Impaired Keratinocyte Proliferative and Clonogenic Potential in Transgenic Mice Overexpressing 14-3-3σ in the Epidermis

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The 14-3-3 protein family controls diverse biochemical processes through interaction with phosphorylated consensus sequences in protein targets. Its epithelial specific member, 14-3-3σ, also known as stratfin, is highly expressed in differentiated keratinocytes, and in vitro evidence indicates that 14-3-3σ downregulation leads to keratinocyte immortalization. To define the role of 14-3-3σ in skin homeostasis in vivo, we generated transgenic mice overexpressing 14-3-3σ in proliferating keratinocytes of the epidermis and hair follicle. Transgenic animals show decreased epidermal thickness and hair follicle density associated with reduced number of proliferating keratinocytes and decreased levels of keratins 14, 5, and 15. Primary keratinocytes isolated from transgenic mice manifest reduced proliferation and migration. Moreover, clonogenicity assessment and label-retaining analysis reveal a reduction in keratinocyte progenitor cell number in transgenic mice. Response to IGF-1 is strongly impaired in cultured transgenic keratinocytes compared with wild-type cells. Consistently, activation of phosphoinositol 3-kinase (PI3K), AKT, and Rac1, all IGF-1 downstream mediators, is reduced. Our results demonstrate that 14-3-3σ controls the in vivo epidermal proliferation-differentiation switch by reducing proliferative potential and forcing keratinocytes to exit the cell cycle, and that this effect associates with inhibition of the IGF-1 pathway.

INTRODUCTION

The balance between keratinocyte proliferation and differentiation has a pivotal role for the maintenance of skin homeostasis. Stem cells in the bulge of the hair follicle and in the basal layer of interfollicular epidermis guarantee epidermal renewal (Watt and Jensen, 2009). These cells can enter into an active proliferating state, generating transient amplifying cells that exit from the cell cycle, move in the suprabasal layers, and differentiate. Several molecular mediators control the keratinocyte proliferation/differentiation switch (Koster and Roop, 2007; Blanpain and Fuchs, 2009). Despite major achievements in the understanding of such regulation over the last years, many aspects remain to be determined.

14-3-3σ, also known as stratfin, is a member of the 14-3-3 protein family specifically expressed in stratified epithelia. Like other 14-3-3 isoforms, 14-3-3σ controls diverse biochemical processes by binding to many different ligands via a consensus motif phosphorylated on serine or threonine residues. A targeted proteomic analysis identified several 14-3-3σ-binding partners belonging to subclasses involved in the regulation of cytoskeletal dynamics, polarity, adhesion, mitogenetic signaling, and motility (Benzinger et al., 2005).

Several lines of evidence suggest that 14-3-3σ exerts an important role in cell cycle progression control. First, 14-3-3σ expression is positively regulated by p53 following DNA damage, leading to a stable G2/M mitotic arrest (Hermeking et al., 1997). Moreover, 14-3-3σ expression is silenced following promoter hypermethylation in different carcinomas (Lodygin and Hermeking, 2006), whereas ectopic 14-3-3σ expression in tumor cells leads to G2/M arrest (Hermeking et al., 1997; Ferguson et al., 2000).

In the epidermis, 14-3-3σ expression is low in the basal compartment and highly upregulated in suprabasal keratinocytes (Dellambra et al., 2000). Downregulation of 14-3-3σ prevents senescence and leads to immortalization of cultured keratinocytes (Dellambra et al., 2000). In vivo,
epigenetic 14-3-3-σ silencing has been shown in epidermal tumors of the basal-cell compartment (Lodygin et al., 2003). Moreover, a mutation in 14-3-3-σ, resulting in a truncated protein potentially acting as a dominant-negative, was found in the repeated epilation mice (Herron et al., 2005; Li et al., 2005) that show a hyperproliferative epidermis lacking a cornified envelope and, as heterozygous, display repeated hair loss, and a tendency to develop skin tumors (Holbrook et al., 1982; Lutzner et al., 1985). Together, these findings support the concept that 14-3-3-σ promotes keratinocyte cell cycle withdrawal and differentiation.

