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Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis

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To elucidate the biological role of Stat3 in the skin, conditional gene targeting using the Cre-*loxP* system was performed as germline *Stat3* ablation leads to embryonic lethality. *K5-Cre;Stat3*$^{flox/-}$ transgenic mice, whose epidermal and follicular keratinocytes lack functional Stat3, were viable and the development of epidermis and hair follicles appeared normal. However, hair cycle and wound healing processes were severely compromised. Furthermore, mutant mice expressed sparse hair and developed spontaneously occurring ulcers with age. Growth factor-dependent *in vitro* migration of *Stat3*-disrupted keratinocytes was impaired despite normal proliferative responses. We therefore conclude that Stat3 plays a crucial role in transducing a signal required for migration but not for proliferation of keratinocytes, and that Stat3 is essential for skin remodeling, including hair cycle and wound healing.

Keywords: conditional gene targeting/hair cycle/skin remodeling/Stat3/wound healing

Introduction

STATs (signal transducers and activators of transcription) belong to a family of cytoplasmic proteins that are activated by a large number of extracellular signaling molecules including cytokines, growth factors and hormones (Schindler and Darnell, 1995; Ihle, 1996; Darnell, 1997; O'Shea, 1997). Activation of STATs is accomplished by their phosphorylation on a tyrosine residue mediating their dimerization through reciprocal Src homology 2 (SH2) phosphotyrosine interactions, leading to nuclear translocation and hence transcriptional regulation of target genes. STATs are phosphorylated either by receptor tyrosine kinases such as receptors for epidermal growth factor (EGF), platelet derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1), or by Janus kinases (JAKs) which are non-covalently associated to receptors. Cytokine receptors lacking an intrinsic kinase activity mediate signals to STATs through the activation of JAKs.

Seven STATs have now been identified in mammals: Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B and Stat6. Each of these Stats is differentially activated by various extracellular ligands, thereby allowing differential intracellular processing of signals transduced across the membrane. The biological role of each of the Stats has been delineated by investigating the phenotype of mice deficient for each individual Stat. Innate immune responses to viral or bacterial infections were impaired in *Stat1* mutant mice, reflecting its role in mediating the action of interferon (Durbin *et al.*, 1996). Stat4 is activated in T cells by IL-12 stimulation, which leads to development of T helper-1 (TH1) cells. *Stat4* knockout mice are accordingly deficient in TH1 function (Kaplan *et al.*, 1996). On the other hand, *Stat6* knockout mice have deranged IL-4 signaling and cannot generate a TH2 response (Takeda *et al.*, 1996). Whereas Stat4 and Stat6 are activated by a limited number of ligands, Stat5A and Stat5B have a broad activation profile by various ligands including prolactin, growth hormone, erythropoietin, granulocyte-macrophage colony-stimulating factor and IL-2. *Stat5A* knockout mice, however, develop normally except for the inability of females to lactate after parturition because of a failure in mammary gland differentiation (Liu *et al.*, 1997). *Stat5B* knockout mice show a phenotype related to growth hormone signaling (Udy *et al.*, 1997).

Stat3 is activated by cytokines of the IL-6 family such as IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M and cardiotropin 1 (reviewed by Akira, 1997). Non-cytokine ligands such as EGF, PDGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF) and leptin have also been shown to activate Stat3 (Darnell 1997; Boccaccio *et al.*, 1998). The phenotype of *Stat3* knockout mice is unique in that it leads to an early embryonic lethality (Takeda *et al.*, 1997). Thus, the absence of Stat3 is not compensated for by any other Stats expressed in the visceral endoderm during 6.5 and 7.5 days of development. We envisage that Stat3 may be spatio-temporally activated by certain combinations of ligands and receptors. Because embryonic lethality ensues in Stat3-null mice generated by conventional gene targeting technology, a conditional, tissue-specific and/or temporal, gene knockout strategy was required for assessing its later biological role. Takeda *et al.* (1998, 1999) have recently generated *Stat3* knockout mice specifically in T lymphocytes or in macrophages/neutrophils by using a Cre-*loxP* strategy (Rajewsky *et al.*, 1996), and delineated distinct roles of Stat3 for the respective cell lineage.

The growth and differentiation of keratinocytes is regulated by many growth factors and cytokines including EGF, transforming-growth factor (TGF)-α, heparin binding EGF-like factor (HB-EGF), amphiregulin, keratinocyte growth factor (KGF), TGF-β, insulin-like growth factor, PDGF, HGF, IL-6, IL-1 and TNF-α (reviewed by Bennett

and Schultz, 1993; Peus and Pittelkow, 1996; Martin, 1997). EGF receptor (R) knockout mice demonstrated an abnormality in skin structure and hair development (Miettinen *et al.*, 1995; Murillas *et al.*, 1995; Sibilia and Wagner, 1995). A similar phenotype in skin and hair was observed in TGF-α knockout mice (Luetteke *et al.*, 1993), suggesting that TGF-α, an autocrine growth factor of the EGF family (Coffey *et al.*, 1987), plays a crucial role in skin development. Interestingly, Stat3 is activated by factors of the EGF family as well as by HGF, PDGF or IL-6 (Zhong *et al.*, 1994; Akira, 1997; Boccaccio *et al.*, 1998), which are all involved in growth/differentiation of keratinocytes (Rheinwald and Green, 1977; Grossman *et al.*, 1989; Matsumoto *et al.*, 1991; Martin, 1997). Successful ablation of a floxed *Stat3* allele using Cre recombinase driven by keratin 5 promoter allowed us to generate keratinocyte-specific *Stat3* knockout mice. A comprehensive analysis of skin development as well as wound healing and hair cycling in these keratinocyte-specific Stat3-null mice revealed that Stat3 plays pivotal roles in skin remodeling.

Results

Stat3 deficiency in keratinocytes of K5-Cre; Stat3$^{flox/-}$ mice

To elucidate the physiological role of Stat3 in the skin, we specifically disrupted the *Stat3* gene in keratinocytes by crossing *Stat3^{flox}* mice (Takeda *et al.*, 1998) with *keratin5-Cre* transgenic mice (*K5-Cre*) (Tarutani *et al.*, 1997). The keratin 5 promoter directs gene expression in the basal layer of epidermal and follicular keratinocytes, so that a floxed gene is disrupted throughout the epidermis and outer root sheath of hair follicles (Byrne and Fuchs, 1993; Murillas *et al.*, 1995; Tarutani *et al.*, 1997). To increase the efficiency of Cre-mediated *Stat3* disruption and prevent Cre-mediated inter-chromosomal recombination, a *Stat3*-null allele was introduced over the floxed allele (Takeda *et al.*, 1997, 1998). Genotyping of *Stat3* gene for both alleles was carried out by genomic PCR (Figure 1A) and the efficiency and specificity of the Cre-mediated *Stat3* disruption was determined (Figure 1B). As previously described in floxed *Pig-a* gene in *K5-Cre* mice (Tarutani *et al.*, 1997), disruption of floxed *Stat3* in *K5-Cre;Stat3$^{flox/-}$* mice was efficient and specific in keratinocytes. Keratinocytes from *Stat3*-disrupted mice expressed a reduced amount of Stat3 protein whose relative molecular mass was slightly decreased (Takeda *et al.*, 1998), corresponding to the truncated Stat3 (Stat3Δ) (Figure 1C). Stat3Δ together with wild-type Stat3 was detected in keratinocytes from *K5-Cre;Stat3$^{flox/+}$* mice (Figure 1C). Reverse transcriptase (RT)–PCR and DNA sequencing analysis revealed that Stat3Δ lacks the tyrosine residue essential for phosphorylation and the MAP-kinase recognition site (Takeda *et al.*, 1998), both of which are critical for Stat3 activation (Zhang *et al.*, 1995).

