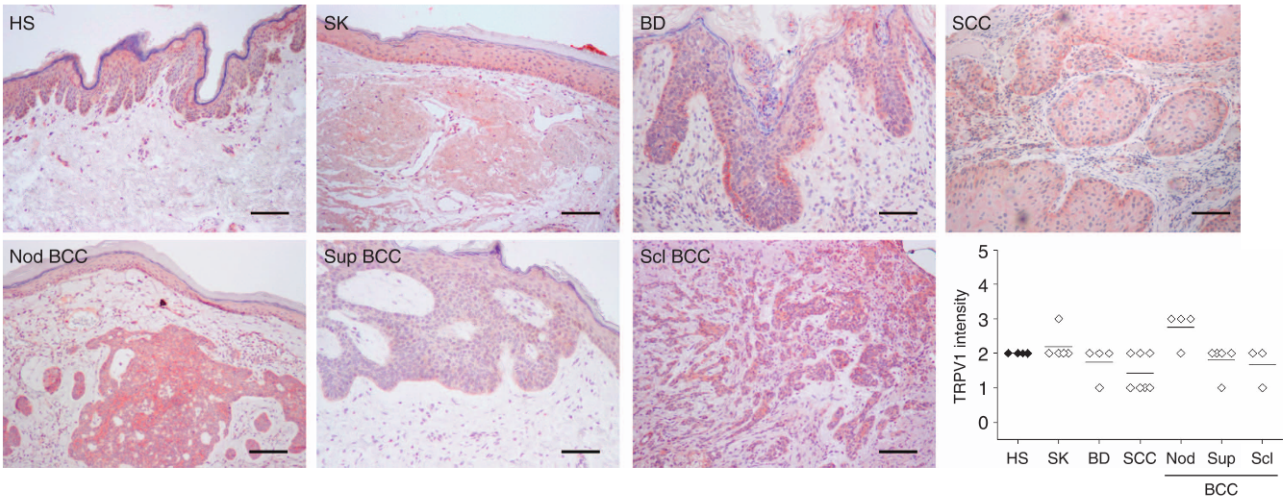

Figure 1. Localization of transient receptor potential vanilloid 1 (TRPV1) in human healthy and cancer skin tissues. Immunohistochemical localization reveals TRPV1 protein staining in the epidermis, basal, and suprabasal epidermal keratinocytes. TRPV1 staining and semiquantitative analysis of skin samples taken from patients suffering from solar keratosis (SK), Bowen's disease (BD), squamous cell carcinoma (SCC), and nodular (Nod), superficial (Sup), and sclerodermiform (Scl) basal cell carcinoma (BCC) do not show a significant difference in protein expression in atypical keratinocytes. Bar = 100 μm.

