

CD271 Mediates Stem Cells to Early Progeny Transition in Human Epidermis

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CD271 is the low-affinity neurotrophin (p75NTR) receptor that belongs to the tumor necrosis factor receptor superfamily. Because in human epidermis, CD271 is predominantly expressed in transit-amplifying (TA) cells, we evaluated the role of this receptor in keratinocyte differentiation and in the transition from keratinocyte stem cells (KSCs) to progeny. Calcium induced an upregulation of CD271 in subconfluent keratinocytes, which was prevented by CD271 small interfering RNA. Furthermore, CD271 overexpression provoked the switch of KSCs to TA cells, whereas silencing CD271 induced TA cells to revert to a KSC phenotype, as shown by the expression of β_1 -integrin and by the increased clonogenic ability. CD271⁺ keratinocytes sorted from freshly isolated TA cells expressed more survivin and keratin 15 (K15) compared with CD271⁻ cells and displayed a higher proliferative capacity. Early differentiation markers and K15 were more expressed in the skin equivalent generated from CD271⁺ TA than from those derived from CD271⁻ TA cells. By contrast, late differentiation markers were more expressed in skin equivalents from CD271⁻ than in reconstructs from CD271⁺ TA cells. Finally, skin equivalents originated from CD271⁻ TA cells displayed a psoriatic phenotype. These results indicate that CD271 is critical for keratinocyte differentiation and regulates the transition from KSCs to TA cells.

Journal of Investigative Dermatology (2015) **135**, 786–795; doi:10.1038/jid.2014.454; published online 20 November 2014

INTRODUCTION

A fine balance of proliferation, differentiation, and apoptosis is required to maintain epidermal homeostasis (Livshits *et al.*, 2012). Continuous epidermal regeneration is accomplished by adult stem cells that are slow-cycling, possess the capacity of self-renew, and remain undifferentiated (Pincelli and Marconi, 2010). Keratinocyte stem cells (KSCs) reside in the basal layer within a microenvironment called niche (Fuchs and Horsley, 2011) and generate transit-amplifying (TA) cells that undergo a limited number of cell divisions before committing to terminal differentiation (Doupé and Jones, 2012). Differentiation begins when cells withdraw from the cell cycle, exit the niche, and detach from the basement membrane and also as a function of β_1 -integrin levels (Watt, 2002; Mulder *et al.*, 2012). The basal-to-suprabasal switch is associated with a change in keratin

expression and is regulated by a number of genes (Estrach *et al.*, 2008; Fuchs *et al.*, 2009; Suzuki and Senoo, 2012). Yet, the mechanisms triggering the transition from KSCs to TA cells are not fully clarified.

Neurotrophin (NT) functions are mediated by two classes of transmembrane receptors, the low-affinity NT CD271 and the tyrosine kinase (Trk) family of receptors. Although Trk receptors mainly control survival and proliferative functions, CD271 can also act independently of Trk by regulating its own signaling events (Blöchl and Blöchl, 2007). CD271 belongs to the tumor necrosis factor receptor superfamily and shares with the other members of the family the so-called “death domain” that mediates apoptosis (Chao and Bothwell, 2002).

In normal human skin, a NT network fulfills different autocrine and paracrine functions, as most cells produce and release NT and express their receptors (Botchkarev *et al.*, 2006). In human keratinocytes, the NT nerve growth factor is mainly expressed in KSCs (Marconi *et al.*, 2003) and stimulates cell proliferation and survival through TrkA receptor (Pincelli and Marconi, 2000), thus contributing to the maintenance of “stemness”. On the other hand, the NT brain-derived neurotrophic factor and NT-4 induce keratinocyte apoptosis through CD271 (Truzzi *et al.*, 2011).

Psoriasis is a chronic skin disease characterized by keratinocyte hyperproliferation, abnormal differentiation, and reduced apoptosis (Nickoloff *et al.*, 2007), thus representing a suitable model for the study of epidermal homeostasis. Nerve growth factor and TrkA are overexpressed (Fantini *et al.*, 1995), whereas CD271 protein is absent in psoriatic lesions (Truzzi *et al.*, 2011).

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Abbreviations: CD271⁺ TA cell, CD271⁻ transit-amplifying cell; CD271⁻ TA cell, CD271⁻ transit-amplifying cell; CD271, p75 neurotrophin receptor; KSC, keratinocyte stem cell; K10, keratin 10; K15, keratin 15; TA cell, transit-amplifying cell

Received 21 February 2014; revised 16 September 2014; accepted 5 October 2014; accepted article preview online 20 October 2014; published online 20 November 2014

Because CD271 is predominantly expressed in TA (Truzzi *et al.*, 2011), we reasoned that CD271 could be involved in KSC/TA transition and differentiation. We present evidence that CD271 is expressed in a population of “early” TA cells and regulates keratinocyte differentiation. Moreover, CD271 drives the transition from KSCs to TA cells. Finally, CD271⁺ TA cells exhibit a differentiative capacity in three-dimensional skin reconstructs, whereas CD271⁻ TA cells generate a psoriasiform skin equivalent model.

RESULTS

CD271 mediates keratinocyte differentiation *in vitro*

To evaluate CD271 expression during keratinocyte differentiation, primary keratinocytes were cultured in serum-containing medium and studied under subconfluent, preconfluent, confluent, and post-confluent conditions. Culture confluency, in the presence of serum, was associated with irreversible growth arrest and commitment to differentiation (Supplementary Figure S1 online). CD271 protein levels were absent in subconfluent cells, appeared in low levels in preconfluent keratinocytes, and markedly increased in confluent cells, whereas they decreased in post-confluent keratinocytes. This was paralleled by the upregulation of the differentiation markers cytokeratin 10 (K10) and involucrin in confluent keratinocytes. Moreover, TrkA receptor, which is mainly expressed by proliferating keratinocytes, appeared to be downregulated when cells

