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Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair

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Recently we demonstrated a strong induction of activin expression after skin injury, suggesting a function of this transforming growth factor-β family member in wound repair. To test this possibility, we generated transgenic mice that overexpress the activin βA chain in the epidermis under the control of a keratin 14 promoter. The transgenic mice were significantly smaller than control littermates, and they had smaller ears and shorter tails. In their skin, the fatty tissue was replaced by connective tissue and a severe thickening of the epidermis was found. The spinous cell layer was significantly increased, and the epidermal architecture was highly disorganized. These histological abnormalities seem to result from increased proliferation of the basal keratinocytes and abnormalities in the program of keratinocyte differentiation. After skin injury, a significant enhancement of granulation tissue formation was detected in the activin-overexpressing mice, possibly as a result of premature induction of fibronectin and tenascin-C expression. These data reveal novel activities of activin in the regulation of keratinocyte proliferation and differentiation as well as in dermal fibrosis and cutaneous wound repair.

Keywords: activin/epidermis/granulation tissue/keratinocyte/wound

Introduction

Morphogenesis of the skin during embryonic development and wound repair in the adult are controlled by a wide variety of growth and differentiation factors, which have only partially been identified. Among the key players are polypeptides of the transforming growth factor β (TGF-β) family, which in mammals include, for example, three types of TGF-β (TGF-β1, β2 and β3), various bone morphogenetic proteins (BMPs), Mullerian inhibiting substance, inhibins and activins (for a review, see Massagué, 1990). A series of studies has provided insight into the important function of TGF-βs in skin morphogenesis and wound repair (for reviews, see Roberts and Sporn, 1996; O'Kane and Ferguson, 1997). However, an *in vivo* function of activin in these processes has as yet not been demonstrated.

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 (βAβA) and activin B (βBβB), as well as the heterodimeric activin AB (βAβB), have been described (reviewed by Massagué, 1990; Vale *et al.*, 1990). Recently, βC, βD and βE chains have been discovered (Hötten *et al.*, 1995; Oda *et al.*, 1995; Fang *et al.*, 1996), although the corresponding proteins have not been characterized yet. In most assays, activin A, AB and B have similar and equipotent activities, although slight differences between the three isoforms have also been reported.

The biological activities of activins are mediated by heteromeric receptor complexes consisting of two different types of receptor, the type I (ActRIA and ActRIB) and the type II (ActRII and ActRIIB) receptors, which are all characterized by the presence of an intracellular serine/threonine kinase domain (for a review, see Mathews and Vale, 1993). The formation of heteromers of type I and type II receptors is essential for signal transduction (Attisano *et al.*, 1996). Besides these transmembrane receptors, soluble activin-binding proteins, follistatins, have been discovered, which bind to activin and thereby inhibit its biological effects (Nakamura *et al.*, 1990; de Winter *et al.*, 1996).

The first evidence for a possible role of activin in the skin resulted from the phenotypes of knockout mice. Besides various defects that caused death within the first 24 h after birth, mice lacking the activin βA chain lacked whiskers and whisker follicles were abnormal (Matzuk *et al.*, 1995a), and mice deficient in follistatin showed disturbed whisker development and hyperkeratotic skin (Matzuk *et al.*, 1995b). To further determine the function of activin in normal and wounded skin, we recently analyzed the expression of activin and its receptors during the wound healing process. We found a strong induction of activin βA and βB mRNA expression in the granulation tissue and in suprabasal keratinocytes of the hyperproliferative epithelium after skin injury (Hübner *et al.*, 1996; G.Hübner and S.Werner, unpublished data). Furthermore, all known activin receptors were expressed in the

mesenchymal and epithelial compartments of normal and wounded skin, although their expression was not induced after injury (Hübner *et al.*, 1996). To gain insight into the effects of activin in skin morphogenesis and wound repair, we used a keratin 14 (K14) promoter to express the activin βA chain ectopically in basal keratinocytes of the epidermis as well as in the outer root sheath of the hair follicles. The increased levels of mature activin protein significantly affected the morphogenesis of the skin, and a striking effect on the wound healing process was observed.

Results

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

To determine the function of activin in the skin, we overexpressed the activin βA chain in the epidermis of transgenic mice. For this purpose we used a human K14 promoter that has been shown to target the expression of transgenes to the non-differentiated basal cells of the epidermis and to the keratinocytes of the outer root sheath of the hair follicles (Vassar *et al.*, 1989; K.Ongena and D.Huylebroeck, unpublished data). The human activin βA cDNA, including an in-frame epitope of the influenza virus hemagglutinin (IVH), was used as a transgene. Human activin has been shown to be fully active on rodent cells (Yasuda *et al.*, 1993). The βA chain was chosen since this variant is particularly strongly induced during cutaneous wound repair (Hübner *et al.*, 1996). 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 express high levels of the transgene and that secrete a protein of the expected size into the culture medium. Interestingly, these cells revealed characteristics of differentiated cells, such as growth to higher cell density and expression of the differentiation-specific keratin 10 (data not shown). These findings correlate with the recently demonstrated ability of activin to induce differentiation of primary keratinocytes *in vitro* (Seishima *et al.*, 1999). Taken together, these *in vitro* results provided evidence for the suitability of the transgene construct for expression of biologically active activin in keratinocytes.

Generation of transgenic mice that express high levels of human activin A in the skin

Eight transgenic mouse lines with different integration sites of the transgene were generated. Four of these lines expressed high levels of the transgene in the skin. In these lines the expression levels of the transgene in the back skin were similar or even higher compared with the levels of endogenous activin seen in wounded skin of control mice (compare Figure 7A). Two other lines expressed very low levels of the transgene. In these mice the expression levels of the transgene in the skin of the back were significantly lower compared with the expression levels of endogenous activin in wounded skin (data not shown). Two lines did not express the transgene.

The highest levels of the transgene mRNA were found in the skin of the back and the tail (Figure 1B) as well as in the ear (not shown). Low levels of expression were also detected

