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ORIGINAL ARTICLE

Embryonic stem cell factors undifferentiated transcription factor-1 (UFT-1) and reduced expression protein-1 (REX-1) are widely expressed in human skin and may be involved in cutaneous differentiation but not in stem cell fate determination

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Summary

Undifferentiated transcription factor-1 (UTF-1) and reduced expression protein-1 (REX-1) are used as markers for the undifferentiated state of pluripotent stem cells. Because no highly specific cytochemical marker for epidermal stem cells has yet been identified, we investigated the expression pattern of these markers in human epidermis and skin tumours by immunohistochemistry and in keratinocyte cell cultures. Both presumed stem cell markers were widely expressed in the epidermis and skin appendages. Distinct expression was found in the matrix cells of the hair shaft. Differentiation of human primary keratinocytes (KC) *in vitro* strongly downregulated UTF-1 and REX-1 expression. In addition, REX-1 was upregulated in squamous cell carcinomas, indicating a possible role of this transcription factor in malignant tumour formation. Our data point to a role for these proteins not only in maintaining KC stem cell populations, but also in proliferation and differentiation of matrix cells of the shaft and also suprabasal KC.

Keywords
carcinoma, keratinocyte, reduced expression protein-1, stem cells, undifferentiated transcription factor-1

The integrity of the skin is maintained by epidermal stem cells that self-renew and generate daughter cells that undergo terminal differentiation (Fuchs 2008; Giangreco *et al.* 2008). These specialized adult stem cells are required for tissue replacement throughout the lifespan of an organism. In addition, they are involved in the development of squamous cell carcinomas (SCCs) and possibly benign skin tumours (Kamstrup *et al.* 2008). The potential therapeutic application of epidermal adult stem cells in various clinical settings has led to increasing interest in their exact localization within the basal layer of the epidermis and skin appendages, and in identifying reliable marker molecules for use in their isolation.

Bulge cells were the first adult stem cells of the hair follicle to be identified and were later shown to be capable of forming hair follicles, interfollicular epidermis and sebaceous glands (Ohyama 2007). In addition, according to the epidermal proliferating unit (EPU) model, stem cells located at the base of the interfollicular epidermis may give rise to distinct cell populations called EPUs. Epidermal proliferating units are well documented in murine thin epidermis and were recently also described in human skin preparations (Ghazizadeh & Taichman 2005; Strachan & Ghadially 2008). A third stem cell population resides at the base of sebaceous glands (Jones *et al.* 2007; Fuchs 2008; Giangreco *et al.* 2008). Although these stem cell populations have been investigated in detail, no pure epidermal stem cell cultures are available and no highly specific cytochemical marker has yet been identified.

To identify possible stem cell compartments of the skin, we looked for presumed markers of pluripotency, which may also be expressed in multipotent adult progenitor cells

(Jiang *et al.* 2002a,b). Undifferentiated transcription factor-1 (UTF-1) and reduced expression protein-1 (REX-1) are known to be highly and almost exclusively expressed during early embryogenesis and in human adult testes and germ cell neoplasms (Hosler *et al.* 1989; Okuda *et al.* 1998; Kristensen *et al.* 2008). Because their expression seems to be associated with stemness properties, they have been used extensively to characterize the undifferentiated stem cell state of cell populations *in vitro* (Domogatskaya *et al.* 2008; Jo *et al.* 2008; Kopp *et al.* 2008; Lee *et al.* 2008; Poloni *et al.* 2008).

The expression of UTF-1, REX-1, Sox-2 and Nanog of murine keratinocytes (KC) has been reported to be induced by transfection with the pluripotency regulator, Oct-4 (Grinnell *et al.* 2007). In addition, factors secreted from embryonic stem cells can stimulate the expression of Oct-4 and its target transcripts in murine KC (Grinnell & Bickenbach 2007), indicating a role of these pluripotency markers in skin homeostasis. Recently, expression of hRex-1 mRNA was detected in normal human epidermal KC, SCCs as well as in a variety of other epithelial cell types and tumour cell lines (Mongan *et al.* 2006). However, the expression pattern of these stem cell markers in human skin is still unknown. In this study, we set out to investigate the expression pattern of the presumed pluripotency markers UTF-1 and REX-1 in regular and inflamed epidermis as well as in skin tumours.