In apparent contrast to this data, 14-3-3-σ has been found to be highly expressed in conditions associated with keratinocyte hyperproliferation, such as in squamous cell carcinomas and psoriasis (Lodygin et al., 2003). Moreover, 14-3-3-σ expression is increased in the upper layers of hyperproliferative epidermis in keratin 10 (K10)-null mice (Reichelt and Magin, 2002). These findings suggest that high 14-3-3-σ expression levels may be not generally required for cell cycle withdrawal, and point to the necessity to further investigate the role and mechanism of action of 14-3-3-σ in vivo.

To analyze 14-3-3-σ role in epidermal homeostasis, we generated transgenic mice overexpressing 14-3-3-σ under the control of a K14 promoter (K14-σ transgenic mice), which results in high expression in basal epidermal keratinocytes and keratinocytes of the outer root sheath in the hair follicle.

RESULTS

Overt skin phenotype of K14-σ transgenic mice

Two transgenic lines overexpressing human 14-3-3-σ under the control of a K14 promoter were generated. The first one (line 7) showed patchy desquamating skin lesions early in life (Supplementary Figure S1 online) and sparse hair loss with age (not shown). However, because of breeding difficulties, susceptibility to skin infections leading to poor health conditions and short life span, only a preliminary characterization of this line could be performed. The other line (line 44) only displayed early wrinkle appearance starting from 8–10 weeks of age (Figure 1). Upon wax depilation, a high percentage of transgenic mice from line 44 manifested excoriated skin lesions (77.8% versus 30% in wild-type mice) and incomplete hair regrowth (72.7%; Figure 1). Despite the less severe phenotype, this line manifested similar features to line 7, and thus was chosen for the phenotypic characterization of 14-3-3-σ overexpression. Line 44 contained around 14 transgene copies.

In situ hybridization, immunohistochemistry, and western blot analysis revealed a strong increase of 14-3-3-σ expression in the keratinocytes of the basal layer and of the most external layer of the hair follicle (the outer root sheath) in transgenic mice compared with wild-type controls (Supplementary Figure S2 online).

Reduced thickness and expression of proliferating keratinocyte markers in the epidermis of transgenic mice

Histological analysis revealed that the epidermis of transgenic mice was thinner than that of wild-type controls (Figures 2a–e). Epidermal hypoplasia started to be evident in the skin of adult mice and became more marked with increasing age (Figures 2c and d). A reduction in hair follicle density was also observed (Figures 2f and g).

The decrease in epidermal thickness suggested that 14-3-3-σ overexpression may interfere with differentiation. To examine this aspect, immunohistochemistry was performed on newborn back skin and adult tail skin with the following markers: K14, K15, K6, K10, involucrin, and loricrin. No obvious alterations in expression pattern could be detected for K10, involucrin, loricrin, K15 (Supplementary Figure S3A online), or K6 (data not shown). However, K14 staining was strongly reduced in basal keratinocytes of interfollicular epidermis and hair follicle outer root sheath in transgenic mice (Figure 3a).

Western blot analysis on protein extracts from isolated keratinocytes confirmed the strong reduction in K14 protein levels in transgenic animals (0.19 ± 0.08 relative amount versus wild type). Expression levels of K5, the K14 partner in proliferating keratinocytes, were also strongly decreased (0.29 ± 0.02). In addition, the level of K15, which detects hair follicle stem cells and, in newborn mice, also basal proliferating keratinocytes (Liu et al., 2003), was significantly reduced (0.47 ± 0.11), whereas involucrin and loricrin, markers of suprabasal differentiated keratinocytes, were increased (1.56 ± 0.19 and 1.80 ± 0.27, respectively; Supplementary Figure S3B online). K10 and K6 protein levels were not different between wild-type and transgenic keratinocytes (0.86 ± 0.17 and 0.97 ± 0.62, respectively; Supplementary Figure S3B online). The expression of K14 and K5 mRNAs was also analyzed by real-time RT-PCR and shown to be significantly decreased in transgenic mice as compared with wild-type ones (K14: 0.49 ± 0.002; K5: 0.48 ± 0.01; Figures 3c and d).

