Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis

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Recently, we demonstrated a strong upregulation of activin expression after skin injury. Furthermore, overexpression of this transforming growth factor β family member in the skin of transgenic mice caused dermal fibrosis, epidermal hyperthickening and enhanced wound repair. However, the role of endogenous activin in wound healing has not been determined. To address this question we overexpressed the soluble activin antagonist follistatin in the epidermis of transgenic mice. These animals were born with open eyes, and the adult mice had larger ears, longer tails and reduced body weight compared with non-transgenic littermates. Their skin was characterized by a mild dermal and epidermal atrophy. After injury, a severe delay in wound healing was observed. In particular, granulation tissue formation was significantly reduced, leading to a major reduction in wound breaking strength. The wounds, however, finally healed, and the resulting scar area was smaller than in control animals. These results implicate an important function of endogenous activin in the control of wound repair and scar formation.

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Introduction

Cutaneous wound repair is a complex process involving blood clotting, inflammation, new tissue formation and finally tissue remodelling. These processes are controlled by cell–cell and cell–matrix interactions and by a wide variety of different growth factors and cytokines. Although expression of many of these factors has been observed in the healing wound, their functional roles have been poorly characterized. Recent studies have provided

evidence for a particularly important role of transforming growth factor β (TGF- β) family members in cutaneous wound repair. This family includes, in mammals, three types of TGF- β (TGF- β 1, β 2 and β 3), various bone morphogenetic proteins (BMPs), Mullerian inhibiting substance, inhibins and activins (for review see Massagué, 1990). Whereas the function of the three TGF- β s in skin morphogenesis and wound healing has been well characterized (for a review see Roberts and Sporn, 1996; O’Kane and Ferguson, 1997), a role for the other family members in this process has only recently emerged. For example, BMP-6 is expressed at high levels in the hyperproliferative wound epithelium, where it seems to be involved in the re-differentiation of the keratinocytes at later stages of the healing process (Kaiser *et al.*, 1998). Furthermore, several studies from our laboratory have provided evidence for a novel role of activin in skin wound healing.

Like the majority of other members of the TGF- β family, activins are dimeric proteins, the monomeric polypeptides of which are connected by disulfide linkage. Three different forms of activin, the homodimeric activin A (β A β A) and activin B (β B β B), as well as the heterodimeric activin AB (β A β B) have been described (reviewed by Massagué, 1990; Vale *et al.*, 1990). Furthermore, β C, β D and β E chains have been discovered (Hötten *et al.*, 1995; Oda *et al.*, 1995; Fang *et al.*, 1996), but they are not expressed in the skin.

The biological activities of activins are mediated by heteromeric receptor complexes consisting of a type II receptor (ActRII or ActRIIB) and a type I receptor (ActRIA or ActRIB), which are characterized by the presence of an intracellular serine/threonine kinase domain. The formation of heteromers of type I and type II receptors is essential for efficient ligand binding and signal transduction (reviewed by Mathews and Vale, 1993). Besides these transmembrane signalling receptors, an activin-binding protein, follistatin, has been discovered, which binds to activins with high affinity and thereby inhibits their biological effects *in vitro* and *in vivo* (Nakamura *et al.*, 1990; de Winter *et al.*, 1996). Two different variants of follistatin have been identified which are generated by alternative splicing at the 3’ end of the transcript with the option of retaining exon 5. The longer and most abundant variant, FS-315, and the smaller variant, FS-288, are both secreted glycoproteins, and their affinities for activins are almost identical. However, the biological activity of FS-288 is higher due to its greater affinity for heparan sulfate (Inouye *et al.*, 1991; Sugino *et al.*, 1993; reviewed by Patel, 1998). In addition to activins, follistatin binds to BMP-2, -4, -7 and -11, although with significantly lower affinity compared with activin (K_D 23 nM versus 0.5–0.7 nM) (Iemura *et al.*, 1998; Gamer *et al.*, 1999). Therefore, it seems likely that

the major function of follistatin is the inhibition of activin action.

We have previously shown a strikingly increased expression of the activin β A chain and a minor increase in the expression of the β B chain after skin injury in mice. *In situ* hybridization and immunohistochemistry revealed the highest levels of activin mRNAs and proteins in the granulation tissue and in keratinocytes of the migrating epithelial tongue and the hyperproliferative wound epithelium. Furthermore, all known activin receptors were expressed in the mesenchymal and epithelial compartments of normal and wounded skin, although their expression did not increase after injury (Hübner *et al.*, 1996a; our unpublished data).

To gain insight into the biological activities of activin in wound repair, we recently generated and analysed transgenic mice that overexpress the activin β A chain in basal keratinocytes of the epidermis and in outer root sheath keratinocytes of the hair follicles. Interestingly, the activin-overexpressing mice were characterized by epidermal hyperplasia as a result of increased keratinocyte proliferation and differentiation abnormalities. Furthermore, fibrosis in the underlying dermis was observed. Most importantly, granulation tissue formation and wound re-epithelialization were strongly enhanced in these animals, demonstrating that the overexpressed activin stimulates the wound healing process (Munz *et al.*, 1999).

