Triterpenes Promote Keratinocyte Differentiation *In Vitro, Ex Vivo* and *In Vivo*: A Role for the Transient Receptor Potential Canonical (subtype) 6

Ute Woelfle[1], Melanie N. Laszczyk[1,2,5], Margarethe Kraus[3], Kristina Leuner[3], Astrid Kersten[4], Birgit Simon-Haarhaus[1], Armin Scheffler[2], Stefan F. Martin[5], Walter E. Müller[3], Dorothee Nashan[6] and Christoph M. Schempp[1]

It has been shown recently that triterpenes inhibit cancer cell growth of various cell types *in vitro*. In this work, the effect of highly purified triterpenes (TE) with betulin as the major compound (>80% w/w) on cell proliferation, apoptosis, and differentiation of human keratinocytes was analyzed *in vitro*, *ex vivo,* and *in vivo*. *In vitro*, TE increased calcium influx into primary keratinocytes and upregulated various differentiation markers including keratin 10. TE also specifically increased the expression of the non-selective transient receptor potential canonical (subtype) 6 (TRPC6) in keratinocytes, and knocking down TRPC6 inhibited keratin 10 upregulation. *Ex vivo*, in human skin explants TE induced the expression of TRPC6 in the epidermis and increased DNA fragmentation of terminally differentiating keratinocytes. Topical treatment with TE of actinic keratoses, that represent *in situ* squamous cell carcinomas with disturbed epithelial differentiation, resulted in downgrading of aberrant Ki67 expression and upregulation of keratin 10 *in vivo*. Our data indicate that TE promotes keratinocyte differentiation *in vitro* and *in vivo*. This effect seems to be mediated at least in part by TRPC6.

Journal of Investigative Dermatology (2010) **130,** 113–123; doi:10.1038/jid.2009.248; published online 13 August 2009

INTRODUCTION

Pentacyclic triterpenes of the lupan type such as betulin, betulinic acid, and lupeol display anticarcinogenic effects in various cell types (Fulda *et al.*, 1998; Liu, 2005; Alakurtti *et al.*, 2006; Chaturvedi *et al.*, 2008). Furthermore, betulinic acid has been shown to induce terminal differentiation in human primary keratinocytes (hPK) (Pisha *et al.*, 1995; Selzer *et al.*, 2000; Eiznhamer and Xu, 2004; Galgon *et al.*, 2005). Recently, we reported that highly purified triterpenes from birch bark (TE) with betulin as the major compound (>80% w/w) display dose-dependent cytotoxic and apoptosis-inducing effects in immortalized human HaCaT keratinocytes and skin cancer cells (Laszczyk *et al.*, 2006). We also found that a TE-oleogel is effective in the topical treatment of actinic keratoses (AK) in a prospective, randomized comparative clinical phase 2a study. The clearing rate with TE-oleogel was comparable to standard treatment, that is, cryotherapy. In contrast to other established therapies of AK the skin tolerance of TE-oleogel was excellent (Huyke *et al.*, 2009).

AK are common dermatological diseases that are caused by chronic ultraviolet-induced damage of epidermal keratinocytes (Frost and Green, 1994; Marks and Motley, 1995; Green *et al.*, 1999; Moy, 2000). Owing to similar histological characteristics, AK are considered to represent *in situ* squamous cell carcinomas (Marks *et al.*, 1988; Tucci *et al.*, 1998; Cockerell, 2000; Ortonne, 2002). The strictly regulated balance of proliferation, differentiation, and desquamation of keratinocytes in the epidermis is profoundly disturbed in AK (Markey *et al.*, 1991; Smit *et al.*, 2004; Aslan *et al.*, 2006; Raj *et al.*, 2006), and epidermal cell proliferation is not confined to the basal layers, but can also be detected in supra-basal regions of the epidermis (Cockerell, 2000; Smit *et al.*, 2004). Abnormal proliferation of keratinocytes in AK can be visualized by immunohistochemical staining for the proliferation marker Ki67. Increased expression of Ki67 usually correlates with the degree of dysplasia in AK (Cockerell, 2000; Smit *et al.*, 2004).

The aim of this work was to gain more detailed insight into the effects and mode of action of TE in hPK *in vitro*, *ex vivo,* and *in vivo* in AK. When performing *in vitro* studies with hPK it must be kept in mind that hPK in normal skin undergo highly

[1]*Department of Dermatology, Competence Center skintegral, University Medical Center Freiburg, Germany;* [2]*Birken GmbH, Niefern-Öschelbronn, Germany;* [3]*Department of Pharmacology, Biocenter, N260, University of Frankfurt, Germany;* [4]*Dermatohistopathology Dr Laaff, Freiburg, Germany;* [5]*Allergy Research Group, Department of Dermatology, University Medical Center Freiburg, Germany and* [6]*Department of Dermatology, Study Center Dermato-Oncology, University Medical Center Freiburg, Germany*

Correspondence: Professor Christoph M. Schempp, Department of Dermatology, Competence Center skintegral, University Medical Center Freiburg, Hauptstr. 7, D-79102 Freiburg, Germany.
E-mail: christoph.schempp@uniklinik-freiburg.de

Abbreviations: AK, actinic keratose; DSGC, distal stratum granulosum cell; hPK, human primary keratinocyte; INV, involucrin; TE, triterpenes; TGM, transglutaminase

Received 9 March 2009; revised 22 June 2009; accepted 25 June 2009; published online 13 August 2009

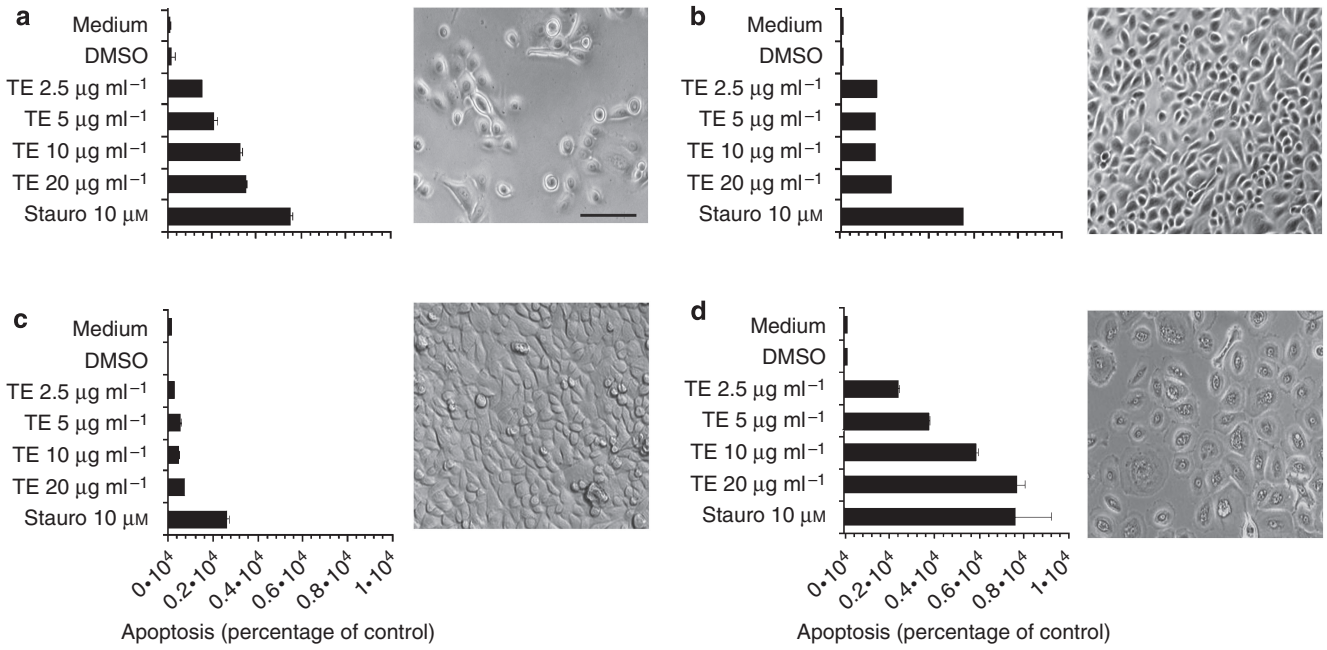

Figure 1. Pro-apoptotic effects of TE in different types of hPK. (**a**) Proliferating cells; (**b**) early confluent cells; (**c**) late confluent cells; (**d**) senescent cells. The left panel shows apoptosis rates in different types of hPK. Apoptosis was evaluated by measuring oligonucleosomes in the cell culture supernatants using a cell death detection ELISA. Data represent mean ± SD of three independent experiments. The right panel illustrates the appearance of different types of hPK in the phase contrast microscope. Cells were generated by different culture conditions as described in (Perera *et al.*, 2006). Scale bar = 15 μm.