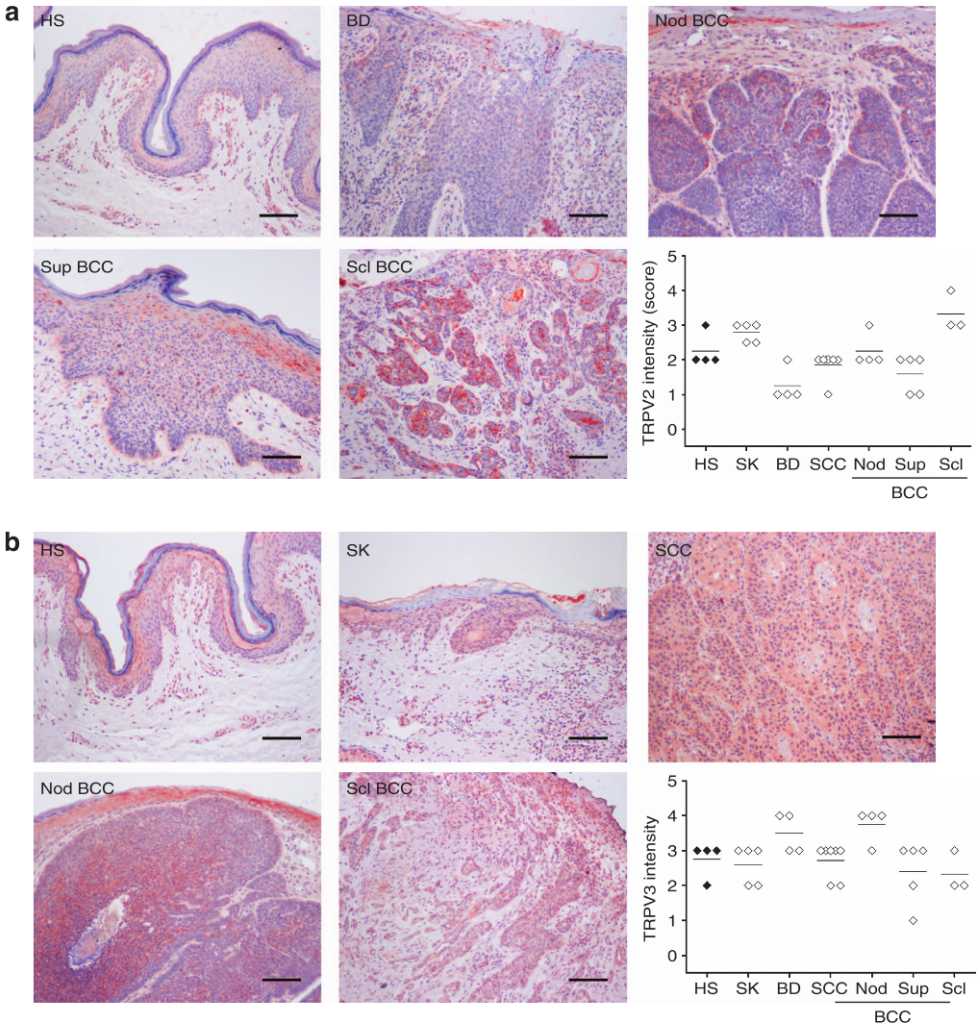

Figure 2. Localization of transient receptor potential vanilloid 2 and 3 (TRPV2 and TRPV3) in human healthy and cancer skin tissues. Immunohistochemical localization reveals TRPV2 (**a**) and TRPV3 (**b**) protein staining in basal and suprabasal keratinocytes, endothelial cells, and neuronal structures. TRPV2 and TRPV3 staining and semiquantitative analysis of skin samples taken from patients suffering from solar keratosis (SK), Bowen's disease (BD), squamous cell carcinoma (SCC), and nodular (Nod), superficial (Sup), and sclerodermiform (Scl) basal cell carcinoma (BCC) do not show a significant difference in protein in atypical keratinocytes. Bar = 100 μm.

adnexal structures. Intense immunostaining was evident in the epidermal and dermal part of the eccrine sweat gland ducts. The secretory portion of sweat glands showed staining in single secretory and myoepithelial cells. Endothelial cells decorating dermal blood vessels were also TRPV4 positive (Figure 3). TRPA1 immunoreactivity was detected in the basal layer of the epidermis in healthy skin (Figure 4). Specificity of staining is indicated from previous studies (Nassini *et al.*, 2012; Sulk *et al.*, 2012) and from negative controls performed in the presence of an excess of the respective immunizing peptide (Supplementary Figure S1 online).

Differential expression of TRPs in skin cancer tissues

Immunohistochemistry was used to determine semiquantitatively the level of expression of TRPV1, TRPV2, TRPV3, TRPV4, and TRPA1 in keratinocytes of healthy skin and cancer skin samples. As the expression of all the channels was predominant in keratinocytes, the analysis was limited to this cutaneous cell type. TRPV1, TRPV2, and TRPV3 expression was similar in atypical keratinocytes of SK, BD, SCC, BCC, and in keratinocytes of healthy skin (Figure 1 and Figure 2a and b). However, analysis of TRPV4 staining showed from remarkable reduction to complete abrogation of protein expression in skin samples of BD, SCC, and BCC of different histotypes as compared with healthy skin samples (Figure 3a and b). Reduction in TRPV4 protein staining was detected in keratinocytes in all the tested conditions. In contrast, other cells, such as those of the sweat gland ducts in the various types of cancers, exhibited a TRPV4 staining intensity, unchanged as compared with that observed in control samples (as an example, see SCC in Figure 3). In SK samples, the apparently reduced expression of TRPV4 staining did not