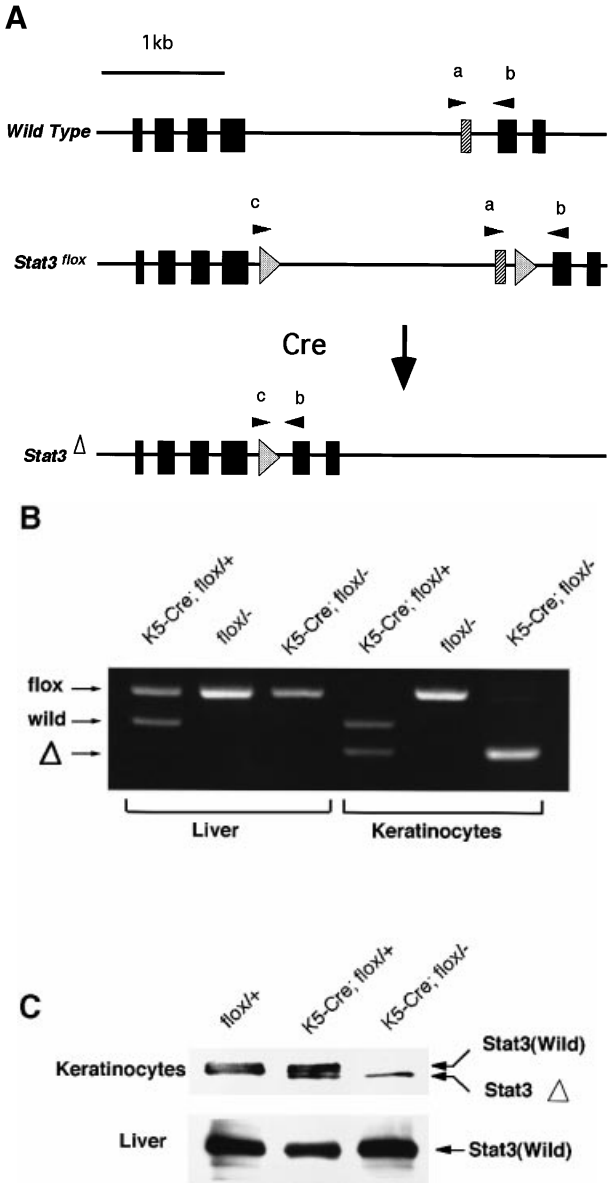

Fig. 1. Keratinocyte-specific disruption of the *Stat3* gene using the Cre-*loxP* system. (**A**) Gene organization in *Stat3^{flox}* mice before and after Cre-mediated disruption. The exon 21 (shaded rectangle) of Stat3 was flanked by two identically oriented loxP sites (dotted triangle). 'a', 'b' and 'c' indicate primers for detection of wild, floxed and disrupted (Δ) Stat3 allele. (**B**) Keratinocyte-specific disruption of the *Stat3* gene in *K5-Cre;Stat3$^{flox/-}$* mice. To evaluate the efficiency of *Stat3* disruption, allele-specific PCR was performed using the three primers shown in Figure 1A. *Stat3$^{flox/-}$* indicates that one allele is a floxed *Stat3* and the other a *Stat3*-null, which was not amplified in the allele-specific PCR because of deletion at exons 20–22 (Takeda *et al.*, 1998). (**C**) Analysis of Stat3 protein in the keratinocytes and liver cells. Stat3 protein from Stat3Δ allele was seen as a truncated form with slightly decreased mol. wt. Note that the keratinocytes of *K5-Cre; Stat3$^{flox/+}$* mice expressed duplex Stat3 proteins, a wild and a truncated form. Wild-type Stat3 (~90 kDa) was exclusively shown in the liver cells from both *K5-Cre;Stat3$^{flox/+}$* and *K5-Cre;Stat3$^{flox/-}$* mice.

Inability of Stat3 activation in K5-Cre;Stat3$^{flox/-}$ keratinocytes

Stimulation with EGF gave rise to Stat3 phosphorylation in the keratinocytes of *Stat3$^{flox/+}$* and *K5-Cre;Stat3$^{flox/+}$* but not of *K5-Cre;Stat3$^{flox/-}$* mice (Figure 2A). Additionally, Stat3Δ in *K5-Cre;Stat3$^{flox/+}$* keratinocytes did not induce a dominant-negative effect on wild-type Stat3 phosphorylation. This contrasts with the finding that wild-type Stat3 was inhibited by Stat3Δ in thymocytes of *Lck-Cre; Stat3$^{flox/+}$* mice (Takeda *et al.*, 1998).

Immunohistochemical analysis with an anti-Stat3 antibody revealed that in wild-type keratinocytes, Stat3 was clearly translocated to the nucleus after EGF or HGF stimulation (Figure 2B). However, it should be noted that

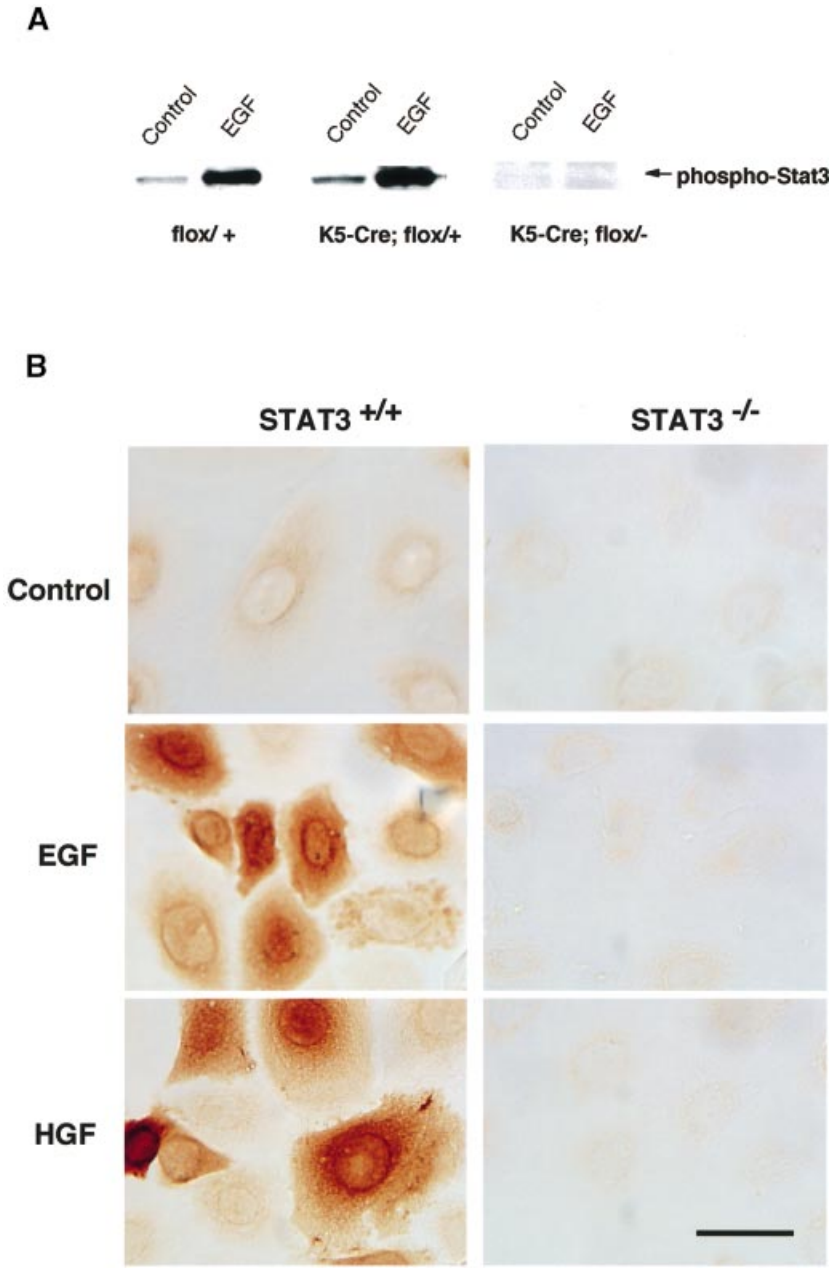

Fig. 2. Lack of Stat3 activation in the keratinocytes of *K5-Cre;Stat3*$^{flox/-}$ mice. (**A**) Western blotting with anti-phosphorylated Stat3 (Tyr705) detected the activated state of Stat3 in the cell lysates prepared from cultured keratinocytes of *Stat3*$^{flox/+}$ and *K5-Cre;Stat3*$^{flox/+}$ mice after EGF treatment (100 ng/ml, 20 min). On the other hand, no phosphorylated Stat3 was detected from *K5-Cre;Stat3*$^{flox/-}$ keratinocytes. (**B**) Immunocytochemical examination with anti-Stat3 revealed that EGF or HGF induced nuclear translocation of Stat3 in wild-type keratinocytes (+/+). In contrast, Stat3 was faintly stained in *Stat3*-disrupted keratinocytes (–/–) irrespective of the stimulation. Scale bar, 25 μm.

enhanced immunoreactivity to the anti-Stat3 antibody was simultaneously observed in the activated wild-type keratinocytes. On the other hand, Stat3 was weakly stained in keratinocytes of *K5-Cre;Stat3*$^{flox/-}$ mice irrespective of the stimulation, corresponding to the result of Western blotting shown in Figure 1C, in which the amount of Stat3Δ appeared to be reduced.

Skin development is intact in K5-Cre;Stat3$^{flox/-}$ *mice*

Despite functional ablation of Stat3 in the keratinocytes of *K5-Cre;Stat3*$^{flox/-}$ mice, these mice were viable and displayed no developmental alterations in the epidermis and hair follicles by postnatal day 11 (PD11) (Figure 3). Histologically, the structure of horny, spinous and basal cell layers of the epidermis were similar and devoid of any abnormality in these *Stat3*-disrupted mice. Such *Stat3*-disrupted mice also showed no gross alterations in the length and texture of the first coat of skin (data not shown). Growth and differentiation markers of keratinocytes such as keratin 6, 10, 14 and involcrin were appropriately expressed in the epidermis and hair follicles (data not shown).