were induced to differentiate (Figure 1a and Supplementary Figure S2A online). Furthermore, calcium exposure (Yuspa *et al.*, 1988) in serum-free medium induced a morphological change in subconfluent keratinocytes that exhibited a cuboidal shape with close packing suggestive of cell–cell contact formation (Figure 1b). Calcium also triggered the upregulation of K10 and involucrin, as shown by western blotting (WB). On the other hand, confluent keratinocytes, cultured under low calcium conditions, appeared to be less differentiated, as confirmed by the low levels of K10 and involucrin. Markers of proliferation, such as cytokeratin 15 (K15) and survivin were downregulated both in subconfluent keratinocytes with calcium and in confluent keratinocytes. CD271 was upregulated in subconfluent keratinocytes under high calcium conditions, similarly to other differentiation markers. CD271 was also upregulated in confluent keratinocytes, although to a lesser extent, mostly because of the lack of calcium (Figure 1c and Supplementary Figure S2B online). Calcium-induced CD271 overexpression in subconfluent keratinocytes was confirmed by real-time PCR (Figure 1d) and by flow cytometry (Figure 1e). When CD271 was silenced by specific CD271 small interfering RNA (siRNA), calcium treatment only partially induced keratinocyte differentiation, as shown by the reduced levels of involucrin, K10, and epidermal fatty acid-binding protein (Figure 1f and Supplementary Figure S2C online). In addition, upon

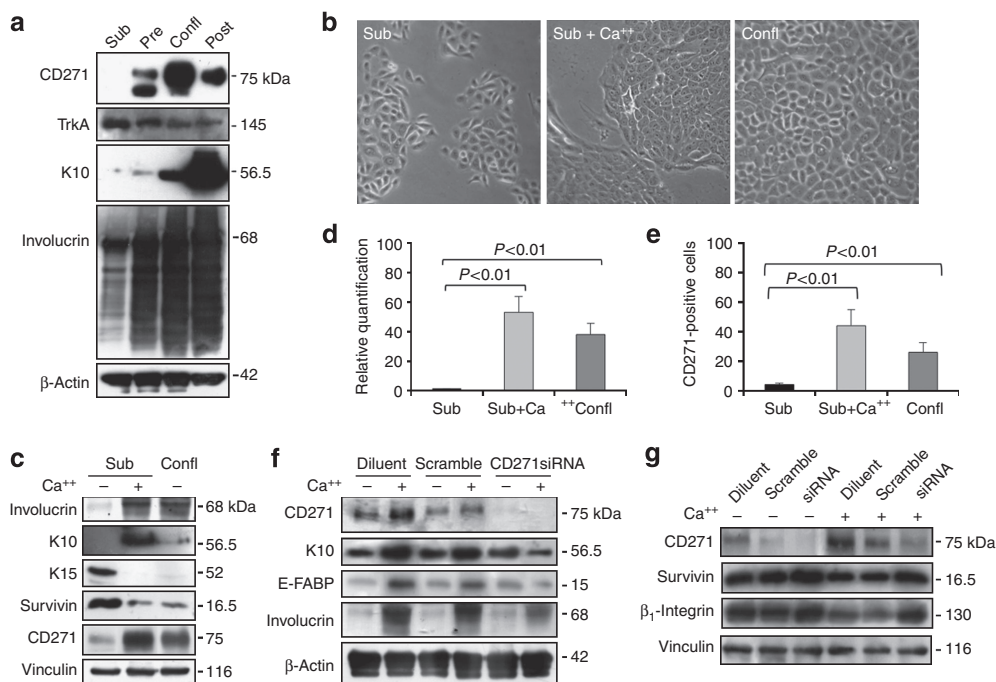


Figure 1. CD271 regulates keratinocyte differentiation *in vitro*. (a) Keratinocytes were cultured in DMEM containing 10% fetal bovine serum. Protein extracts from the subconfluent (sub), preconfluent (pre), confluent (confl), and post-confluent (post) keratinocytes were immunoblotted. (b) Keratinocytes were cultured with keratinocyte growth medium, and subconfluent cells were induced to differentiate with 1.8 mM Ca⁺⁺ for 48 hours. (c) Protein extracts from the different culture conditions were immunoblotted. (d) A real-time PCR was performed on RNA extracts from the three culture conditions by using primers for CD271. Subconfluent keratinocytes were used as a calibrator. Student's *t*-test was performed between samples and calibrator. (e) Keratinocyte were stained with mouse anti-CD271 mAb and analyzed by flow cytometry. (f, g) Keratinocytes were transiently transfected with 50 nM CD271 small interfering RNA and treated with 1.8 mM Ca⁺⁺ for 48 hours. Protein extracts were immunoblotted. E-FABP, epidermal fatty acid-binding protein; K10, keratin 10; K15, keratin 15; Trk, tyrosine kinase.

treatment with CD271, siRNA levels of survivin and β_1 -integrin were partially increased (Figure 1g and Supplementary Figure S2C online). When keratinocytes were given calcium and silenced with CD271 siRNA, they appeared less cuboidal and packed in shape (Supplementary Figure S1B online). Taken together, these results suggest that CD271 mediates human keratinocyte differentiation.

CD271 mediates KSC-progeny and progeny-KSC transition

Because CD271 is mostly expressed in TA cells (Truzzi et al., 2011) and mediates keratinocyte differentiation, we wanted to evaluate whether it is involved in the early steps of KSC transition to progeny. Overexpression of CD271 induced KSC to turn to a TA phenotype, as shown by the upregulation of K10 and involucrin, and by the downregulation of survivin and β_1 -integrin, whereas K15 remained unchanged (Figure 2a and Supplementary Figure S3A online). On the other hand, silencing of CD271 converted TA cells into a KSC phenotype, as shown by the upregulation of β_1 -integrin, survivin, and K15, and the downregulation of K10 and involucrin (Figure 2b and Supplementary Figure S3B online). In addition, TA cells silenced for CD271 displayed a higher clonogenic ability, if compared with mock-treated TA cells (Figure 2c and d). These results indicate that CD271 has a critical role in KSC–TA transition.