Methods

Cell culture

Primary human KC (Lonza, Basel, Switzerland) were cultured as described previously (Mildner *et al.* 2006). As a positive control for the expression of stem cell markers, we used the human embryonic carcinoma cell line NTera-2.

Tissue samples

Formalin-fixed, paraffin-embedded tissue of regular skin ($n = 6$), psoriasis ($n = 6$), eczema ($n = 6$), basal cell carcinoma ($n = 15$) and SCC ($n = 19$) were derived from the files of the Institute for Clinical Pathology and Department of Dermatology, Medical University of Vienna.

Immunohistochemistry

Samples were immunostained for UTF-1 and REX-1 using monoclonal antibodies MAB4337 (Chemicon, Billerica, MA, USA) and MAB3598 (R&D, Minneapolis, MN, USA), respectively. Antigen retrieval was performed by boiling sections in a microwave oven (600 W) in 1 mM EDTA buffer (pH 8.0) for 20 min. Primary antibody was applied at 1:300 (UTF-1) and 1:2000 (REX-1) dilutions overnight, followed by anti-mouse or anti-rat biotinylated IgG (Dako, Glostrup, Denmark) and subsequently a streptavidin/AP kit (Dako). The enzyme reaction was developed with an alkaline phosphatase substrate kit (Biogenex, San Ramon, CA, USA). Negative controls were carried out on consecutive tissue sections using isotype-matched control reagents [normal mouse IgG and normal rat IgG-HRP, both Santa Cruz Biotechnology, Santa Cruz, CA 95060, USA and streptavidin/HRP kit (Dako)]. A semi-quantitative assessment of the intensity of expression was performed independently by two investigators (no staining = 0; weakly positive = 1; moderately positive = 2; strongly positive = 3).

RNA isolation and cDNA preparation

Cells were seeded in 12-well plates (Corning Incorporated, Corning, NY, USA) and were harvested at 50–80% confluence (preconfluent) or 1–4 days after reaching confluence (postconfluent). After lysis by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA extraction following the manufacturer's instructions, cDNAs were transcribed with the GeneAmp Kit using MuLV-reverse transcriptase and oligo(dT) primers (Applied Biosystems, Foster City, CA, USA) as indicated in the instruction manual.

Quantitative real-time PCR

Reduced expression protein-1 mRNA expression was quantified by real-time PCR with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. The primers for REX-1 (forward: 5′-ACTACTCGGCCCTGGAG AAG-3′, reverse: 5′-AAAGCCCCTTCTCCTCACTC-3′) and β-2-microglobulin (B2M, forward: 5′-GATGAGTATGCCT GCCGTGTG-3′, reverse: 5′-CAATCCAAATGCGGCATCT-3′) were designed as described previously (Kadl *et al.* 2002). The relative expression of the target genes was calculated by comparison with the housekeeping gene B2M using a formula described by Wellmann *et al.* (2001). The efficiencies of the primer pairs were determined as described in Kadl *et al.* (2002).

Western blotting

Cells seeded in six-well plates (Corning Incorporated) were lysed in SDS–PAGE loading buffer either at 50–80% confluence (preconfluent) or 1–4 days after reaching confluence (postconfluent). Alternatively, KC differentiation was induced by the addition of 1.2 mM $CaCl_2$ to KC growing at 30% confluency for 24 h.

Nuclear and cytoplasmic extracts were prepared as described previously (Harant *et al.* 1996). Western blot analysis was performed as described previously (Mildner *et al.* 1999). Briefly, lysates were electrophoresed through an 8–18% gradient polyacrylamide gel (GE Healthcare, Solingen, Germany) and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Anti-UTF-1 (1:500), anti-REX-1 (1:500) and anti-filaggrin (1:1000; Neomarkers, Lab Vision Corporation, Fremont, CA, USA) monoclonal antibodies were used as first-step antibodies, and HRP-conjugated goat anti-mouse IgG (Amersham Life Science, Amersham, UK) or goat anti-rat IgG (Pierce, Rockford, IL,

USA) were used as second-step reagent. Reaction products were detected with the ChemiGlow reagent (Biozyme Laboratories, South Wales, UK) according to the manufacturer's instructions.