Impairment of the second hair cycle in Stat3-disrupted mice

Because *Stat3* is disrupted in keratinocytes of the outer root sheath, the hair cycle in *Stat3*-disrupted mice was

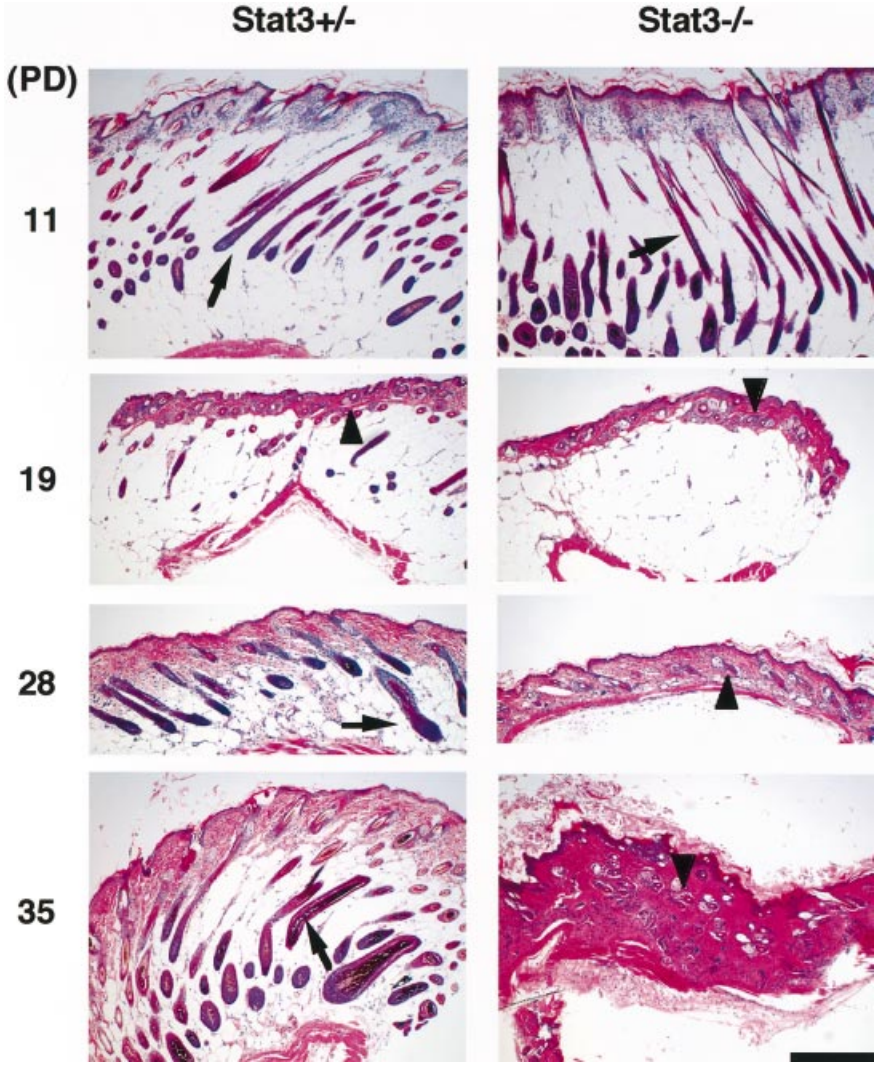

Fig. 3. Impaired second hair cycle in *Stat3*-disrupted mice. Histological appearance of the skin at various times of control littermates (Stat3+/–, left panels) and *Stat3*-disrupted mice (Stat3–/–, right panels). Hair follicles are at the anagen on PD11 and the telogen stage by PD19 in both control and *Stat3*-disrupted mice. Hair follicles in the control mice enter the second anagen by PD28 (PD28, left panel) whereas those of *Stat3*-disrupted mice remain at telogen (PD28, right panel). Even at late anagen stage (PD35) in control mice, *Stat3*-disrupted mice stay in telogen (PD35, right panel). Scale bars, 250 μm. Arrows and arrowheads show the anagen and the telogen hair follicles, respectively. The number of mice examined at PD11, PD19, PD28, PD35 was 11 (seven controls, four Stat3–/– mice), eight (five controls, three Stat3–/– mice), 10 (six controls, four Stat3–/– mice) and eight (five controls, three Stat3–/– mice), respectively. There was no difference in hair cycles between Stat3+/+ and +/– mice (data not shown).

examined. Hair follicles elongate to reach the deep adipose tissue at the anagen stage (Figure 3, PD11), then undergo gradual cellular quiescence (catagen) and finally, around PD19 undergo complete rest (telogen). Until this stage there was no observed difference between control littermates and *Stat3*-disrupted mice. However, the second anagen was not observed in the mutant mice (Figure 3, right panel of PD28). In contrast, the control mice entered the anagen, expressing thickened and elongated hair follicles far down into the adipose tissue (Figure 3, left panel of PD28). *Stat3*-disrupted mice remained in a telogen stage even by PD35 when the control littermates were at the late anagen stage. It is interesting to note that at this stage the *Stat3*-disrupted skin displayed pronounced dermal fibrosis together with infiltration of inflammatory cells and an atrophic change in adipose tissue. Thus, we suggest that Stat3 regulates the second and subsequent hair cycles whereas the first hair cycle, which starts around 14.5 days post coitus (d.p.c.) (Kashiwagi *et al.*, 1997), is Stat3 independent.

Retardation of skin wound healing in Stat3-disrupted mice

Stat3-disrupted and control littermates were wounded with a biopsy punch and then the process of healing was monitored. Wound healing was markedly retarded in the *Stat3*-disrupted mice while re-epithelialization was completed in the control littermates on day 8 after excision wounding (Figure 4A). Histological examination revealed an ulcer of the skin in the *Stat3*-disrupted mice, whereas at this time point the wound had completely healed in the control littermates (Figure 4B). However, no difference was shown between the *Stat3*-disrupted and control mice in the dermal responses to wound such as granulation, inflammation and neovascularization. Thus the retarded wound healing in the *Stat3*-disrupted mice was due not

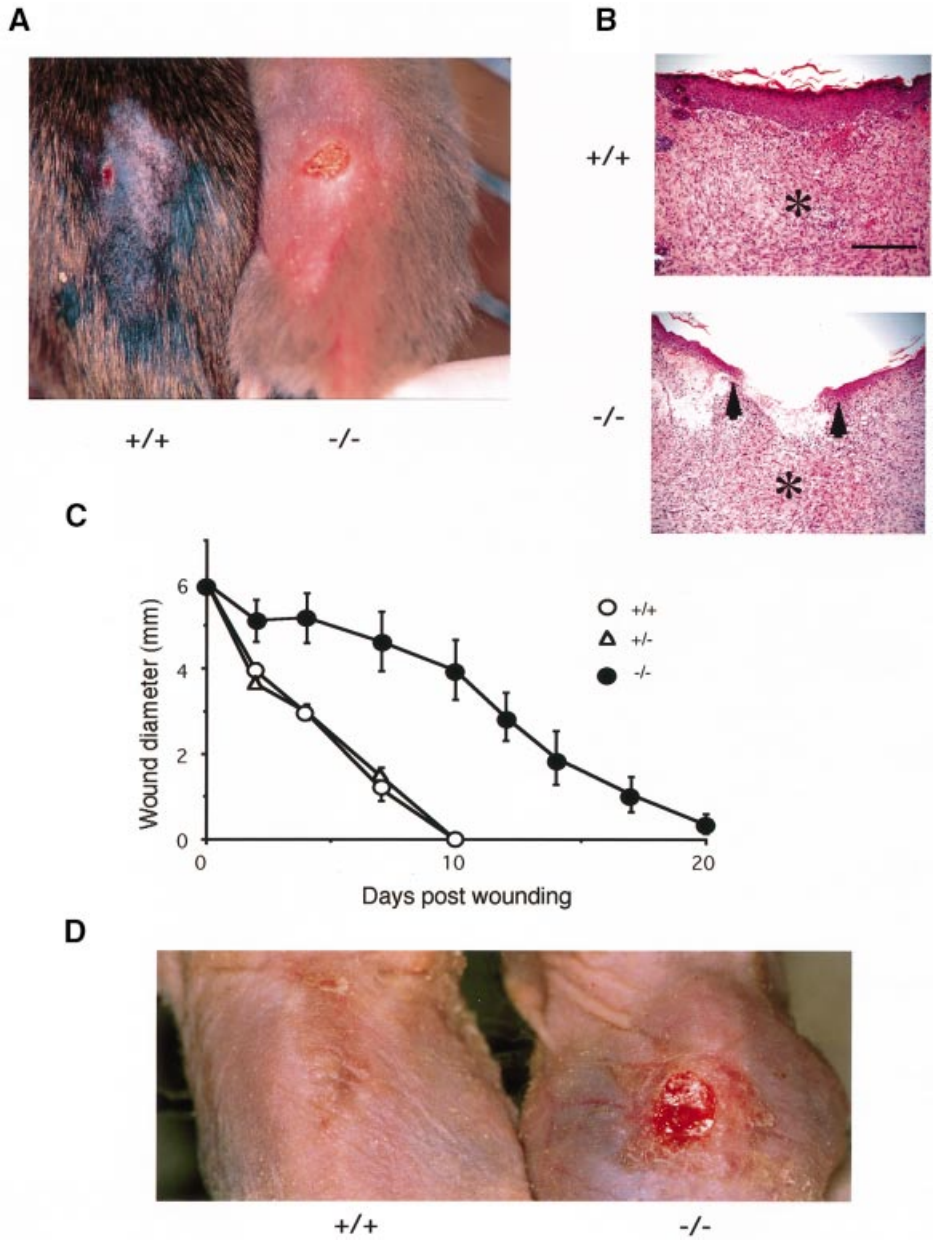

Fig. 4. Retardation of skin wound healing in *Stat3*-disrupted mice. (**A**) Comparison of skin wound healing in a *Stat3*-disrupted mouse (–/–, right) and a control littermate (+/+, left). The photograph was taken on day 8 after wounding. (**B**) Histology of the wounds. On day 12 after wounding the skin was completely repaired in a control littermate (+/+, upper) whereas the re-epithelization was not finished in a *Stat3*-disrupted mouse (–/–, lower). There was no difference in the wound-associated dermal reaction including granulation, inflammation and neovascularization (*) between control and Stat3-disrupted mice. Scale bar, 300 μm. Arrowheads indicate epidermal wound edges. (**C**) Quantitative difference in wound healing processes between controls and *Stat3*-disrupted mice. A full-thickness skin excision was made on the back using a biopsy punch of 6 mm diameter, and the diameters of the wounds were calibrated daily. Wound closure in *Stat3*-disrupted (*K5-Cre;Stat3*$^{flox/-}$) mice (●, $n = 7$) was strikingly retarded, whereas control mice of +/+ (○, $n = 9$, including wild, *K5-Cre*;*Stat3*$^{flox/+}$) and +/– (△, $n = 11$, including *K5-Cre;Stat3*$^{flox/+}$, *Stat3*$^{flox/-}$) had healed by day 10. Values indicate the mean diameter ± SE. (**D**) Wound healing of grafted skin. Retarded wound healing was reproduced in the skin from a *Stat3*-disrupted mouse transplanted on to nude mice (right), whereas the control graft (left) was completely healed. This photograph was taken on day 10 after excisional wounding (4 mm diameter) of the grafted skin. One of the representatives is shown.

to an impairment in secondary development of dermal components but rather to a fault in epidermal regeneration.