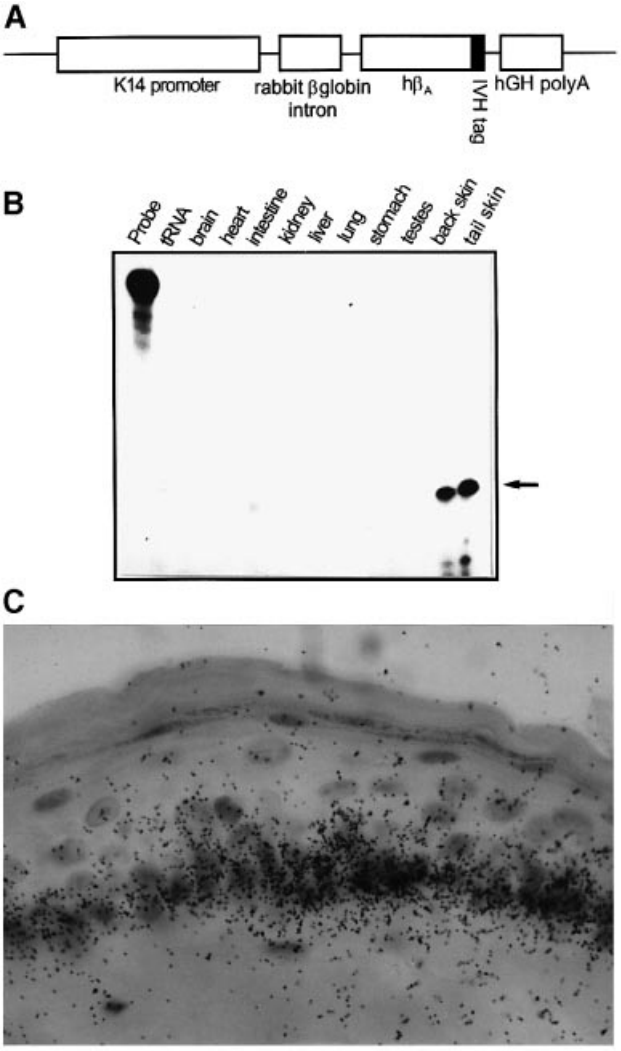

Fig. 1. (**A**) Diagram of the transgene construct. Functional elements include the human K14 promoter, the rabbit β-globin intron, the human activin βA cDNA fused in-frame to the IVH-epitope tag, and the human growth hormone (hGH) poly(A). (**B**) Tissue-specific expression of the transgene. RNA was isolated from various tissues of the transgenic mice. Total cellular RNA (20 μg) was analyzed for the presence of activin βA mRNA by RNase protection assay using the probe described in Figure 7A; 20 μg of tRNA were used as a negative control. The hybridization probe (1000 c.p.m.) was loaded in the lane labeled 'probe' and used as a size marker. (**C**) *In situ* localization of the human activin βA mRNA in the tail skin from a 3-month-old heterozygous transgenic mouse. Tail skin was separated from the bone, fixed in 4% paraformaldehyde and paraffin embedded. Sections (6 μm) were hybridized with a ^{35}S-labeled riboprobe complementary to the human activin βA mRNA. They were coated with NBT nuclear emulsion, exposed for 2 weeks and counterstained with hematoxylin–eosin. Silver grains appear as black dots. Magnification 400×.

in the tongue, the oral epithelium and the thymus (data not shown), whereas other tissues, including brain, heart, intestine, kidney, liver, lung, stomach and testes, were negative (Figure 1B). The transgene was expressed at high levels in basal keratinocytes (Figure 1C) and in outer root sheath cells of the hair follicles (data not shown). These data are consistent with the expected activity of the K14 promoter (Vassar *et al.*, 1989).

Transgenic mice appear macroscopically abnormal

Immediately after birth, the transgenic mice appeared normal with the exception of a necrotic tip of the tail.

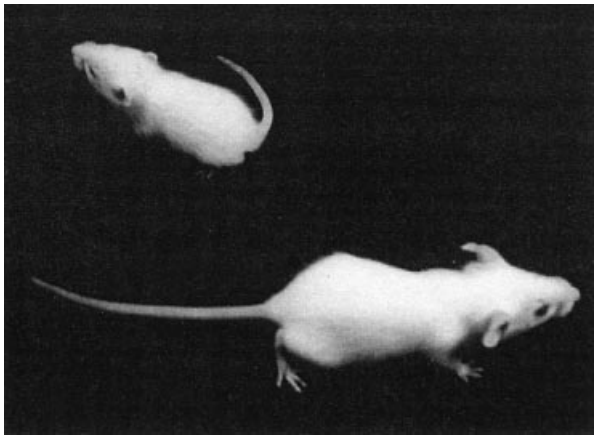

Fig. 2. The macroscopic phenotype of the activin-overexpressing mice. A female control mouse (3 weeks old) is shown on the right, a female homozygous transgenic littermate is shown on the left. Note the small size of the transgenic mouse and the short tail and small ears.

However, within 3 weeks after birth, obvious abnormalities appeared in the four lines that expressed high levels of the transgene. These mice had significantly shorter ears and tails. Furthermore, they were smaller compared with their control littermates (Figure 2). This could be due to the expression of the transgene in the tongue, the oral epithelium and/or the esophagus where K14 is also expressed (Vassar *et al.*, 1989). Indeed, a histological analysis of the tongue revealed a severe hyperthickening of the tongue epithelium and an increase in the number of papillae (data not shown). These abnormalities might inhibit food intake and result in a nutritional defect. In addition, the activin expressed in stratified epithelia might enter the bloodstream and thus cause systemic effects apart from those in the skin. To test this possibility, we determined the activin levels in the serum using a specific two-site immunoassay that detects free activin A and activin A bound to carrier proteins, but not activin B or inhibins (Knight *et al.*, 1996). Interestingly, the serum of 13 mice from two different lines contained between 20 and 150 ng/ml of activin (average 53 ng/ml), whereas activin levels were below the detection limit of 76 pg/ml in the control mice ($n = 25$), indicating that the activin expressed in stratified epithelia does indeed enter the bloodstream. At the age of 3 months, the differences in body size and weight were no longer observed, although the ears and the tail remained smaller compared with non-transgenic littermates. No macroscopic differences were found in the transgenic lines that expressed very low levels of activin or that lacked expression of the transgene (data not shown).

Transgenic mice are characterized by epidermal thickening and dermal fibrosis

The transgenic mice were further examined for phenotypic abnormalities in the skin. All experiments were performed with adult mice (10–14 weeks of age). Only littermates of the same sex were used for a direct comparison. A significant histological phenotype was seen in the tail skin of the four lines that express high levels of the transgene (Figure 3B and E, heterozygous mouse; Figure 3C and F, homozygous mouse), but not in the lines that only weakly express the transgene or that lack transgene expression (data not shown). The epidermis of the transgenic mice was much thicker compared with the skin of control animals. The spinous layer was greatly increased compared with normal epidermis, but little parakeratosis was observed in the cornified layer (Figure 3E and F). Furthermore, the epidermal architecture was highly disorganized, the alternating pattern of ortho- and parakeratinized epidermis that is normally seen in mouse tail skin was changed, and there was an irregular border between the dermis and the epidermis in the transgenic mice. Apart from epidermal changes, the subcutaneous fatty tissue was replaced by connective tissue in the transgenic mice (Figure 3B and C). Similar abnormalities were also seen in the skin of the ear and to a lesser extent in the skin of the back (data not shown).

The phenotypic abnormalities revealed a strong correlation with the expression level of the transgene, as demonstrated by the difference between heterozygous and homozygous mice of each line (Figure 3, middle and lower panel) and by comparison of different lines that expressed different levels of the transgene (data not shown). In general, abnormalities were seen only in the lines in which the mRNA levels of the transgene in non-wounded skin were at least comparable to the mRNA levels of endogenous activin seen in wounded skin of control mice (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 by mRNA expression analysis of the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α, which were found to be expressed at equally low levels in the skin of transgenic mice and control mice (not shown).

Overexpression of activin affects keratinocyte proliferation and differentiation

The severe hyperthickening of the epidermis was reminiscent of the phenotype seen in hyperproliferative skin disease. We therefore determined the proliferation status of the keratinocytes by labeling the proliferating cells with 5-bromodeoxyuridine (BrdU) *in vivo* and subsequent staining of the skin with an antibody directed against BrdU. As in control mice (Figure 4A), proliferation was restricted to the basal layer of the epidermis of transgenic mice (Figure 4B). However, the number of proliferating basal cells was 2.5-fold higher in the epidermis of transgenic mice ($n = 10$) compared with control mice ($n = 10$), demonstrating that the overexpression of activin stimulates proliferation of basal keratinocytes.