organized morphological and functional changes during their way from the proliferative stage (basal layer) via the growth arrested early and late differentiation stages (spinous and granular layer) to the dead outermost cornified layer (Perera *et al.*, 2006). Perera *et al.* (2006) demonstrated that proliferating, early and late confluent as well as senescent hPK can be generated under defined cell culture conditions, and that these cell culture stages correspond to the above-mentioned layers of the epidermis (Perera *et al.*, 2006). As proliferating and early-differentiated cells represent the dysplastic cells in most types of AK, we studied subconfluent hPK *in vitro*. Calcium has a key role in the differentiation process of keratinocytes. Several TRPC (transient receptor potential canonical) channels (for example, TRPC1, −4, −6) and the TRPV6 channel (vanilloid transient receptor potential) have previously been shown to be involved in calcium-mediated keratinocyte differentiation (Cai *et al.*, 2005; Lehen'kyi *et al.*, 2007; Beck *et al.*, 2008; Muller *et al.*, 2008). Therefore, one focus of this work was to elucidate the involvement of these receptors in the effect of TE on hPK.

We found that treatment of subconfluent hPK with TE *in vitro* induced the expression of differentiation markers (for example, keratin 10 (KRT10)), specifically enhanced the expression of TRPC6 and increased calcium influx into the cells. *Ex vivo*, treatment with TE of skin explants did not result in quantitative apoptosis throughout the epidermis, as one would expect with primarily cytotoxic compounds, but increased DNA fragmentation of distal stratum granulosum cells (DSGC) indicative for enhanced terminal differentiation of hPK. *In vivo*, topical treatment of AK with TE-oleogel resulted in normalization of skin morphology, reduction of aberrant Ki67 expression and increased expression of the differentiation marker KRT10.

RESULTS

TE displays pro-apoptotic effects on hPK *in vitro*

We analyzed the effect of TE on various keratinocyte types present in the different layers of the epidermis (basal, spinous, granular, and cornified layer). These specific types of keratinocytes can be generated *in vitro* (proliferating, early and late confluent as well as senescent hPK) using defined cell culture conditions as described by Perera *et al.* (2006). As shown in Figure 1 proliferating and early confluent hPK were relatively small with prominent nuclei (Figure 1a and b, right panel), late confluent cells were more flat with less prominent nuclei (Figure 1c, right panel), and senescent hPK, in contrast to confluent hPK, were larger, round and more flattened with cytoplastic vacuoles (Figure 1d, right panel). To determine if TE displays pro-apoptotic effects on hPK, the various types of hPK were incubated with TE for 24 h and apoptosis was measured with an oligonucleosome cell death detection ELISA. TE-induced apoptosis in all types of hPK in a dose-dependent manner (Figure 1a–e, left panel). Senescent hPK were most susceptible to apoptosis induced by TE. As senescent hPK reflect DSGC that are prone to apoptosis/cornification our findings fit to the situation in living skin. In contrast, proliferating cells showed less apoptosis, and late confluent cells showed the least apoptosis rates. Therefore, in all further experiments we used hPK between the proliferating and early confluent stage (subconfluent or 70% confluent cells). These hPK together with proliferating basal hPK, correspond to stratum basale and stratum spinosum keratinocytes *in situ*, representing the dysplastic cells in AK and should therefore be particularly susceptible to TE treatment. Subconfluent hPK were treated with 10 μg/ml TE, a concentration that only caused moderate apoptosis rates (Figure 1).

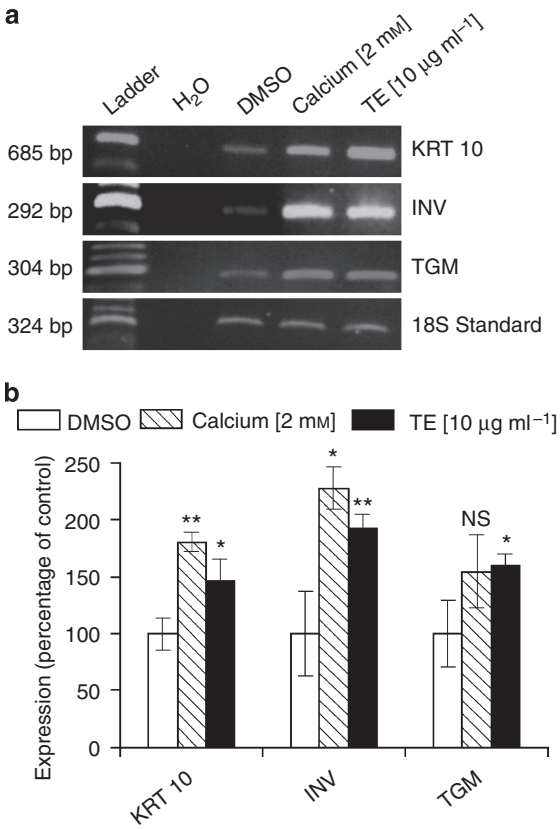

Figure 2. TE induces differentiation in hPK. hPK were incubated for 24 h in low calcium medium (0.1 mM, control), 2 mM calcium (positive control) or 10 µg ml^{-1} TE. (**a**) Total RNA of hPK was isolated, reverse transcribed and subjected to TGM, INV, KRT10 semi-quantitative RT-PCR. (**b**) The histogram shows the relative expression level of TGM, INV, or KRT10 compared to their normalized expression level in untreated control cells. Asterisks denote statistical significance compared to DMSO ($n=3$, mean ± SD) (*$P<0.05$; **$P<0.01$; NS, not significant).

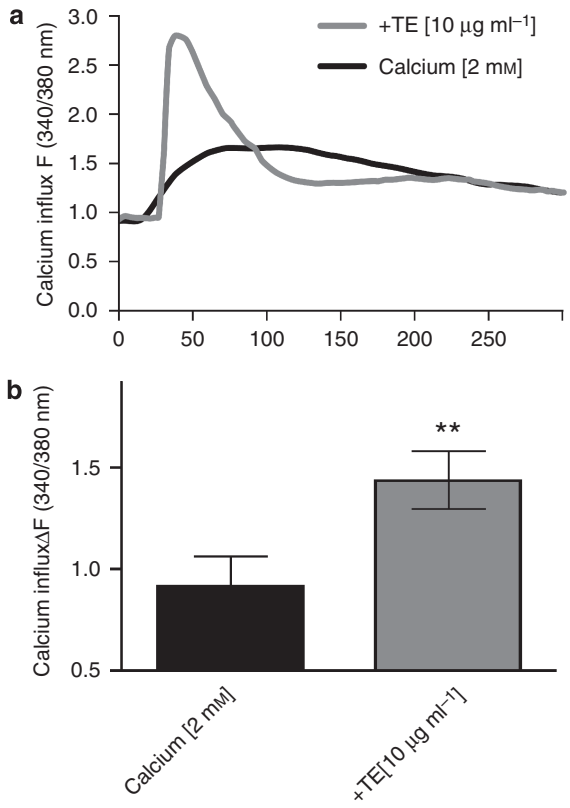

Figure 3. TE increases high [Ca2 +]$_{ex}$ induced calcium-influx in hPK. The cells were incubated with TE (10 µg ml ×) or medium (control) for 24 h. Subsequently the TE-treated and the control hPK were stimulated with 2 mM calcium and calcium influx was determined. (**a**) Representative time traces show TE-induced changes in [Ca^{2+}]$_i$ after acute stimulation with 2 mM calcium in fura-2-loaded hPK cells. (**b**) Histogram of high [Ca^{2+}]$_{ex}$ induced cation influx in TE-incubated and control hPK ($n=6$, mean ± SD) (**$P<0.01$).