To evaluate quantitative differences in the wound healing process, wound diameter was sequentially measured. Wild-type, *K5-Cre* transgenic *Stat3*$^{flox/+}$ (referred to as +/+) and *K5-Cre;Stat3*$^{flox/+}$, *Stat3*$^{flox/-}$ (+/–) mice had completely healed by 10 days after wounding (Figure 3C). At the same time point, the wound in *K5-Cre;Stat3*$^{flox/-}$ mice (–/–) was still open (average wound 4 mm), and usually healed at ~20 days after wounding (Figure 3C).

Disruption of the floxed *Stat3* allele occurred in a keratinocyte-specific manner (Figure 1B and C) with very little, if any, disruption in epithelial cell-containing organs such as stomach and oesophagus (Tarutani *et al.*, 1997). Thus, it is possible that the retarded wound healing in *K5-Cre;Stat3*$^{flox/-}$ mice might be due to disruption of *Stat3* in other tissue(s). In order to exclude this possibility and to confirm that the primary cause of the retarded wound healing in the *Stat3*-disrupted mice was impaired regeneration of the epidermis, the skin was transplanted on to

nude mice. The retarded phenotype was reproduced in the *Stat3*-disrupted skin (Figure 3D), compounding the idea that the primary cause of retarded wound healing in the *Stat3*-disrupted mice resides within the mutant skin itself.

Although retarded, wound healing was eventually completed in the *K5-Cre;Stat3$^{flox/-}$* mice (Figure 3C). It should be noted that dermal contraction could draw the wound edges together (Martin, 1997), and hence contribute to wound closure in these mice.

Migration defect of the Stat3-disrupted keratinocytes accounts for the phenotype

Cell migration and proliferation are critical events in re-epithelialization of cutaneous wounds (reviewed by Bennett and Schultz, 1993; Martin, 1997) and hair growth (reviewed by Peus and Pittelkow, 1996). Therefore, the motility and growth of keratinocytes *in vitro* were examined in the *Stat3*-deficient keratinocytes. *In vitro* wounds were inflicted in subconfluent keratinocyte cultures pretreated with mitomycin C to prevent cell proliferation. Following wounding, closure of the cell-free area by migrating cells was examined in the presence of EGF, TGF-α, HGF, IL-6 and keratinocyte growth factor (KGF), all of which have been reported to promote epithelial cell motility (Barrandon and Green, 1987; Matsumoto *et al.*, 1991; Nishida *et al.*, 1992; Werner *et al*., 1994; Sato *et al.*, 1995). EGF, TGF-α, HGF and IL-6, all of which activate Stat3 (Figure 2, and Zhong *et al*., 1994; Boccaccio *et al.*, 1998), promote the migration of Stat3+/– keratinocytes (*Stat3$^{flox/-}$*) (Figure 5A). In contrast, the migration of *Stat3*-disrupted keratinocytes was severely impaired in the presence of these ligands (Figure 5A). KGF, a paracrine stimulator for keratinocyte migration as well as proliferation (Werner *et al.*, 1994; Sato *et al.*, 1995), did not activate Stat3 in keratinocytes (data not shown) nor promote the migration of keratinocytes from either control or *Stat3*-disrupted mice (Figure 5A). Our results support the finding that KGF acts as a mitogen, and not a migration stimulator for keratinocytes (McCawley *et al.*, 1998). Figure 5B demonstrates a quantitative difference of ligand-dependent migration between the control and *Stat3*-disrupted keratinocytes. Thus, functional Stat3 is required for the migration and accordingly re-epithelialization of keratinocytes.

The involvement of Stat3 in keratinocyte proliferation was also examined. Growth factor-dependent proliferation of the *Stat3*-disrupted keratinocytes *in vitro* was appreciable and comparable to the control in terms of dose dependency of exogenous ligands (Figure 5C). Ki67, which is a proliferation-associated antigen (Gerdes *et al.*, 1983), was expressed in keratinocytes at the wound edge and hair follicles of *Stat3*-disrupted as well as control mice *in vivo* (data not shown). Thus, keratinocyte proliferation is mediated through Stat3-independent pathway(s). Taken together, the retarded *in vivo* wound healing in the *K5-Cre;Stat3$^{flox/-}$* mice could be attributed to the poor motility of *Stat3*-disrupted keratinocytes.

Spontaneous skin alterations in aged Stat3-disrupted mice

Despite the normal appearance of the skin in *Stat3*-disrupted mice at birth, *Stat3*-disrupted mice older than 2 months had sparse hair and developed scale and crusts on an apparently rough skin surface (Figure 6A). Histologically, they harbored spontaneous ulcers and erosions with a decreased number of hair follicles (Figure 6B). Additionally, the mutant mice expressed marked hyperplasia of the epidermis (acanthosis) with hyperkeratosis and scale-crusts. There was pronounced inflammatory infiltration and fibrosis throughout the dermis. While most of the hair follicles were at the telogen phase, some were at anagen. Furthermore, there were aberrant hair follicles; for example, small clusterings of matrix cells, which resembled undifferentiated hair germs (Figure 6C), distorted or curved hair shafts (Figure 6D) or gigantic hair bulbs with bizarre shapes (Figure 6E). The phenotype expressed in aged *Stat3*-disrupted mice appears to be consequent to the impaired wound healing and disorganized hair cycling. Thus Stat3 plays a crucial role in the remodeling of the skin and hence in the structural integrity of the tegument in mice.

Discussion

STATs are latent cytoplasmic proteins that deliver signals from extracellular stimuli to the nucleus in order to regulate the transcription of many different genes (reviewed by Schindler and Darnell, 1995; Ihle, 1996; Darnell, 1997; O'Shea, 1997). Despite complexity in the determination of STAT availability for specific cell lineages, phenotypes revealed through the knockouts of specific STAT family member highlight their predominant biological functions *in vivo*. Stat3 is activated by a number of ligands including IL-6-type cytokines (IL-6, CNTF, LIF, oncostatin M, IL-11, cardiotropin 1) and EGF, PDGF, HGF, G-CSF, IFN-α/β, IL-2, IL-10, CSF-1, angiotensin II, leptin, erythropoietin and thrombopoietin (Akira, 1997). Knockout of *Stat3* in the germline results in embryonic lethality (Takeda *et al.*, 1997), implying that Stat3 has global and critical effects on development. Therefore, conditional gene targeting in the specific lineage was required for assessing its later biological roles.

Cre-loxP system enables analysis of Stat3 function in keratinocytes

The Cre-*loxP* system used here specifically removes the tyrosine phosphorylation site together with the mitogen-activated protein kinase (MAPK) recognition site (Takeda *et al.*, 1998), both of which are important for Stat3 activation (Zhang *et al*., 1995). Using the Cre-*loxP* system with the same floxed *Stat3* construct, Takeda *et al.* (1998) have recently established mice that have specifically ablated *Stat3* in T cells or macrophages/neutrophils. T cells from *Lck-Cre;Stat3 $^{flox/-}$* mice do not proliferate in response to IL-6 probably because these T cells are unable to prevent apoptosis upon IL-6 stimulation. Mice devoid of Stat3 in macrophages/neutrophils developed chronic enterocolitis revealed by skewed Th1 predominance because of a lack of IL-10 production (Takeda *et al*., 1999). In the present study, keratinocytes devoid of Stat3 failed to respond to EGF, HGF or IL-6, resulting in their impaired migration (Figure 5A and B). Thus, in contrast to the outcome of gene targeting of a *Stat* family member having a narrow activation profile such as Stat6, the *Stat3* knockout results in various alterations in a cell-lineage-specific fashion.