To determine the effect of activin on keratinocyte differentiation, we analyzed the expression of differentiation-specific proteins in control and transgenic mouse tail epidermis by immunofluorescence. In normal epidermis, keratins 5 and 14 (K5 and K14) are expressed at high levels in the basal layer, but not in the suprabasal layers (reviewed by Fuchs, 1990). When basal cells become committed to terminal differentiation and move to the suprabasal layer, they synthesize a new pair of keratins, K1 and K10 (reviewed by Fuchs, 1990). Expression of K14 was detected in basal cells in the epidermis of both normal (Figure 5A) and transgenic mice (Figure 5B), and the staining persisted throughout the whole epithelium. In transgenic mice, however, the signal obtained with the K14

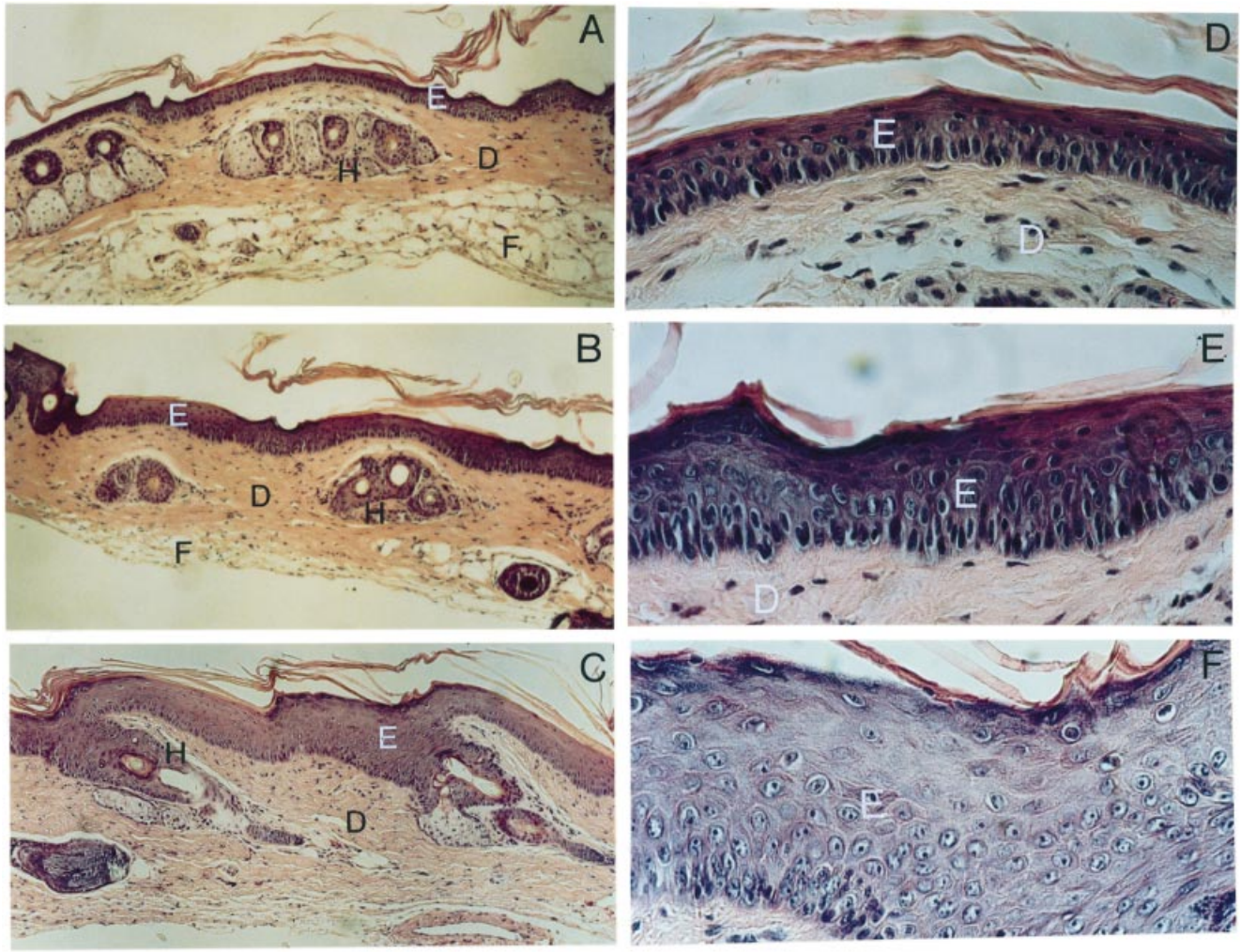

Fig. 3. Histology of tail skin from a control mouse (**A** and **D**), a heterozygous transgenic mouse (**B** and **E**) and a homozygous transgenic mouse (**C** and **F**) (all 3 months old). Paraffin sections (6 μm) from the tail were stained with hematoxylin–eosin. D, dermis; E, epidermis; H, hair follicle; F, fatty tissue. Note the presence of several suprabasal layers of nucleated cells in the epidermis of transgenic mice, their high degree of disorganization, the lack of a clear border between the dermis and the epidermis, and the replacement of fatty tissue by connective tissue. Magnification 200× (A, B and C) and 400× (D, E and F).

antibody appeared generally weaker, and the distribution of this keratin within the basal cells was altered compared with control mice. Thus, a stronger signal was seen at the basolateral side of the basal keratinocytes compared with the apical side (Figure 5B). Keratin 10 expression started in the first suprabasal layer in normal and transgenic mice (Figure 5C and D), demonstrating that the additional layers seen in the epidermis of the transgenic mice consist of at least partially differentiated cells.

To determine the stage of differentiation of these additional K10-positive suprabasal cells, we stained the skin simultaneously with an antibody against loricrin, the major protein of the cornified envelope (Mehrel *et al.*, 1990), and with a monoclonal antibody against K14 to visualize the basal layer. As shown in Figure 5G and H, loricrin (green color) was appropriately synthesized in the granular layer of normal (Figure 5G) and transgenic (Figure 5H) mouse epidermis, whereas the additional suprabasal K10-positive keratinocytes did not express this protein in the transgenic mice. Finally, we stained the skin with an antibody directed against K16. This keratin and its partner K6 are restricted to the hair follicles in normal skin, but are transiently expressed in the proliferating epidermis of wounds and permanently upregulated in the suprabasal epidermal layers of hyperplastic, neoplastic and psoriatic skin (reviewed by Fuchs, 1990). Suprabasal keratinocytes in the epidermis of the transgenic mice expressed this keratin (Figure 5F), although the expression was patchy. The density of the K16-positive cells in the different transgenic mouse lines correlated with the expression level of the transgene (data not shown).