Results

UTF-1 and REX-1 are constitutively expressed in the human epidermis and skin appendages

Undifferentiated transcription factor-1 was constitutively expressed in both basal and suprabasal KC. The staining was confined to nuclei, and the intensity was weak to moderate and mostly homogenous. However, suprabasal KC usually showed a stronger staining than basal cells (Figure 1, panel a).

We found a distinct staining for UTF-1 in the pool of undifferentiated matrix cells in the hair follicle shaft (Figure 1, panel b) that was mainly faint in the adjacent inner and outer root sheath. By contrast, KC of the adjacent upper bulb and lower follicle were negative or only weakly positive for UTF-1. A heterogeneous staining was also found in mesenchymal cells of the dermal papilla (Figure 1, panel b). Notably, UTF-1 expression was not distinctly stronger in the basal layer of the hair shaft of the isthmus region that comprises stem cell populations of the bulge (Figure 1, panel c).

The expression pattern of sebocytes was more variable. Basal cells of these glands expressed UTF-1 predominantly weakly or were negative for this protein, whereas the expression intensity of differentiated sebocytes varied from negative to sometimes distinctly positive (Figure 1, panel d). In addition, nuclei of eccrine glands were negative to weakly positive for UTF-1 and showed a heterogeneous staining for this protein (not shown).

Reduced expression protein-1 staining was also confined to nuclei and weak to moderate in regular epidermis. In contrast to UTF-1 expression, basal KC were negative or weakly positive and suprabasal KC distinctly positive for REX-1 (Figure 2, panel a). Similar to UTF-1 expression, REX-1 was distinctly expressed in matrix cells of the shaft (Figure 2, panel b). A distinct REX-1 staining was also found in cells of the inner and outer root sheath. Basal cells of the hair bulge expressed REX-1 weakly and heterogeneously but were not obviously more strongly positive than other KC of the shaft.

Mesenchymal cells of the dermal papilla were negative or weakly positive. A minority of differentiated sebocytes strongly stained for REX-1, whereas undifferentiated basal cells were mostly weakly positive or negative. Eccrine glands were mostly weakly positive or negative, and luminal cells expressed REX-1 slightly stronger (not shown).

The expression of UTF-1 and REX-1 was not clearly regulated in eczema and psoriasis as compared to normal epidermis (data not shown). Negative controls were carried out on consecutive tissue sections using isotype-matched control reagents and yielded negative results (Figure S1).