To determine a possible role of endogenous activin in skin morphogenesis and wound repair, it was essential to block its function in the skin. Since activin β A knockout mice die from multiple abnormalities within the first 24 h after birth (Matzuk *et al.*, 1995), the analysis of the wound healing process in these animals was not possible. Furthermore, a tissue-specific knockout of this gene in the skin was not suitable for the analysis of activin function in the wound, since activin is expressed in both the mesenchyme and the epidermis, and it is as yet not possible to specifically delete genes in the mesenchymal compartment of the skin. Therefore, we decided to overexpress the activin antagonist follistatin in the epidermis. Since the latter is a soluble protein, it seemed likely that the epidermis-derived follistatin would also diffuse to the underlying mesenchyme and inhibit the activin present in this part of the skin.

The results presented in this study provide insight into the role of endogenous activin in skin morphogenesis, wound healing and scar formation.

Results

Cloning and characterization of a DNA construct for overexpression of follistatin in transgenic mice

To determine the function of endogenous activin in the skin, we expressed the long splice variant of murine follistatin (FS-315) in the epidermis of transgenic mice under the control of a human keratin 14 (K14) promoter. FS-315 was chosen because it is the major follistatin variant. Furthermore, the reduced affinity to heparan sulfate proteoglycans compared with FS-288 indicated that the longer splice variant would diffuse more readily (Sugino *et al.*, 1993). The K14 promoter was chosen because it targets the expression of transgenes to the non-differentiated basal cells of the epidermis and to the

Fig. 1. Expression of FS-315 in HaCaT keratinocytes. (A) Scheme of the transgene construct. Functional elements include the human K14 promoter, the rabbit β -globin intron, the murine FS-315 cDNA, and the human growth hormone poly(A). (B) Expression of murine FS-315 in HaCaT cells. HaCaT cells were stably transfected with the transgene construct (K14-mFS) together with a plasmid that confers G418 resistance or with the resistance plasmid alone (control). Cells were incubated in the presence or absence of tunicamycin for 24 h and analysed by western blotting for the presence of follistatin in the conditioned medium.

keratinocytes of the outer root sheath of the hair follicles (Vassar *et al.*, 1989; Munz *et al.*, 1999). The suitability of the transgene construct (Figure 1A) for expression in keratinocytes was first tested *in vitro* by stable transfection of the immortalized but non-transformed HaCaT keratinocyte cell line (Boukamp *et al.*, 1988). Several cell lines were obtained that secrete a protein of the expected size into the culture medium as determined by western blotting with a follistatin-specific antiserum (Figure 1B). The diffuse band indicated that the protein is glycosylated. Indeed, after a 24 h incubation of the cells in tunicamycin-containing medium, a sharp band of reduced size was detected (Figure 1B). These *in vitro* results demonstrated that keratinocytes transfected with the transgene construct are able to express, glycosylate and secrete the recombinant follistatin.

Generation of transgenic mice that express high levels of follistatin in the skin

Four transgenic founder mice with different integration sites of the transgene (data not shown) were generated. Expression of the transgene was determined by RNase protection assay using a probe that allows endogenous and transgene-derived follistatin to be distinguished between (Figure 2A). Two of these founders expressed high levels of the transgene in the skin (Figure 2B; data not shown). In these mice the expression levels of the transgene in tail skin were 8-fold higher compared with the levels of endogenous follistatin (compare upper and lower bands in the protection assay of Figure 2B). In the skin of another founder, the levels of the transgene-derived follistatin mRNA were 4-fold higher compared with controls, and in the fourth founder, expression of transgene-derived follistatin was identical to that of endogenous follistatin

Fig. 2. Tissue-specific expression of the transgene. **(A)** The riboprobe used for the detection of follistatin RNA by RNase protection assay is shown together with the expected protected fragments. **(B)** RNA was isolated from various tissues of the transgenic mice (tg) and of control littermates (wt). Total cellular RNA (20 µg) was analysed for the presence of follistatin mRNA by RNase protection assay. tRNA (20 µg) was used as a negative control. One thousand c.p.m. of the hybridization probe were loaded in the lane labelled 'probe' and used as a size marker. **(C and D)** Radioactive *in situ* hybridization of tail epidermis from a transgenic mouse (D) and a control littermate (C). Silver grains appear as black dots. BK, basal keratinocytes. Bar = 20 µm.

(data not shown). Interestingly, expression of endogenous follistatin was downregulated in the animals that expressed high levels of the transgene, indicating a compensatory effect (Figure 2B). All founders were used for the generation of transgenic mouse lines. However, one male founder mouse that expressed high levels of the transgene and one female founder mouse that expressed intermediate levels of the transgene were obviously mosaic and did not give rise to transgenic progeny.

The highest levels of transgene mRNA were found in the skin of the back and the tail (Figure 2B), as well as in the ear (not shown) and in the tongue. Low levels of expression were also detected in the lung, heart and brain

(Figure 2B). This expression pattern was observed in all founder mice and also in the progeny of the two transmitting founders. The activity of the promoter in lung, heart and brain is obviously related to the specific construct, since we did not observe this activity in transgenic mice that express other transgenes under the same promoter (Munz *et al.*, 1999; S.Werner, unpublished data).

Within the skin, expression was restricted to the basal layer of the epidermis and to outer root sheath keratinocytes as determined by radioactive *in situ* hybridization of mouse tail skin (Figure 2D; data not shown). This finding confirms the expected activity of the promoter in the skin.