Abnormal distribution of tenascin-C and nidogen in the skin of transgenic mice

The observed replacement of fatty tissue by connective tissue led us to investigate the expression of various extracellular matrix proteins in these animals. Surprisingly, no significant differences in the mRNA expression levels of fibronectin, collagen αI, collagen αIII and of the basement membrane proteins tenascin-C and nidogen were observed (data not shown). However, immunohistochemistry with antibodies against tenascin-C and nidogen revealed significant changes in the distribution of these proteins in the skin of transgenic mice. As shown in

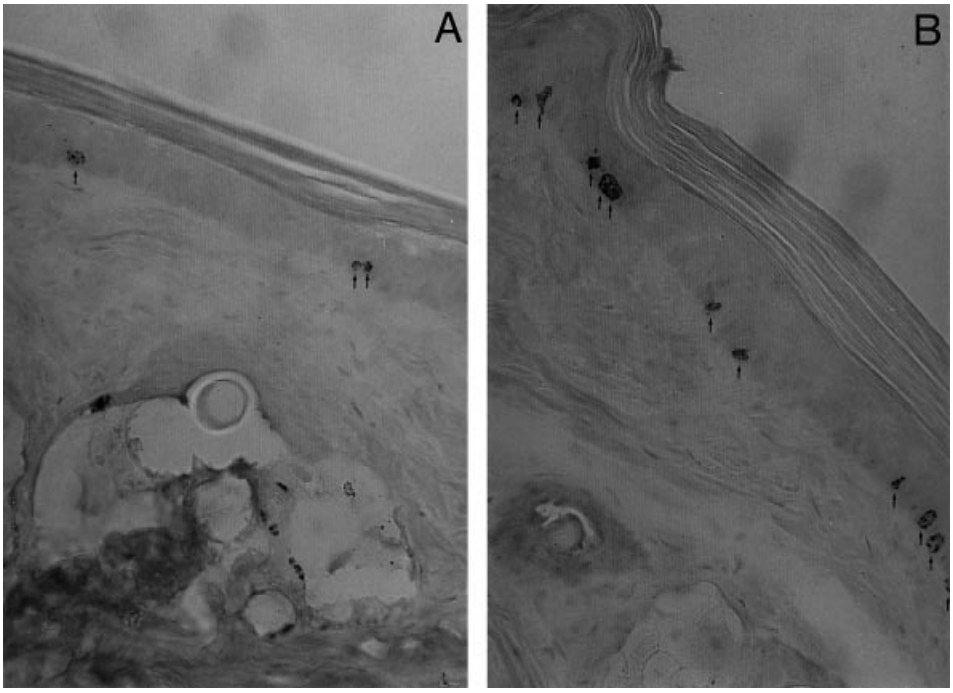

Fig. 4. Detection of proliferating cells in the tail skin of control and transgenic mice. Mice were injected with BrdU and killed 3 h after injection. Frozen sections of the tail skin of control mice (**A**) and transgenic mice (**B**) were incubated with a peroxidase-conjugated antibody against BrdU and stained with the diaminobenzidine–peroxidase staining kit. Stained nuclei are indicated by arrows. Magnification 400×.

Figure 6 for tenascin-C, the staining was confined to the basement membrane zones in tail skin of control mice (Figure 6A). In contrast, a strong tenascin-C signal was found in interfollicular dermis of the transgenic mice (Figure 6B). Nidogen was seen in the dermis as well as in the basal and the first suprabasal layer of the transgenic mouse epidermis, whereas this protein was restricted to the basement membrane in control animals (not shown). By contrast, collagen type IV was confined to the basement membrane in normal and transgenic mice (data not shown), indicating that the basement membrane of the activin-overexpressing mice is not generally distorted.

Enhanced wound healing in activin transgenic mice

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. As expected from the activity of the K14 promoter, *in situ* hybridization revealed a strong expression of human activin βA mRNA in the hyperproliferative epithelium at the wound edge and in the epidermis adjacent to the wound (data not shown). Most importantly, the K14 promoter-driven expression of the human activin βA mRNA (lower bands in Figure 7A) was significantly higher compared with endogenous activin βA (higher band in Figure 7A), although the latter was strongly induced in both normal and transgenic mice (Figure 7A).

The epitope-tagged activin βA monomer of ~17 kDa was detected in the lysates from non-wounded and from wounded skin of the transgenic mice that expressed high levels of the transgene (l.24) as determined by Western blot analysis after SDS–PAGE under reducing conditions with a monoclonal antibody directed against the IVH epitope (Figure 7B). A protein of the same size was also found in the supernatant of the HaCaT cells transfected with the human activin-βA–IVH cDNA (data not shown), demonstrating that this band does indeed correspond to the processed and secreted activin βA protein, which was reduced to the monomer under our experimental conditions.

We subsequently performed a detailed histological analysis of 5-day wounds from three independent transgenic mouse lines that express high levels of the transgene (line 24: 10 control mice, nine heterozygous transgenic mice, three homozygous transgenic mice; line 12: three control mice, five heterozygous transgenic mice; line 31: three control mice, three heterozygous transgenic mice). This time point was chosen because differences in the extent of re-epithelialization and granulation tissue formation can easily be detected at this stage after injury. Furthermore, activin levels are particularly high at day 5 after wounding (Hübner *et al.*, 1996). Histological characterization of the wounds revealed a remarkable increase in the amount of granulation tissue in the wounds of the transgenic mice (Figure 8B) compared with control mice (Figure 8A). The cell density in the granulation tissue of the transgenic animals was significantly higher compared with control animals (Figure 8B) and there was a remarkable deposition of connective tissue, particularly below the epithelial tongue (Figure 8D). Finally, there was no clear border between the hyperproliferative epithelium and the underlying dermis or granulation tissue (indicated by arrows). The extent of re-epithelialization was increased in some of the wounds of the activin-overexpressing mice, although this was not consistently observed. These findings demonstrate that the increased levels of activin in the wounds of transgenic mice stimulate the wound healing process.