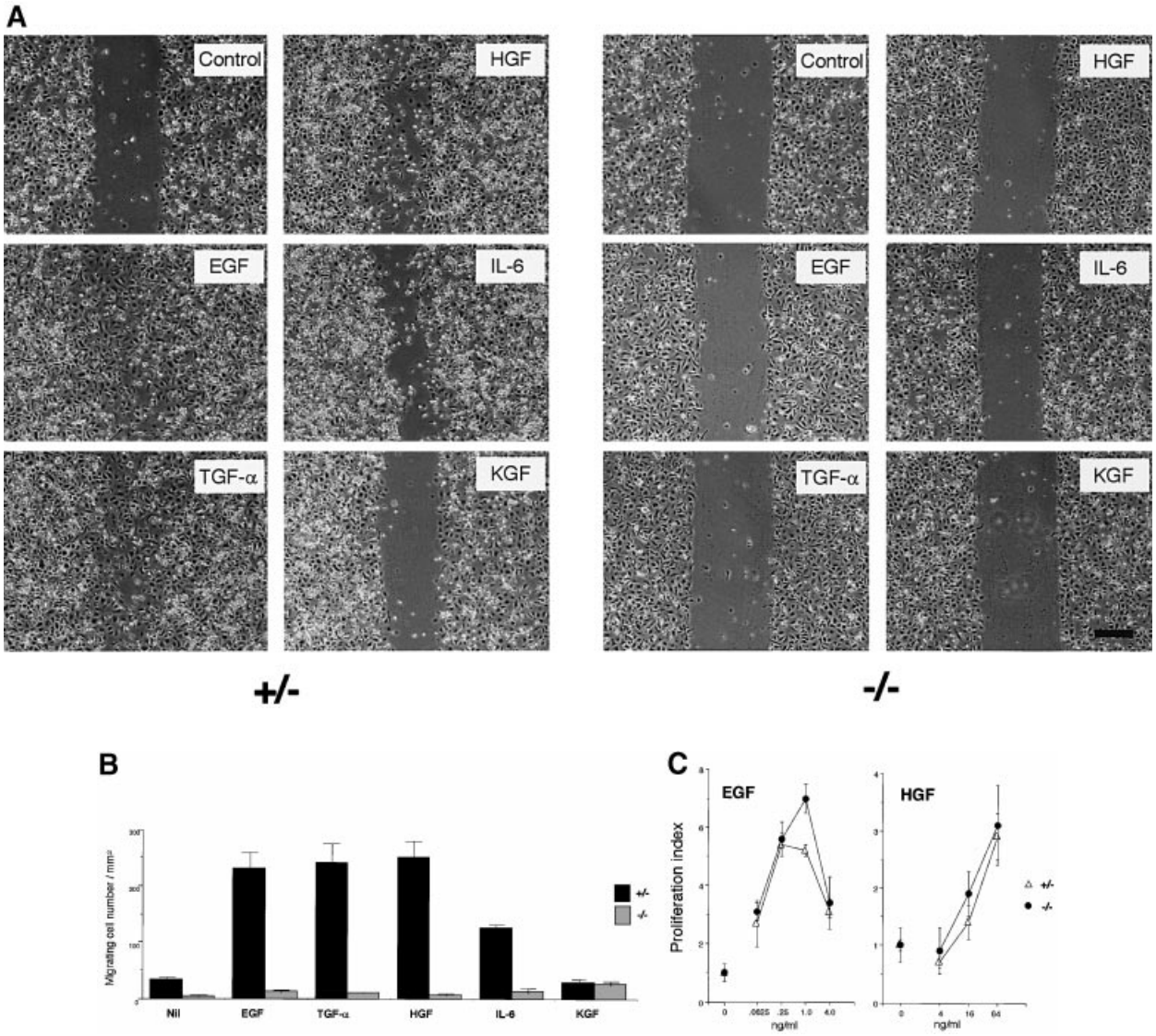

Fig. 5. Impaired migration of *Stat3*-disrupted keratinocytes. (**A**) *In vitro* wounds were introduced in cultured keratinocytes at subconfluency derived from control (*Stat3*$^{flox/-}$, left panels) and *Stat3*-disrupted mice (right panels), and the keratinocytes were cultured for another 48 h in the presence of indicated ligands. The control keratinocytes (+/–) migrated on stimulation with EGF, TGF-α, HGF and IL-6, whereas *Stat3*-disrupted keratinocytes (–/–) did not. Scale bar, 200 μm. (**B**) Quantitative evaluation of keratinocyte migration in the responses to ligands. Migrating keratinocytes from *Stat3*$^{flox/-}$ (black bars) and *Stat3*-disrupted (gray bars) mice were quantitatively assessed as described in Materials and methods. Migrating cells are shown as mean number per mm^2 beyond the wound edge. (**C**) Appreciable proliferation of *Stat3*-disrupted keratinocytes to ligands. The proliferation indices represent ratios of [^{3}H]thymidine incorporation in the presence of growth factor at indicated concentrations to those in medium alone. The mean of triplicate determinations ± SD is shown. No virtual difference is obtained in the proliferation indices in response to EGF or HGF at various concentrations between control (*K5-Cre;Stat3*$^{flox/+}$, △) and *Stat3*-disrupted mice (●).

The basal layer of the epidermis expresses keratin 5 (K5) which pairs with K14. When keratinocytes differentiate from basal to spinous cells, the expression of K5/K14 decreases and that of K1/K10 is initiated. K5 is also expressed in the outer root sheath cells of hair follicles (Byrne and Fuchs 1993), namely follicular keratinocytes. In *K5-Cre;Stat3*$^{flox/-}$ mice, *Stat3* would therefore be disrupted throughout the epidermis and outer root sheath of hair follicles. The alteration of hair architecture in these mice is likely to be due to the lack of *Stat3* in the outer root sheath cells.

Disruption of the EGFR by homologous recombination (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995), or by introducing a dominant-negative mutant as a transgene in mice under the control of the K5 promoter (Murillas *et al.*, 1995), demonstrates rudimentary curly whiskers and waved pelage hair. The hair follicles in these mice fail to enter into the catagen phase, and are arrested at the anagen stage. In organ cultures of embryonic skin, exogenous EGF inhibited the formation of hair follicles at an early stage in the development of hair rudiments (Kashiwagi *et al.*, 1997). Administration of excessive doses of EGF to newborn mice cause follicles to enter catagen phase (Moore *et al.*, 1981). Taken together, these data suggest that EGFR signaling controls hair follicle morphogenesis and subsequent hair cycles through an inhibitory effect on the growth of hair follicles. The observation that EGFR expression is down-regulated at

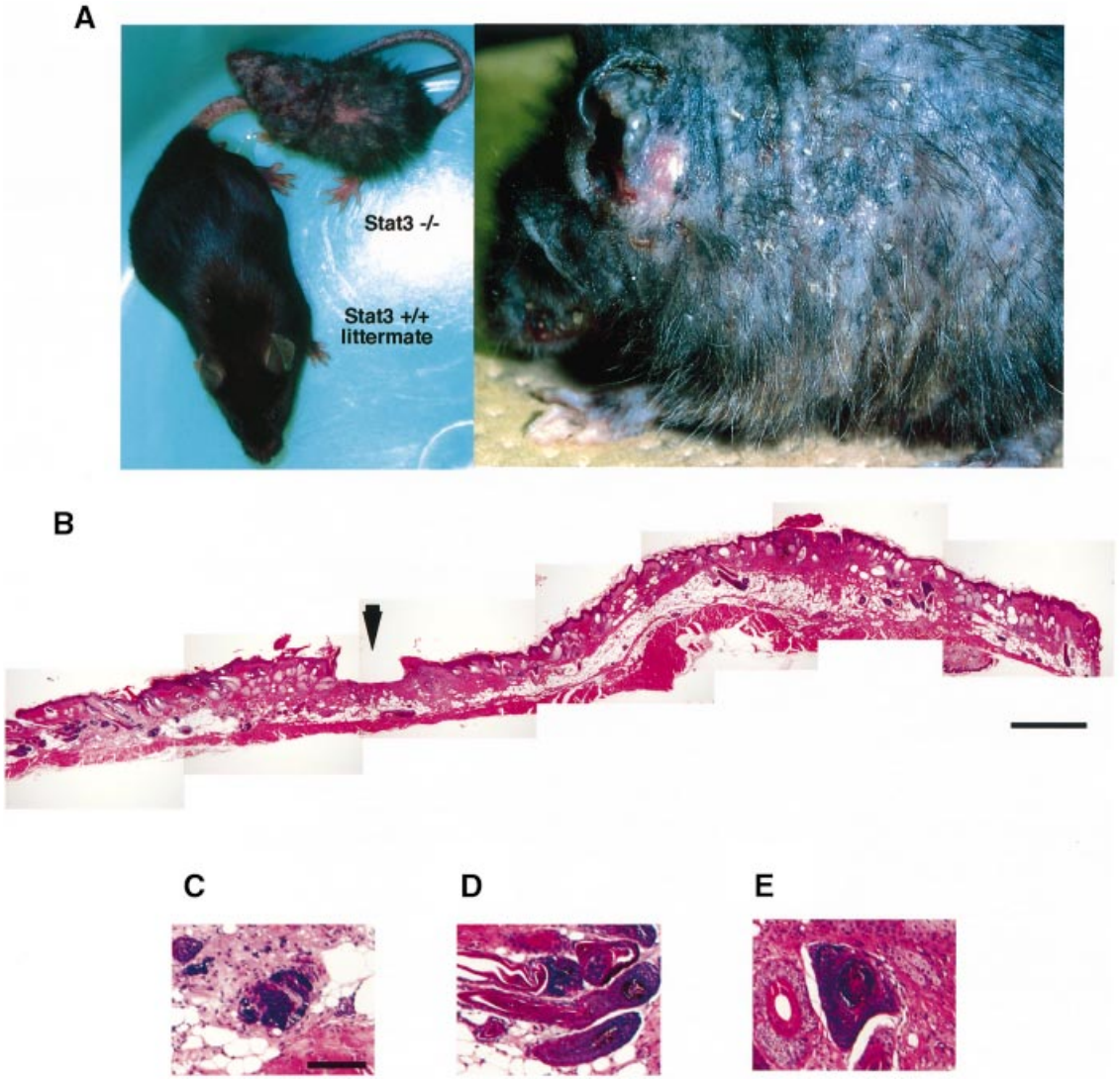

Fig. 6. Alterations in aged *Stat3*-disrupted mice. (**A**) A 5-month-old *Stat3*-disrupted mouse and a wild-type sibling (left), and a close-up view of the *Stat3*-disrupted mouse (right). (**B**) Histological appearance. Ulcer (arrowhead), epidermal hyperplasia and aberrant hair follicles are noted in the *Stat3*-disrupted mouse. Bar, 800 μm. (**C**–**E**) Aberrant hair follicles in the *Stat3*-disrupted mouse. Bar, 100 μm.

the tip of the migrating front of embryonic hair germ and anagen follicles (Green and Couchman, 1984) may, therefore, account for this machinery being refractory to growth factors of the EGF family.