To evaluate whether apoptosis contributes to the decrease in epidermal thickness, a TUNEL staining was performed. In both K14-σ and wild-type mice, a small and comparable percentage of keratinocytes of the most differentiated layers were stained (1.79% ± 1.05, wild-type mice; 1.96% ± 1.10, transgenic mice; Supplementary Figure S4 online).
Impaired proliferation of keratinocytes overexpressing 14-3-3σ

To analyze the effect of 14-3-3σ overexpression on keratinocyte proliferation, BrdU nuclear labeling was examined following a single injection into mice. A significant reduction in BrdU-positive interfollicular basal cells was found in transgenic mice (11.37% ± 2.64) compared with wild-type littermates (17.70% ± 3.48; Figures 4a–c). Similarly, cultured primary keratinocytes from transgenic mice displayed a significant decrease in BrdU incorporation compared with wild-type controls (Figure 4d).

Moreover, scrape wounds were mechanically made in confluent keratinocyte cultures, and gap closure was analyzed (Figure 4e). A reduced capacity in filling the gap was observed in keratinocyte monolayers from transgenic mice as compared with wild-type ones. As this capacity depends on both proliferation and migration, and 14-3-3σ was found to interact with several proteins involved in cell motility (Benzinger et al., 2005), keratinocyte migration was also investigated using a Boyden chamber assay. This revealed that keratinocytes overexpressing 14-3-3σ manifest significantly reduced motility (Figure 4f).

As 14-3-3σ has been shown to potentially bind several proteins regulating cell adhesion, the adhesiveness of transgenic keratinocytes was evaluated by a specific in vitro assay. Wild-type and 14-3-3σ-overexpressing keratinocytes plated on type IV collagen manifested similar adhesive properties (Figure 4g).

Reduced keratinocyte progenitor cell number in K14-σ transgenic mice

We, then, tested whether the colony-forming capacity of keratinocytes isolated from K14-σ mice was impaired. Colony-forming assays on primary keratinocytes showed that the number of colonies, indicative of number of proliferating cells, was strongly decreased in transgenic mice compared with controls (Figures 5a and b). Average colony size was also reduced, as the majority of colonies from transgenic keratinocytes (75.3%) showed an area below 1 mm² after...
1 week of culture with respect to 35.5% of colonies from wild-type keratinocytes (Figure 5c). These small colonies resembled the keratinocyte paraclones, the increase of which is a measure of in vitro stem cell depletion (Barrandon and Green, 1987; Dellambra et al., 2000).

To analyze whether the progenitor cell number was affected in transgenic mice, an in vivo BrdU-labeling long-term analysis was performed by multiple injections of BrdU in 10-day-old mice. Indeed, epidermal progenitors are slow cycling cells and retain BrdU for a longer period than actively proliferating cells. To assess the efficiency of BrdU labeling, a group of mice (n = 3) was killed 2 days after the final BrdU injection. A high and similar percentage of cells within the basal interfollicular epidermis and hair follicle

Figure 3. Altered epidermal differentiation in transgenic mice overexpressing 14-3-3σ. (a) Immunohistochemistry for keratin 14 (K14) in the skin of newborn mice. Arrows indicate the interfollicular keratinocytes of the basal layer, whereas arrowheads indicate follicular keratinocytes of the outer root sheath. Bars = 100 μm. (b) Western blot for K14 and K5 on protein extracts from keratinocytes isolated from the skin of newborn mice. Numbers above western panels indicate the fold change in transgenic mice relative to wild-type controls (**P < 0.01; n = 4). (c, d) Real-time PCR on RNA extracted from keratinocytes freshly isolated from the skin of newborn mice. Values are expressed as mean ± SD of two different experiments with three mice each (**P < 0.005). Tg, transgenic; Tub, tubulin; WT, wild type.