Fig. 3. FS-315-overexpressing mice are macroscopically abnormal. A female control mouse (wild type; 2 months old) is shown at the top in (A), and on the right hand side in (B); a female heterozygous transgenic littermate (tg) with severe phenotypic abnormalities is shown at the bottom in (A), and on the left hand side in (B). A newborn wild-type animal is shown in (C), and a newborn transgenic littermate is shown in (D).

Follistatin-overexpressing mice are macroscopically abnormal

Immediately after birth, the transgenic mice appeared grossly normal with the exception of open eyes (Figure 3D). However, within 3 weeks of birth, obvious abnormalities appeared in the two founders which expressed high levels of the transgene, and in the transgenic progeny of the high-expressing, transmitting founder (line 50) (Figure 3A and B; a heterozygous transgenic mouse with a particularly severe phenotype is shown). These mice had significantly larger ears (Figure 3B) and longer tails (Figure 3A) relative to their body size, and their coat appeared rough and irregular. In most animals, the eyes became white and narrowed (Figure 3B). Finally, these transgenic mice were significantly smaller compared with their control littermates, and their body shape appeared abnormal (Figure 3A and B). The smaller size of the transgenic mice was reflected by reduced body weight. At the age of 10–16 weeks, the body weight of female transgenic mice was in average 21 g ($n = 26$), whereas the body weight of female control littermates was in average 25 g ($n = 26$). Similar differences were seen for the male animals.

In contrast to the high-expressor line, the body weight of the mice that expressed low levels of the transgene (line 5) was not significantly altered. Thus, the phenotypic

abnormalities correlated with the expression level of the transgene. This was also demonstrated by the increased severity of the phenotype in the homozygous progeny of the founder (data not shown). Interestingly, homozygous animals of the strongly expressing mouse line died within the first 2–4 weeks after birth, demonstrating that such high levels of follistatin are obviously lethal.

Minor skin abnormalities in the follistatin-overexpressing mice

The transgenic founder mice and their progeny were further examined for phenotypic abnormalities in the skin. Only littermates of the same sex were used for a direct comparison. As shown by Masson trichrome staining of tail and back skin of adult mice (Figure 4A; data not shown), the thickness and density of the connective tissue were reduced in the animals that expressed high levels of the transgene. This reduced connective tissue is not the result of reduced mRNA expression of fibronectin, collagen $\alpha 1(I)$ and $\alpha 1(III)$, as determined by RNase protection assay of back and tail skin RNA (data not shown). In addition, the epidermis was slightly thinner, and most of the hair follicles were smaller in the transgenic animals (Figure 4; data not shown). No signs of inflammation were observed in the dermal or epidermal compartment of the transgenic mice as assessed by histological analysis and mRNA analysis of the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α), which were found to be expressed at equally low levels in the skin of transgenic mice and control mice (Figure 4; data not shown).

Overexpression of follistatin does not affect keratinocyte proliferation and differentiation

To determine a potential effect of follistatin overexpression on keratinocyte proliferation, we labelled the proliferating cells with 5'-bromodeoxyuridine (BrdU) *in vivo* and subsequently stained the skin with an antibody directed against BrdU. As in the control mice (Figure 4B, left panel), proliferation was restricted to the basal layer of the epidermis of transgenic mice (Figure 4B, right panel, line 50), and the number of proliferating basal cells was similar in the tail epidermis of transgenic mice ($n = 3$) and control mice ($n = 3$).

To determine the effect of follistatin on keratinocyte differentiation, we analysed the expression of differentiation-specific proteins in the tail epidermis by indirect immunofluorescence. Expression of K14 was detected in basal cells of the epidermis of both normal and transgenic mice (Figure 5, top panel), and the staining persisted throughout the whole epidermis. Keratin 10 (K10) expression started in the first suprabasal layer in normal and transgenic mice (Figure 5, second panel). Furthermore, loricrin, the major protein of the cornified envelope (Mehrel *et al.*, 1990) was appropriately expressed in the granular and cornified layers (data not shown). Keratin 6 (K6) is restricted to the hair follicles in normal skin, but is transiently expressed in the hyperproliferative epidermis of wounds and permanently upregulated in the suprabasal epidermal layers of hyperplastic, neoplastic and psoriatic skin (reviewed by Fuchs, 1990). This keratin was not detectable in interfollicular epidermis of any mice, independent of their genotype (Figure 5, third panel). Taken

Fig. 4. Normal epidermal architecture and keratinocyte proliferation in the follistatin-transgenic mice. (A) Paraffin sections (6 μ m) from the tail were stained using the Masson trichrome protocol. D, dermis; E, epidermis; F, fatty tissue; HF, hair follicle. (B) Detection of proliferating cells in the tail skin of control mice and transgenic mice. Paraffin sections of the tail skin of BrdU-injected control mice (left panel) and transgenic mice (right panel) were incubated with a peroxidase-conjugated anti-BrdU antibody, stained with the diaminobenzidine peroxidase-staining kit, and counterstained with hematoxylin. Stained nuclei are indicated by arrows. E, epidermis; D, dermis; HF, hair follicle. Bar indicates 50 μ m in (A) and 30 μ m in (B).

together, these results demonstrate that the overexpression of follistatin in the epidermis does not affect epidermal keratinocyte proliferation or differentiation.