TE induces differentiation of hPK *in vitro*

The differentiation-promoting effect of TE was investigated by incubating subconfluent hPK with 10 µg/ml TE for 24 h. As positive control we used 2 mM calcium, referred to as high [Ca^{2+}]$_{ex}$. It is well established that calcium induces keratinocyte differentiation and the expression of differentiation markers (Cai *et al.*, 2005). Gene expression was investigated in hPK for the early differentiation markers involucrin (INV) and KRT10 as well as the late differentiation marker transglutaminase (TGM) using semi-quantitative RT-PCR. Low KRT10, INV, and TGM mRNA concentrations were detected in hPK cultured in medium containing 0.1 mM [Ca^{2+}]$_{ex}$ (Figure 2a and b). KRT10, INV, and TGM mRNA levels were increased in cells cultured in the presence of either high [Ca^{2+}]$_{ex}$ or TE, as quantification of the RT-PCR signals clearly showed (Figure 2b). We confirmed these results by real time PCR and obtained a sixfold increase of KRT10 expression (Supplementary Table 1). It has been shown that an increased expression of KRT10 and INV can be caused by the transmembrane receptors Notch1 and 2 during the keratinocyte differentiation process (Rangarajan *et al.*, 2001). Cytospin experiments revealed that high [Ca^{2+}]$_{ex}$ as well as TE treatment induced Notch2 expression in hPK, whereas in untreated hPK the Notch receptor was not or only weakly expressed (Supplementary Figure 1a and b). However, the γ secretase inhibitor I GSI inhibited KRT10 expression in both solvent-treated and Calcium as well as TE-treated cells. Therefore, a direct proof that TE induces KRT10 expression via Notch could not be shown (Supplementary Figure 1c and d). To clearly determine the role of Notch2 in mediating TE-induced keratinocyte differentiation knockdown experiments would be necessary.

TE increases calcium influx in hPK induced by high [Ca^{2+}]$_{ex}$

As high [Ca^{2+}]$_{ex}$ triggers the differentiation process of hPK, we analyzed if calcium influx is also involved in TE-induced differentiation. First, calcium influx was measured in hPK after short-term treatment with TE. TE itself did not induce calcium influx immediately (data not shown). To investigate if TE might interfere with calcium influx which is triggered by high [Ca^{2+}]$_{ex}$, we incubated hPK over 24 h with TE and measured high [Ca^{2+}]$_{ex}$ induced calcium influx. Pre-incubation of hPK for 24 h with TE (10 µg ml^{-1}) resulted in a significantly increased calcium influx as measured by elevation of calcium-dependent fluorescence in fura-2-loaded hPK compared with untreated control cells (Figure 3a and b).

TE specifically induces TRPC6 expression in hPK *in vitro*

Calcium-permeable channels are potential candidates involved in the increased calcium influx observed in hPK upon treatment with TE. Especially interesting is the transient receptor potential superfamily of cation channels. The involvement of these channels in the induction of keratinocyte differentiation was already shown for TRPC1 (Cai *et al.*, 2005; Beck *et al.*, 2008), TRPC4 (Beck *et al.*, 2008), TRPC6

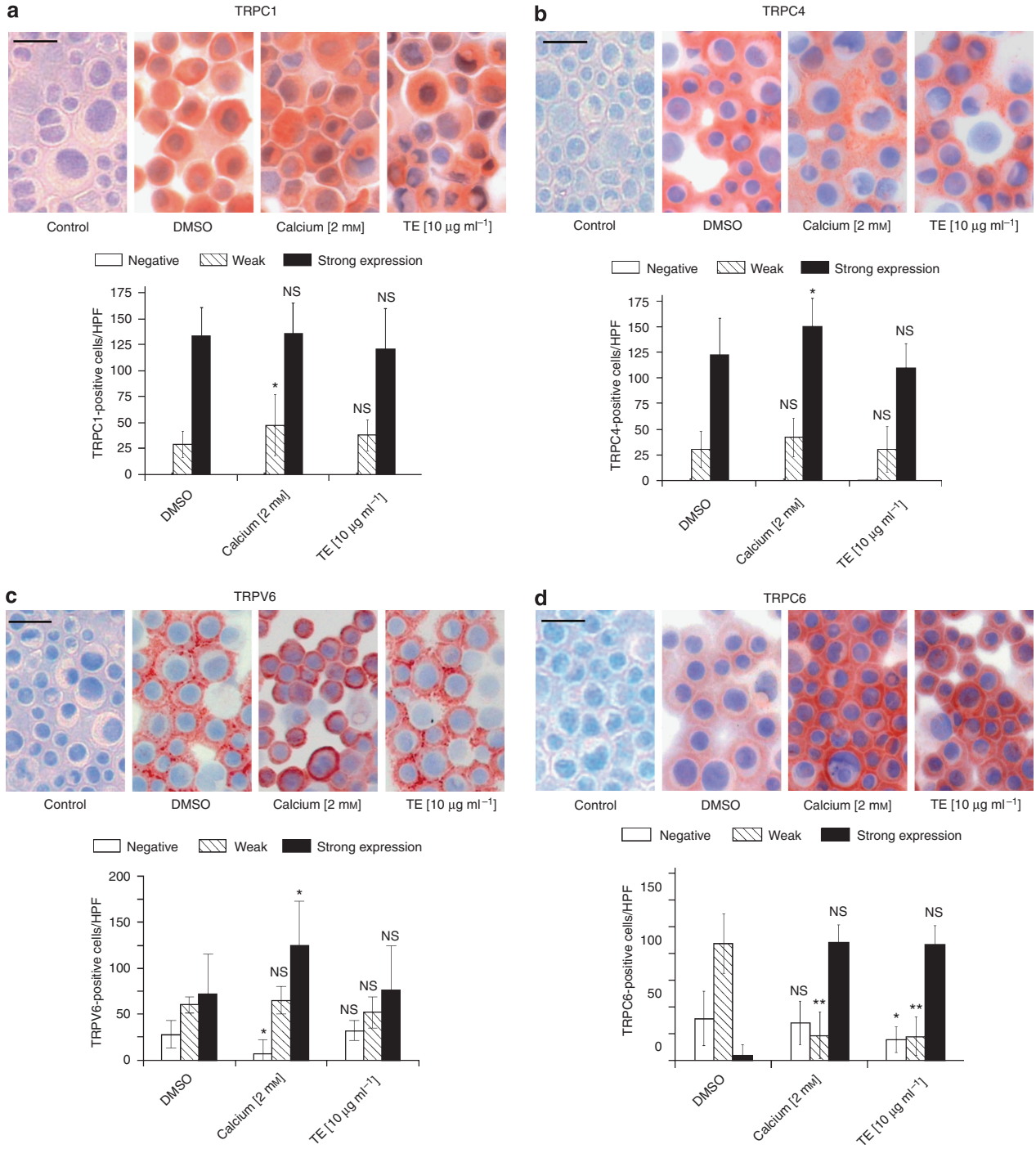

Figure 4. TRP channel expression in hPK. hPK were incubated for 24 h in low calcium medium (0.1 mM), 2 mM calcium (positive control) or 10 μg ml^{-1} TE. After centrifugation onto cytospin slides, the cells were stained for TRPC1 (**a**), TRPC4 (**b**), TRPV6 (**c**), and TRPC6 (**d**) expression (controls: without primary antibody). Four random fields of sections from three independent cytospin preparations were counted for TRPC1, TRPC4, TRPV6, and TRPC6-positive hPK at × 400 magnification and representative images are shown. The final count per group represents mean ± SD. *P<0.05; **P<0.01; NS, not significant. Scale bar = 10 μm. (**e**) Total RNA of hPK treated with TE was subjected to RT-PCR. TRPC6 expression of untreated hPK was compared with hPK treated with 10 μg ml^{-1} TE or 2 mM calcium. The histogram shows relative expression levels of TRPC6 in relation to control cells. Asterisks denote statistical significance to control hPK (n = 3) (*P<0.05).

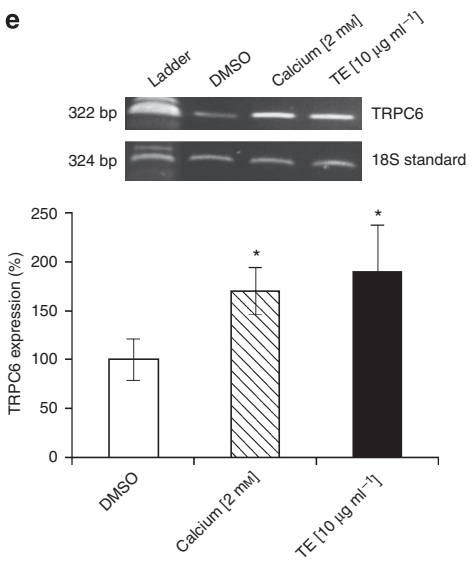

Figure 4. Continued.