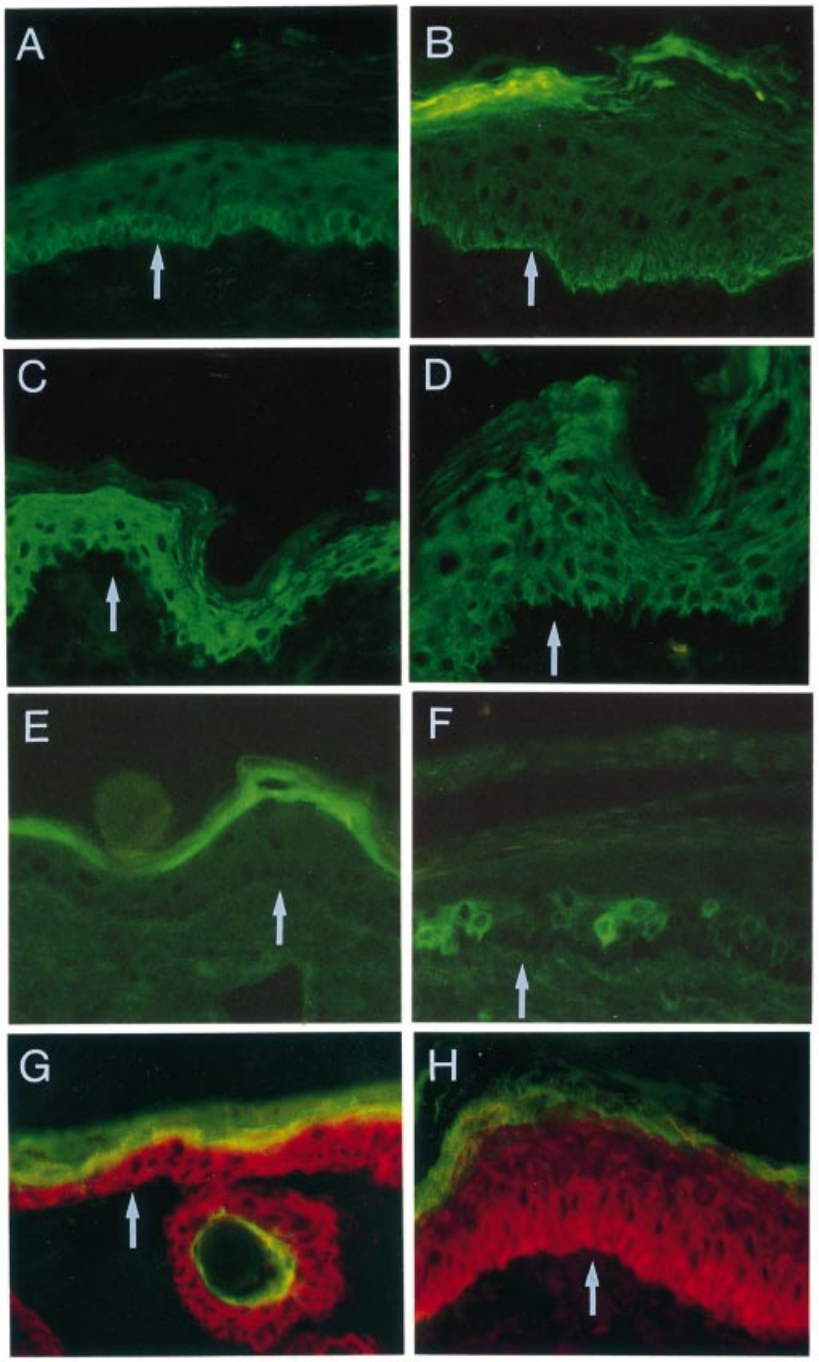

Fig. 5. Expression of differentiation-specific proteins in tail epidermis of control mice (left panels) and heterozygous transgenic mice (right panels). Sections were incubated with antibodies to K14 (**A** and **B**), K10 (**C** and **D**) and K16 (**E** and **F**). The basal layer is indicated by arrows. Double immunofluorescence with antibodies against K14 (red) and loricrin (green) is shown in (**G**) and (**H**). Magnification 250×.

Altered expression of fibronectin and tenascin-C in the wounds of transgenic mice

To gain insight into the mechanisms underlying the increased granulation tissue formation, we analyzed the mRNA expression of major wound matrix proteins in normal and wounded skin. Both fibronectin and tenascin-C expression were upregulated after skin injury in normal and transgenic mice (Figure 9). However, the most significant upregulation of both transcripts occurred earlier in the transgenic animals. Therefore, these proteins might accumulate in the wound tissue, contributing to enhanced granulation tissue formation. By contrast, collagen αI and αIII mRNA expression was similar in non-wounded and wounded skin of normal and transgenic mice (data not shown). The stimulation of granulation tissue formation is likely to be a direct effect of activin, since the expression of various other TGF-β superfamily members was not significantly altered by overexpression of the transgene in non-wounded and wounded skin (data not shown). Thus, activin seems to be a novel mediator of connective tissue synthesis/deposition in normal skin and during cutaneous wound repair.

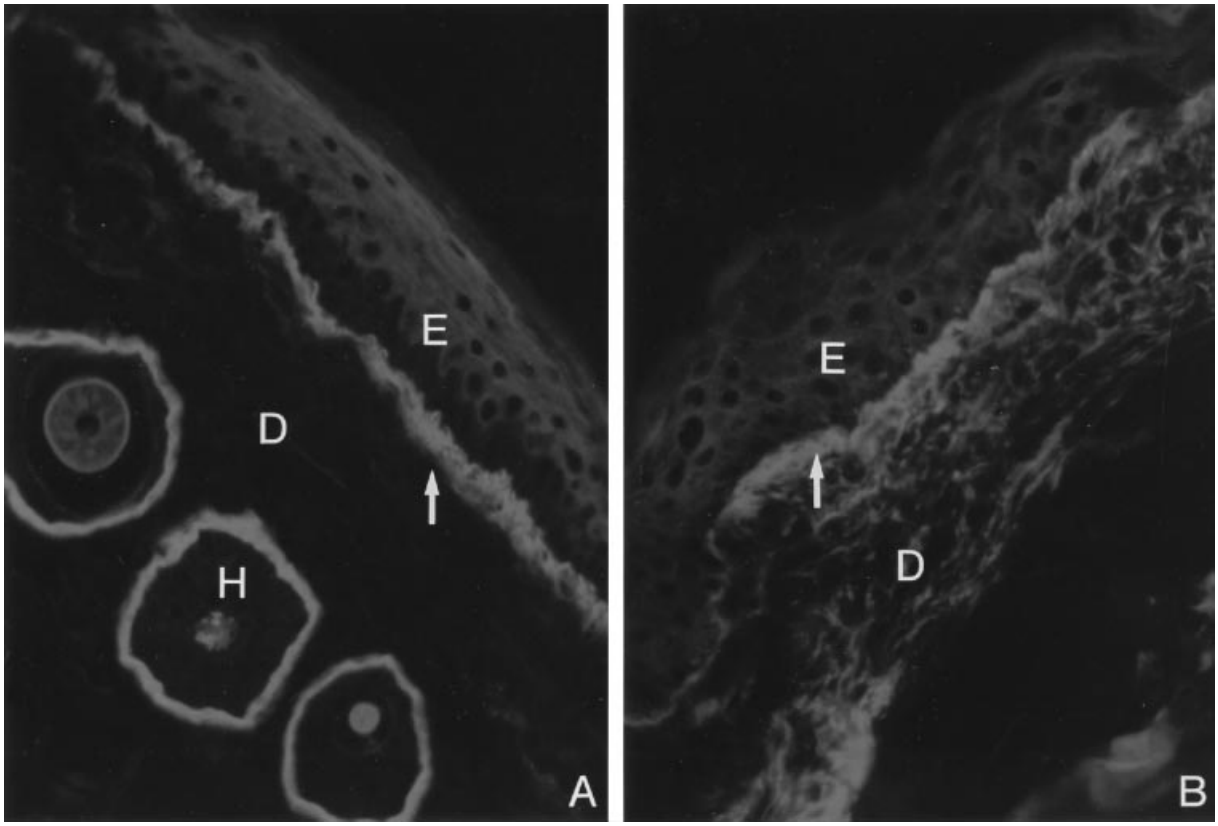

Fig. 6. Aberrant distribution of tenascin-C in the tail skin of transgenic mice. Frozen sections (6 μm) from the tail skin of a control mouse (**A**) and a transgenic mouse (**B**) were incubated with a monoclonal antibody directed against tenascin-C. The basement membrane zones are indicated by arrows. E, epidermis; D, dermis; H, hair follicle. Note the clear basement membrane staining in control epidermis and the strong staining of interfollicular dermis in the transgenic mice. Magnification 400×.

Discussion

To gain insight into the role of activin in skin development and wound repair, we generated transgenic mice that overexpress the activin βA chain in the basal layer of the epidermis. These mice were smaller compared with their control littermates at 3 weeks of age. This could be due to the observed abnormalities in the tongue epithelium, which might result in a nutritional defect. In addition, defects in the oral epithelium and/or the esophagus might be present, since the K14 promoter is also active in these stratified epithelia. Alternatively, the increased activin levels in the blood might cause defects outside of the skin. However, high levels of activin have been reported during pregnancy (Fowler *et al.*, 1998) without obvious effects on various internal organs. Furthermore, such high levels do not obviously affect skin physiology or morphology, indicating that the phenotype seen in the skin of our transgenic mice is a result of the local overexpression of activin and not of serum-derived activin.