In contrast, the hair follicles of keratinocyte-specific *Stat3*-disrupted mice develop normally, with the first hair cycle being normal (Figure 3, PD11, PD19). Thus an EGFR signal independent of the Stat3 pathway is required for the organized morphogenesis of hair follicles and induction of catagen. Despite the normal appearance of the first hair cycle, *Stat3*-disrupted mice are impaired at the onset of the second anagen (Figure 3, PD28). Since the EGFR is absent from the tip of the migrating front of early second anagen follicle (Green and Couchman, 1984), we suggest that Stat3 activation by ligands other than EGF family members is required at this phase. Among a wide variety of ligands which activate Stat3, HGF is the most likely candidate due to the fact that exogenous HGF accelerates and prolongs the anagen phase of hair follicles (Jindo *et al.*, 1998). Although the hair follicles in *Stat3*-disrupted mice remain in telogen at PD35, some of the follicles can enter anagen with age (Figure 6B). Interestingly, the aged follicles are less synchronized and are of an unusual shape (Figure 6C–E). Growth factor(s) whose downstream signals bypass Stat3 could induce anagen albeit less efficiently. During this interruption of the hair cycle, the dermis is thickened with fibrosis and the subcutaneous adipose tissue is replaced by granulomatous tissue resulting in atrophic change (Figure 3, PD28, PD35). Similar alterations in the dermis occurred in mice with EGFR deficiency acquired by transgenesis of a dominant-negative mutant driven by the K5 promoter (Murillas *et al.*, 1995). This implies the presence of pathological epithelium–mesenchyme interactions in the hair follicle (Hardy, 1992).

A migration defect in Stat3-disrupted keratinocytes is responsible for impaired skin remodeling

We found that wound healing in keratinocyte-specific *Stat3*-disrupted mice is retarded not only *in vivo* (Figure 4) but also *in vitro* (Figure 5A and B), such that keratinocyte

migration under the stimulation of growth factors is severely compromised, despite the proliferative response appearing normal (Figure 5C). During skin wound healing, epidermal keratinocytes at the wound edge are stimulated by a wide variety of cytokines and growth factors that promote keratinocyte migration and proliferation, resulting in re-epithelialization (reviewed by Bennett and Schultz, 1993; Martin, 1997). Members of the EGF family, especially TGF-α (Coffey *et al.*, 1987) and HB-EGF (Higashiyama *et al.*, 1991), play a pivotal role in stimulating epidermal keratinocytes in the wound in an autocrine or juxtacrine fashion, respectively. HB-EGF-dependent migration was also compromised in *Stat3*-disrupted keratinocytes (data not shown), suggesting that Stat3 mediates a signal through HER4 as well as HER1. HER4 is activated by HB-EGF; HER4 activation stimulates chemotaxis but not proliferation (Elenius *et al.*, 1997). IL-6 and HGF also activate Stat3, and can promote epithelial cell growth or migration *in vitro* (Grossman *et al.*, 1989; Matsumoto *et al.*, 1991). These cytokines may be generated from wound-associated mesenchymal cells such as activated fibroblasts or macrophages, and therefore stimulate keratinocyte migration *in vivo*. Since wound closure did occur eventually in *K5-Cre;Stat3*$^{flox/-}$ mice (Figure 4C), we suggest that intracellular signaling molecule(s) other than Stat3 may also be involved in keratinocyte migration *in vivo*. Furthermore, the Stat3 pathway has also been shown to cooperate with other intracellular signaling pathways such as ras-MAP kinase to promote epithelialization (Boccaccio *et al.*, 1998).

Cell adhesion to extracellular matrix (ECM) components and their degradation by proteolytic enzymes play important roles in tissue remodeling (Yuspa *et al.*, 1993; Martin, 1997). Growth factors could co-ordinate keratinocyte function in integrin expression and production of proteases such as matrix metalloproteinases (MMPs) during the process of skin remodeling (Yuspa *et al.*, 1993; Peus and Pittelkow 1996; Martin, 1997). Keratinocyte migration requires MMP production in order to degrade ECM components. Recent studies have revealed that EGF or HGF trigger keratinocytes to produce collagenase-1 (Dunsmore *et al.*, 1996; Pilcher *et al.*, 1997) and MMP-9 (McCawley *et al.*, 1998). Transcriptions of MMP13 (murine homologue to collagenase-1) and MMP9 in *Stat3*-disrupted and control keratinocytes were enhanced by EGF or HGF (data not shown), suggesting that Stat3 is not involved in promoting the transcription of these protease genes. α2 integrin forms a heterodimeric receptor with β1 integrin specific for type-I collagen (Chen *et al.*, 1993). However, we obtained no difference in EGF-upregulated α2 integrin expression between *Stat3*-disrupted and control keratinocytes (data not shown). We cannot exclude the possibility that *Stat3*-disrupted keratinocytes are compromised in the expression of integrins other than α2 or in subsequent signaling after binding to specific ECM molecules essential for migration.

Since epidermal and follicular keratinocytes (outer root sheath cells) share features (Lenoir *et al.*, 1988; Byrne and Fuchs, 1993), it is likely that hair cycle impairment in *Stat3*-disrupted mice is also due to a failure in the migration of follicular keratinocytes. Indeed, an anagen progression involves downward migration of hair follicle keratinocytes responding to an as yet undefined 'dermal message' (Hardy, 1992).

Morphogenesis and remodeling in the skin

In 14.5 d.p.c. mice embryonic skin, the pilosebaceous units develop from epidermal downgrowths (Kashiwagi *et al.*, 1997) under the influence of specific mesenchymal cell condensations, which supply permissive and instructive signals that govern the position and type of hairs and other appendages (reviewed by Hardy, 1992; Stenn *et al.*, 1996). These signals are regulated by the expression of patterning genes, such as *Wnt*-catenin-LEF-1 signaling (Gat *et al.*, 1998), sonic hedgehog (St-Jacques *et al.*, 1998), bone morphogenetic protein families (Blessing *et al.*, 1993) and many others (reviewed by Stenn *et al.*, 1996), leading to development of hair buds. Since K5 is expressed in the epithelia earlier than the onset of hair morphogenesis (Ramirez *et al.*, 1994), the *Stat3* gene must be disrupted in outer root sheath throughout the developing processes. Moreover, we found that disruption of *Stat3* was almost complete in neonatal keratinocytes from *K5-Cre;Stat3*$^{flox/-}$ mice (Figure 1B). Thus, Stat3 in keratinocytes is not involved in the morphogenesis of skin and hair follicle since keratinocyte-specific *Stat3*-disrupted mice show no anomalies at birth. It is highly likely that compromised migration of *Stat3*-disrupted keratinocytes is responsible for the failure to enter the ordered second anagen which is initiated by the dermal mesenchymal signal, namely cytokines/growth factors emanating from dermal papilla (Hardy, 1992; Stenn *et al.*, 1996). Data presented here are in agreement with the concept that the first hair cycle is distinct from all the subsequent hair cycles in its cellular origin and morphological sequence, such that the first hair cycle might be regarded as a neogenic event (Wilson *et al.*, 1994). Taken collectively, these observations suggest that Stat3 activation is indispensable for the progression of the second hair cycle onwards, which represents one of remodeling phases of the skin, although the morphogenesis of hair follicles is independent of Stat3. Based on this, a hypothetical model of the hair cycle is depicted in Figure 7.

Throughout life, epidermal keratinocytes continuously grow, differentiate and die. Keratinocytes are provided from stem cells located in the interfollicular epidermis and in a bulge region of the lower outer root sheath of the follicles (Cotsarelis *et al.*, 1990). Spontaneous wounds and sparse hair development in the aged *Stat3*-disrupted mice (Figure 6A and B) may be due to an inability of bulge-derived stem cells to migrate and serve as new stem cells at the wound site and in the outer root sheath. Stem cells in the latter may be necessary to form organized anagen follicles. Thus keratinocyte Stat3 appears to play a critical role in the process of skin homeostasis and remodeling. This role in skin remodeling recapitulates its involvement in liver regeneration, where IL-6, EGF-family members and HGF, all of which activate Stat3, increase in the serum after partial hepatectomy. These cytokines are essential for liver regeneration (Michalopoulos and DeFrances, 1997). In conclusion, Stat3 contributes significantly to keratinocyte activity especially in skin remodeling including wound healing and hair cycling processes.