Figure 4. Reduced keratinocyte proliferation in keratin 14-σ transgenic mice. (a, b) Immunohistochemistry for BrdU in the tail skin of adult mice. Arrows indicate positive keratinocytes in the basal layer. Bars = 50 μm. (c) Morphometric analysis of BrdU-positive basal cells in the epidermis (n = 7). (d) BrdU incorporation in cultured keratinocytes from newborn mice (n = 5). (e) Representative scratch assay images taken at 0 and 48 hours after wounding (n = 4). (f) Boyden chamber assay on cultured keratinocytes (*P < 0.05; n = 4). (g) Adhesion assay with keratinocytes seeded at different density (black: 5 x 10⁴ cells; gray: 10⁵ cells; and white: 2 x 10⁵ cells; n = 4). Tg, transgenic; WT, wild type.
outer root sheath were labeled in wild-type and transgenic mice (interfollicular epidermis: wild type (WT) 71.38 ± 1.04, transgenic (Tg) 71.77 ± 0.77; hair follicle: WT 75.88 ± 1.64, Tg 76.80 ± 2.84), indicating that the multiple BrdU chases were able to label most of proliferating cells. Forty-five days after BrdU injection, a reduction of >30% in label-retaining cells was observed in the interfollicular basal layer in transgenic mice compared with wild-type controls (Figure 5d). Similar results were obtained by counting BrdU-positive outer root sheath keratinocytes in the hair follicles (Figure 5e).}

**Response to IGF-1 is strongly reduced in keratinocytes of K14-σ transgenic mice**

To identify possible 14-3-3σ target pathways responsible for the observed effects on transgenic keratinocytes, the IGF-1 signaling was analyzed. We focused on this pathway because several findings indicate an important function for IGF-1 in regulating epidermal proliferation, motility, and progenitor cell behavior (Haase et al., 2003; Sadagurski et al., 2006; Stachelscheid et al., 2008).

BrdU incorporation was measured in keratinocytes from transgenic and wild-type mice treated or not with IGF-1. The mitogenic response upon IGF-1 stimulation was strongly reduced in cultured K14-σ keratinocytes as compared with control cells (83% in wild-type keratinocytes and 16% in transgenic keratinocytes; Figure 6a).

The responsiveness to IGF-1 was further tested by treating scratched keratinocyte monolayers and analyzing gap closure (Figure 6b). Forty-eight hours after wounding, starved wild-type keratinocytes treated with IGF-1 were able to colonize the cell-free zone similarly to those cultured in complete medium (WT + IGF-1: 65.30% of initial wound area ± 10.75 SEM and WT complete medium: 72.52% ± 8.71), whereas keratinocytes from K14-σ mice treated with IGF-1 behaved similarly to starved cells (Tg + IGF-1: 16.49% ± 11.72; Tg complete medium: 21.81% ± 6.83; Tg starved 18.09% ± 9.32; and WT starved 10.22% ± 5.51), indicating a striking impairment in the proliferation and migration capacity of K14-σ keratinocytes following IGF-1 treatment.

**IGF-1R signaling is impaired in transgenic mice overexpressing 14-3-3σ**

To examine the mechanisms by which 14-3-3σ-overexpressing keratinocytes show a reduced response to IGF, we first analyzed the expression of IGF-1 receptor (IGF-1R) in keratinocytes isolated from newborn mice. A significant reduction in IGF-1R expression was found at the RNA (0.39 ± 0.05 relative amount; n = 4; P = 0.0016) and protein level (0.55 ± 0.18; Figure 6c) in 14-3-3σ-overexpressing keratinocytes as compared with controls.

IGF-1R stimulates phosphoinositol 3-kinase (PI3K) in keratinocytes, thereby activating its downstream pathway (Haase et al., 2003; Sadagurski et al., 2006). Following IGF-1 stimulation, a reduced induction in phosphorylated PI3K with respect to total PI3K was observed in keratinocytes overexpressing 14-3-3σ (2.49 ± 1.10-fold increase) compared with controls (6.75 ± 1.04-fold increase; Figure 6d).

PI3K directly phosphorylates and thus activates the serine/threonine protein kinase Akt (Burgering and Coffier, 1995; Franke et al., 1995). Akt phosphorylation was strongly reduced upon IGF-1 stimulation in keratinocytes from transgenic mice compared with wild-type ones (Figure 6e).