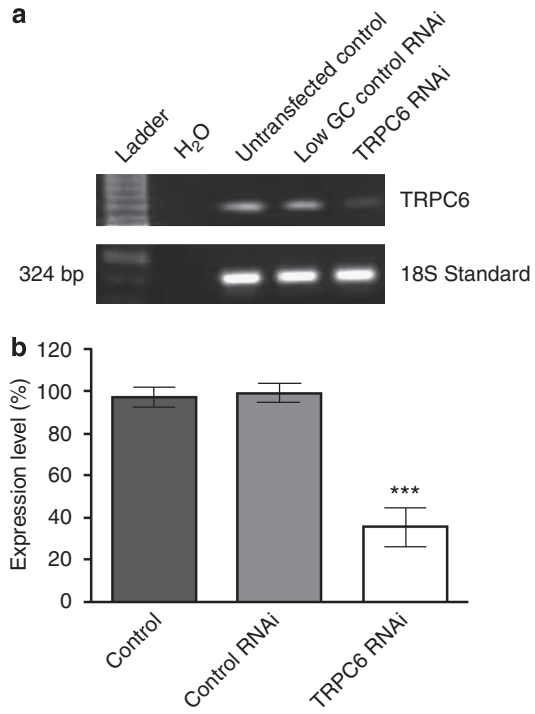

Figure 5. TRPC6 silencing. (**a**) hPK were transfected with anti-TRPC6 RNAi or control RNAi with low GC content. The effectiveness of RNAi transfection was determined with RT-PCR analyses. (**b**) Histogram depicting relative expression levels of TRPC6 normalized to its expression level in untransfected control cells. Asterisks denote statistical significance compared with control hPK ($n = 3$, mean ± SD) (***$P < 0.001$).

(Muller *et al.*, 2008), and TRPV6 (Lehen'kyi *et al.*, 2007). To evaluate if these TRP channels are also involved in TE-induced differentiation of hPK, we performed cytospins of subconfluent hPK using high $[Ca^{2+}]_{ex}$ as positive control. As shown in Figure 4, the basal level of TRPC1 (a) and TRPC4 (b) was already very high in the untreated controls and could not further be increased with TE or high $[Ca^{2+}]_{ex}$. TRPV6 was also highly expressed in untreated subconfluent hPK (Figure 4c). High $[Ca^{2+}]_{ex}$ additionally increased TRPV6 expression, but TE showed no effect. TRPC6 was only weakly expressed in control hPK (Figure 4d). High $[Ca^{2+}]_{ex}$ as well as 10 µg/ml TE clearly up-regulated TRPC6 expression. TRPC6-positive hPK showed staining of the cell membrane and the cytoplasm. To confirm the immunohistochemical results we also performed flow cytometry and RT-PCR analysis of TRPC6 in high $[Ca^{2+}]_{ex}$ or TE-treated hPK. RT-PCR (Figure 4e) clearly confirmed an increased TRPC6 expression in TE-treated cells. Similarly, flow cytometry confirmed this finding (Supplementary Figure 2).

TE-induced KRT10 expression depends on TRPC6

To test whether TE-induced TRPC6 expression is a prerequisite for keratinocyte differentiation we specifically knocked down TRPC6 in hPK. hPK were transfected with TRPC6 RNAi or control RNAi with corresponding low GC content. The silencing of TRPC6 was confirmed with RT-PCR analysis. Anti-TRPC6 RNAi reduced TRPC6 expression, whereas the low GC control RNAi had no effect (Figure 5a and b). The silencing of TRPC6 was also verified with a western blot against TRPC6 showing a reduced protein level of TRPC6 in TRPC6-silenced cells (Supplementary Figure 3).

RNAi-transfected cells were pre-incubated for 24 h with TE (10 µg ml^{-1}) before KRT10 expression was measured. The relative expression level of KRT10 in anti-TRPC6 RNAi-transfected cells was reduced to background levels in comparison to the normalized expression level in TE-treated untransfected cells (Figure 6a and b). In the low GC RNAi-transfected control cells KRT10 expression was upregulated after TE treatment, but the effect was slightly weaker compared with the untransfected controls. These results indicate that TE-induced KRT10 expression depends on TRPC6. Similar results were seen with the expression of TGM1 (Supplementary Figure 4).

TE induces TRPC6 expression in human skin explants

To evaluate the effect of TE on the expression of TRPC6 *in situ*, we incubated living human skin *ex vivo* with 10 µg ml^{-1} TE for 24 h. As shown in Figure 7, TE clearly increased the expression of TRPC6 in hPK over the constitutive level. The control without the primary antibody showed no unspecific background. TRPC6-positive keratinocytes showed staining of the cell membrane and the cytoplasm, comparable to the *in vitro* findings. In skin explants, TRPC6 was primarily expressed by stratum spinosum and stratum granulosum keratinocytes. In contrast, basal keratinocytes were negative for TRPC6 staining. This indicates that only keratinocytes entering into epidermal differentiation are susceptible for triggering TRPC6 expression. Interestingly, there was a lamellar accumulation of TRPC6 reactivity in the distal stratum granulosum where keratinocytes physiologically flatten, dissolve their nucleus, and disintegrate during their terminal differentiation. Positive staining of the stratum corneum indicated that the TRPC6 protein is not degraded, but accumulates in the keratin envelope of the epidermis. In contrast to the *in vitro* findings we could not find

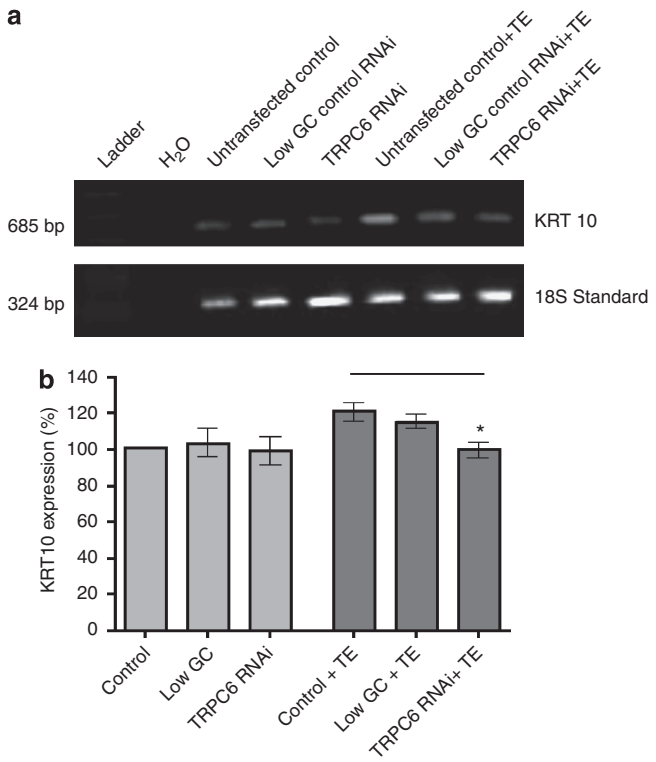

Figure 6. TRPC6 mediates TE-induced differentiation. hPK were transfected with anti-TRCP6 RNAi or control RNAi with low GC content and incubated for 24 h with TE (10 μg ml^{-1}). (**a**) Expression of KRT10 in untreated (untransfected, TRPC6 RNAi or control RNAi transfected) hPK and 10 μg ml^{-1} TE-treated (untransfected, TRPC6 RNAi or control RNAi transfected) cells was determined in RT-PCR analysis. (**b**) The histogram reflects the relative expression level of KRT10 compared with its normalized expression levels in untransfected, untreated hPK. Asterisk denotes statistical significance compared with control + TE ($n = 3$, mean ± SD) (*$P < 0.05$).