As expected from the experimental design, which involved the K14 promoter, the major phenotypic abnormalities were observed in the skin. The thickness of the epidermis was significantly increased, resembling the hyperplastic neonatal mouse epidermis. Interestingly, we recently observed a higher expression level of the activin βA chain in newborn back skin compared with adult back skin (data not shown), indicating a role of activin in the hyperplasia seen in neonatal skin. Consistent with the appearance of the epidermis, the rate of proliferating keratinocytes was 2.5-fold higher in the skin of transgenic animals compared with the controls. The observed stimulation of keratinocyte proliferation by activin is likely to be indirect, since activin inhibited proliferation of human HaCaT keratinocytes (Shimizu *et al.*, 1998) and primary keratinocytes *in vitro* (Seishimi *et al.*, 1999). Irrespective of whether the growth-stimulatory effect was direct or indirect, the signals transmitted by activin and by other TGF-β family members to keratinocytes are different, since TGF-β2 inhibited HaCaT cell proliferation at much lower concentrations than activin A (Shimizu *et al.*, 1998). Furthermore, overexpression of TGF-β1 or BMP-6 in the epidermis of transgenic mice resulted in phenotypic abnormalities that were strikingly different to those observed in our activin transgenic mice (Sellheyer *et al.*, 1993; Cui *et al.*, 1995; Blessing *et al.*, 1996; Fowlis *et al.*, 1996). These results demonstrate that different members of the TGF-β family can exert unique activities in the skin.

In addition to the stimulation of keratinocyte proliferation, the increased levels of activin also affected the program of keratinocyte differentiation. The normal pattern of ortho- and parakeratinized epidermis in the tail skin was changed, resembling the effect of retinoic acid treatment of normal tail skin (Schweizer *et al.*, 1987). Consistent with the observed hyperproliferation, we found expression of K16 in interfollicular epidermis. Surprisingly, the levels of K14 appeared lower in the basal cells of the transgenic mice. These reduced K14 levels are likely to be the result of inefficient translation of the mRNA and/or reduced stability of the protein, since no differences were seen in

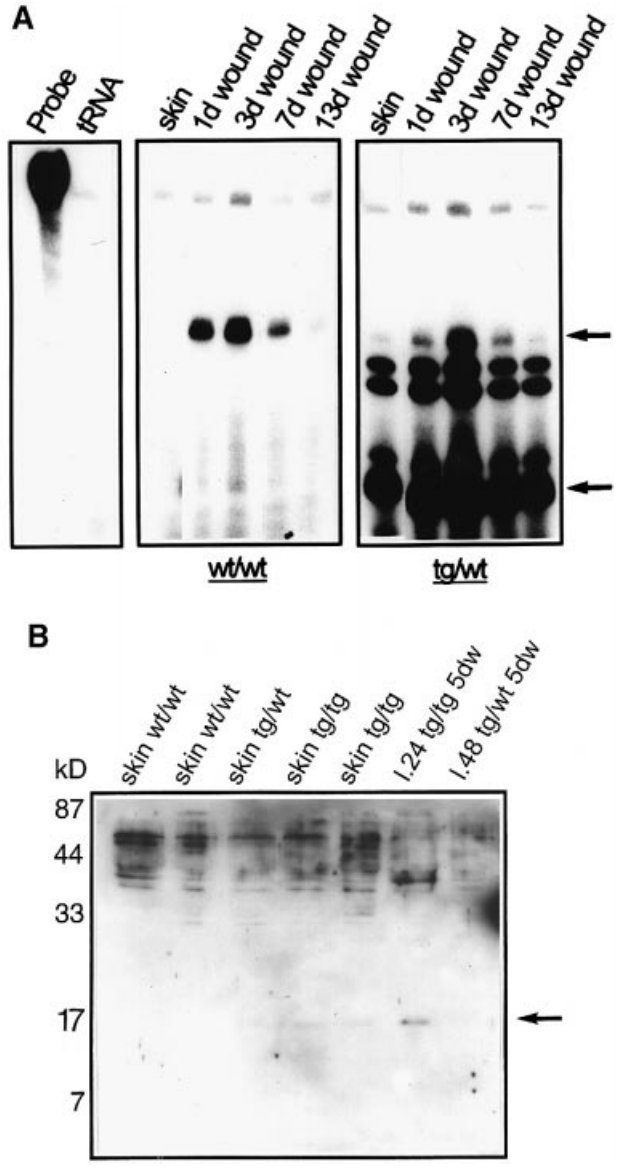

Fig. 7. (**A**) Expression of endogenous activin βA mRNA and of RNA derived from the transgene in normal and wounded skin. RNA was isolated from control back skin and from wounds of several stages, and analyzed by RNase protection assay for the presence of activin βA mRNA. A 150 bp murine βA cDNA was used as a template. Owing to an 85 bp identity of the human and murine sequences, an 85 bp protected fragment was generated by RNA derived from the transgene (lower arrow). Note the exclusive presence of mouse activin βA mRNA (upper arrow) in the control mice (wt/wt), the presence of both mouse and human βA mRNAs in the transgenic mice (tg/wt), and the strong induction of endogenous activin βA expression after injury in control and transgenic mice. The additional bands that are not indicated by arrows result from incomplete digestion of partially matching sequences. (**B**) Expression of the recombinant activin A fusion protein in tail skin (skin) and 5 day wounds (5dw) of normal (wt/wt) and transgenic (tg/wt) mice. Total protein (20 μg) from skin and wound lysates was analyzed by Western blotting under reducing conditions for the presence of the recombinant activin fusion protein using an antibody against the IVH epitope and the ECL detection system. The recombinant protein (17 kDa) is indicated by the arrow. l.24, transgenic mouse line expressing high levels of the transgene; l.48, transgenic mouse line expressing very low levels of the transgene.

the K14 mRNA levels (data not shown) between normal and transgenic mice. The latter result demonstrates that the phenotype is not a result of transcription factor depletion due to the presence of the exogenous keratin 14 promoter. The most striking observation was that, despite an increased proliferation rate, expression of the differentiation-specific K10 was detected in the first suprabasal cell layers, demonstrating that the cells have entered a differentiation program, although the process of terminal differentiation seems to be delayed, as reflected by the lack of loricrin expression in the nucleated K10-positive cells. Loricrin was, however, expressed normally in the granular layer, demonstrating that terminal differentiation was not inhibited. It seems possible that activin is involved in the initiation of differentiation in a hyperproliferative epithelium, as indicated by the presence of a large number of K10-positive cells in exponentially growing activin-transfected HaCaT cells (data not shown) under conditions where K10 expression is normally suppressed (Ryle *et al.*, 1989). Participation of activin in the early induction of keratinocyte differentiation is further supported by the localization of activin mRNA and protein in the suprabasal but not in the basal cells of the hyperproliferative epithelium at the wound edge (Hübner *et al.*, 1996 and G.Hübner and S.Werner, unpublished data). Induction of keratinocyte differentiation by activin A has also recently been shown in primary keratinocytes *in vitro* (Seishima *et al.*, 1999). In contrast to our *in vivo* results, however, the process of terminal differentiation was also enhanced by activin in these cells. These discrepancies could be due to the presence of other factors present *in vivo* in a hyperproliferative epithelium that delay the later differentiation steps.