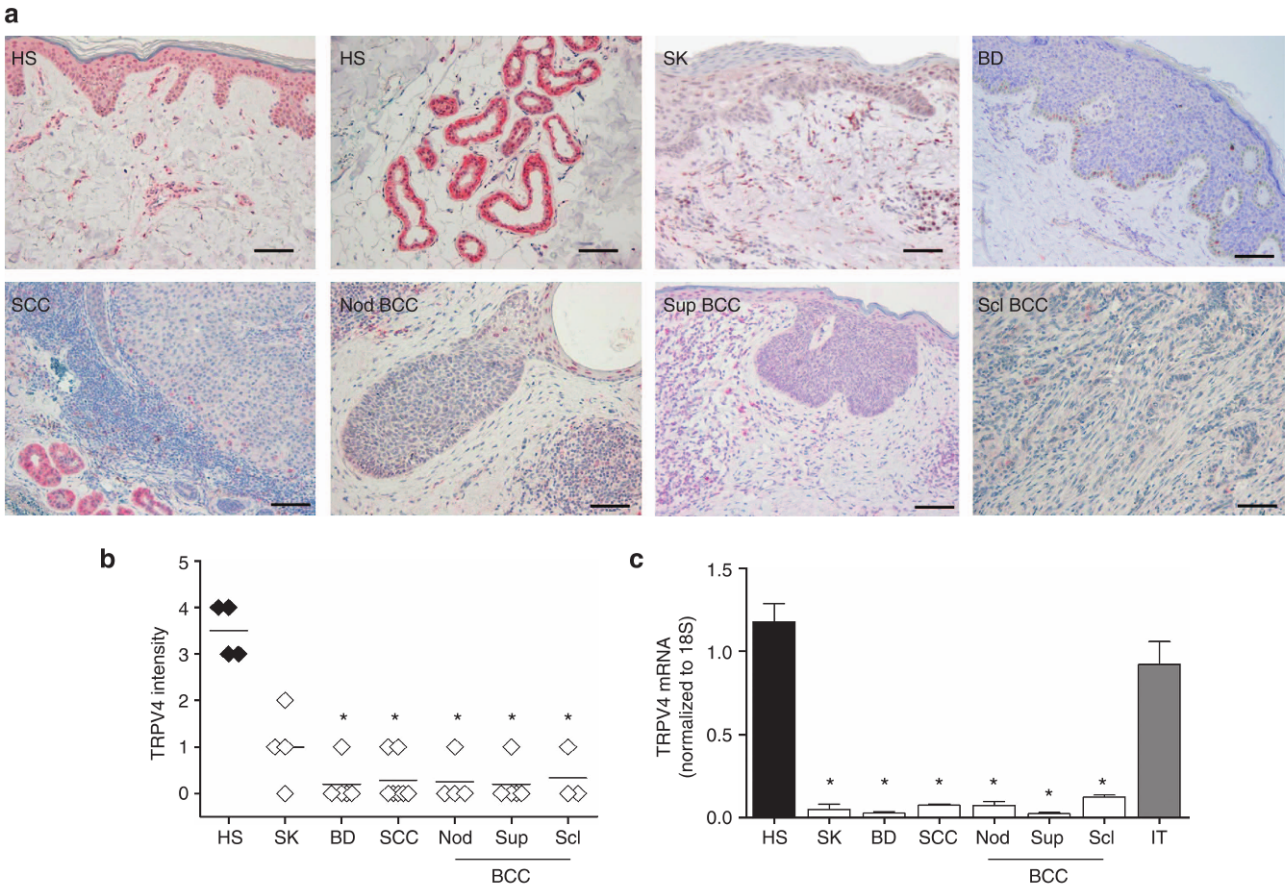

Figure 3. Transient receptor potential vanilloid 4 (TRPV4) protein and messenger RNA (mRNA) results downregulated in different premalignant and invasive non-melanoma skin cancers of different histotypes compared to human healthy skin. (**a**) Immunohistochemical localization reveals TRPV4 protein staining in basal and suprabasal epidermal keratinocytes, and in adnexal structures in samples of healthy skin. TRPV4 staining (**a**) and semiquantitative analysis (**b**) of skin samples taken from patients suffering from solar keratosis (SK), Bowen's disease (BD), squamous cell carcinoma (SCC), and nodular (Nod), superficial (Sup), and sclerodermiform (Scl) basal cell carcinoma (BCC) show a significant downregulation of protein in atypical keratinocytes. (**c**) TRPV4 mRNA analysis of samples taken from SK, BD, SCC, and BCC (Nod, Sup, and Scl) paraffin-embedded tissues shows a significant downregulation of TRPV4 mRNA compared with healthy skin (HS). Samples of dermatitis (inflamed tissues, IT) show levels of TRPV4 mRNA similar to those of HS. Values are expressed as percentage compared with 18S mRNA. *$P<0.05$ versus HS (nonparametric, two-tailed Mann–Whitney test in **b** and analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test in **c**). Bar = 100 μm.

reach the significance level (Figure 3a and b). In contrast, expression of TRPA1 protein was significantly higher in SK as compared with healthy skin, whereas no significant difference was noted in any other skin cancer type (Figure 4). We further tested TRPV4 messenger RNA (mRNA) expression by real-time PCR in the same paraffin-embedded samples used for immunohistochemical studies. Remarkable downregulation of TRPV4 mRNA was observed in all the cancer types compared with the level of TRPV4 mRNA measured in healthy skin samples (Figure 3c). TRPV4 mRNA was detected in the whole paraffin-embedded slice, and its decreased level paralleled the decrease in protein staining observed in atypical or neoplastic keratinocytes. As other cells (sweat gland cells) did not show any reduction in TRPV4 protein, it is plausible that keratinocytes from normal adnexal structures contribute only marginally to the bulk of channel mRNA in the present human skin tissues.

Inflammatory mediators downregulate TRPV4 mRNA expression

Several cytokines have been shown to contribute to the biology of skin neoplasms, including BCC and SCC (Elamin *et al.*, 2008). Keratinocytes in response to different stimuli, including UVB radiation, may release additional proinflammatory mediators such as prostaglandin E2 (PGE_2), which has been proposed to promote skin tumor development (Pei *et al.*, 1998; Countryman *et al.*, 2000). The expression of TRPV4 (Becker *et al.*, 2005) and TRPV1 (Southall *et al.*, 2003) channels in HaCaT has been previously reported. Moreover, stimulation of HaCaT cells with the selective TRPV1 agonist, capsaicin, caused the release of IL-8 (Southall *et al.*, 2003). By using western immunoblotting and real-time PCR, we confirmed TRPV4 expression in the HaCaT cell line (Figure 5a). Human embryonic kidney 293 and human bronchial smooth muscle cells have been used as negative and positive control (Jia *et al.*, 2004), respectively (Figure 5a).