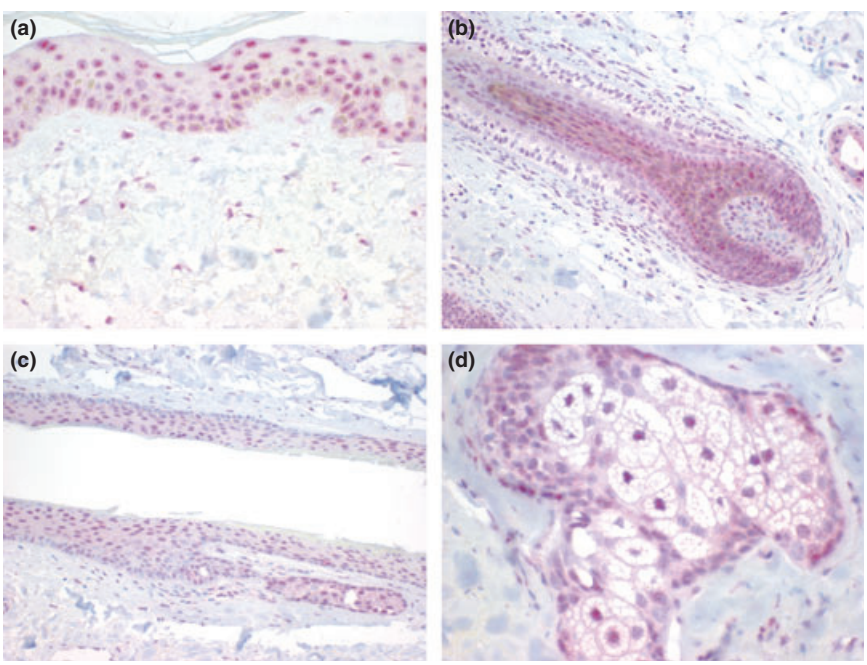

Figure 1 Undifferentiated transcription factor-1 (UTF-1) is homogeneously expressed in human skin. Normal epidermal keratinocytes express UTF-1 constitutively, and this expression is sometimes stronger suprabasally (a). Undifferentiated matrix cells of the hair shaft are strongly positive for UTF-1 (b), whereas cells of the bulge region are negative or weakly positive for UTF-1 (c). In addition to undifferentiated basal cells, UTF-1 is also expressed in sebocytes (d).

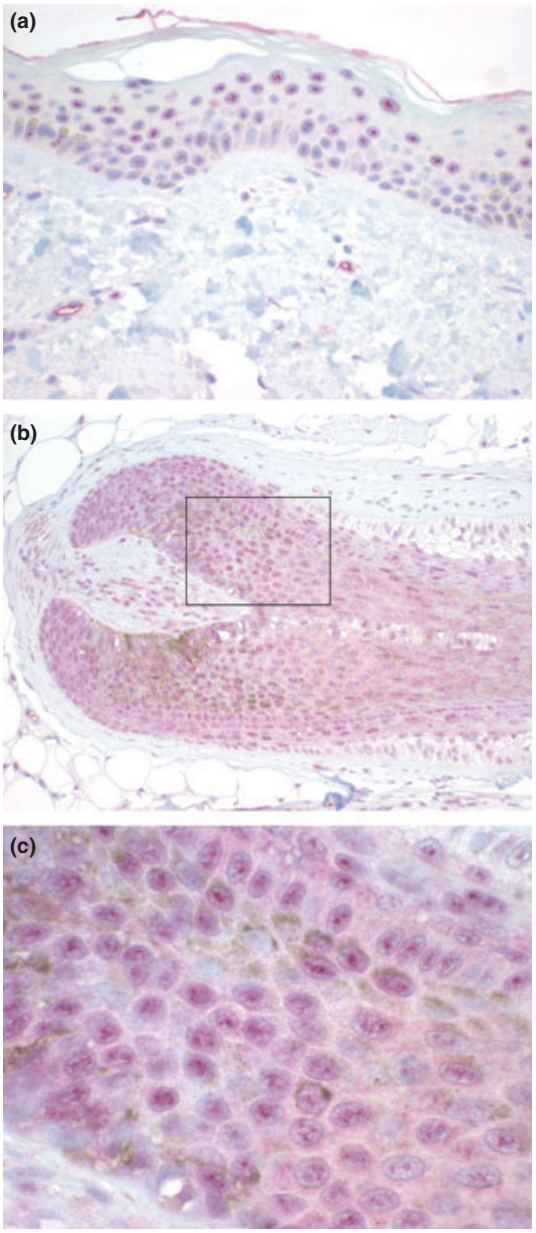

Figure 2 Expression of REX-1 in human skin. Suprabasal keratinocytes (KC) are distinctly positive for REX-1 in contrast to basal KC (a). Similarly, REX-1 is distinctly expressed in matrix cells of the hair shaft (b). A detailed view of a part of the upper image illustrates that undifferentiated shaft matrix cell KC (left upper region) show stronger REX-1 expression than KC of the upper bulb (right side of the picture (c).