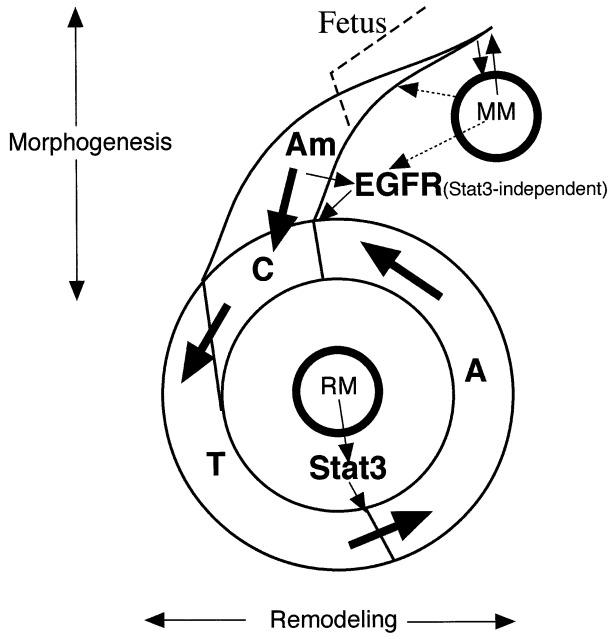

Fig. 7. Hypothetical model of hair cycle. Follicular morphogenesis starts at 14.5 d.p.c. through morphogenic mesenchymal (MM)–epithelial interactions. Hair follicular rudiments grow downwards and differentiate to develop the complex hair structure until around PD17 when EGFR signal (Stat3 independent) exerts an inhibitory effect on the growth. Then, follicles undergo cellular quiescent process (catagen) and finally, the telogen phase. Around PD21, the second anagen is initiated in response to remodeling mesenchymal (RM) signal, which requires Stat3. Thus, the hair cycle is depicted as a number 6-shaped loop with the top ingress representing the morphogenetic phase when the follicle is initially developed (morphogenic anagen, Am) and the subsequent circle represents the remodeling phase in which Stat3 signaling is involved. C, catagen; T, telogen; A, anagen.

Materials and methods

Generation of gene targeting mice using Cre-loxP strategy and PCR

Generation of *K5-Cre* transgenic mice (Tarutani *et al.*, 1997), *Stat3*-null heterozygous mice (Takeda *et al.*, 1997) and *Stat3*$^{flox/flox}$ mice (Takeda *et al.*, 1998) were previously described. *K5-Cre* transgenic mice were bred with *Stat3*-null heterozygous mice to generate mice carrying the *K5-Cre* transgene and a *Stat3*-null allele (*K5-Cre;Stat3*$^{+/-}$). These mice were then mated with *Stat3*$^{flox/flox}$ mice. Offspring carrying a *floxed Stat3* allele and/or *K5-Cre* transgene (*K5-Cre;Stat3*$^{flox/+}$, *K5-Cre;Stat3*$^{flox/-}$, *Stat3*$^{flox/+}$, *Stat3*$^{flox/-}$) were used for further analyses. Allele-specific PCR was carried out as illustrated in Figure 1A. The primers used, 'a' (5′-CCTGAAGACCAAGTTCATCTGTGTGAC-3′), 'b' (5′-CACACA-AGCCATCAAACTCTGGTCTCC-3′) and 'c' (5′-GATTTGAGTCA-GGGATCCTTATCTTCG-3′) are specific for exon 21, exon 22 of the *Stat3* gene and the 5′ *loxP* site of the targeted construct, respectively. Using a mixture of these primers, PCR was performed with 35 cycles of a reaction consisting of 1 min of denaturation at 93°C, 1 min of annealing at 65°C and 2 min of elongation at 72°C. With primers 'a' and 'b', PCR products were ~250 and 350 bp specific for wild-type, and floxed *Stat3* allele, respectively. With primers 'b' and 'c', they were 150 bp specific for the *Stat3*Δ allele.

Preparation of keratinocytes

Full-thickness skin taken from newborn to 5-day-old mice was treated with 250 U/ml of dispase (Godo Shusei, Tokyo, Japan) overnight at 4°C, and the epidermis was peeled off from the dermis and trypsinized in order to yield single cells. It should be noted that epidermal keratinocytes prepared by this method are inevitably contaminated with follicular keratinocytes from outer root sheath (Kitano and Okada, 1983). Mice were killed by intraperitoneal injection of sodium pentobarbital.

Western blotting

Keratinocytes of equivalent numbers or liver tissue of equivalent wet weight was lysed with ice-cold lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_3$ and 5 μg/ml aprotinin. Whole-cell lysates were incubated with anti-Stat3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) followed by protein G–Sepharose (Pharmacia, Uppsala, Sweden) overnight at 4°C. Immunoprecipitates were separated on SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with the same antibody, followed by treatment with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin (Amersham, Buckinghamshire, UK), and then visualized with an enhanced chemiluminescence (ECL) system (Amersham). To examine the phosphorylation of Stat3, keratinocytes were cultured in MCDB153 medium (Kyokuto, Tokyo, Japan), supplemented with 5 μg/ml insulin, 0.1 mM monoethanolamine, 0.1 mM phosphoryl ethanolamine and 0.5 μM hydrocortisone at 37°C under an atmosphere with 5% CO_2, and allowed to grow for 3 days in the presence of 1% bovine pituitary extract (BPE, prepared by mincing of bovine pituitary glands). After starvation for 24 h, cells were treated with or without 100 ng/ml EGF (Upstate Biotechnology, Lake Placid, NY) for 20 min, subject to cell lysis. Cell lysates of epidermal keratinocytes of equivalent numbers were subjected to Western blotting as described above, except that anti-phosphorylated Stat3 (Tyr705; New England Biolabs, Beverly, MA) was used for blotting.

Immunocytochemical staining

Keratinocytes were treated with or without 100 ng/ml EGF or HGF (Collaborative Biolamedical Products, Bedford, MA) for 40 min. They were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100 for 5 min and incubated with anti-Stat3 (C-20; Santa Cruz Biotechnology), followed by treatment with biotinylated anti-rabbit IgG and horseradish-peroxidase-conjugated ABC reagent (Vector Laboratories, Buringame, CA).

Wound healing assay

These experiments were carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals. Under anesthesia with sodium pentobarbital, full-thickness skin excisions were made on the back of 6- to 9-week-old mice using a biopsy punch of 6 mm diameter. In graft experiments, pieces of full-thickness skin (2–3 cm^2) obtained from mice of indicated genotypes were transplanted on to the back of Balb/c nu/nu mice (10-week-old), whose back skin had been ablated with a razor and then fixed tightly with adhesive bandages for a week. Two weeks after transplantation the grafted skin was wounded with a biopsy punch of 4 mm diameter. The punched-out grafted skin was subject to PCR to confirm the donor's genotype.

In vitro migration assay

Keratinocytes were cultured in dishes precoated with type-I collagen (Iwaki Glass, Tokyo, Japan) until they reached subconfluency in the presence of BPE, then starved for 24 h, treated with 10 μg/ml mitomycin C for 2 h to avoid the proliferative effect on the cells and finally subjected to *in vitro* wound closure assay. A cell-free area was introduced by scraping the monolayer with a yellow pipette tip. Cell migration to the cell-free area for another 48 h was evaluated in the absence of exogenous ligands or in the presence of 30 ng/ml EGF, TGF-α (Otsuka Pharmaceutical, Tokushima, Japan), HGF, KGF (PeproTech, London, UK) and IL-6 (R&D Systems, Minneapolis, MN). Photographs were taken using a phase-contrast microscope (DIAPHOT 300; Nikon, Tokyo, Japan). The number of migrating keratinocytes were counted after taking photographs of four non-overlapping fields. Values represent the mean ± SE of migrating cells per mm^2 beyond the frontiers of the *in vitro* wound edge.

Proliferation assay

Keratinocytes (10^4) were plated in a well of a 96-well type I collagen-precoated plate (Iwaki Glass), and cultured in the presence of EGF or HGF at the concentrations indicated for 72 h. They were pulsed with 0.5 μCi [^{3}H]thymidine (Amersham) for the final 8 h of the culture, and then precipitated with trichloroacetate, and radioactivity was measured by a γ-scintillation counter.

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References

Akira,S. (1997) IL-6-regulated transcription factors. *Int. J. Biochem. Cell Biol.*, **29**, 1401–1418.

Barrandon,Y. and Green H. (1987) Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-alpha and epidermal growth factor. *Cell*, **50**, 1131–1137.

Bennett,N.T. and Schultz,G.S. (1993) Growth factors and wound healing: biochemical properties of growth factors and their receptors. *Am. J. Surg.*, **165**, 728–737.

Blessing,M., Nanney,L.B., King,L.E., Jones,C.M. and Hogan,B.L.M. (1993) Transgenic mice as a model to study the role of TGF-β-related molecules in hair follicles. *Genes Dev.*, **7**, 204–215.

Boccaccio,C., Ando,M., Tamagnone,L., Bardelli,A., Michieli,P., Battistini,C. and Comoglio,P.M. (1998) Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature*, **391**, 285–288.

Byrne,C. and Fuchs,E. (1993) Probing keratinocyte and differentiation specificity of the human K5 promoter in vitro and in transgenic mice. *Mol. Cell. Biol.*, **13**, 3176–3190.

Chen,J.D., Kim,J.P., Zhang,K., Sarret,Y., Wynn,K.C., Kramer,R.H. and Woodley,D.T. (1993) Epidermal growth factor (EGF) promotes human keratinocyte locomotion on collagen by increasing the alpha 2 integrin subunit. *Exp. Cell Res.*, **209**, 216–223.