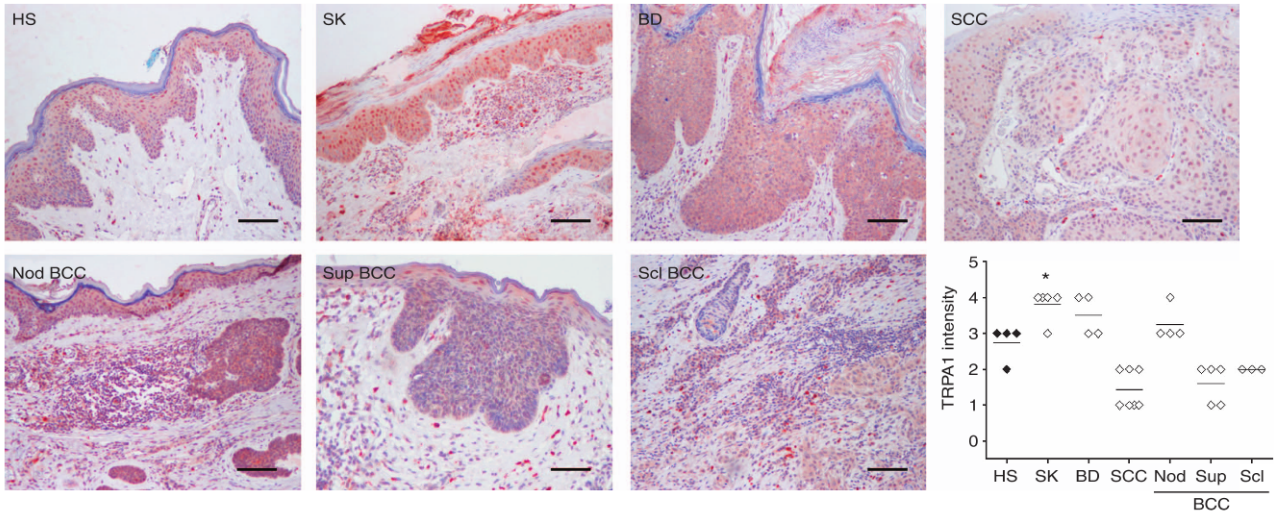

Figure 4. Localization of transient receptor potential ankyrin 1 (TRPA1) in human healthy and cancer skin tissues. Immunohistochemical localization of TRPA1 reveals protein staining in the basal layer of the epidermis in healthy skin. TRPA1 semiquantitative analysis of skin samples taken from patients suffering from solar keratosis (SK), Bowen's disease (BD), squamous cell carcinoma (SCC), and nodular (Nod), superficial (Sup), and sclerodermiform (Scl) basal cell carcinoma (BCC) show a significant increase in TRPA1 staining only in atypical keratinocytes in samples of SK, whereas in different cases of BD, SCC, and BCC, irrespective of different histotypes, TRPA1 expression does not display any significant difference in staining compared with healthy skin (HS). *$P<0.05$ versus HS (nonparametric, two-tailed Mann–Whitney test). Bar = 100 μm.

Expression of TRPV4 in HaCaT cells was downregulated by incubation for 24 hours with IL-8, IL-1β, tumor necrosis factor-α (TNF-α), or PGE_2 (Figure 5b–d).

IL-8 release and TRPV4 downregulation

To investigate the role of TRPV4 in the regulation of proinflammatory mediators in keratinocytes, we measured the release of IL-8, IL-1β, TNF-α, and PGE_2 from HaCaT cells after exposure to the selective TRPV4 agonist 4α-phorbol-12,13-didecanoate (4αPDD). 4αPDD evoked a concentration-dependent release of IL-8 (Figure 5e). The response to 4αPDD was abrogated in the presence of the TRPV4-selective antagonist HC-067047 (Everaerts *et al.*, 2010; Figure 5e). In contrast, exposure of HaCaT to 4αPDD (1–3–10 μM) did not affect the release of IL-1β, TNF-α, and PGE_2 (Figure 5g–i). In addition to 4αPDD, we tested the effect of a highly selective and potent TRPV4 agonist GSK1016790A (GSK; Thorneloe *et al.*, 2008). Exposure of HaCaT to GSK (0.5–1–5 μM) produced a concentration-dependent increase in IL-8 release, an effect abated in the presence of HC-067047 (Figure 5f). Overnight exposure to 4αPDD or to GSK (10 or 5 μM, respectively) did not affect cell viability (Figure 5j and k).

On the basis of these results, to investigate the possible relationship between TRPV4 downregulation and IL-8, we have measured the IL-8 mRNA level in healthy skin and tumor tissues by real-time PCR. In addition, we included in this part of the study some tissues characterized by neutrophilic inflammation (pustular psoriasis, neutrophilic folliculitis, and leucocytoclastic vasculitis) where presumably IL-8 should be increased. In these samples, we measured mRNA for TRPV4 and IL-8 (Figure 5l). As expected, IL-8 mRNA expression was high in inflamed tissues, whereas it was totally absent in normal skin (Figure 5l). TRPV4 expression was similarly elevated in both inflamed and normal skin (Figure 3c). SK, BD, and malignant cancer samples exhibited variable levels of IL-8 expression, between the almost undetectable value of normal skin and the high level of inflamed tissues (Figure 5l).

DISCUSSION

In line with previous reports that described the distribution of certain TRP proteins in epidermal keratinocytes in human skin (Chung *et al.*, 2003; Bodo *et al.*, 2005; Atoyan *et al.*, 2009; Axelsson *et al.*, 2009; Earley *et al.*, 2010; Radtke *et al.*, 2011), we confirmed that in human healthy skin samples TRPV1, TRPV2, TRPV3, TRPV4, and TRPA1 proteins are expressed in basal and suprabasal epidermal keratinocytes. For TRPV4 protein, we also observed an intense staining in adnexal structures in the eccrine sweat gland ducts, myoepithelial cells, and endothelial cells. Previous studies established an altered expression and function of one or more TRP proteins belonging to different channel subfamilies in malignant cells of various types of cancers. For instance, expression levels of members of the TRP melastatin (Duncan *et al.*, 2001; Tsavaler *et al.*, 2001; Oancea *et al.*, 2009) and TRPV (Fixemer *et al.*, 2003; Lazzeri *et al.*, 2005; Bode *et al.*, 2009; Santoni *et al.*, 2012) subfamilies have been proposed to be associated with the induction/progression of the different tumors. Thus, it is possible that in human skin, and particularly in keratinocytes, thermo-TRPs may undergo changes during the carcinogenesis process.