Although the transgene is only expressed in the epidermis and in hair follicle keratinocytes, we also observed abnormalities in the interfollicular dermis, which was characterized by a replacement of fatty tissue by connective tissue. This might be due to the diffusion of activin from the keratinocytes into the underlying dermis, possibly facilitated by abnormalities in the basement membrane zone. Further evidence for diffusion of activin into the underlying mesenchyme comes from the histological analysis of the ear cartilage, which was severely hypertrophic (data not shown). In addition, a high diffusibility of activin has previously been demonstrated in the *Xenopus* embryo, where this TGF-β family member was shown to have long-range signaling activity, thereby forming a concentration gradient by diffusion (McDowell *et al.*, 1997). Within the dermis, long-term stimulation by activin could gradually alter extracellular matrix molecule metabolism, which might explain the observed substitution of fatty tissue by connective tissue. Consistent with this hypothesis, we recently observed upregulation of fibronectin mRNA expression by short-term stimulation with activin in cultured murine 3T3 fibroblasts (G.Hübner and S.Werner, unpublished data) and others have shown increased expression of collagen type I mRNA by activin in cultured murine kidney fibroblasts (Sugiyama *et al.*, 1998). Surprisingly, we could not detect any significant differences in the levels of mRNAs encoding several matrix proteins. However, long-term stimulation with activin may gradually lead to a low level of extracellular matrix accumulation, as indicated by the presence of high levels of immunoreactive tenascin-C in intrafollicular dermis. In addition, expression

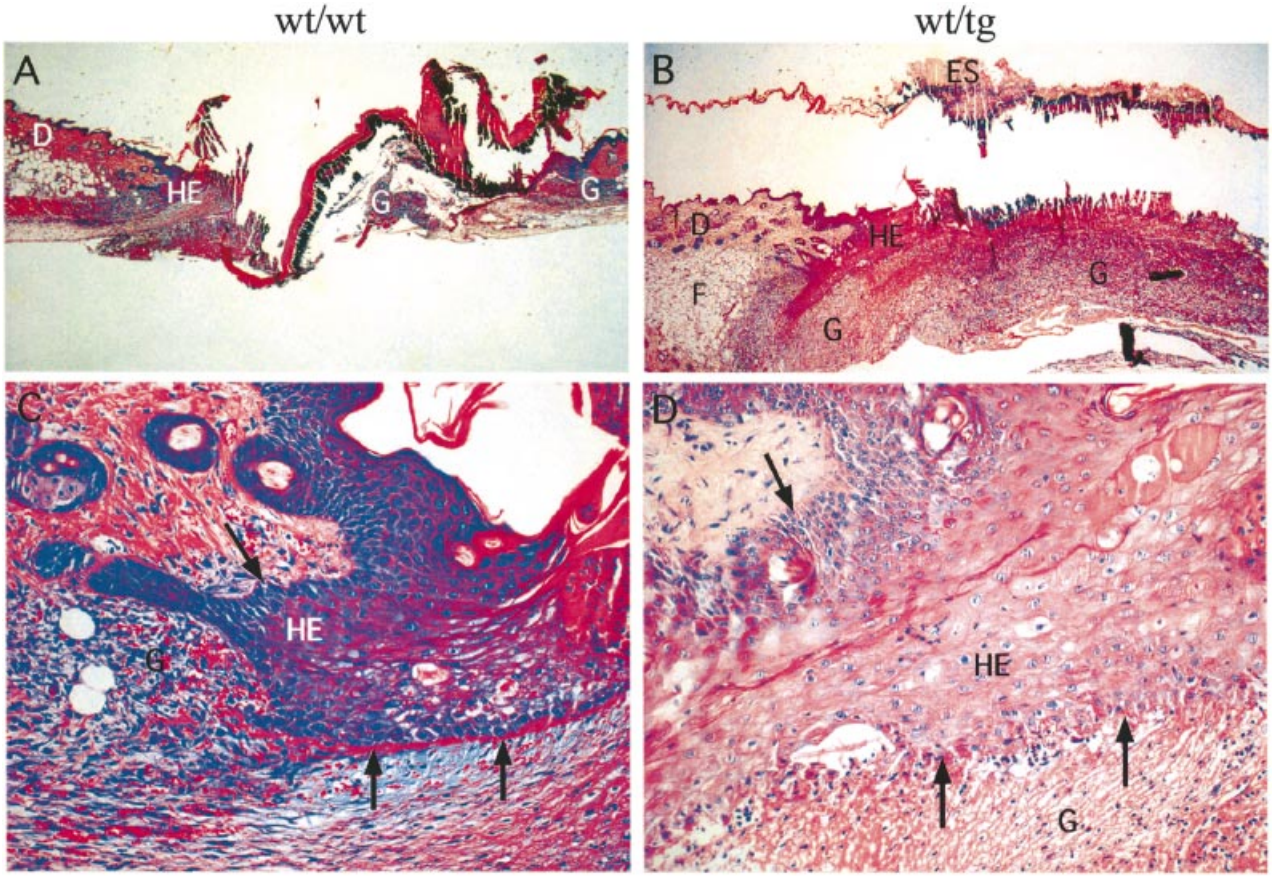

Fig. 8. The wound healing phenotype of activin-overexpressing mice. Full-thickness excisional wounds were made on the back of female transgenic mice (wt/tg) and female control littermates (wt/wt) (3 months old). Mice were killed at day 5 after injury. Sections (6 μm) from the middle of the wounds were stained with hematoxylin–eosin. (**A**) and (**C**) Wounds of a control mouse; (**B**) and (**D**) wounds of a heterozygous transgenic mouse. G, granulation tissue; D, dermis; HE, hyperproliferative epithelium; ES, eschar; M, muscle (panniculus carnosus); F, fatty tissue. Note the larger area of granulation tissue in the transgenic mice (compare A and B), the accumulation of extracellular matrix below the hyperproliferative epithelium in the transgenic mice (D), and the lack of a clear border between the dermis or granulation tissue and the epidermis (indicated by arrows). Magnification 25× (A and B) and 200× (C and D).

of these matrix molecules might be upregulated in the transgenic mice during an earlier phase of skin development, leading to excessive deposition of these proteins. Our results indicate a role for activin in the induction of fibrotic processes and this hypothesis is strongly supported by the detection of high levels of activin in fibrotic kidneys (de Bleser *et al.*, 1997; Sugiyama *et al.*, 1998), cirrhotic livers (Matsuse *et al.*, 1995, 1996) and in arteriosclerosis (Inoue *et al.*, 1994; Pawlowski *et al.*, 1997). Thus, in addition to TGF-β (for a review, see Lawrence, 1996), activin might be a novel player in fibrotic disease.