CD271⁺ TA cells are early differentiated keratinocytes

We have previously shown that CD271 displays an irregular pattern of expression in basal keratinocytes (Truzzi et al., 2011). Here, we demonstrated that, in normal human epidermis, CD271⁺ cells partially express KSC markers, similar to survivin and K15 (Figure 3a). This seems to suggest that CD271 identifies a subpopulation of basal cells that still retain some features of KSCs. To better characterize this subpopulation, we sorted CD271⁺ keratinocytes from freshly isolated TA cells by flow cytometry and compared them with CD271⁻ TA cells or with freshly isolated KSCs. As shown by confocal analysis, only few K10 cells were detected in the CD271⁺ TA cells and in KSC population. On the contrary, most CD271⁻ TA cells expressed K10. K15 and survivin were more expressed in CD271⁺ TA cells and in KSCs than in CD271⁻ TA cells (Figure 3b and c). WB confirmed that freshly isolated CD271⁺ TA cells contain lower levels of K10 than the CD271⁻ TA keratinocytes. Finally, freshly isolated CD271⁺ TA cells expressed more survivin and K15 compared with CD271⁻ TA cells (Figure 3d and e). Taken together, these results suggest that CD271⁺ TA cells have just started the differentiation process, whereas CD271 is lost as keratinocytes proceed toward terminal differentiation (Figure 3f).

CD271⁺ TA cells display a high proliferative potential

In normal human epidermis, very few CD271⁺ cells express Ki67 (Figure 4a), possibly indicating that most CD271⁺ keratinocytes are still quiescent cells *in vivo*. To better analyze the function of CD271, freshly isolated CD271⁺ and CD271⁻ TA cells were compared with freshly isolated KSCs or with total TA cells, used as a control. CD271⁺ TA cells showed a lower proliferative potential compared with KSCs.

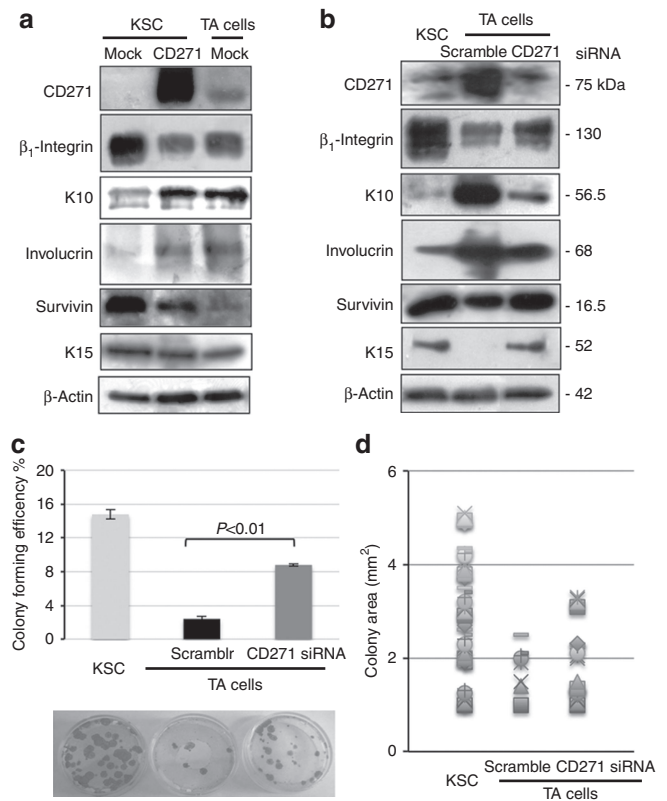


Figure 2. CD271 regulates keratinocyte stem cell (KSC)-progeny and progeny-KSC transition. (a) KSCs were infected with mock or CD271 retroviral vector, and cell lysates were immunoblotted. (b) Transit-amplifying (TA) cells were transiently transfected with 50 nM CD271 small interfering RNA (siRNA) and protein extracts were immunoblotted. Results were compared with KSC protein levels. (c, d) Colony forming efficiency and colony areas were determined on freshly isolated KSCs, siRNA, or scrambled CD271-transfected TA cells, as described in Materials and Methods section. K10, keratin 10; K15, keratin 15.

Yet, proliferative capacity was significantly higher in CD271⁺ TA cells than in CD271⁻ TA cells or in total TA cells, as shown by colony number and area (Figure 4b and c). Moreover, CD271⁺ TA cells proliferated significantly more compared with CD271⁻ TA cells or total TA cells (Figure 4d) and yielded a higher number of cells during long-term proliferation assay (Figure 4e). Human basal keratinocytes produce biologically active NTs and express TrkA and TrkC (Marconi et al., 2003). TrkA and TrkC that mediate proliferation in keratinocytes were slightly more expressed in KSCs than in TA cells, whereas they tended to decrease in post-mitotic keratinocytes. TrkB was equally expressed in KSCs and TA cells, whereas it decreased in post-mitotic cells (Figure 4f and Supplementary Figure S7A online). TrkA and TrkC were more expressed in CD271⁺ TA cells than in the negative cells, whereas TrkB was equally expressed in the subpopulations (Figure 4g and Supplementary Figure S7B online). Survivin and cyclin B expression is higher in CD271⁺ than in CD271⁻ TA cells. The addition of NTs caused CD271⁺ TA cells to proliferate more than CD271⁻ TA cells, as shown by the upregulation of survivin and cyclinB (Figure 4h and Supplementary Figure S7C online). This is