REX-1 but not UTF-1 is upregulated in human epidermal neoplasias

Undifferentiated transcription factor-1 expression in basal cell carcinomas was homogeneous but weak in 7/13 and moderate in a further six samples. Expression of UTF-1 in SCC was moderate and similar to regular epidermis in all tested specimens (weak: $n = 4$, moderate: $n = 11$, strong: $n = 4$, compare Figure 3a,b). Basal cells were distinctly positive, whereas termi-

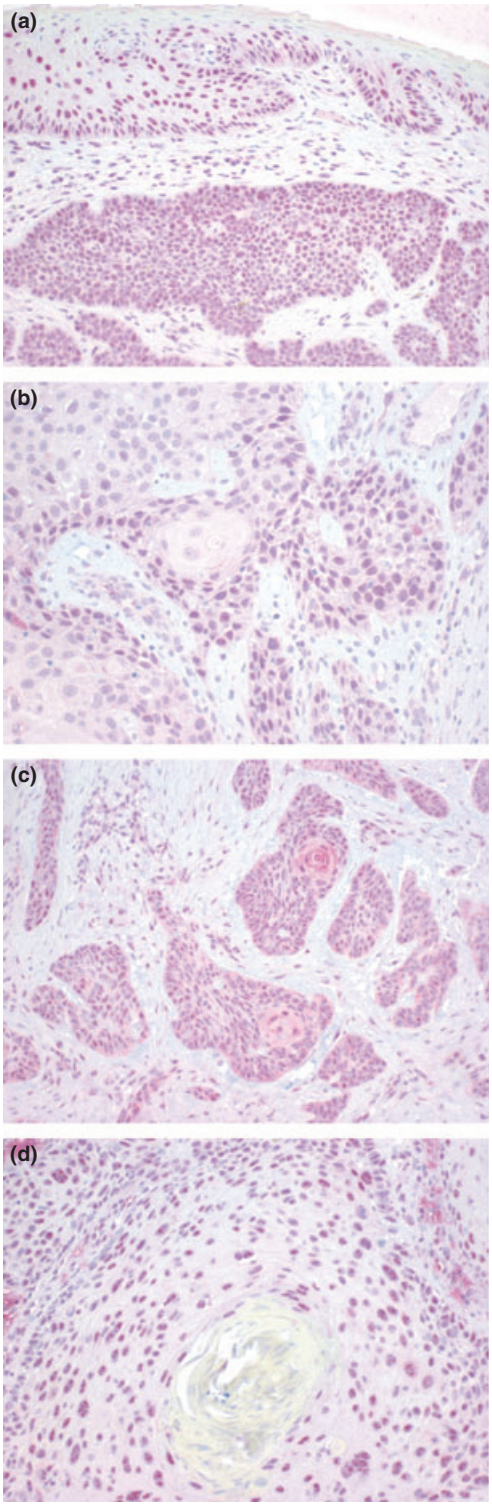

Figure 3 REX-1 but not undifferentiated transcription factor-1 (UTF-1) is upregulated in squamous cell carcinomas (SCCs). Undifferentiated transcription factor-1 is expressed in basal cell (a) and SCCs (b), the latter with a distribution similar to regular epidermis. Note that differentiating tumour cells become negative for UTF-1 (b). Moderate expression of REX-1 can be found constitutively in all basal cell (c) and SCCs (d) investigated, and its expression is focally strong in carcinomas.

nally differentiated tumour cells were mostly negative (Figure 3, panel b). Mitotic cells showed strong cytoplasmic staining for UTF-1 as has been described for the stem cell marker BMI-1 (Reinisch *et al.* 2007; not shown).

Whereas the expression intensity of REX-1 in basal cell carcinomas was slightly weaker or identical to that of suprabasal KC in 4/15 tumours, moderate expression was found in 9/15 (Figure 3, panel c) and strong expression in 2/15 basal cell carcinomas. By contrast, REX-1 staining was strong in 10/19 SCCs. In these tumours, REX-1 was distinctly upregulated as compared to normal skin (Figure 3, panel d), whereas in one carcinoma, only weak REX-1 expression was found. No correlation was found between grading and expression of UTF-1 and REX-1 (not shown).

Differentiation of human primary KC in vitro *downregulates UTF-1 and REX-1 expression*

Undifferentiated transcription factor-1 and REX-1 were constitutively expressed in human KC *in vitro* (Figure 4).