Finally, the small GTPase Rac-1, a target of epidermal IGF-1R signaling having an important role in the regulation of keratinocyte proliferative potential and stemness (Stachelscheid et al., 2008), was also analyzed. Following IGF treatment, a more than 2-fold increase (2.07 ± 0.27) in activated Rac/total Rac was detected in wild-type keratinocytes, whereas in cells from transgenic mice, the amount of activated Rac was only slightly augmented (1.12 ± 0.20; Figure 6f). Together, these results show a strong reduction in IGF-1-mediated signaling in 14-3-3σ-expressing keratinocytes.
14-3-3σ in Epidermal Homeostasis

**DISCUSSION**

To define the *in vivo* role of 14-3-3σ in controlling epidermal homeostasis, we generated transgenic mice that express this protein at high levels in basal-proliferating keratinocytes. Our analysis revealed that 14-3-3σ overexpression forces basal keratinocytes to exit the cell cycle and to differentiate prematurely, resulting in a reduction of both epidermal proliferative capacity and progenitor stem cell number.

The decreased proliferation potential parallels with reduced K14 and K5 gene expression both at mRNA and protein level. As these keratins identify highly proliferating basal keratinocytes (Byrne et al., 1994), these findings indicate that 14-3-3σ levels have an impact on proliferative potential of these cells.

Our *in vivo* data are consistent with previously reported *in vitro* findings that suggested that the downregulation of 14-3-3σ is a crucial step in keratinocyte immortalization (Dellambra et al., 2000). Our observations also agree with a tumor suppressor function of 14-3-3σ, which was shown to be silenced in cutaneous basal cell carcinomas by promoter hypermethylation (Lodygin et al., 2003) and mutated in the naturally occurring tumor prone repeated epilation mouse model (Herron et al., 2005; Li et al., 2005).

In apparent contrast to our data, high levels of 14-3-3σ are expressed in cutaneous squamous cell carcinomas and hyperproliferative epidemics of inflammatory skin disorders (Lodygin et al., 2003). One possible explanation is that 14-3-3σ has additional roles in pathological conditions, such as the prevention of apoptosis and promotion of cell survival (Samuel et al., 2001; Oh et al., 2009). Another possibility could be that 14-3-3σ is induced under hyperproliferative stress to counteract uncontrolled proliferation.

To assess the molecular alterations underlying the proliferative defect and the reduced progenitor stem cell number in transgenic mice, IGF-1R signaling pathway was investigated. Basal keratinocytes express IGF-1R and are thought to respond to fibroblast-released IGF-1 with an increase in cell proliferation and migration. Stachelscheid et al. (2008) showed that IGF-1R signaling has a central role in the regulation of progenitor cell behavior in the interfollicular epidermis and in promoting keratinocyte proliferation, in part, through the small GTPase Rac-1. Our findings clearly show that keratinocytes of transgenic mice are less responsive to IGF-1.

A reduction in the IGF-1 signaling pathway was observed, as the activities of PI3K, Akt, and Rac, key downstream targets, were all blunted in 14-3-3σ transgenic keratinocytes. The 14-3-3σ has been described to physically associate with and inhibit Akt (Yang et al., 2006). In line with this finding, we observed a strong downregulation of Akt activity. The decrease in IGF-1R/PI3K activity is likely to be responsible for the impaired migration observed in 14-3-3σ keratinocytes, as PI3K was shown to also regulate human keratinocyte migration (Haase et al., 2003).
In disagreement with our data, Zhang et al. (2004) reported that 14-3-3σ was upregulated in a breast cancer cell line treated with IGF-1 and that the increased 14-3-3σ levels were necessary to mediate the mitogenic effect of IGF-1. Again, this apparent discrepancy could reflect the antiapoptotic and cell survival functions that 14-3-3σ might have in a pathological model such as tumor cells (Samuel et al., 2001).

As 14-3-3σ interacts with a great number of proteins, it is conceivable that other molecular pathways are also responsible for decreased keratinocyte proliferation and progenitor stem cell number in transgenic mice. However, the strongly reduced responsiveness to IGF-1 points to a relevant role of this signaling route in determining the epidermal phenotype of K14-σ mice.

Taken together, our results provide evidence that 14-3-3σ has a crucial role in the control of the proliferation/differentiation switch in keratinocytes and indicate that increased 14-3-3σ levels inhibit IGF-1 cell responsiveness, leading to decreased keratinocyte proliferation and progenitor cell number.