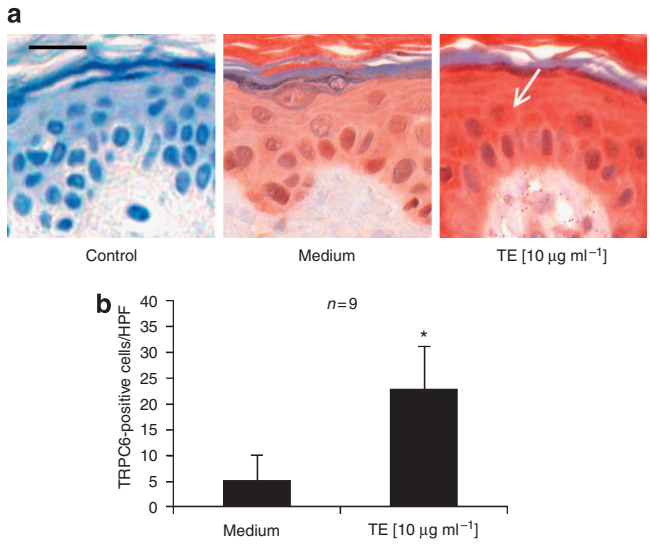

Figure 7. TE induces TRPC6 expression in living human skin ex vivo. Split-thickness skin organ cultures (skin explants) obtained from dermatome-separated human skin were incubated for 24 h in low calcium medium (0.1 mM, control) or 10 μg ml^{-1} TE. (**a**) Sections (3 μm) were stained for TRPC6 (control: without primary antibody). Representative sections of high power fields are shown. Arrow indicates TRPC6-positive cell. Scale bar = 20 μm. (**b**) Five random fields of sections from nine independent skin explants were counted for TRPC6-positive keratinocytes at ×400 magnification. The final count per group represents mean ± SD (*$P < 0.05$). Note the absence of TRPC6 expression in basal keratinocytes but strong positivity of stratum spinosum keratinocytes in TE-treated skin.

TE-induced increased expression of differentiation markers in the skin explants because of the high constitutive expression levels (data not shown).

TE induces DNA fragmentation of distal stratum granulosum cells *ex vivo*

Our *in vitro* experiments on hPK had already shown that TE enhances apoptosis in specific types of hPK (senescent hPK). Therefore, we also examined the effect of TE on keratinocyte apoptosis *in situ* by incubating skin explants with TE for 24 h. Subsequently DNA fragmentation was measured by *in situ* end labeling with terminal deoxynucleotidyl-transferase (TUNEL technique) in treated *versus* untreated skin explants (Figure 8). In untreated skin, only sparse DNA fragmentation could be seen, mainly in the upper layers of the epidermis (Figure 8a). The dashed lines in Figure 8 indicate the border between the dead corneocytes of the stratum corneum and the living cells of the stratum granulosum. TE primarily induced DNA fragmentation in the DSGC that are terminally differentiating into corneocytes. Only little DNA fragmentation could be detected throughout the other layers of the epidermis (Figure 8c). In contrast, the positive control staurosporine-induced quantitative DNA fragmentation in all epidermal layers without accumulation of fragmented DNA in the distal stratum granulosum (Figure 8b).

TE promotes keratinocyte differentiation in AK after topical treatment *in vivo*

To evaluate the effect of TE on epidermal cells *in vivo*, we investigated punch biopsies obtained from patients ($n = 4$) with AK treated with TE-oleogel twice a day for 3 months (Huyke *et al.*, 2009). Skin biopsies were taken from AK before and at the end of treatment and 3 μm sections were stained for TRPC6, Ki67, and KRT10. With immunohistochemistry, no clear modification of the constitutive TRPC6 expression could be observed after 3 months of treatment (data not shown). However, the typical aberrant expression of the proliferation marker Ki67 in untreated AK (Figure 9a, middle) was reduced to the basal layers after 3 months of treatment with TE-oleogel in all biopsy pairs (Figure 9a, right panel and Figure 9b). As increased proliferation of AK keratinocytes is often accompanied by disturbed differentiation we also analyzed the expression of KRT10 in the biopsies. Before treatment, dysplastic cells were present up to the stratum granulosum, and all dysplastic cells were KRT10-negative (Figure 10a, middle). Treatment with TE-oleogel resulted in an increased expression of KRT10 (Figure 10a, right panel and Figure 10b) similar to the staining pattern of healthy skin. In summary, these immunohistochemical data confirm the clinical improvement of AK observed after treatment with TE-oleogel.

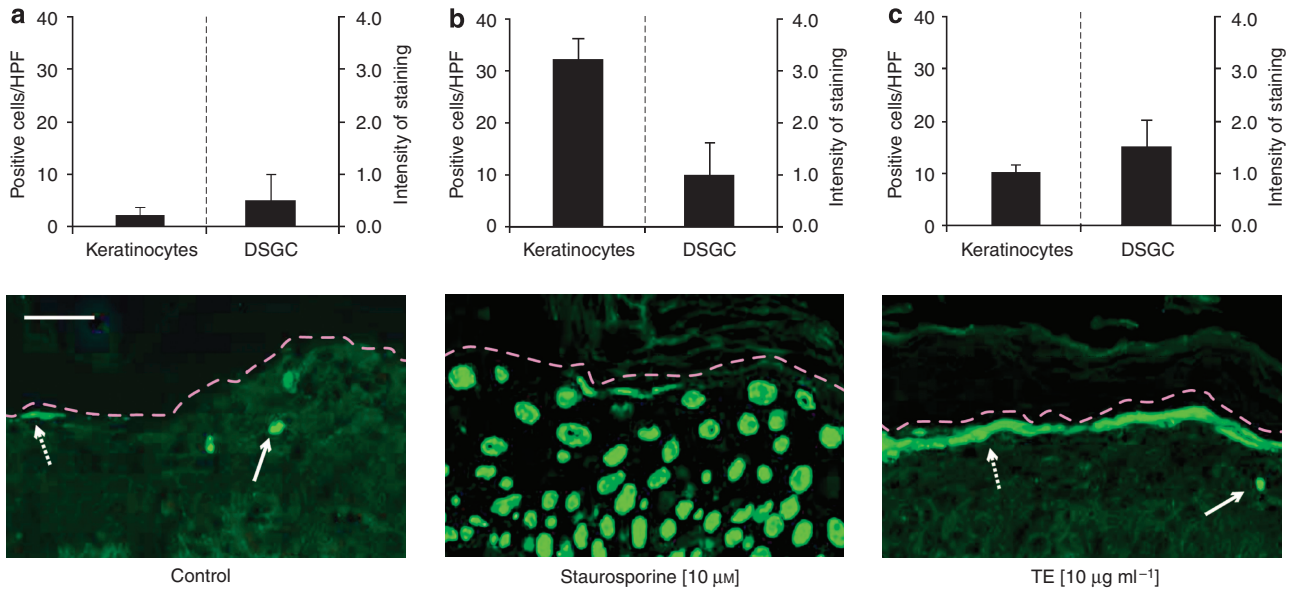

Figure 8. TE induces DNA fragmentation in human skin explants ex vivo. Split-thickness skin organ culture (skin explants) obtained from dermatome-separated human skin were incubated for 24 h in low calcium medium (0.01 mM, control) ($n=9$) (**a**), staurosporine (10 μM) ($n=3$) (**b**) or TE (10 μg ml^{-1}) ($n=9$) (**c**). Sections (3 μm) were stained for apoptotic DNA fragmentation using a fluorescence-based TUNEL kit. The histograms show five random fields of sections from 3–9 independent skin explants counted for TUNEL positive keratinocytes (left bars) and relative intensity of distal stratum granulosum cells (DSGC) staining (right bars, for grading see Methods) at ×400 magnification. The final count per group represents mean ± SD. In the lower panel arrows indicate sporadic DNA fragmentation of stratum granulosum keratinocytes (solid arrows) and of DSGC (dashed arrows). Scale bar = 20 μm.

DISCUSSION

Normal epidermis consists of three nucleated layers, that is, the basal (stratum basale), spinous (stratum spinulosum), and granular layer (stratum granulosum), and one anucleated cornified layer (stratum corneum). Under normal conditions hPK proliferate only in the basal layers, and the divided daughter cells stay either in the basal layer as an epidermal stem cell or move up to the skin surface to give rise to gradually differentiated cells through the spinous, granular, and cornified layers (Segre, 2006).