The FS-315 transgene is highly expressed in the epidermis of wounded skin

To determine the effect of the transgene on the wound healing process, full-thickness excisional wounds were generated on the back of transgenic mice and control littermates of the same sex at the age of 10–12 weeks. Non-radioactive *in situ* hybridization of day 5 wound sections revealed a strong expression of follistatin mRNA in the hyperproliferative epithelium and in injured hair follicles at the wound edge and in the epidermis adjacent to the wound (Figure 6C). The sensitivity of the hybridization was not sufficient to determine the endogenous follistatin mRNA, as demonstrated by the lack of signal in control animals (Figure 6B). Thus, the signal seen in the wound of the transgenic animals is exclusively due to the presence of the transgene mRNA. No signal was observed with the sense probe (Figure 6D).

Most importantly, the K14 promoter-driven expression of FS-315 mRNA (lower band in Figure 6A) was significantly higher compared with endogenous follistatin (higher band in Figure 6A), as assessed by RNase protection assay using the riboprobe described in Figure 2A. Consistent with previous studies (Hübner *et al.*, 1996a), expression of endogenous follistatin was not significantly regulated by skin injury.

Fig. 5. Expression of differentiation-specific proteins in tail epidermis of control mice (wt, left panels) and heterozygous transgenic mice (tg, right panels). Sections were incubated with antibodies to K14 (upper panel), K10 (second panel) and K6 (third panel), or DNA was stained with Hoechst (lower panel; co-staining with K6). The basal layer is indicated by arrows. Bar = 30 μ m.

Fig. 6. Expression of follistatin mRNA in normal and wounded skin. (A) RNA was isolated from control back skin and from wounds at several stages and analysed by RNase protection assay for the presence of follistatin mRNA using the probe described in Figure 2A. Hybridization of the same set of RNAs with a GAPDH riboprobe was used to confirm equal loading. One thousand c.p.m. of the hybridization probes were loaded in the lanes labelled 'probe' and used as a size marker. tRNA (20 µg) was used as a negative control. (B–D) Frozen sections from day 5 wounds of (B) control mice and (C) follistatin-transgenic mice were analysed by non-radioactive *in situ* hybridization for the presence of follistatin mRNA. (D) Hybridization with the sense probe. D, dermis; G, granulation tissue; HE, hyperproliferative epidermis; HF, hair follicle. Bar = 30 µm.

Upregulation of activin β A and downregulation of BMP expression during wound healing in normal and transgenic mice

To determine the follistatin binding partners that might be targets of follistatin in the skin of the transgenic mice, we analysed the expression of activin β A and β B and of BMP-2, -4, -7 and -11 mRNAs in non-wounded tail and

Fig. 7. Expression of follistatin binding partners in normal and wounded skin of follistatin-overexpressing mice and control mice. Total cellular RNA (20 µg) isolated from normal and wounded back skin of control (wt) and transgenic mice (tg) was analysed by RNase protection assay for the expression of activin β A, β B, BMP-2, BMP-4 and BMP-7 mRNAs. One thousand c.p.m. of the hybridization probes were loaded in the lanes labelled 'probe' and used as size markers. tRNA (20 µg) was used as a negative control. Hybridization of the same set of RNAs with a GAPDH riboprobe was used to confirm equal loading.

back skin and during the wound healing process in normal and transgenic mice (Figure 7; data not shown). For this purpose, we performed RNase protection assays with RNAs from skin and complete wounds. We found low levels of activin β A mRNA and slightly higher levels of activin β B mRNAs in back skin (Figure 7) and tail skin (data not shown). Furthermore, BMP-2, -4 and -7 mRNAs were found to be expressed, whereas BMP-11 mRNA was hardly detectable.

As recently demonstrated for BALB/c and CD-1 mice (Hübner *et al.*, 1996a; Munz *et al.*, 1999), expression of activin β A mRNA, and to a lesser extent β B mRNA, was induced after skin injury in the B6D2F2 hybrid mice used in the present study. In contrast to activins, expression of the follistatin-binding BMPs during wound healing has as yet not been determined. As shown in Figure 7, the mRNA levels of BMP-2, -4 and -7 declined after skin injury. BMP-7 but not BMP-2 and -4 mRNA levels had returned to basal levels after completion of the proliferative phase (day 13 after wounding). Expression of BMP-11 mRNA was hardly detectable in wounded skin. The upregulation

of activin expression and the downregulation of BMP expression strongly suggests that the observed wound healing abnormalities are primarily due to the inhibition of activin function. Expression of the follistatin transgene did not affect the levels of activin or BMP mRNAs in normal or wounded skin, indicating that overexpression of follistatin does not lead to a compensatory induction of any of its binding partners.

Although TGF- β s are not binding partners of follistatin, we analysed their expression in normal and wounded skin of the transgenic mice. As described previously (Frank *et al.*, 1996), the mRNA expression of all three isoforms was upregulated after skin injury, but no difference was observed between control and follistatin-overexpressing mice (data not shown). Consistent with the normal TGF- β expression, the mRNA levels of connective tissue growth factor (CTGF), a potent fibroblast mitogen and matrix-inducing factor that is regulated by TGF- β (Duncan *et al.*, 1999), were not altered in the transgenic mice (data not shown). Furthermore, BMP-6, which does not bind to follistatin, was expressed at equal levels in follistatin-overexpressing mice and control littermates, and no induction of this gene was seen after injury, either in control B6D2F2 mice or in transgenic animals (data not shown).