Coffey,R.J.,Jr, Derynck,R., Wilcox,J.N., Bringman,T.S., Goustin,A.S., Moses,H.L. and Pittelkow,M.R. (1987) Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature*, **328**, 817–820.

Cotsarelis,G., Sun,T.T. and Lavker,R.M. (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, **61**, 1329–1337.

Darnell,J.E.,Jr (1997) STATs and gene regulation. *Science*, **277**, 1630–1635.

Dunsmore,S.E., Rubin,J.S., Kovacs,S.O., Chedid,M., Parks,W.C. and Welgus,H.G. (1996) Mechanisms of hepatocyte growth factor stimulation of keratinocyte metalloproteinase production. *J. Biol. Chem.*, **271**, 24576–24582.

Durbin,J.E., Hackenmiller,R., Simon,M.C. and Levy,D.E. (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*, **84**, 443–450.

Elenius,K., Paul,S., Allison,G., Sun,J. and Klagsbrun,M. (1997) Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J.*, **16**, 1268–1278.

Gat,U., DasGupta,R., Degenstein,L. and Fuchs,E. (1998) *De novo* hair follicle morphogenesis and hair tumors in mice expressing a truncated β-catenin in skin. *Cell*, **95**, 605–614.

Gerdes,J., Schwab,U., Lemke,H. and Stein,H. (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer*, **31**, 13–20.

Green,M.R. and Couchman,J.R. (1984) Distribution of epidermal growth factor receptors in rat tissues during embryonic skin development, hair formation, and the adult hair growth cycle. *J. Invest. Dermatol.*, **83**, 118–123.

Grossman,R.M., Krueger,J., Yourish,D., Granelli-Piperno,A., Murphy, D.P., May,L.T., Kupper,T.S., Sehgal,P.B. and Gottlieb,A.B. (1989) Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proc. Natl Acad. Sci. USA*, **86**, 6367–6371.

Hardy,M.H. (1992) The secret life of the hair follicle. *Trends Genet.*, **8**, 55–61.

Higashiyama,S., Abraham,J.A., Miller,J., Fiddes,J.C. and Klagsbrun,M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*, **251**, 936–939.

Ihle,J.N. (1996) STATs: signal transducers and activators of transcription. *Cell*, **84**, 331–334.

Jindo,T., Tsuboi,R., Takamori,K. and Ogawa,H. (1998) Local injection of hepatocyte growth factor/scatter factor (HGF/SF) alters cyclic growth of murine hair follicles. *J. Invest. Dermatol.*, **110**, 338–342.

Kaplan,M.H., Sun,Y.L., Hoey,T. and Grusby,M.J. (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*, **382**, 174–177.

Kashiwagi,M., Kuroki,T. and Huh,N. (1997) Specific inhibition of hair follicle formation by epidermal growth factor in an organ culture of developing mouse skin. *Dev. Biol.*, **189**, 22–32.

Kitano,Y. and Okada,N. (1983) Separation of the epidermal sheet by dispase. *Br. J. Dermatol.*, **108**, 555–560.

Lenoir,M.C., Bernard,B.A., Pautrat,G., Darmon,M. and Shroot,B. (1988) Outer root sheath cells of human hair follicle are able to regenerate a fully differentiated epidermis *in vitro*. *Dev. Biol.*, **130**, 610–620.

Liu, X., Robinson,G.W., Wagner,K.U., Garrett,L., Wynshaw-Boris,A. and Hennighausen,L. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.*, **11**, 179–186.

Luetteke,N.C., Qiu,T.H., Peiffer,R.L., Oliver,P., Smithies,O. and Lee,D.C. (1993) TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell*, **73**, 263–278.

Martin,P. (1997) Wound healing—aiming for perfect skin regeneration. *Science*, **276**, 75–81.

Matsumoto,K., Hashimoto,K., Yoshikawa,K. and Nakamura,T. (1991) Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. *Exp. Cell Res.*, **196**, 114–120.

McCawley,L.J., O'Brien,P. and Hudson,L.G. (1998) Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)-mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. *J. Cell Physiol.*, **176**, 255–265.

Michalopoulos,G.K. and DeFrances,M.C. (1997) Liver regeneration. *Science*, **276**, 60–66.

Miettinen,P.J., Berger,J.E., Meneses,J., Phung,Y., Pedersen,R.A., Werb,Z. and Derynck,R. (1995) Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*, **376**, 337–341.

Moore,G.P., Panaretto,B.A. and Robertson,D. (1981) Effects of epidermal growth factor on hair growth in the mouse. *J. Endocrinol.*, **88**, 293–299.

Murillas,R., Larcher,F., Conti,C.J., Santos,M., Ullrich,A. and Jorcano,J.L. (1995) Expression of a dominant negative mutant of epidermal growth factor receptor in the epidermis of transgenic mice elicits striking alterations in hair follicle development and skin structure. *EMBO J.*, **14**, 5216–5223.

Nishida,T., Nakamura,M., Mishima,H. and Otori,T. (1992) Interleukin 6 promotes epithelial migration by a fibronectin-dependent mechanism. *J. Cell Physiol.*, **153**, 1–5.

O'Shea,J.J. (1997) Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? *Immunity*, **7**, 1–11.

Peus,D. and Pittelkow,M.R. (1996) Growth factors in hair organ development and the hair growth cycle. *Dermatol. Clin.*, **14**, 559–572.

Pilcher,B.K., Dumin,J.A., Sudbeck,B.D., Krane,S.M., Welgus,H.G. and Parks,W.C. (1997) The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J. Cell Biol.*, **137**, 1445–1457.

Rajewsky,K., Gu,H., Kuhn,R., Betz,U.A., Muller,W., Roes,J. and Schwenk,F. (1996) Conditional gene targeting. *J. Clin. Invest.*, **98**, 600–603.

Ramirez,A., Bravo,A., Jorcano,J.L. and Vidal,M. (1994) Sequences 5′ of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. *Differentiation*, **58**, 53–64.

Rheinwald,J.G. and Green,H. (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature*, **265**, 421–424.

St-Jacques,B., Dassule,H.R., Karavanova,I., Botchkavev,V.A., Li,J., Danielian,P.S., McMahon,J.A., Lewis,P.M., Paus,R. and McMahon, A.P. (1998) Sonic hedgehog signaling is essential for hair development. *Curr. Biol.*, **8**, 1058–1068.

Sato,C., Tsuboi,R., Shi,C.M., Rubin,J.S. and Ogawa,H. (1995) Comparative study of hepatocyte growth factor/scatter factor and keratinocyte growth factor effects on human keratinocytes. *J. Invest. Dermatol.*, **104**, 958–963.

Schindler,C. and Darnell,J.E.,Jr (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.*, **64**, 621–651.

Sibilia,M. and Wagner,E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*, **269**, 234–238.

Stenn,K.S., Combates,N.J., Eilertsen,K.J., Gordon,J.S., Pardinas,J.R., Parimoo,S. and Prouty,S.M. (1996) Hair follicle growth controls. *Dermatol. Clin.*, **14**, 543–558.

Takeda,K., Tanaka,T., Shi,W., Matsumoto,M., Minami,M., Kashiwamura, S., Nakanishi,K., Yoshida,N., Kishimoto,T. and Akira,S. (1996) Essential role of Stat6 in IL-4 signalling. *Nature*, **380**, 627–630.

Takeda,K., Noguchi,K., Shi,W., Tanaka,T., Matsumoto,M., Yoshida,N., Kishimoto,T. and Akira,S. (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl Acad. Sci. USA*, **94**, 3801–3804.

Takeda,K., Kaisho,T., Yoshida,N., Takeda,J., Kishimoto,T. and Akira,S. (1998) Stat3 activation is responsible for IL-6 dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J. Immunol.*, **161**, 4652–4660.

Takeda,K., Clausen,B.E., Kaisho,T., Tsujimura,T., Terada,N., Forster,I. and Akira,S. (1999) Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity*, **10**, 39–49.

Tarutani,M., Itami,S., Okabe,M., Ikawa,M., Tezuka,T., Yoshikawa,K., Kinoshita,T. and Takeda.J. (1997) Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. *Proc. Natl Acad. Sci. USA*, **94**, 7400–7405.

Udy,G.B., Towers,R.P., Snell,R.G., Wilkins,R.J., Park,S.H., Ram,P.A., Waxman,D.J. and Davey,H.W. (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl Acad. Sci. USA*, **94**, 7239–7244.

Werner,S., Smola,H., Liao,X., Longaker,M.T., Krieg,T., Hofschneider, P.H. and Williams,L.T. (1994) The function of KGF in morphogenesis of epithelium and re-epithelialization of wounds. *Science*, **266**, 819–822.

Wilson,C., Cotsarelis,G., Wei,Z.-G., Fryer,E., Margolis-Fryer,J., Ostead,M., Tokarek,R., Sun,T.-T. and Lavker,R.M. (1994) Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. *Differentiation*, **55**, 127–136.

Yuspa,S.H., Wang,Q., Weinberg,W.C., Goodman,L., Ledbetter,S., Dooley,T. and Lichti,U. (1993) Regulation of hair follicle development: an *in vitro* model for hair follicle invasion of dermis and associated connective tissue remodeling. *J. Invest. Dermatol.*, **101**, 27s–32s.

Zhang,X., Blenis,J., Li,H.C., Schindler,C. and Kiang,S.C. (1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science*, **267**, 1990–1994.

Zhong,Z., Wen,Z. and Darnell,J.E.,Jr (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, **264**, 95–98.

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