Different types of hPK (for example, proliferating *versus* differentiating hPK) can be distinguished by the expression of apoptosis- and differentiation-associated proteins (Perera *et al.*, 2006). In healthy epidermis, the proliferation marker Ki67 is only expressed in the basal layers, and KRT1 and KRT10 are expressed in the spinous and granular layer. Calcium is involved in the differentiation process of keratinocytes by sequentially turning on and off genes that are required for the differentiation process (for example, INV, TGM, and KRT10) (Pillai *et al.*, 1990; Tu *et al.*, 2004). This differentiation process is steered by the calcium gradient in the skin from a low concentration in the stratum basale, progressively increasing toward the stratum granulosum and the skin surface, in which the proteins critical for barrier function are accumulating (Pillai *et al.*, 1990; Lehen'kyi *et al.*, 2007; Leinonen *et al.*, 2009). In tissue culture, differentiation can be triggered by experimentally increasing the extracellular calcium concentration above 0.1 mM (Eckert *et al.*, 1997). Upon increased external calcium concentrations calcium-sensing receptors get activated and trigger phospholipase C that generates diacylglycerol and inositol triphosphate. Inositol triphosphate releases calcium from intracellular stores (for example, from the ER) and diacylglycerol directly activates receptor-operated calcium channels (Cai *et al.*, 2005) such as several TRPC (canonical transient receptor potential) channels. TRPC1 (Cai *et al.*, 2005; Beck *et al.*, 2008), TRPC4 (Beck *et al.*, 2008), TRPC6 (Muller *et al.*, 2008), and TRPV6 (Lehen'kyi *et al.*, 2007) are discussed to have a role in calcium-mediated keratinocyte differentiation.

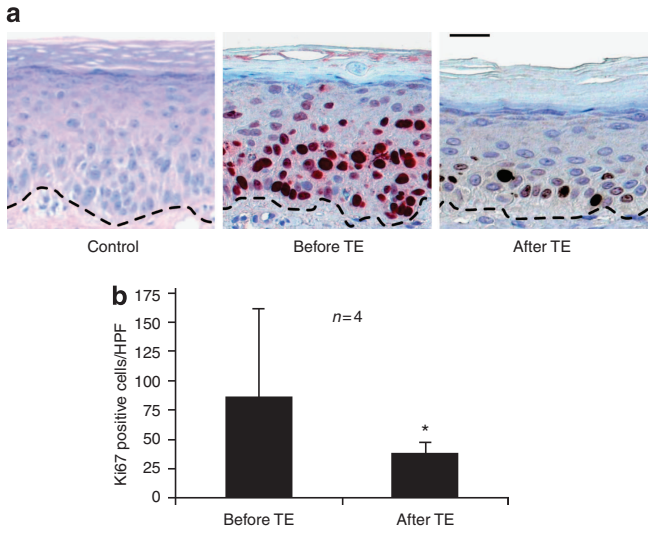

Figure 9. TE normalizes aberrant Ki67 expression in AK *in vivo*. Punch biopsies obtained from patients ($n=4$) with AK were treated twice a day for 3 months with TE. (**a**) 3 μm sections were stained for Ki67 and representative sections are shown (control: without primary antibody). Scale bar = 20 μm. (**b**) Five random field sections of four independent biopsy pairs were counted for Ki67-positive keratinocytes at ×400 magnification. The final count per group represents mean ± SD (*$P<0.05$).

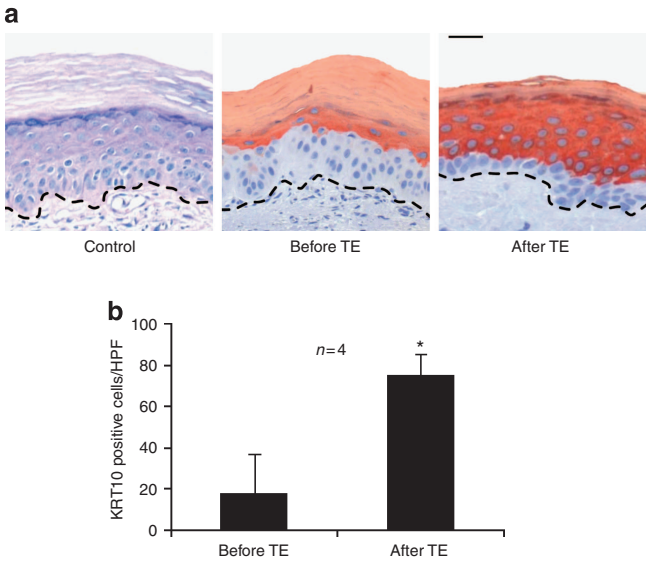

Figure 10. TE induces KRT10 expression in AK *in vivo*. Punch biopsies obtained from patients ($n=4$) with AK were treated twice a day for 3 months with TE. (**a**) Sections (3 μm) were stained for KRT10 and representative sections are shown (control: without primary antibody). Scale bar = 20 μm. (**b**) Five random field sections of four independent biopsy pairs were counted for KRT10-positive keratinocytes at × 400 magnification. The final count per group represents mean ± SD (*$P< 0.05$).

In this work we have investigated the effect of TE on the differentiation process of hPK in various readout systems *in vitro, ex vivo,* and *in vivo*. In cell culture TE differentially induced apoptosis in specific types of hPK. Especially senescent hPK were susceptible to the apoptotis-inducing effect of TE. As senescent hPKs correspond to distal stratum granulosum cells that are prone to apoptosis/cornification, our findings fit to the situation in living skin, in which these cells are also most susceptible to apoptosis (Nickoloff *et al.*, 2002a). We have shown a differentiation-promoting effect of TE on early confluent hPK by an increased expression of early and late differentiation markers as well as an increased protein expression of the receptor Notch. Notch has been proposed to induce early differentiation markers in hPK, such as KRT 1 and 10 (Nickoloff *et al.*, 2002b). However, to clearly show the role of Notch in TE-induced KRT10 expression the activation of Notch by TE must be determined, as well as the effect of TE on the expression of early differentiation markers (for example, KRT10) in hPK with silenced Notch expression.

To elucidate whether TRP channels are involved in the differentiation-inducing effect of TE we analyzed the expression of TRPC1, TRPC4, TRPC6, and TRPV6 channels that have been suggested to have a key role in keratinocyte differentiation. Lehen'kyi *et al.* (2007) described the TRPV6 channel as a key molecule in calcium-induced differentiation of hPK. We could confirm this but saw no increased TRPV6 expression upon TE treatment. For TRPC1 and TRPC4, we observed a high basal expression that could neither be increased by $[Ca^{2+}]_{ex}$ nor by TE. Although Beck *et al.* (2008) described a differentiation-promoting effect of these channels in keratinocytes , different types of hPK might account for this discrepancy to our findings. In this work, our data provide strong evidence that TE induces differentiation of hPK via upregulation and indirect activation of TRPC6 by high $[Ca^{2+}]_{ex}$. We have observed that the cytoplasmatic staining of TRPC6 can be explained as follows. First, the used TRPC6 antibody from Santa Cruz (C-13) is raised against a peptide mapping the cytoplasmic C terminus of TRPC6 of human origin and may therefore partly show cytoplasmic staining. Second, receptors like the TRPC6 are transported from the ER to the Golgi apparatus and processed and packaged in secretory vesicles, before they are inserted in the plasma membrane by a fusion step of these secretory vesicles with the membrane. TRPC6 can therefore also be detectable in the cytoplasma. Third, if most of the cell membrane is covered with the antibody the staining might appear to be intracellular although it sticks more or less to the cell surface. TRPC6 silencing inhibited TE-induced expression of KRT10 and TGM *in vitro*. This is in line with our recently published findings that TRPC6 channel activation is essential for hPK differentiation (Muller *et al.*, 2008).

As apoptosis is involved in an epidermal turnover, stratum corneum formation, and removal of ultraviolet-damaged premalignant cells (Bowen *et al.*, 2003), we investigated the apoptosis-inducing effect of TE in skin explants *ex vivo*. Lippens *et al.* (2005) described DNA fragmentation in the distal epidermis as ''terminal differentiation'' leading to cornification of keratinocytes that establish a tight barrier of dead cells . They suggested this is different from classical ''programmed cell death'' (apoptosis), the suicidal programme aimed at eliminating individual cells. We observed that TE, in contrast to the cytotoxic apoptosis-inducing compound staurosporine, does not induce quantitative apoptosis throughout the epidermis but, in accordance with the terminal differentiation concept, specifically promotes DNA fragmentation of distal stratum granulosum keratinocytes. These cells correspond to senescent hPK *in vitro* that are highly susceptible to TE-induced apoptosis. This might explain the clearing of AK without pronounced side effects observed upon topical treatment with TE-oleogel *in vivo* (Huyke *et al.*, 2009). In contrast to TE-oleogel cytotoxic compounds established in the treatment of AK such as 5-fluorouracil induce quantitative destruction of the epidermis leading to inflammation and exudation before regeneration starts from the basal cell layers.