Impaired wound healing in follistatin-overexpressing mice

We subsequently performed a detailed histological analysis of full-thickness excisional wounds from the follistatin-overexpressing mice and control littermates of the same sex. For this purpose we only used transgenic mice that had a <20% weight reduction compared with control littermates, whereas particularly small and weak animals (see Figure 3A) were excluded. No differences were observed between control mice and transgenic mice at day 1 after injury (data not shown). Day 5 after wounding is characterized by massive granulation tissue formation and re-epithelialization. Furthermore, activin levels are particularly high at day 5 after wounding (Hübner *et al.*, 1996a). At this time point, we observed a remarkable reduction in the amount of granulation tissue in the wounds of the transgenic mice that expressed high levels of the transgene (Figure 8B) compared with control mice (Figure 8A; results from two wounds each of 17 transgenic mice and 23 wild-type mice). The cell density in the granulation tissue of the control animals was significantly higher compared with transgenic animals, and the connective tissue in the transgenic mice appeared less dense (compare Figure 8E and F). BrdU incorporation studies revealed a reduction in the total number of proliferating cells in the wounds of transgenic mice, but the density of BrdU-positive cells was not significantly different (data not shown). The reduced granulation tissue was not due to differences in the mRNA expression of collagens type I and III and fibronectin as revealed by RNase protection assay (data not shown).

In spite of the reduced granulation tissue formation in the transgenic animals, the number of blood vessels was not altered, and the vessels in the granulation tissue were even enlarged in the transgenic mice (Figure 8E and F). This was also confirmed by immunofluorescence with an antibody against the endothelial cell-specific protein

PECAM (Figure 8G and H, shown in red). Furthermore, the hyperproliferative wound epithelium was thinner in the transgenic animals, although the extent of re-epithelialization was not significantly altered (Figure 8C and D). Similar abnormalities were also observed in the mice that express low levels of the transgene (results from 10 transgenic and three wild-type mice), although the phenotype was less pronounced in these animals (data not shown). These findings demonstrate that the increased levels of follistatin in the wounds of transgenic mice cause a delay in the wound healing process.

To determine whether the reduced granulation tissue formation seen in the transgenic mice leads to a reduction in tensile strength of the wound, two full-thickness incisions were made at one anterior and one posterior dorsal site of the skin. Tensile strength of full-thickness incisional wounds in follistatin-transgenic ($n = 14$) and wild-type mice ($n = 7$) was measured on day 5 after wounding. Interestingly, the *in situ* wound strength of the transgenic mice that expressed high levels of the transgene (Figure 9, line 50, $n = 9$) was reduced by 42%, demonstrating that the observed histological abnormalities are functionally important. A 24% reduction in tensile strength was also found in the animals that express low levels of the transgene (Figure 9, line 5, $n = 5$) and that lack systemic abnormalities. This finding strongly suggests that the wound healing abnormalities are not caused by systemic defects but rather by the increased levels of follistatin in the skin.

Finally, we analysed the wounds of control and transgenic animals at a late stage of repair. At day 13 after injury, wounds of control mice had lost their eschar, but the latter was still present on most of the wounds of the transgenic animals that expressed high levels of the transgene (data not shown). A histological analysis of the wounds at that stage revealed that the wounds of both wild-type and transgenic mice were re-epithelialized, but the epidermis was much thinner in the transgenic mice than in controls. Most importantly, a much less extended scar tissue was seen in the transgenic animals, and the connective tissue was less dense (compare Figure 10A and B). These studies demonstrate that the presence of high levels of follistatin delay the wound healing process but reduce the area of the residual scar.

Discussion

Previous studies have suggested a major effect of activin on the wound healing process. However, since activin β A knockout mice die shortly after birth, the role of endogenous activin in postnatal skin development and in the healing response has as yet not been determined. To address these questions, we generated transgenic mice that overexpress the activin antagonist follistatin in the basal layer of the epidermis. Surprisingly, the mice that express high levels of follistatin were smaller compared with their control littermates, and their body weight was significantly reduced. In the case of the homozygous animals of the high-expressing line, this weight reduction was particularly severe, and the animals died within a few weeks of birth. The reduced weight could be due to the expression of the transgene in the tongue, oral epithelium and/or oesophagus, where K14 is also expressed (Vassar *et al.*,

Fig. 8. Histological abnormalities in the wounds of follistatin-transgenic mice. Full-thickness excisional wounds were made on the back of transgenic mice (tg) and control littermates (wt) (3 months old). Paraformaldehyde-fixed paraffin sections (6 μ m) from the middle of day 5 wounds were stained by the Masson Trichrome procedure (A, B, E and F) or with hematoxylin/eosin (C and D). Alternatively, ethanol/acetic acid fixed paraffin sections were analysed by immunofluorescence with antibodies against K14 (green) and the endothelial cell-specific marker protein PECAM (red) using a confocal microscope (G and H). (A), (C), (E) and (G) show wounds of a control mouse; (B), (D), (F) and (H) show wounds of a heterozygous transgenic mouse. BV, blood vessel; D, dermis; E, epidermis; Es, eschar; F, fatty tissue; G, granulation tissue; HE, hyperproliferative epithelium; HF, hair follicle; M, muscle (*panniculus carnosus*). Bar indicates 500 μ m in (A) and (B), 50 μ m in (C) and (D) and 40 μ m in (E)–(H).