Here, we provide evidence that in skin samples obtained from biopsies of premalignant lesions of NMSC, such as SK and BD, and in malignant BCC and SCC, among the series of TRPs analyzed, TRPA1 protein expression was increased in SK and, more importantly, TRPV4 protein displays a remarkably

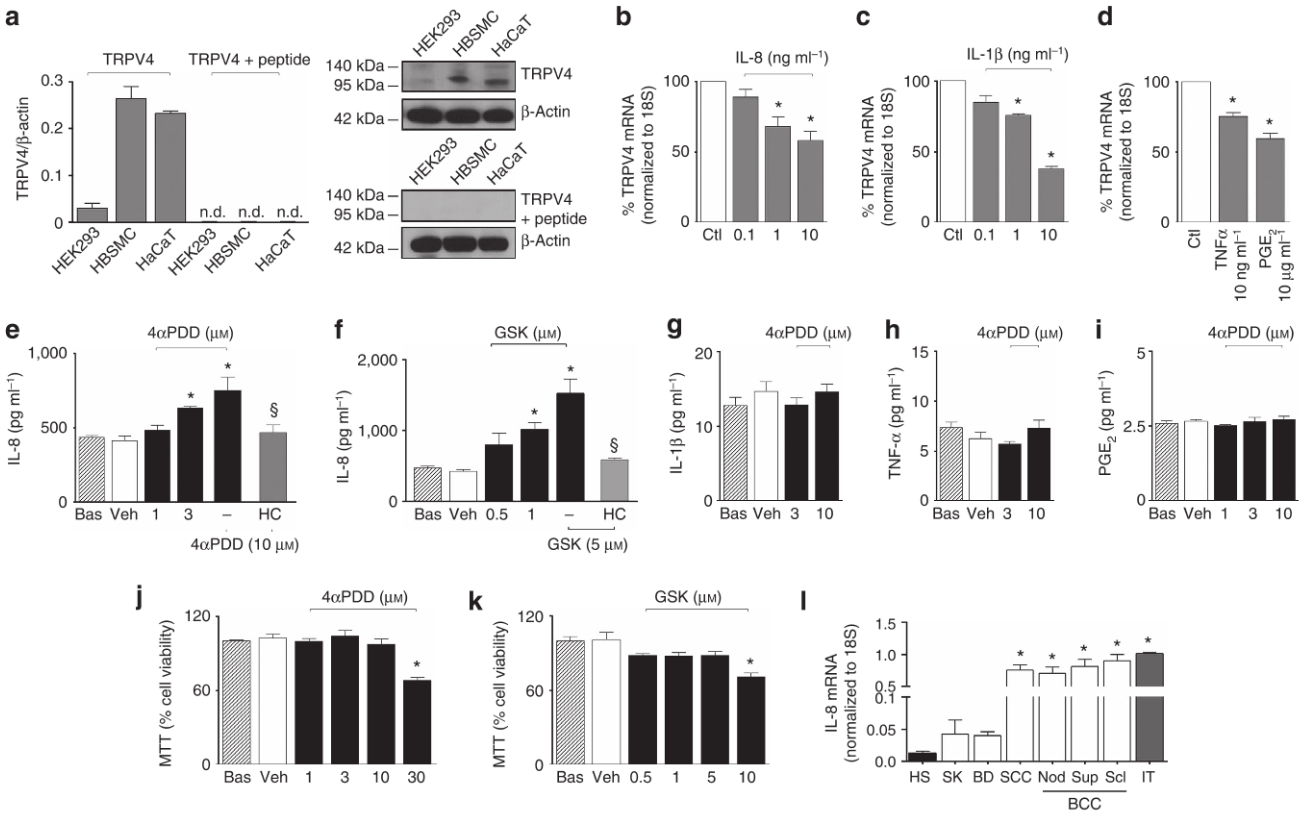

Figure 5. Effect of proinflammatory mediators on transient receptor potential vanilloid 4 (TRPV4) messenger RNA (mRNA) expression and release of IL-8 by TRPV4 stimulation in human keratinocytes. The protein levels of TRPV4 have been determined by western blot in different cellular lines including the human keratinocyte cell line HaCaT (**a**) compared with human embryonic kidney 293 (HEK293) and human bronchial smooth muscle cells (HBSMCs) as negative and positive control, respectively. Overnight exposure to increased concentrations (0.1–10 ng ml^{-1}) of IL-8 (**b**) and IL-1β (**c**) induces a significant reduction in the TRPV4 mRNA levels in HaCaT. Values are expressed as percentage compared with 18S mRNA. A statistically significant reduction in TRPV4 mRNA level has also been obtained after incubation of HaCaT cells with 10 ng ml^{-1} TNF-α and 10 μg ml^{-1} prostaglandin E2 (PGE_2) (**d**). Each column represents the mean ± SEM of at least four independent experiments. *$P<0.05$ versus control group (Ctl) (analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test). Overnight exposure to 4α-phorbol-12,13-didecanoate (4αPDD) (**e**) and GSK1016790A (GSK) (**f**) induces IL-8 release from HaCaT in a concentration-dependent manner. IL-8 release evoked by both TRPV4 agonist 4αPDD (10 μM) and GSK (5 μM) is reduced by pretreatment with the selective TRPV4 antagonist HC-067047 (HC, 10 μM). Overnight exposure to 4αPDD did not increase the release of IL-1β (**g**), TNF-α (**h**), and PGE_2 (**i**). Each column represents the mean ± SEM of at least three independent experiments. *$P<0.05$ versus basal group; $^{§}P<0.05$ versus 4αPDD (10 μM) and versus GSK (5 μM). Effect of 4αPDD and GSK exposure on cell viability evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test in HaCaT (**j**, **k**). Each column represents the mean ± SEM of at least three independent experiments. *$P<0.05$ versus basal group (Bas) (ANOVA followed by Bonferroni's *post hoc* test). (**l**) Real-time PCR for the measurement of IL-8 mRNA has been performed in the healthy skin (HS), solar keratosis (SK), Bowen's disease (BD), squamous cell carcinoma (SCC), and nodular (Nod), superficial (Sup), and sclerodermiform (Scl) basal cell carcinoma (BCC), as well as in samples of dermatitis (inflamed tissues, IT). Values are expressed as percentage compared with 18S mRNA. *$P<0.05$ versus HS, ANOVA followed by Bonferroni's *post hoc* test.