TRPC6 expression was increased after short-term incubation of skin explants with TE, eventually enhancing the normal epidermal differentiation process. Long-term treatment of AK with TE *in vivo* resulted in downgrading of dysplasia and normalization of the epidermal morphology (Huyke *et al.*, 2009). In AK the number of cells that enter mitosis is increased (Haake and Polakowska, 1993) and the expression of the differentiation marker KRT10 is diminished. As we have shown here, topical treatment of AK with TE for 3 months enhanced the expression of KRT10 and normalized the aberrant Ki67 expression, eventually leading to the elimination of neoplastic cells.

In summary, the findings from this work, together with the results of a previously published clinical study (Huyke *et al.*, 2009), suggest that in the treatment of epithelial skin cancer,

that is, AK, TE might represent a therapeutic strategy that acts by promoting keratinocyte differentiation. In this process, at least in part, upregulation of the non-selective cation channel TRPC6 seems to have a role.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies and dilutions were used for immunohistochemical stainings or FACS analysis: the primary polyclonal TRPC6 antibody (Chemicon, Schwalbach, Germany), 1:200; the polyclonal TRPV6 antibody (Chemicon), 1:500; the polyclonal TRPC4 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), 1:50; the polyclonal TRPC1 antibody (Santa Cruz Biotechnology), 1:500; the monoclonal KRT10 antibody (Santa Cruz Biotechnology), 1:1,000; the polyclonal Ki-67 antibody (DCS, Hamburg, Germany), 1:200; The secondary antibody multi-link-biotin, the streptavidin-HRP-label and the AEC-substrate were from Dako (Glostrup, Denmark) and were used according to the manufacturer's instruction. Another secondary antibody was the goat anti-rabbit-Cy 5 antibody (Dianova, Hamburg, Germany).

TE and TE-oleogel were prepared from birch bark as described (Laszczyk *et al.*, 2006) and were provided by Birken GmbH (Niefern-Öschelbronn, Germany). TE derived from birch bark mainly consists of betulin (⩾80% w/w), but also contains other triterpenes, such as betulinic acid (3%) and lupeol (2%) (Laszczyk *et al.*, 2006). Stock solutions of TE were prepared with dimethyl sulfoxide (Sigma-Aldrich, Taufkirchen, Germany). Staurosporine (Sigma-Aldrich) was dissolved in EtOH 70% v/v and was used at a concentration of 10 μM. All stock solutions were stored at −20 °C until use. Test concentrations were freshly prepared for each cell culture experiment using final non-toxic concentrations of 0.25% dimethyl sulfoxide or 0.5% EtOH in cell culture medium. The control was treated with solvent only. hPK were grown in keratinocyte medium (SFM medium) (Promo Cell, Heidelberg, Germany) containing 0.1 mM calcium.

Cell Culture

Human primary keratinocyte (hPK) were prepared from adult skin obtained from dermatological surgery and cultured according to the method of Rheinwald and Green (1975) in SFM medium. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. For calcium imaging cells were seeded in 6-well plates on glass cover slips. For RT-PCR studies, FACS or cytospin experiments, cells were allowed to attach for 24 h after trypsinization. Subsequently, SFM medium was replaced by SFM medium with 2 mM calcium or TE (10 μg ml^{-1}). After 24 h RT-PCR, FACS- or cytospin stainings were performed. Cytotoxicity and apoptosis assays with hPK were performed in 96-well plates according to the manufacturer's instructions. Cell culture conditions to generate proliferative, early confluent, late confluent and senescent hPK were adapted from Perera *et al.* (2006). After generation of the different hPK cell types, the cells were incubated for 24 h with 2 mM calcium, TE (10 μg ml^{-1}) or 10 μM staurosporine before the different assays were performed.

Apoptosis assay

Cells were evaluated for apoptosis using a cell death detection ELISA (Cell Death Detection ELISA^{PLUS}, Roche Molecular Biochemicals, Mannheim, Germany) as described (Schempp *et al.*, 2002). The principle of this test is the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates using biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies. All data are expressed as mean ± SD of at least three independent experiments.

Cytospins

Cells were trypsinized, washed twice in PBS, and centrifuged onto coated microscope slides using a cytospin centrifuge (Thermo Shandon, Astmoor, UK). For immunocytochemical analyses the cells were fixed with 4% formaldehyde and acetone, permeabilized with 0.5% Tween 20 (all from Merck KG, Darmstadt, Germany) and stained for TRPC1, TRPC4, TRPC6, TRPV6, and KRT10, using the LSAB method as described (Schempp *et al.*, 2005). Stainings without the primary antibodies served as control. Four random high power fields were enumerated at × 400 magnification. The final count per group represents mean ± SD.

Cell transfection

For TRPC6 knock-down studies with siRNA, hPK were cultured in 6-well plates on glass cover slips for 24 h and transiently transfected by addition of transfection cocktail containing 100 nM TRPC6 siRNA and 2.5 μg ml^{-1} lipofectamine 2,000 in 250 μl of Opti-MEM medium. As a control 100 nM siRNA control sequence with low GC content with its complementary sequences were transfected in the same procedure (all from Invitrogen, Karlsruhe, Germany). RT-PCR analysis was performed 2 days after transfection.

Reverse transcription PCR

Reverse transcription PCR was performed as described (Muller *et al.*, 2008). In brief, total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. First-strand cDNA was synthesized from 2 μg total RNA in 20 μl final volume using the Omniscript kit (Qiagen, Hilden, Germany) with random hexamer primers (Invitrogen). 2 μl aliquots of the reverse transcription solution were used as a template for specific PCR. The PCR primers (20 pmol each) used to amplify keratin 10 (KRT10), INV, TGM, and TRPC6 were already published (Muller *et al.*, 2008). Commercially available 18S rRNA primers (Ambion, Huntington, UK) were used as internal loading control and the predicted 18S (Classic II) band size was 324 bp. Changes in relative mRNA levels were obtained by relating each PCR product to its internal control. After gel electrophoresis quantification was archived with Easywin 32 software (Herolab) or ImageJ.

Fluorescence measurements

The intracellular calcium concentration $[Ca^{2+}]_i$ measurements in single cells were carried out using the fluorescence indicators fura-2-AM in combination with a monochromator-based imaging system (TILL Photonics, Martinsried, Germany or Attofluor Ratio Vision system) attached to an inverted microscope (Axiovert 100, Carl Zeiss, Oberkochen, Germany) as described earlier(Muller *et al.*, 2008). Fluorescence was excited at 340 and 380 nm. After correction for background fluorescence, the fluorescence ratio F_{340}/F_{380} was calculated.

Split-thickness skin organ culture

Punch biopsies (6-mm) containing epidermis and papillary dermis were prepared from dermatome-separated normal human skin,

obtained from dermatological surgery. The skin explants were floated in six-well plates in SFM medium (negative control), 2 mM calcium (positive control) or TE (10 μg ml^{-1}). After 24 h the cultures were terminated, fixed in paraformaldehyde and embedded in paraffin.

Skin biopsies from AK

Punch biopsies (4-mm) were obtained from AK lesions of four patients before therapy and after 3 months of treatment with TE-oleogel during a previously published study (Huyke *et al.*, 2009). In this trial TE-oleogel was applied extensively by the patients twice daily. The study was conducted according to the Helsinki Guidelines for good clinical practice. All patients gave their written informed consent and the protocol was approved by the ethics committee of the University of Freiburg, Germany and the German health authority (BfArM, Bonn, Germany). The biopsies were fixed in paraformaldehyde, embedded in paraffin and stored at room temperature until immunohistochemical analysis.

Detection of apoptosis in histological sections

Apoptotic DNA fragmentation was detected in paraffin-embedded skin explants using an *in situ* cell death detection kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). DNA fragmentation was monitored by labeling of DNA-3′-ends using the TUNEL method. To quantify the extent of apoptosis, five random high power fields of 3 μm tissue sections were counted for positively stained epidermal cells with a fluorescence microscope (Axioscope, Zeiss, Germany; ×400 magnification). In addition, the staining intensity of the flattened band-like DSGC was separately graded as follows: 0, negative; 1, focal staining; 2, moderate band-like staining; 3, vigorous band-like staining. The final count per group represents mean ± SD.