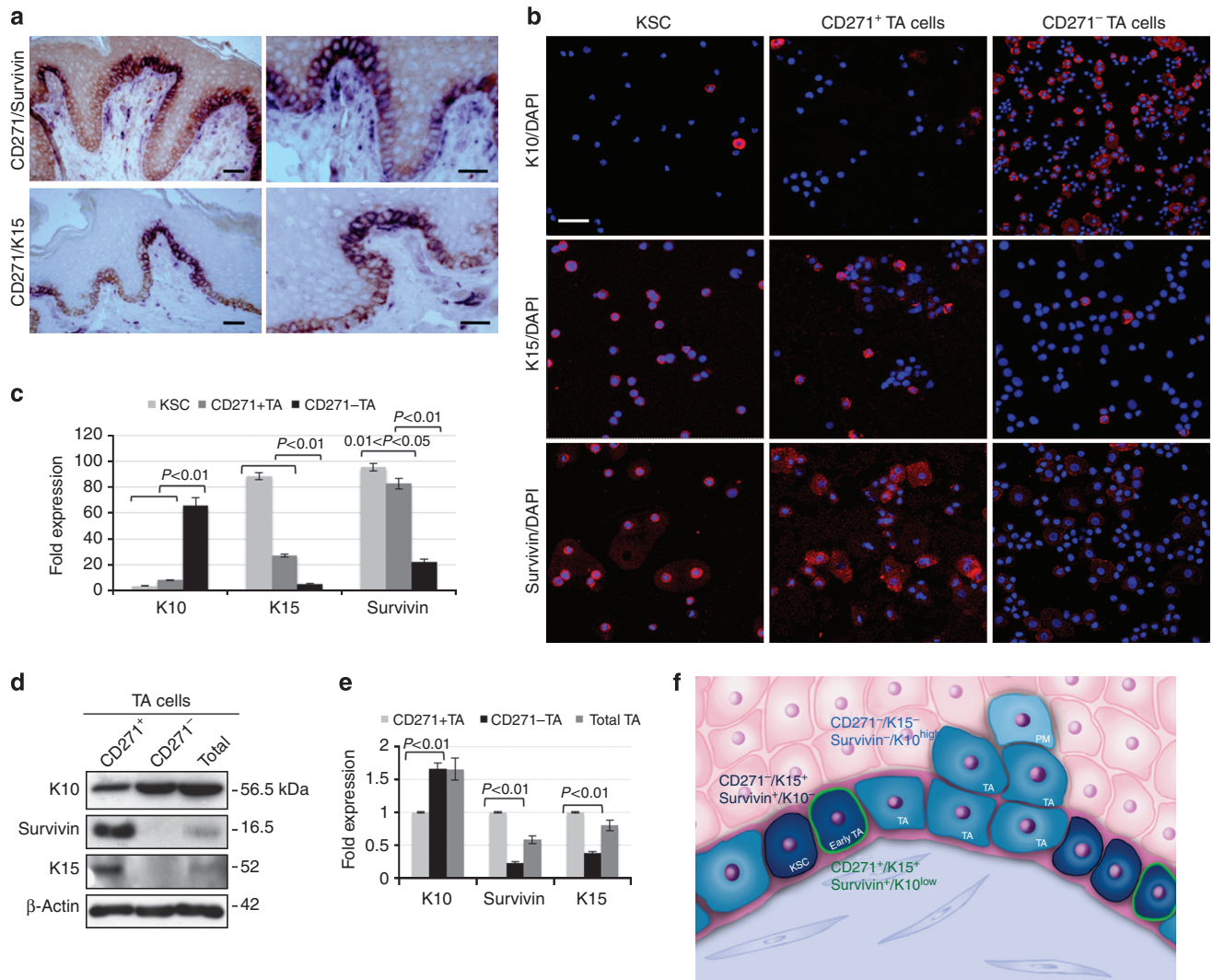


Figure 3. CD271⁺ transit-amplifying cells (CD271⁺ TA cells) are early differentiated keratinocytes. (a) Paraffin-embedded sections of normal skin were double stained: fast blue was used as chromogen for CD271, whereas survivin and K15 staining were revealed by carbazol. Bars = 120 μ m. (b, c) Freshly isolated KSCs, CD271⁺, and CD271⁻ TA cells were isolated, fixed in 4% paraformaldehyde, spun, and immunostained. Cells were analyzed by confocal scanning laser microscopy and positive cells were calculated. Bar = 12 μ m. (d, e) Protein extracts from freshly isolated total, CD271⁺, and CD271⁻ TA cells were immunoblotted. Protein expression was measured by densitometry. (f) Keratinocyte stem cells (KSCs) generate TA cells with different proliferative capacities that eventually undergo terminal differentiation. CD271 acts as “switch on-off” protein, and it marks the stem cell progeny (early TA cells) that initiate the differentiation process. DAPI, 4',6-diamidino-2-phenylindole; K10, keratin 10; K15, keratin 15.

consistent with the co-receptor function of CD271 that increases Trk high affinity for NTs. Within the sorted population, NTs slightly modulate these markers. This is likely due to the autocrine NT release (Marconi *et al.*, 2003) that levels the difference when NTs are exogenously given.

These data confirm that CD271⁺ TA cells have a higher proliferative potential compared with CD271⁻ TA cells, possibly enhanced by the paracrine and autocrine NT released from keratinocytes.

CD271⁺ TA cells reconstitute human epidermis with early differentiative features

To further understand the role of CD271, freshly isolated TA cells were seeded onto dermal equivalents in order to obtain

three-dimensional skin reconstructs. Skin equivalents originated from freshly isolated CD271⁺ TA cells were compared with skin equivalents originated from CD271⁻ TA cells or from freshly isolated KSCs (Figure 5a). Freshly isolated KSCs generated a morphologically normal epidermis with a well-organized and polarized basal layer and differentiated suprabasal layers. Both CD271⁺ and CD271⁻ TA cells were capable of regenerating a multilayered epidermis with differentiated suprabasal layers. However, involucrin and epidermal fatty acid-binding protein were much more expressed in skin equivalents from CD271⁻ than in reconstructs from CD271⁺ TA cells. Loricrin, which is normally expressed in the stratum corneum of healthy skin, was detected only in skin equivalent originated from KSCs or CD271⁺ TA cells.