reduced expression in skin cancer. Immunohistochemical findings are strengthened by real-time PCR results. Remarkable downregulation of the TRPV4 mRNA was found in the same samples of skin cancer where TRPV4 protein expression was also reduced. These results indicate that, regardless of the initiating mechanism, TRPV4 downregulation occurs already at the transcriptional level. The observation that such a marked reduction, observed in cancer samples, also occurs in a precancer condition such as SK suggests that epigenetic or other factors that govern channel downregulation act similarly in both atypical and malignant skin phenotypes.

Skin carcinogenesis has been associated with UV irradiation and the ensuing increase in oxidative stress (Mukhtar and Elmets, 1996), and TRPA1 exhibits a peculiar sensitivity for oxidative stress by-products (Trevisani *et al.*, 2007; Andersson *et al.*, 2008; Materazzi *et al.*, 2008; Sawada *et al.*, 2008). UV radiation represents the major causative factor for melanoma (Boniol *et al.*, 2012; Mulliken *et al.*, 2012). Increasing information is available regarding the ability of UV to activate TRPA1. A previous study has shown that, in a TRPA1-expressing recombinant system, UVA, most probably *via* the generation of reactive oxygen species, induces channel activation (Hill and Schaefer, 2009). More recently, it has been reported that UVA radiation stimulates TRPA1 in melanocytes, thus suggesting a role for the TRPA1 channel in the phototransduction process in melanocytes, and possibly in melanin synthesis (Bellono *et al.*, 2013). TRPA1 has also been detected in human keratinocytes, where it modulates the expression of an array of

genes involved in cell differentiation, metabolism, signaling, and transcription (Atoyan *et al.*, 2009). As SK is a condition that is markedly associated with sun exposure, further research may explore whether UV radiation is responsible for the upregulation of TRPA1 in SK keratinocytes. However, a very recent study reported that, at variance with TRPV4, in both human and mouse skin samples local transcription of TRPA1 is not detectable (Liu *et al.*, 2013).

However, the major finding of the present study relates to TRPV4. Transformation from healthy to atypical, but not yet malignant, keratinocytes in SK was associated with a tendency (which, however, did not reach the significance level) to a reduced TRPV4 expression, which, however, was fully evident and significant in BD and in malignant forms of cancer such as SCC. This suggests a sort of progressive reduction in TRPV4 protein expression from healthy to precancerous and finally malignant skin cancer phenotypes. The implications of the present results are 2-fold. First, we confirm that in human skin TRPV4 expression is not solely confined to cutaneous nociceptors, but it is abundantly distributed in different non-neuronal cutaneous cell types, and prevalently in keratinocytes. Second, loss of TRPV4 expression in keratinocytes seems to be specifically associated with the transition from a healthy phenotype to a cancer phenotype. TRPV4 has been claimed to produce various functions in keratinocytes. Although keratinocyte TRPV4 may respond to warm temperatures (Chung *et al.*, 2003), TRPV3 appears to mainly contribute to the transmission of warm temperature sensation, via ATP release in these cells (Mandadi *et al.*, 2009). Additional proposed roles of TRPV4 in keratinocytes are represented by the control of the skin permeability barrier (Denda *et al.*, 2007), and the promotion of Ca^{2+}-dependent phenomena as the development and maturation of cell–cell junctions (Sokabe *et al.*, 2010). Cell proliferation and differentiation are regulated by Ca^{2+} ions, as high extracellular Ca^{2+} favors differentiation and low extracellular Ca^{2+} maintains an undifferentiated state. It is possible that the TRPV4 channel contributes to these phenomena in keratinocytes (Lee and Caterina, 2005). A recent paper has reported that UVB exposure causes direct TRPV4 activation in keratinocytes, and that epidermal TRPV4 orchestrates UVB-evoked skin tissue damage, thus increasing the expression of the proalgesic mediator, endothelin-1, and producing pain behavior in mice; all these effects were reduced by TRPV4 inhibition (Moore *et al.*, 2013). In addition, also in human specimens, the expression of epidermal TRPV4 and endothelin-1 is enhanced by sunburn (Moore *et al.*, 2013). Together, these data strongly highlight the role of TRPV4 expressed in keratinocytes as a therapeutic target for UVB-evoked skin tissue damage and sunburn-related pain.