MATERIALS AND METHODS

Generation of transgenic mice

Transgene was constructed by cloning the full-length coding sequence of the human 14-3-3σ complementary DNA into a K14 cassette expression vector (Vassar et al., 1989). Transgenic mice were generated by pronuclear injection of fertilized oocytes from a C57Bl/6xDBA/2 mouse cross, and transgenic lines were established on a BDF1 background. Mice were screened by tail tip DNA PCR analysis using the following primers: forward 5'-GGACCTCTACAAAGACAGCA-3', located in the 14-3-3σ-coding sequence; reverse 5'-CTCCTAGGGACCTCTAGAGG-3', in the vector polyA sequence. To quantify the number of inserted copies, mouse genomic DNA was ScaI digested and analyzed by Southern blotting.

Animal experiments were approved by the IDI-IRCCS Animal Care and Use Committee.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded skin sections were processed, as described (Failla et al., 2000). The following antibodies were used: anti-human 14-3-3σ (PC482 Calbiochem, Merck Chemicals, Nottingham, UK); anti-mouse K14 (MS-115-P1; Lab Vision, Cheshire, UK); anti-mouse K5, K10, K6, involucrin, and loricrin (respectively, PRB-160P, PRB-159P, PRB-169P, PRB-140C, and PRB-145P; Covance, Berkeley, CA); and anti-mouse K15 (MS-1068-P1, Lab Vision). All antibodies were used at a concentration of 2 μg ml⁻¹.

In situ hybridization

In situ hybridization was performed, as previously described (Failla et al., 2000). To generate the riboprobe, a complementary DNA fragment was obtained by amplifying the transgenic vector with the reverse primer used for genotyping mice and a forward primer located 370 bp upstream. Complementary DNA was cloned into the pCRII-TOPO vector (Invitrogen, Paisley, UK) and transcribed in the presence of [³⁵S]UTP (GE Healthcare, Milan, Italy).

Apoptosis analysis

The Fluorometric DeadEnd TUNEL System (Promega, Madison, WI) was used on paraffin-embedded skin sections following manufacturer instructions. A total of 10 fields per section (× 100 magnification) were analyzed using the Axiovision analysis system (Carl Zeiss, Göttingen, Germany) and counting the number of positive nuclei with respect to total keratinocytes analyzed.

Cell cultures

Mouse keratinocytes were isolated from the skin of newborn mice, as described (Hager et al., 1999), except that the epidermis was dissociated following 0.05% DNase addition (Sigma-Aldrich, Milan, Italy). Keratinocytes were plated on mouse type IV collagen (BD Biosciences, Erembodegen, Belgium) and grown in Cnt-57 progenitor cell-targeted medium (CELLnTEC, Bern, Switzerland).

Western blot

Keratinocytes isolated, as described, for cell cultures were lysed in RIPA buffer and boiled in SDS sample buffer. Samples were separated in SDS-PAGE gels and transferred to PVDF membranes (Millipore, Milan, Italy). Membranes were incubated with the same antibodies used for immunohistochemistry at a concentration of 1 μg ml⁻¹, or with an anti-human 14-3-3σ polyclonal antibody 1:8000 (PRIMM, Milan, Italy; Dellambra et al., 2000), or with 2 μg ml⁻¹ of an anti-mouse IGF-1R antibody (sc-712, Santa Cruz Biotechnology, Heidelberg, Germany).

For analysis on cultured cells, subconfluent keratinocytes were starved for 4 hours in Cnt-57 basal medium (CELLnTEC), stimulated for 30 minutes with 100 ng ml⁻¹ of recombinant mouse IGF-1 (Peprotech, Rocky Hill, NJ) or with basal medium alone as control, and protein extracts were separated by SDS-PAGE. For PI3K and Akt detection, the following antibodies were used: anti-mouse Akt and pAkt, (sc-8312 and sc-7985, respectively, Santa Cruz Biotechnology), at a concentration of 1 μg ml⁻¹; anti-mouse PI3K (4292, Cell Signaling), at a concentration of 0.1 μg ml⁻¹; pPI3K (sc-12929, Santa Cruz Biotechnology), at a concentration of 1 μg ml⁻¹.

Anti-actin or anti-tubulin (sc-5286 and sc-1616, respectively, Santa Cruz Biotechnology) were used as loading control at a concentration of 0.2 μg ml⁻¹. Detection was performed using the ECL Western blotting detection reagents (GE Healthcare).