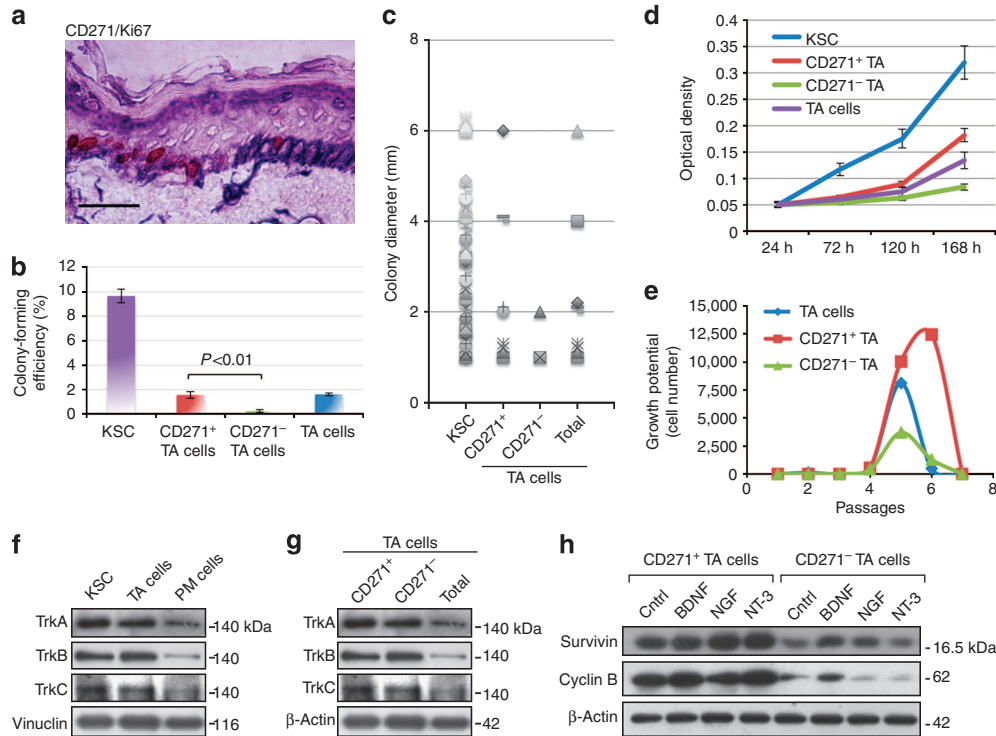


Figure 4. Proliferative capacity of CD271⁺ transit-amplifying cells (CD271⁺ TA) cells. (a) Paraffin-embedded sections of normal skin were double stained: fast blue and carbazol were used for CD271 and Ki67, respectively. Bar = 120 μm. (b, c) Colony forming efficiency and colony areas were determined on freshly isolated keratinocyte stem cells (KSCs), on total, and CD271⁺ and CD271⁻ TA cells, as described in Materials and Methods section. (d) Keratinocyte subpopulations were cultured up to 168 hours after seeding. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed at different times. (e) Long-term assay was performed on freshly isolated keratinocyte subpopulations, as described in Materials and Methods section. (f) Protein extracts from freshly isolated KSCs, TA cells, and post-mitotic (PM) cells were immunoblotted. (g) Protein extracts from freshly isolated CD271⁺, CD271⁻, or total TA cells were immunoblotted. (h) CD271⁺ or CD271⁻ TA cells were treated with brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), or neurotrophin-3 (NT-3) for 48 hours. Cell lysates were blotted. Trk, tyrosine kinase.

Moreover, K10 was expressed in all suprabasal layers of skin equivalents originated from CD271⁺ TA cells, whereas it was only detected in the upper epidermal layers of skin equivalents originated from CD271⁻ TA cells. Finally, K15 was detected only in some cells of the basal layer in skin equivalent originated from CD271⁺ TA cells, whereas no K15-positive cells were detected in skin equivalent originated from CD271⁻ TA cells (Figure 5a and b).

Skin equivalents originated from CD271⁻ TA cells display a psoriatic phenotype

Because CD271 modulates keratinocyte differentiation and is absent in lesional psoriatic skin (Truzzi *et al.*, 2011), we reasoned that skin equivalents originated from freshly isolated CD271⁻ TA cells might display a psoriasis-like phenotype. As predicted, Ki67, which is normally increased in lesional psoriatic skin, was more expressed in skin equivalents from CD271⁻ TA cells than in CD271⁺ TA cells, as shown by counting the percentage of positive nuclei (Figure 6a and b). Only skin equivalents obtained from CD271⁻ TA cells expressed psoriasin and phosphorylated STAT3, which are typically increased in lesional psoriatic epidermis. This was demonstrated by counting the stained areas and the

percentage of positive nuclei for psoriasin and phosphorylated STAT3, respectively. Finally, K16 was slightly more expressed in skin equivalents originated from CD271⁻ TA cells than in reconstructs from CD271⁺ TA cells (Figure 6a and b). When CD271⁺ TA cells were seeded onto dermal equivalent and thus induced to proliferate, they generated a skin reconstruct where CD271 was not expressed (Figure 6c), indicating that during proliferative conditions, such as during the development of psoriatic lesion, CD271 protein is lost. To confirm whether the absence of CD271 is critical to the development of a psoriasiform phenotype, we generated skin equivalents with keratinocytes silenced for CD271. As expected, knock-down of CD271 resulted in the increase of involucrin, epidermal fatty acid-binding protein, psoriasin, and K16, which is in line with the results obtained in CD271⁻ TA cells (Figure 6d).

DISCUSSION

Although a huge body of evidence has demonstrated the critical role of KSC and its progeny in governing epidermal renewal, studies have been hampered by the lack of a definite marker able to distinguish KSCs from TA cells and by the largely unknown mechanisms underlying the transition from