It has been widely reported that both cancer cells and surrounding cells release a wide array of cytokines in order to establish a tumor microenvironment that affects cancer cell growth differently, attenuating apoptosis and promoting tissue invasion and metastasis (Elamin *et al.*, 2008; St John *et al.*, 2009). Several proinflammatory and immunomodulatory cytokines are induced in epidermal cells after exposure to one of the most prominent cutaneous tumorigenic agents, UV radiation, including IL-1β, IL-6, IL-8, and TNF-α (Kupper *et al.*, 1987; Takashima and Bergstresser, 1996; Strickland *et al.*, 1997). TNF-α mediates UV induction of adhesion molecule expression (Krutmann *et al.*, 1992) and Langerhans cell migration (Vincek *et al.*, 1993). IL-8 is a chemokine that induces an inflammatory cell infiltrate after UV radiation (Strickland *et al.*, 1997); IL-1β acts as a chemoattractant, induces TNF-α (Corsini *et al.*, 1995), and enhances keratinocyte prostaglandin synthesis (Pentland and Mahoney, 1990); and one prostaglandin, PGE_2, increases in epidermal keratinocytes after UV irradiation (Rundhaug *et al.*, 2011). To explore the mechanism responsible for the marked reduction in TRPV4 expression in tumor keratinocytes, we measured channel mRNA expression in a keratinocyte cell line (HaCaT cells) which has been reported to express TRPV4 (Becker *et al.*, 2005) by real-time PCR, after exposure to IL-1β, IL-6, IL-8, and TNF-α and PGE_2. All these stimuli induced a dose-dependent reduction in TRPV4 mRNA levels, thus suggesting that cytokines and prostaglandins, released within the tumor milieu, have the potential to reduce or suppress *TRPV4* gene expression in keratinocytes. Next, we explored the ability of TRPV4 to release the proinflammatory mediators that downregulate channel expression. Present data demonstrate that TRPV4 stimulation by the selective agonist 4αPDD did not affect the release of IL-1β, TNF-α, and PGE_2 from HaCaT cells. However, TRPV4 stimulation by 4αPDD increased IL-8 release, which should be specifically dependent on channel activation, as it was abrogated by the selective TRPV4 antagonist HC-067047. Previous studies have reported that TRP channel stimulation causes IL-8 release. TRPV1 stimulation has been shown to release IL-8 from HaCaT cells (Southall *et al.*, 2003), and it modulates IL-8 release in human corneal epithelial cells (Wang *et al.*, 2011). Recently, we reported that TRPA1 activation releases IL-8 from TRPA1 expressed in non-neuronal airway cells (Nassini *et al.*, 2012). IL-8 release can be considered as part of a defense response orchestrated by keratinocytes, via TRPV4, to limit cancer progression. However, it is also possible that IL-8, downregulating TRPV4, may progressively reduce keratinocyte potential to release IL-8 itself. To better address this issue, we studied the expression of IL-8 mRNA in healthy skin and cancer tissues and both IL-8 and TRPV4 mRNA in skin dermatitis biopsies. The observation that, in inflamed tissues, IL-8 mRNA was, as expected, elevated, and TRPV4 expression was high and comparable to that of normal skin, indicates that increased IL-8 levels do not warrant TRPV4 downregulation. Thus, the most parsimonious explanation is that the marked reduction in TRPV4 expression documented in skin cancers is independent from, and not the consequence of, the variable and moderate increase in IL-8 observed in these conditions.

A constraint of the present study is represented by the limited number of cases analyzed, a factor that may underestimate differences in TRPV1, TRPV2, TRPV3, and TRPA1 expression in the various types of cancer. Nevertheless, our findings, showing a remarkable channel downregulation, point to TRPV4 as an early biomarker of cutaneous cancers. The ability of IL-8 to downregulate TRPV4 and of TRPV4 to release IL-8 depict a possible autocrine circuitry carried out by

keratinocytes to regulate skin homeostasis and contribute to pathophysiological events. However, the observation that TRPV4 expression is not affected by high IL-8 levels, as found in inflamed tissues, strengthens the hypothesis that TRPV4 is upstream to IL-8 and not *vice versa*. An additional hypothesis is that the remarkable TRPV4 downregulation in cancer, a condition that is most likely associated with cancer onset and progression, may contribute to a poor defensive inflammatory response in skin cancer.

MATERIALS AND METHODS

Tissue collection

The study series included skin biopsies of human healthy skin ($n=4$), SK ($n=5$), BD ($n=5$), invasive cutaneous SCC ($n=7$), and BCC of different histotypes, including nodular ($n=4$), superficial ($n=5$), and sclerodermiform/morphea-like types ($n=3$). To examine the expression of mRNA of TRPV4 and IL-8 skin biopsies of pustular psoriasis ($n=3$), neutrophilic folliculitis ($n=3$) and leucocytoclastic vasculitis ($n=3$) were also included in the study. Paraffin-embedded skin specimens were retrospectively retrieved from the archive of the Division of Pathology, Department of Surgery and Translational Medicine, the University of Florence, Italy. Patients' data, including age, sex, and anatomic tumor site, were collected. The median age of patients with SK and BD was 67 (range, 53–79) and 73.3 (range, 71–81) years, respectively. For both premalignant lesions, three patients were female and two patients were male. Tumor site distribution was the head and neck, upper extremities, and trunk. The median age of patients with SCC was 80 years (range 73–88 years). Six patients were male and one was female, and the tumor site distribution was upper extremities. The median age of patients with nodular BCC was 71 years (range, 39–89 years), eight patients were male and four were female, and the tumor site distribution was upper, lower, and trunk extremities. Ethical approval for the experiments performed on human tissue was obtained from the internal institutional review board.