BrdU cell-labeling assays

For detecting proliferating keratinocytes in vivo, adult mice were injected intraperitoneally with BrdU (Sigma-Aldrich) at 100 mg per kg bodyweight and killed following a 24-hour chase period. Skin samples were fixed in paraformaldehyde and embedded in paraffin, and sections were stained with an anti-BrdU antibody (M0744 clone Bu20a, DakoCytomation, Milan, Italy). Ten fields per section (× 100 magnification) were analyzed counting the number of interfollicular basal keratinocyte-positive nuclei with respect to the total basal-cell number.

For detecting BrdU-retaining stem cells, 10-day-old mice were injected intraperitoneally with 50 mg kg⁻¹ BrdU for three consecutive days. Following 45-day chase period, mice were killed and back skin excised, fixed, and analyzed by immunohistochemistry, as described above.

In vitro proliferation rate was determined by measuring BrdU incorporation with an ELISA kit (Roche, Indianapolis, IN). Subconfluent
keratinocytes were treated with 1 μM BrdU for 16 hours, and BrdU incorporation was evaluated following manufacturer's instructions.

For BrdU incorporation following IGF-1 treatment, subconfluent keratinocytes were starved for 16 hours in Cnt-57 basal medium, and for the following 24 hours were (1) stimulated with 100 ng ml⁻¹ of mouse recombinant IGF-1 (Peprotech), or (2) cultured in Cnt-57 complete medium (positive control), or (3) maintained in basal medium (negative control). Cells were treated with BrdU for the last 16 hours of IGF-1 treatment and analyzed, as described above.

Scratch assay
Confluent keratinocytes were washed in phosphate-buffered saline and cell monolayers were then scratched with a P-200 tip (Labcon, Hannover, Germany). Wounded monolayers were observed at different time points after injury and pictures taken with a digital camera. The residual gap between migrating keratinocytes was measured with a computer-assisted analysis system and expressed as percentage of the initial scratched area.

To test IGF-1 effect, confluent monolayers were starved overnight in Cnt-57 basal medium and then (1) maintained in basal medium (negative control), or (2) incubated with 100 ng ml⁻¹ of recombinant mouse IGF-1 (Peprotech), or (3) cultured in complete medium (positive control).

Adhesion assay
Freshly isolated keratinocytes were seeded at different concentrations (5 × 10⁴, 10⁵, or 2 × 10⁵ cells per well) in a 24-well plate previously coated with 0.125 mg ml⁻¹ of mouse type IV collagen. Cells were allowed to attach for 12 hours, washed twice with phosphate-buffered saline to remove unattached ones, fixed for 30 minutes with paraformaldehyde, and stained with crystal violet. Four random fields for each well were counted under microscope.

Colony-forming assay
Four × 10⁴ keratinocytes were seeded in triplicate in 6-well plates and cultured for 7 days. Cells were then fixed with 1% paraformaldehyde for 15 minutes and stained with crystal violet (0.05% in phosphate-buffered saline) for 1 hour. The number of colonies was counted, and colony dimension analyzed by using a computer-assisted image analysis.

Rac activation assay
Rac activation was analyzed on cultured keratinocytes treated for 1 hour with 100 ng ml⁻¹ of rmIGF-1 (Peprotech) following the procedure described by Tunggal et al. (2005) and using an anti Rac1 antibody (610650, Becton Dickinson, Erembodegen, Belgium) diluted 1:1,000.

Boyden chamber assay
Keratinocyte migration was analyzed using Boyden chambers (Neuroprobe, Gaithersburg, MD) equipped with 8-μm pore diameter polycarbonate filters (Nucleopore, Whatman, Clifton, NJ) coated with 0.125 mg ml⁻¹ of murine collagen type VI (BD), as described (Cianfarani et al., 2006). In all, 5 × 10⁵ cells were suspended in Cnt-57 keratinocyte medium and loaded into the upper compartment of the Boyden chambers. Analysis was performed after 16 hours of incubation. Experiments were carried out on three different cultures per genotype in triplicate.

Statistical analysis
Numerical differences were analyzed by the unpaired, two-tailed Student's t-test. P-values < 0.05 were considered significant. If not differently specified, values are expressed as mean ± SD.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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

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