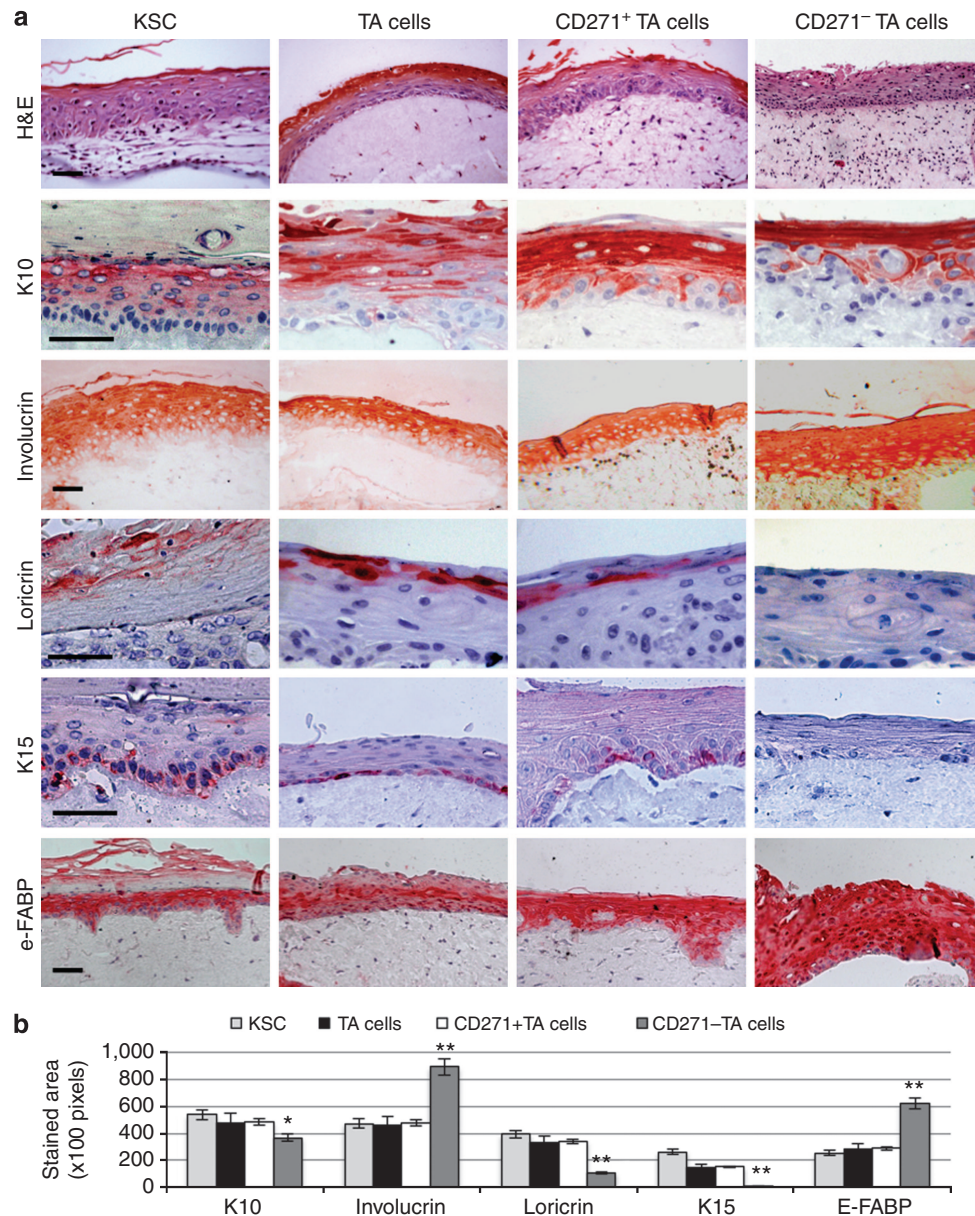


Figure 5. Role of CD271 in skin equivalent differentiation. (a) Skin equivalents obtained by seeding freshly isolated keratinocyte stem cells (KSCs) or total and CD271⁺ or CD271⁻ transit-amplifying (TA) cells on dermal equivalent were paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E) and different markers; fast red was used as chromogen. (b) Stained areas were evaluated by image pixel count using ImageJ64. Experiments were conducted at least in triplicate from different samples. Bars = 120 μm. *0.01 < P < 0.05, **P < 0.01. K10, keratin 10; K15, keratin 15.

KSCs to more differentiated keratinocytes. According to the main model, epidermal homeostasis is maintained by long-lived KSCs that generate TA cells; by contrast, a more recent concept seems to indicate the existence of a single committed progenitor with different stochastic fate (Ghazizadeh and Taichman, 2005; Clayton *et al.*, 2007; Youssef *et al.*, 2012). The present study sheds more light on these mechanisms and on the relationship between KSCs and TA cells. First, this work points to CD271 as a relevant molecule in the differentiation program of human epidermis. In keratinocyte cultures, calcium addition induces an increase in intracellular

calcium concentration, resulting in the upregulation of genes correlated with differentiation, as well as in the downregulation of proliferation markers (Tu and Bikle, 2013). Here, we show that CD271 is induced by calcium together with other differentiation proteins in subconfluent keratinocytes. Moreover, when KSCs are induced to overexpress CD271, they display a TA phenotype, characterized by increased differentiation markers. This indicates that CD271 triggers the switch between KSCs and TA cells. On the other hand, CD271 siRNA-treated TA cells display reduced levels of differentiation markers and increased expression of K15,