1989). Furthermore, teeth abnormalities were observed with significantly deformed and longer incisors. These abnormalities might inhibit food intake and result in a

nutrition defect. In addition, systemic abnormalities due to the expression of follistatin in the lung and the heart, or to diffusion of follistatin into the blood stream cannot be

Fig. 9. Reduced *in situ* wound strength in the transgenic mice. Tensile strength of full-thickness incisional wounds in follistatin transgenic (high expressing line = line 50, $n = 9$; low expressing line = line 5, $n = 5$) and wild-type mice ($n = 7$) was measured on day 5 after wounding according to the manufacturer's protocol for the non-human disruptive linear incision analysis. *In situ* wound strength was measured as maximum amount of negative pressure (mm Hg) required for failure. Bars represent mean values $\pm$ SEM within each group of animals. P values are derived from Student's t -test.

excluded. Unfortunately, the serum levels of follistatin cannot be determined due to the lack of an immunoassay for mouse follistatin. However, the normal body weight observed in mice that overexpress follistatin under the control of the metallothionein promoter, which is highly active in many tissues, including the liver, argues against a role of serum-derived follistatin in the observed weight reduction (Guo *et al.*, 1998).

In addition to the reduced body weight, we observed several other macroscopic abnormalities. The animals were born with open eyes, a feature that was also observed in activin β B knockout mice (Schrewe *et al.*, 1994). Other obvious defects were the enlarged size of the ears and the particularly long tail. This is opposite to the phenotype observed in our activin β A-overexpressing mice, which were characterized by short tails and small ears (Munz *et al.*, 1999), possibly as a result of the severe cartilage hypertrophy. The complementary phenotypes observed in the activin β A-overexpressing mice on the one hand and the activin β B knockout mice and the follistatin-transgenic animals on the other hand strongly suggest that the overexpressed follistatin does indeed inhibit activin action.

The strong phenotype observed in the skin of activin-overexpressing mice (Munz *et al.*, 1999) suggested a role of activin in skin morphogenesis. Indeed, a slightly thinner epidermis and a reduced density of the dermal matrix were observed, indicating that endogenous activin does indeed affect skin development and/or homeostasis. However, the balance between keratinocyte proliferation and differentiation was obviously not disturbed. This finding confirms previous results demonstrating that, in contrast to TGF- β , relatively high levels of activin are required to inhibit keratinocyte proliferation (Shimizu *et al.*, 1998; Seishima *et al.*, 1999). Thus, the low levels of activin observed in normal adult skin are obviously of minor importance for the regulation of keratinocyte proliferation/differentiation. In contrast, activin appears to be more important in pathological situations as indicated by the dermal fibrosis and epidermal hypertrophy seen in the activin-over-

expressing mice (Munz *et al.*, 1999). Indeed, preliminary findings from our laboratory suggest that increased levels of activin are a feature of human skin disease characterized by either fibrosis or epithelial hyperproliferation.

An additional abnormality of the follistatin-transgenic mice was a rough and irregular coat. This finding is consistent with the observations of other investigators who overexpressed follistatin under the control of the metallothionein promoter (Guo *et al.*, 1998). The abnormal appearance of the coat is most likely due to differences in hair length, which was particularly obvious when we shaved the animals. These differences were also reflected at the histological level, where a significant variation in the size of the hair follicles was observed, with most follicles being smaller in the transgenic animals compared with controls. These findings, as well as the lack of whiskers and the abnormal whisker follicles in mice lacking the activin β A chain (Matzuk *et al.*, 1995), indicate a role of activin in the regulation of hair growth. In addition, it seems possible that the high levels of follistatin in the skin of our transgenic animals also inhibit the functions of BMPs, which have been shown to play an important role in hair follicle morphogenesis and hair growth (Blessing *et al.*, 1993; Botchkarev *et al.*, 1999; Kulesa *et al.*, 2000). A role of BMPs in the observed hair abnormalities is further supported by the normal hair in the animals that express low levels of follistatin in the skin. Thus, these levels might be sufficient to inhibit activin, which binds follistatin with high affinity. However, much higher follistatin levels, as seen in the high-expressor line, appear to be required to neutralize BMPs.

The most striking finding of our study was the severely impaired wound healing seen in our follistatin-over-expressing mice. In the mice that expressed high levels of the transgene, this defect was macroscopically visible, since the transgenic mice lost their eschars later than controls. At the histological level, a thinner hyperproliferative epithelium and particularly a reduced granulation tissue were observed. Most importantly, the dermal organization of the newly deposited collagen was abnormal as demonstrated by the significant reduction in wound breaking strength. Therefore, our results demonstrate that the increased levels of follistatin have severe functional consequences for the wound healing process.

In spite of the reduced granulation tissue formation, the density of blood vessels below the hyperproliferative wound epithelium was not reduced in the transgenic mice, and the individual vessels were even enlarged. VEGF, angiopoietin-1 and angiopoietin-2, which have key roles in angiogenesis, are obviously not responsible for this effect on the vessels, since the mRNA expression of these growth factors was identical in normal skin and during wound healing of transgenic and control mice (data not shown). In contrast, it seems likely that the enlarged blood vessels are a direct result of follistatin action, since the latter has previously been shown to be angiogenic (Kozian *et al.*, 1997) due to the inhibition of the anti-angiogenic protein activin (Breit *et al.*, 2000).