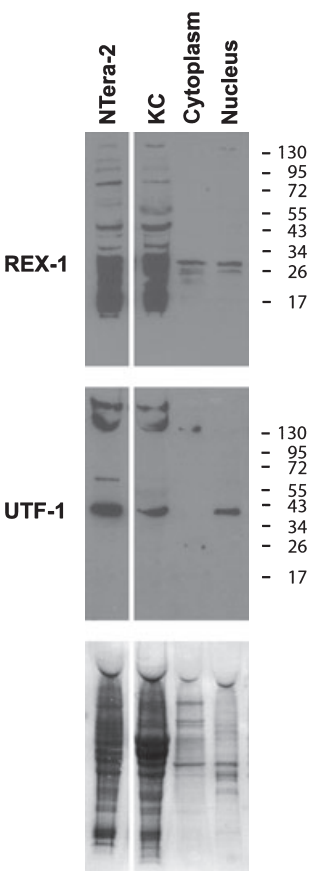

Figure 4 Undifferentiated transcription factor-1 (UTF-1) and REX-1 are regularly expressed in human keratinocytes (KC) *in vitro*. Whereas REX-1 is expressed in both the cytoplasm and nuclear fraction (upper panel), UTF-1 expression is only found in the nuclear fraction of KC by Western blotting (middle panel). Ponceau staining of the membranes demonstrates loading of equal protein amounts (lower panel). One representative experiment of two is shown.

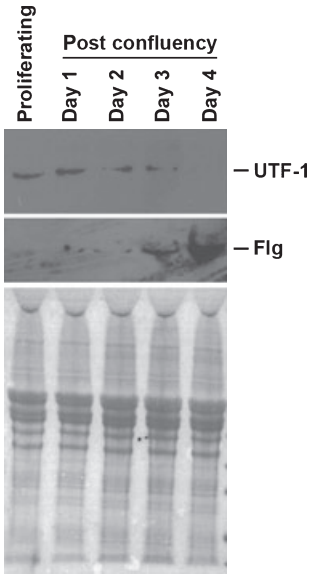

Figure 5 Undifferentiated transcription factor-1 (UTF-1) expression by keratinocytes (KC) is downregulated by confluency. Differentiation of KC results in downregulation of UTF-1 (upper panel) and upregulation of pro-filaggrin (middle panel). Ponceau staining of the membranes demonstrates loading of equal protein amounts (lower panel). One representative experiment of two is shown.

Whereas REX-1 was found in both the cytoplasm and the nucleus, localization of UTF-1 was restricted to the nucleus. The embryonal carcinoma cell line NTera-2 served as a positive control. The strong background observed in Western blots of REX-1 was also found using isotype-matched control IgG (data not shown). Differentiation of KC was induced by confluency as described previously (Chaturvedi *et al.* 1999). UTF-1 was distinctly downregulated at 2 days

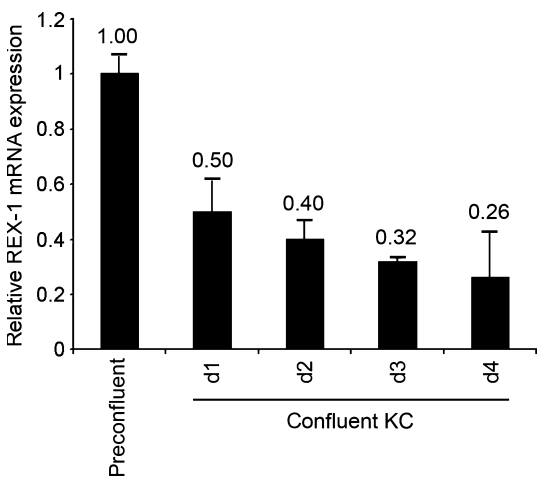

Figure 6 REX-1 mRNA is downregulated in differentiating keratinocytes (KC). Confluency of primary KC results in a distinct downregulation of REX-1 mRNA as detected by rtPCR. One representative experiment of two is shown.

postconfluency and was even undetectable at 4 days postconfluency (Figure 5). The induction of differentiation of KC by confluency was confirmed by expression of filaggrin, a component of the granular cell layer of the epidermis. Because Western blotting of whole cell lysates for REX-1 resulted in distinct background staining, the regulation of REX-1 expression was investigated at the mRNA level by quantitative real-time PCR. As shown in Figure 6, REX-1 mRNA was already strongly downregulated at 2 days postconfluency. Similarly, the addition of $CaCl_2$ to KC growing at low density led to a strong downregulation of both UTF-1 and REX-1 in a further experiment (data not shown), confirming that indeed differentiation of KC and not contact inhibition induced the downregulation of these stem cell markers.