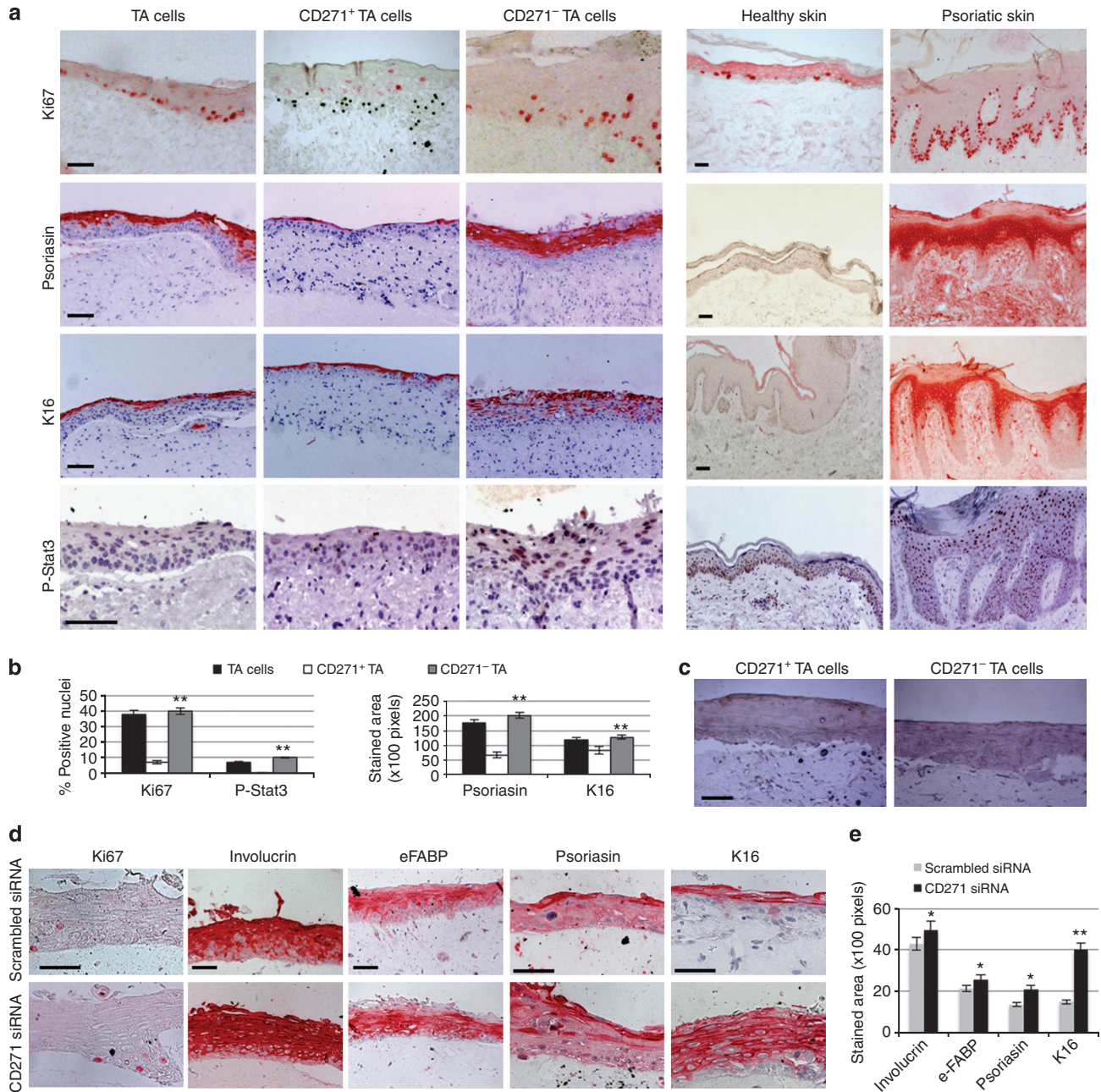


Figure 6. CD271⁻ transit-amplifying cells (CD271⁻ TA) cells generate skin equivalents with a psoriatic phenotype. (a) Paraffin-embedded sections of skin equivalent obtained from freshly isolated total, CD271⁺, or CD271⁻ TA cells, and sections of normal and psoriatic skin were stained with different markers; carbazol was used as chromogen for phosphorylated STAT3 (p-STAT3), whereas fast red was used for Ki67, psoriasis, and K16. (b) Positive nuclei for Ki67 and p-STAT3 were counted, whereas pixel stained areas for psoriasis and K16 were measured. (c) Paraffin-embedded sections of skin equivalents obtained from freshly isolated CD271⁺ and CD271⁻ TA cells were stained with anti-CD271 antibody; fast red was used as chromogen. Experiments were conducted at least in triplicate from different samples. (d) Paraffin-embedded sections of skin equivalents, obtained from keratinocytes treated with scrambled or CD271 small interfering RNA (siRNA), were stained with different markers; fast red was used as a chromogen. Bars = 120 μm. (e) Stained areas of Figure 6d were evaluated by image pixel count using ImageJ64. *0.01 < P < 0.05, **P < 0.01. E-FABP, epidermal fatty acid-binding protein; K16, keratin 16.

survivin, and β₁-integrin, while exhibiting a high colony-forming efficiency, features associated with KSCs. This indicates that CD271 also induces progeny to revert to their precursor cell phenotype. Reprogramming differentiated cells into KSCs has been previously shown in mice. Genetic

labeling and lineage-tracing studies provided evidence that differentiated cells can regenerate skin (Mannik *et al.*, 2010). In addition, paracrine fibroblast growth factor induced a reversion of differentiated human keratinocytes into dedifferentiated cells that displayed the function and the

markers of stem keratinocytes (Sun *et al.*, 2011). The present study suggests that CD271, predominantly expressed in TA cells, acts as a “switch on–off” molecule of early keratinocyte differentiation.

We had previously shown that CD271 induces apoptosis in normal human keratinocytes. When KSCs exit the niche, they can either undergo apoptosis (Tiberio *et al.*, 2002; Marconi *et al.*, 2007; Livshits *et al.*, 2012) or differentiation (Watt, 2002), resulting in different cell fates according to the tissue microenvironment. In prostate cancer cells, CD271 acts as a tumor suppressor by mediating apoptosis and by inducing differentiation (Nalbandian *et al.*, 2005). Although the work by Nalbandian *et al.* (2005) do not address the possible ligand involved in CD271 activity, the present paper, in keeping with a previous article (Truzzi *et al.*, 2011), indicates a possible role for NTs that are produced by keratinocyte and other skin cells. Consistent with the role of NT and their receptors in epidermal homeostasis, TrkA and TrkC are more expressed in KSCs than in TA cells. Furthermore, CD271⁺ TA cells express more TrkA and TrkC compared with CD271⁻ TA cells, suggesting that TA cells expressing CD271 are closer to KSCs and possibly involved in the early differentiation process. Although TA cells are found in both the basal and suprabasal layers (Watt, 2001; Fuchs, 2008), the present work shows that CD271 is confined mostly to the basal layer. In addition, freshly isolated TA cells sorted for CD271 express little K10, as compared with CD271⁻ TA cells. As K10 is normally located in the immediate suprabasal layers (Porter and Lane 2003), CD271 could be expressed earlier and act as a first trigger of differentiation from KSC. This is partially confirmed by the higher amount of survivin, a KSC marker (Sun *et al.*, 2011) in CD271⁺ than in CD271⁻ TA cells. The same holds true for K15 that is predominantly expressed in CD271⁺ TA cells and in skin equivalents generated from CD271⁺ TA. The latter finding is even more interesting in light of previous works, demonstrating that K15 is expressed in slowly cycling and mature basal cells (Porter *et al.*, 2000). Furthermore, a recent work associates K15 expression with the loss of homeostasis and the initiation of epidermal differentiation (Troy *et al.*, 2011). These data seem to indicate that CD271 is capable of triggering keratinocyte differentiation and of reprogramming TA into KSC at a very early stage.