The wound healing phenotype seen in the follistatin-overexpressing mice was complementary to the one observed in the activin-transgenic mice, which were characterized by increased granulation tissue formation and re-epithelialization (Munz *et al.*, 1999) and by a

Fig. 10. Reduced scar tissue in day 13 wounds of follistatin-overexpressing mice. Full-thickness excisional wounds were made on the back of (B) transgenic mice (tg) and (A) control littermates (wt) (3 months old). Mice were killed at day 13 after injury. Paraformaldehyde-fixed paraffin sections (6 μ m) from the middle of the wounds were stained with hematoxylin/eosin. D, dermis; E, epidermis; G, granulation tissue; HF, hair follicle; M, muscle (*panniculus carnosus*). Bar = 300 μ m.

remarkable increase in scar tissue (S.Werner, unpublished data). These findings suggest that the observed abnormalities in the follistatin-overexpressing mice are indeed a result of the inhibition of endogenous activin action. In contrast, BMPs seem to play a minor role in this process, since (i) their expression was downregulated after skin injury and (ii) the affinity of follistatin to BMPs is much lower compared with that of activins (Iemura *et al.*, 1998). To further address this question, we generated B6D2F2 double-transgenic mice that overexpress both activin and follistatin under the control of the K14 promoter. The first double-transgenic mice we obtained revealed normal wound healing at the histological level (data not shown), whereas the characteristic abnormalities were observed in single-transgenic mice overexpressing activin (back-crossed into the B6D2F2 background; Munz *et al.*, 1999) or follistatin (this study). These preliminary findings suggest that the wound healing phenotype seen in activin-transgenic mice can be reversed by the simultaneous presence of follistatin. In contrast, a blockade of BMP action might underlie the hair abnormalities observed in the mice that overexpress high levels of follistatin, since these abnormalities were still visible in our activin/follistatin double-transgenic animals.

Interestingly, the phenotype seen in the follistatin-overexpressing mice was strikingly different from those lacking the TGF- β /activin signal-transducing molecule Smad3. In the latter mice, wound closure and re-epithelialization were significantly enhanced (Ashcroft *et al.*, 1999). This discrepancy suggests that Smad3 is not the predominant signal transducer used by activin in keratinocytes and possibly in other cells of the wound. In contrast, Smad3 appears to be more important in the response to TGF- β which, unlike activin, is a potent inhibitor of keratinocyte proliferation. Thus, the different TGF- β family members appear to exert distinct functions in wound repair that are mediated by different signalling pathways.

In summary, the present and our previous study (Munz *et al.*, 1999) reveal that the levels of bioactive activin

present in the wound tissue are crucial for the outcome of the repair process. On the one hand, increased activin levels appear to enhance the healing rate, but lead to increased scarring. On the other hand, inhibition of activin function delays the repair process, but the outcome of the healed wound appears to be improved. These data suggest that modification of the levels of activin in the wound could be therapeutically explored to modify wound healing. Thus, overexpression of activin or inhibition of follistatin might be useful for the improvement of impaired wound healing, whereas inhibition of activin action by follistatin or other molecules could be employed to prevent excessive matrix deposition as observed in hypertrophic scars and keloids as well as in human fibrotic disease.

Materials and methods

Plasmid construction

The full-length murine FS-315 cDNA (1.1 kb) was amplified from cDNA of mouse skin wounds, subcloned and completely sequenced. It was then inserted into an expression cassette which includes the pBluescript KSII⁺ cloning vector (Stratagene, La Jolla, CA), a 2 kb human K14 promoter (Munz *et al.*, 1999), followed by a 0.65 kb rabbit β -globin intron, and a transcription termination/polyadenylation fragment [poly(A), 0.65 kb] of the human growth hormone gene. The FS-315 cDNA was inserted between the intron and the poly(A) fragment (Figure 1A).

Generation and analysis of stably transfected HaCaT cells

HaCaT keratinocytes were co-transfected with the follistatin expression construct described above and the pSV2-neo plasmid, which confers resistance to geneticin (G418), using the lipofectin transfection system (Gibco-BRL, Paisley, UK). Transfected cells were selected in 400 μ g/ml G418 (Gibco-BRL). Colonies of resistant cells were isolated and analysed for the presence of murine follistatin mRNA by RNase protection assay. Serum-free conditioned medium from these cells was subsequently analysed for the presence of the follistatin protein by western blotting using a rabbit polyclonal antibody directed against the C-terminus of follistatin (NH₂-EQPELEVQYQGR-COOH). For the analysis of N-linked glycosylation, tunicamycin (Sigma Biochemicals, Munich, Germany) dissolved in dimethyl sulfoxide (DMSO; 10 mg/ml stock solution) was added to cell culture media to a final concentration of 5 μ g/ml. Cells were incubated in tunicamycin-containing medium for 24 h before harvesting. Control cells were incubated with the same amount of the solvent DMSO.