Immunohistochemistry

Sections (3 μm) of paraffin-embedded samples (biopsies or skin explants) were stained for Ki67, KRT10, and TRPC6 with the above listed primary and secondary antibodies using the LSAB method (DCS, Hamburg, Germany) as described (Schempp *et al.*, 2005). Stainings without the primary antibodies served as control. Five random fields of sections from ⩾3 independent skin explants or biopsies were enumerated at ×400 magnification. The final count per group represents mean ± SD.

Statistical analysis

Data were compared using the unpaired Student's *t*-test (two-tailed). Statistical significance was established at $P \leqslant 0.05$ (*), 0.01 (**) and 0.001 (***). Data are expressed as mean ± SD of at least three independent experiments.

CONFLICT OF INTEREST

MN Laszczyk and A Scheffler are company employees of Birken GmbH. CM Schempp is a consultant for Birken GmbH. The other authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Markus Braun-Falco for immunohistological photographs. The Competence Center skintegral is supported by grants from Software AG-Stiftung, Zukunftsstiftung Gesundheit, Dr Hauschka-Stiftung and WALA Heilmittel GmbH.

SUPPLEMENTARY MATERIAL

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

REFERENCES

Alakurtti S, Makela T, Koskimies S, Yli-Kauhaluoma J (2006) Pharmacological properties of the ubiquitous natural product betulin. *Eur J Pharm Sci* 29:1–13

Aslan F, Demirkesen C, Cagatay P, Tuzuner N (2006) Expression of cytokeratin subtypes in intraepidermal malignancies: a guide for differentiation. *J Cutan Pathol* 33:531–8

Beck B, Lehen'kyi V, Roudbaraki M, Flourakis M, Charveron M, Bordat P *et al.* (2008) TRPC channels determine human keratinocyte differentiation: new insight into basal cell carcinoma. *Cell Calcium* 43:492–505

Bowen AR, Hanks AN, Allen SM, Alexander A, Diedrich MJ, Grossman D (2003) Apoptosis regulators and responses in human melanocytic and keratinocytic cells. *J Invest Dermatol* 120:48–55

Cai S, Fatherazi S, Presland RB, Belton CM, Izutsu KT (2005) TRPC channel expression during calcium-induced differentiation of human gingival keratinocytes. *J Dermatol Sci* 40:21–8

Chaturvedi PK, Bhui K, Shukla Y (2008) Lupeol: connotations for chemoprevention. *Cancer Lett* 263:1–13

Cockerell CJ (2000) Histopathology of incipient intraepidermal squamous cell carcinoma ("actinic keratosis"). *J Am Acad Dermatol* 42:11–7

Eckert RL, Crish JF, Robinson NA (1997) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* 77:397–424

Eiznhamer DA, Xu ZQ (2004) Betulinic acid: a promising anticancer candidate. *IDrugs* 7:359–73

Frost CA, Green AC (1994) Epidemiology of solar keratoses. *Br J Dermatol* 131:455–64

Fulda S, Scaffidi C, Susin SA, Krammer PH, Kroemer G, Peter ME *et al.* (1998) Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. *J Biol Chem* 273:33942–8

Galgon T, Wohlrab W, Drager B (2005) Betulinic acid induces apoptosis in skin cancer cells and differentiation in normal human keratinocytes. *Exp Dermatol* 14:736–43

Green A, Whiteman D, Frost C, Battistutta D (1999) Sun exposure, skin cancers and related skin conditions. *J Epidemiol* 9:S7–13

Haake AR, Polakowska RR (1993) Cell death by apoptosis in epidermal biology. *J Invest Dermatol* 101:107–12

Huyke C, Reuter J, Rodig M, Kersten A, Laszczyk M, Scheffler A *et al.* (2009) Treatment of actinic keratoses with a novel betulin-based oleogel. A prospective, randomized, comparative pilot study. *J Dtsch Dermatol Ges* 7:128–34

Laszczyk M, Jager S, Simon-Haarhaus B, Scheffler A, Schempp CM (2006) Physical, chemical and pharmacological characterization of a new oleogel-forming triterpene extract from the outer bark of birch (betulae cortex). *Planta Med* 72:1389–95

Lehen'kyi V, Beck B, Polakowska R, Charveron M, Bordat P, Skryma R *et al.* (2007) TRPV6 is a Ca2+ entry channel essential for Ca2+-induced differentiation of human keratinocytes. *J Biol Chem* 282:22582–91

Leinonen PT, Hagg PM, Peltonen S, Jouhilahti EM, Melkko J, Korkiamaki T *et al.* (2009) Reevaluation of the normal epidermal calcium gradient, and analysis of calcium levels and ATP receptors in Hailey-Hailey and Darier epidermis. *J Invest Dermatol* 129:1379–87

Lippens S, Denecker G, Ovaere P, Vandenabeele P, Declercq W (2005) Death penalty for keratinocytes: apoptosis *versus* cornification. *Cell Death Differ* 12(Suppl 2):1497–508

Liu J (2005) Oleanolic acid and ursolic acid: research perspectives. *J Ethnopharmacol* 100:92–4

Markey AC, Lane EB, Churchill LJ, MacDonald DM, Leigh IM (1991) Expression of simple epithelial keratins 8 and 18 in epidermal neoplasia. *J Invest Dermatol* 97:763–70

Marks R, Motley RJ (1995) Skin cancer. Recognition and treatment. *Drugs* 50:48–61

Marks R, Rennie G, Selwood TS (1988) Malignant transformation of solar keratoses to squamous cell carcinoma. *Lancet* 1:795–7

Moy RL (2000) Clinical presentation of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol* 42:8–10

Muller M, Essin K, Hill K, Beschmann H, Rubant S, Schempp CM *et al.* (2008) Specific TRPC6 channel activation, a novel approach to stimulate keratinocyte differentiation. *J Biol Chem* 283:33942–54

Nickoloff BJ, Qin JZ, Chaturvedi V, Bacon P, Panella J, Denning MF (2002a) Life and death signaling pathways contributing to skin cancer. *J Investig Dermatol Symp Proc* 7:27–35

Nickoloff BJ, Qin JZ, Chaturvedi V, Denning MF, Bonish B, Miele L (2002b) Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* 9:842–55

Ortonne JP (2002) From actinic keratosis to squamous cell carcinoma. *Br J Dermatol* 146(Suppl 61):20–3

Perera RJ, Koo S, Bennett CF, Dean NM, Gupta N, Qin JZ *et al.* (2006) Defining the transcriptome of accelerated and replicatively senescent keratinocytes reveals links to differentiation, interferon signaling, and Notch related pathways. *J Cell Biochem* 98:394–408

Pillai S, Bikle DD, Mancianti ML, Cline P, Hincenbergs M (1990) Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. *J Cell Physiol* 143:294–302

Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA *et al.* (1995) Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat Med* 1:1046–51

Raj D, Brash DE, Grossman D (2006) Keratinocyte apoptosis in epidermal development and disease. *J Invest Dermatol* 126:243–57

Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H *et al.* (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO J* 20:3427–36

Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–43

Schempp CM, Kirkin V, Simon-Haarhaus B, Kersten A, Kiss J, Termeer CC *et al.* (2002) Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis. *Oncogene* 21:1242–50

Schempp CM, Kiss J, Kirkin V, Averbeck M, Simon-Haarhaus B, Kremer B *et al.* (2005) Hyperforin acts as an angiogenesis inhibitor. *Planta Med* 71:999–1004

Segre JA (2006) Epidermal barrier formation and recovery in skin disorders. *J Clin Invest* 116:1150–8

Selzer E, Pimentel E, Wacheck V, Schlegel W, Pehamberger H, Jansen B *et al.* (2000) Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. *J Invest Dermatol* 114:935–40

Smit JV, de Sevaux RG, Blokx WA, van de Kerkhof PC, Hoitsma AJ, de Jong EM (2004) Acitretin treatment in (pre)malignant skin disorders of renal transplant recipients: histologic and immunohistochemical effects. *J Am Acad Dermatol* 50:189–96

Tu CL, Oda Y, Komuves L, Bikle DD (2004) The role of the calcium-sensing receptor in epidermal differentiation. *Cell Calcium* 35:265–73

Tucci MG, Offidani A, Lucarini G, Simonelli L, Amati S, Cellini A *et al.* (1998) Advances in the understanding of malignant transformation of keratinocytes: an immunohistochemical study. *J Eur Acad Dermatol Venereol* 10:118–24