Mascreé *et al.* (2012) have recently described by clonal fate analysis the existence of two hierarchically distinct proliferative keratinocyte populations, the slow-cycling stem cells, and the committed progenitor cells. Here we show that within TA cells, there are subpopulations with different proliferative capacities, and that CD271 expression correlates with a high proliferative potential *in vitro*. In normal human epidermis *in vivo*, CD271 is mostly expressed in nonproliferating cells, possibly indicating a different proliferative capacity *in vitro*, and further suggesting that CD271 exerts its activity at the boundary between KSCs and TA cells. Indeed, CD271⁺ TA cells display a proliferative potential superior to CD271⁻ TA cells, whereas NTs stimulate proliferation to a greater extent in CD271⁺ than in CD271⁻ TA cells. This scenario is consistent with the

concept of paracrine and autocrine functions of the NT network at the skin level (Botchkarev *et al.*, 2006).

The presence and function of an early TA cell population have been previously shown both in the hair follicle (Gutiérrez-Rivera *et al.*, 2010) and in the interfollicular epidermis (Li *et al.*, 2004). More recently, using the *in vitro* transplantation assay, Schlüter and Kaur (2013) demonstrated that also early stem cell progeny possesses tissue regenerative capacity. The present study shows that, although all TA cells are capable of reconstituting a full-thickness epidermis in a skin equivalent model, in agreement with previous works (Li *et al.*, 2004), the differential expression of differentiation markers in CD271⁺ as compared with CD271⁻ TA cells supports the concept of CD271 as a marker of early keratinocyte differentiation.

Although psoriasis represents a perfect model of dysregulated epidermal homeostasis, where TA cells appear to carry the main defect (Castelijns *et al.*, 2000; Grabe and Neuber, 2007), CD271 protein is absent in lesional skin *in vivo*, and psoriatic TA keratinocytes express barely detectable levels of the receptor (Truzzi *et al.*, 2011). Consistent with these findings, CD271⁻ TA cells, but not CD271⁺ TA cells, can generate skin equivalents with a psoriasiform phenotype. Interestingly, during the development of skin equivalents generated by CD271⁺ TA cells, when cells are actively proliferating, such as during the development of psoriatic lesion, CD271 protein is lost. One could speculate that, as CD271 is associated with the very initial KSC differentiation, the intrinsic defect in psoriatic epidermis resides in the early TA cells where the absence of the receptor might contribute to the altered keratinocyte differentiation of the disease.

MATERIALS AND METHODS

Cell culture

Human keratinocytes were obtained from neonatal foreskin. For differentiation *in vitro* studies, keratinocytes were cultured in DMEM and Ham's F12 media with serum (Pincelli *et al.*, 1997) or in keratinocyte growth medium (Lonza, Basel, Switzerland) treated with 1.8 mM calcium chloride for 48 hours. Fresh keratinocytes were also divided in KSCs and TA cells on the basis of their ability to adhere to type IV collagen, as described in Supplementary Materials online. Keratinocytes were analyzed immediately after the separation or cultured in serum-free medium (keratinocyte growth medium) and used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, colony forming efficiency, and long-term assay as described in Supplementary Materials online. Patient consent for experiments was not required because Italian laws consider human tissue left over from surgery as discarded material.

TA cell sorting for CD271 expression

Freshly isolated TA cells were sorted for CD271 expression by FACS sorter or by using M-450 epoxy Dynabeads as described in Supplementary Materials online.

Skin reconstructs

Skin reconstructs were obtained by seeding freshly isolated keratinocytes on dermal equivalents generated by fibroblasts-induced type I collagen contraction, as indicated in Supplementary Materials online.

siRNA transfection and infection of keratinocytes

Total keratinocytes, KSCs, and TA cells were plated in keratinocyte growth medium, and 24 hours later cells were siRNA-transfected or infected for CD271 as described in Supplementary Materials online.

Real-time PCR

RNA from total keratinocytes treated or not with 1.8 mM calcium chloride was extracted using TRI Reagent method as previously described (Truzzi *et al.*, 2011). Quantitative real-time PCR was performed as described in Supplementary Materials online.

WB analysis

Cultured or fresh keratinocytes were harvested for protein analysis, and WB was performed as previously described (Tiberio *et al.*, 2002) and indicated in Supplementary Materials online.

FACS analysis

Keratinocytes were incubated with anti-CD271 antibody (Supplementary Table S1 online) for 20 minutes at 4 °C, and then they were labeled with secondary antibody Alexa Fluor anti-mouse 488 (1:50, Life Technologies, Thermo Fisher Scientific Inc, Waltham, MA) for 20 minutes at 4 °C. Cells were analyzed using an Epics XL flow cytometer (Beckman Coulter, Brea, CA).

Immunofluorescence

Freshly isolated keratinocytes were fixed in 4% buffered paraformaldehyde and cytospun onto glass slides. K10, K15, and survivin expression was detected by immunofluorescence as indicated in Supplementary Materials online.

Immunohistochemistry

Paraffin-embedded biopsies (4 μm) of normal human skin, skin reconstructs, and lesional psoriatic human skin were double or single stained for protein analysis by immunohistochemistry, as indicated in Supplementary Materials online. Antibodies utilized were reported in Supplementary Table S1 online.

Statistical analysis

Student's *t*-test was used, as indicated in Supplementary Materials online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was partially supported by Fondazione Cassa di Risparmio di Modena, Italy. We thank Massimiliano Truzzi for the artwork.

SUPPLEMENTARY MATERIAL

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

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