Immunohistochemistry

Sections of 4-μm thickness were cut from tissue blocks of formalin-fixed, paraffin-embedded samples. Immunostaining was performed according to standard procedures. In brief, antigen retrieval was routinely performed by immersing the slides in a thermostat bath containing 10 mM citrate buffer (pH 6.0) for 15 min at 97 °C followed by cooling for 20 minutes at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10 minutes. After blocking with normal horse serum (UltraVision, Bio-Optica, Milan, Italy), sections were incubated overnight at 4 °C with the following rabbit polyclonal antibodies: TRPV1 (1:100, Abcam, Cambridge, UK), TRPV2 (1:200, Acris Antibodies Herford, Germany), TRPV3 (1:100 Acris Antibodies), TRPV4 (1:50, Sigma-Aldrich, Milan, Italy), and TRPA1 (1:250, Novus Biologicals, Cambridge, UK). Bound antibodies were visualized using aminoethylcarbazol as chromogen (Bio-Optica). Nuclei were counterstained with Mayer's hematoxylin. Negative controls were performed by preadsorption with immunizing peptide (overnight 4 °C) for all the antisera. For the semiquantitative image analysis, epidermal intensity and area of immunostained cells were rated on a scale of 0–4 (0 absent, 1 weak/low, 2 moderate, 3 strong, and 4 very strong staining).

Cell culture

The human keratinocyte cell line HaCaT (Cell Line Service, Eppelheim, Germany), human bronchial smooth muscle cell (PromoCell, Heidelberg, Germany), and human embryonic kidney cell 293 (American Type Cell Collection, Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). Cells were cultured in an atmosphere of 95% air and 5% CO2 at 37 °C.

Measurement of cytokines and PGE_2 production by keratinocyte cell culture

Cells were plated at a density of 200,000 cells per well in a 24-well plate and grown for 24 hours to ~80–90% confluence, and then exposed to different concentrations (1–30 μM) of the selective TRPV4 agonist, 4αPDD (Sigma-Aldrich), or its vehicle (0.3% DMSO), in a serum-free medium. In a different set of experiments, HaCaT cells were exposed to GSK (0.5, 1, 5, 10 μM), another potent and selective TRPV4 agonist (Thorneloe *et al.*, 2008), or its vehicle (0.5% DMSO). Selective TRPV4 antagonist HC-067047 (10 μM; Everaerts *et al.*, 2010; Tocris Bioscience, Bristol, UK), or its vehicle (0.1% DMSO), was added 30 minutes before exposure to the agonists. The medium was collected 18 hours after treatment, and human IL-8, IL-6, IL-1β, (TNF-α), and prostaglandin E_2 (PGE_2) were measured using the commercial quantitative ELISA kit (Invitrogen, Carlsbad, CA).

Western immunoblot assay

HaCaT, human bronchial smooth muscle cells, and human embryonic kidney 293 cells were lysed for 30 minutes at 4 °C in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 100 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, and complete protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were used for the TRPV4 protein detection, as described in detail in Supplementary Materials and Methods online.

Reverse transcriptase–PCR and real-time PCR

Extraction of mRNA was performed in paraffin-embedded tissues as described (Kalmar *et al.*, 2013), with some modification. In brief, deparaffinization was performed by adding 1 ml of xylene for 10 minutes twice and 1 ml absolute ethanol for 10 minutes twice. Total RNA has been extracted from the air-dried deparaffinized sections by using the TRIZOL method (Invitrogen).

HaCaT, human bronchial smooth muscle cells, and human embryonic kidney 293 cells were seeded in six-well plates and grown for 24 hours to 90% confluence before stimulation or mRNA extraction. Then, cells were exposed for 24 hours, in a serum-free medium, to different concentrations of several inflammatory mediators, including IL-8, IL-1β, IL-6, TNF-α (all 0.1–10 ng ml^{-1}), and PGE2 10 μg ml^{-1}, or their vehicles (cell medium without stimulus). Total cellular RNA was extracted from stimulated and unstimulated cells by using the TRIZOL method (Invitrogen). Complementary DNA was prepared from total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy), and the real-time PCR was performed to check the TRPV4 and IL-8 mRNA levels as described in more details in Supplementary Materials and Methods online.

Vitality assay by the tetrazolium salt method (MTT reduction assay)

Cytotoxicity of various stimuli, including 4αPDD, GSK, or inflammatory mediators tested on HaCaT cell line, was assessed by using the

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability test, as described in detail in Supplementary Materials and Methods online.

Statistical analysis

For the semiquantitative image analysis, statistical significance was determined by using a nonparametric one-way analysis of variance followed by the Kruskal–Wallis *post hoc* test. All other data are presented as mean ± SEM. Statistical significance was determined by using one-way analysis of variance, followed by Bonferroni's *post hoc* analysis for comparison of multiple groups. $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported in part by the Fondazione Ente Cassa di Risparmio di Firenze to DM and SM, and in part by the Associazione Italiana per la Ricerca sul Cancro (AIRC MFAG, 13336) to RN.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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