Discussion

Epidermal stem cells (ES) are fundamental for maintenance of the skin; however, because of the lack of specific molecular markers, their exact location is still controversial. Undifferentiated transcription factor-1 and REX-1 have been suggested as markers for human ES cells that may also contribute to the 'stemness' phenotype (Richards *et al.* 2004). Therefore, we have here evaluated whether UTF-1 and REX-1 expression may indicate stem cell properties in the epidermis.

We show that UTF-1 and REX-1 are constitutively expressed in both the epidermis and skin appendages. The specificity of our immunohistochemical staining was verified by Western blotting and rtPCR. The widespread expression of these markers demonstrates that they do not indicate stem cell populations or have a function in the maintenance of epidermal stem cells. As the function of UTF-1 and REX-1 in KC has not yet been evaluated, we have to use data obtained in other tissues.

Undifferentiated transcription factor-1 is specifically expressed in the inner cell mass and primitive ectoderm of murine embryos (Okuda *et al.* 1998). The UTF1 protein was shown to repress transcription (Fukushima *et al.* 1999) and to display characteristics expected for a tissue-specific transcriptional coactivator (Okuda *et al.* 1998). It is involved in the maintenance of embryonic stem cells in a stem cell state, can induce rapid proliferation of embryonic stem cells and promote teratoma formation of these cells (Nishimoto *et al.* 2005). In addition, knock-down of UTF1 in embryonal stem and carcinoma cells resulted in a substantial delay or block in differentiation (van den Boom *et al.* 2007). This broad range of functions in the proliferation, differentiation and development of malignancy of ES may similarly play a role in KC; however, the expression pattern found in a large sample of skin diseases does not point to a specific function. A distinct cytoplasmic UTF-1 expression has also been observed in mitotic cell division similar to the strong expression in dividing KC, and the role of this protein in cell division is also not yet clear.

Reduced expression protein-1 was cloned from mouse F9 teratocarcinoma cells and identified as a gene encoding a zinc finger transcription factor exhibiting reduced expression in the presence of retinoic acid (Hosler *et al.* 1989). Furthermore, REX-1 has been identified in human embryonic stem cells (Henderson *et al.* 2002) and in several multipotent adult progenitor cells isolated from bone marrow, muscle and brain (Jiang *et al.* 2002a,b). Reduced expression protein-1 has been shown to play a role in stem cell differentiation (Thompson & Gudas 2002), but is dispensable for both their maintenance and pluripotency in murine embryos (Masui *et al.* 2008).

The expression pattern of UTF-1 and REX-1 in human skin suggests that these proteins may no longer be related to multipotency, but rather to differentiation of matrix cells of the hair shaft and interfollicular KC. Therefore, these proteins functioning in stem cell regulation at the earliest stages of embryogenesis may serve different roles in the skin of adult organism. Epidermal target genes responsible for the role of REX-1 in differentiation have not been identified, but similar to carcinoma stem cells, the modulation of the JAK/STAT pathway (Nishio *et al.* 2001; Xu *et al.* 2008) may be engaged. The distinct REX-1 expression in SCCs is consistent with a function in epithelial tumour development; however, the role of this protein in malignancy requires further investigation.

In summary, we show that the presumed stem cell markers UTF-1 and REX-1 are not specifically restricted to stem cells in the skin, but are widely expressed in both the epidermis and skin appendages. Moreover, our data point to roles for these proteins not in maintaining KC stem cell populations, but rather in differentiation of matrix cells of the shaft as well as in suprabasal KC and sebaceous glands. The upregulation of REX-1 in SCCs points to a role in malignant tumour formation.

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Conflict of interest

We declare that we have no conflict of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. IgG Isotyp control staining with hematoxylin counterstaining.

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