Generation and identification of transgenic mice

Standard procedures were followed in order to generate transgenic mice. Fertilized eggs were obtained following superovulation and mating of B6D2F2 females. The 4.5 kb insert was separated from vector sequences, purified and injected into the pronuclei of one-cell stage embryos. Microinjected zygotes were transferred into the oviducts of pseudopregnant recipient females. Mouse tail DNA was analysed for integration of the transgene by Southern blot (founder analysis) or dot-blot analysis (analysis of progeny). The rabbit β -globin intron fragment was used as a probe, since it does not cross-hybridize with chromosomal mouse DNA.

RNA isolation, RNase protection assay and in situ hybridization

RNA isolation and RNase protection assay were carried out as described by Chomczynski and Sacchi (1987) and Werner *et al.* (1993), respectively. All protection assays were carried out at least in duplicate with different sets of RNA from independent experiments. The following templates were used: a 307 bp fragment corresponding to nucleotides 780–1086 of the murine follistatin cDNA (Albano *et al.*, 1993), mouse activin β A and β B cDNAs (Hübner *et al.*, 1996a); BMP-2 and BMP-4 cDNAs (Vainio *et al.*, 1993); a 194 bp fragment corresponding to nucleotides 889–1082 of the murine BMP-7 cDNA (DDBJ/EMBL/GenBank accession No. X56906.1); a 289 bp fragment corresponding to nucleotides 446–734 of the murine BMP-11 cDNA (DDBJ/EMBL/GenBank accession No. AF092734); a murine VEGF cDNA (Frank *et al.*, 1995); murine angiopoietin-1 and -2 cDNAs (Bloch *et al.*, 2000), murine TGF- β 1, β 2 and β 3 cDNAs (Frank *et al.*, 1996); a murine CTGF cDNA (Dammeier *et al.*, 1998); fibronectin, collagen α 1(I) and α 1(III) cDNAs (Bloch *et al.*, 2000), and murine IL-1 β and TNF- α cDNAs (Hübner *et al.*, 1996b). Quantification of the mRNA expression data was performed with NIH image. Non-radioactive *in situ* hybridization was carried out according to Paratore *et al.* (1999) using the full-length murine follistatin cDNA as a template. Radioactive *in situ* hybridization was performed as described by Wilkinson *et al.* (1987) using the 307 bp fragment described above.

Histological analysis

Back skin and tail skin (separated from the bone) were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Sections (6 μ m) were stained with hematoxylin/eosin or using the Masson trichrome procedure.

Wounding and preparation of wound tissues

Four full-thickness excisional wounds of 0.5 cm diameter were generated on the back of transgenic mice and control mice (10–12 weeks old) by excising skin and *panniculus carnosus* as described by Werner *et al.* (1994). Wounds were left uncovered without a dressing. For expression studies, the complete wounds including 2 mm of the epithelial margins were excised at different time points after injury, immediately frozen in liquid nitrogen and stored at –80°C until used for RNA isolation. Non-wounded back skin served as the control. For histological analysis, the complete wounds with 3 mm of adjacent normal tissue were isolated, bisected, fixed overnight in 4% paraformaldehyde in PBS (for histology) or in 95% ethanol/1% acetic acid (for immunofluorescence) and embedded in paraffin. Sections (6 μ m) from the middle of the wound were stained with hematoxylin/eosin or by the Masson trichrome procedure or used for immunofluorescence. Only littermates of the same sex were used for direct histological comparison. All experiments with animals were carried out with permission from the local authorities.

Tensile strength

Mice were anesthetized by intraperitoneal injection of medetomidin (10 μ g/kg), midazolam (0.1 mg/kg) and fentanyl (1 μ g/kg). Anesthesia was antagonized by subcutaneous injection of atipamezol (0.05 mg/kg), flumazenil (10 μ g/kg) and naloxon (24 μ g/kg). On the day of wounding the dorsal region was shaved and treated with a depilatory agent (Pilca). Two full-thickness incisions (1 cm) were made at one anterior and one posterior dorsal site and the skin margins were closed with strips of a wound plaster (Fixomull stretch, Beiersdorf, Hamburg). Mice were killed on day 5 post-wounding, and the tensile strength of the wounds was determined *in situ* using the BTC-2000™ (SRLI Technologies, Nashville, TN) according to the manufacturer's protocol for the non-human disruptive linear incision analysis. All data are expressed as mean $\pm$ SEM. Statistical significance of differences in tensile strength between the groups was determined by Student's *t*-test.

Immunofluorescence

Acetone-fixed frozen sections (6 μ m) from tail skin or ethanol/acetic acid fixed paraffin sections from wounds were incubated overnight at 4°C with mouse monoclonal antibodies directed against K10 (Dako, Hamburg, Germany) or PECAM (PharMingen, San Diego, CA), or with polyclonal antisera directed against K6, K14, or loricrin (all from BABCO, Richmond, CA). Slides were subsequently incubated for 1 h at room temperature with Cy2™- or Cy3™-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory Inc., West Grove, PA). Analysis was performed with a Zeiss Axiophot microscope or a Leica confocal laser scanning microscope.

Detection of proliferating cells by labelling with BrdU

Mice were injected (intraperitoneally) with BrdU (Sigma; 250 mg/kg in 0.9% NaCl) and killed 2 h after injection. Tail skin and bisected wounds were fixed in acid ethanol (see above) and embedded in paraffin. Sections (6 μ m) were treated with a peroxidase-conjugated monoclonal antibody directed against BrdU (Roche Biochemicals), stained with the diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin.

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