The most striking result of our study was the significant enhancement of the wound healing process in the activin-overexpressing mice. This finding provides further evidence for a novel and important role for activin in wound healing, and suggests that the strong induction of activin expression seen after injury could be of major biological importance. The process of granulation tissue formation was particularly enhanced. Large amounts of extracellular matrix were detected below the activin-producing keratinocytes, indicating that activin stimulates connective tissue synthesis/deposition not only in normal but also in wounded skin. Thereby, autocrine, paracrine and endocrine effects of activin might be involved. Indeed, we found an earlier increase in the mRNA levels of fibronectin and

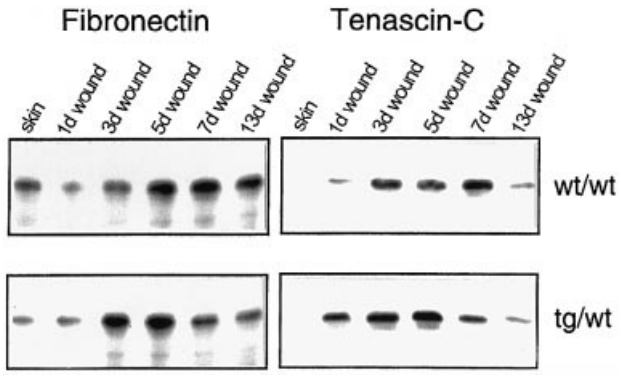

Fig. 9. Activin overexpression alters the mRNA expression levels of fibronectin and tenascin-C. Total cellular RNA was isolated from normal and wounded skin of control mice (wt/wt) and heterozygous transgenic mice (tg/wt). Total cellular RNA (20 μg) was analyzed by RNase protection assay for the presence of fibronectin (left panel) and tenascin-C (right panel). The time after injury is indicated at the top of the lanes. The same batch of RNAs was used for both protection assays. wt/wt, wild-type mice; tg/wt, heterozygous transgenic mice.

tenascin-C after skin injury in the transgenic mice compared with control mice. By contrast, we could not detect significant differences in the levels of collagen α1 and αIII mRNAs between the wounds of normal and transgenic

mice, indicating a selective effect of activin on the mRNA expression of extracellular matrix proteins. Thus, activin seems to differ in this respect from TGF-β, which is also a strong stimulator of collagen gene expression (Roberts *et al.*, 1986). Future studies will reveal whether this selectivity influences the formation of scar tissue and thus the quality of the healed wound. In any case, our results are the first demonstration of a stimulatory effect of activin on the wound healing process, and reveal novel and unique activities of this factor in keratinocyte differentiation, dermal fibrosis, tissue repair and possibly also in human skin disease.

Materials and methods

Plasmid construction

The full-length human activin βA cDNA (1.2 kb) without the stop codon was amplified from cDNA of the HaCaT keratinocyte cell line (Boukamp *et al.*, 1988), and fused in-frame at the 3′ end to a 48 bp sequence encoding an epitope of the IVH followed by a stop codon. This cDNA was sequenced and inserted into an expression cassette that includes the cloning vector pSP72 (Promega), a 2.1 kb human K14 promoter (K.Ongena and D.Huylebroeck, unpublished data), followed by a 0.65 kb rabbit β-globin intron and a transcription termination/polyadenylation fragment [poly(A), 0.63 kb] of the human growth hormone gene (Werner *et al.*, 1993). The activin cDNA was inserted between the intron and the poly(A) fragment (Figure 1A).

Generation of transgenic mice

Standard procedures were followed in order to generate transgenic mice (Hogan *et al.*, 1986). Fertilized eggs were obtained following superovulation and mating of CD1 females. The 4.2 kb insert was separated from vector sequences, purified and injected into the pronuclei of one-cell-stage embryos. Microinjected eggs were transferred at the two-cell stage into the oviducts of pseudopregnant recipient females.

Identification of transgenic mice

Mouse tail DNA was analyzed for integration of the transgene by Southern blot analysis (founder analysis) or dot blot analysis (analysis of progeny). The β-globin intron fragment was used as a probe, since it does not cross-hybridize with chromosomal mouse DNA.

Determination of activin A in the serum

Total activin A was measured in the mouse serum samples using a sensitive and specific enzyme-linked immunosorbent assay developed by Knight *et al.* (1996), with minor modifications as described previously (Riley *et al.*, 1998). The detection limit of the assay was 76 pg/ml. All samples were first assayed at a dilution of 1:8, and then 1:2 for those samples that originally gave results less than the detection limit in the first assay.

RNA isolation, RNase protection assay and in situ hybridization

RNA isolation was carried out as described by Chomczynski and Sacchi (1987). RNase protection analysis was performed as described by Werner *et al.* (1993). As a loading control, 1 μg of all RNA samples was loaded on a 1% agarose gel before hybridization and stained with ethidium bromide. All protection assays were carried out at least in duplicate with different sets of RNA from independent experiments. As a template, we used the mouse activin βA cDNA described by Hübner *et al.* (1996), a 358 bp fragment corresponding to the 3′ end of the human activin βA cDNA (Tanimoto *et al.*, 1992), a 290 bp fragment corresponding to nucleotides 1094–1383 of the mouse tenascin-C cDNA (Weller *et al.*, 1991) and a 162 bp fragment from the 3′ end of the mouse fibronectin cDNA (kindly provided by Dr P.Ekblom). *In situ* hybridization was carried out according to Wilkinson *et al.* (1987) using the human activin βA cDNA fragment as a template. Endogenous mouse activin was not detected with this riboprobe.

Histological analysis

Complete ears and tongues as well as 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 μm) were stained with hematoxylin–eosin.

Wounding and preparation of wound tissues

Full-thickness excisional wounds of 0.5 cm diameter were generated on the back of transgenic 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, we generated six wounds on the back of each mouse. At different time points after injury, the complete wounds including 2 mm of the epithelial margins were excised and immediately frozen in liquid nitrogen until used for RNA isolation. Non-wounded back skin served as a control. For histological analysis, two or four wounds were generated on each mouse. At day 5 after injury, the complete wounds were isolated, bisected, fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin. Sections (6 μm) from the middle of the wound were stained with hematoxylin–eosin. Only littermates of the same sex were used for direct histological comparison. All experiments with animals were carried out with permission from the local government of Bavaria.

Preparation of skin lysates and Western blot analysis

Normal and wounded skin (see above) were frozen in liquid nitrogen. Preparation of tissue lysate was performed as described by Werner *et al.* (1993). In total, 20 μg of protein were analyzed by Western blotting under reducing conditions using a peroxidase-coupled monoclonal antibody directed against the IVH epitope (Roche Biochemicals, Mannheim, Germany) and the enhanced chemiluminescence (ECL) detection system (Amersham, Braunschweig, Germany).

Immunofluorescence

Tail skin was separated from the bone and frozen in tissue freezing medium (Jung, Nussloch, Germany). Acetone-fixed frozen sections (6 μm) were incubated overnight at 4°C with monoclonal antibodies directed against K10 (Dako, Hamburg, Germany), K16 (kindly provided by Dr I.Leigh), K14 (Serotec, Oxford, UK) or tenascin-C (kindly provided by Dr P.Ekblom), or with polyclonal antisera directed against K14 (kindly provided by Dr E.Fuchs), loricrin (BAbCO, Richmond, USA) or nidogen (kindly provided by Dr R.Timpl). Slides were subsequently incubated for 1 h at room temperature with fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies.

Detection of proliferating cells by labeling with BrdU

Mice were injected (i.p.) with BrdU (Sigma, 250 mg/kg in 0.9% NaCl) and killed 3 h after injection. Tail skin was frozen in tissue freezing medium. Sections (6 μm) were treated with a peroxidase-conjugated monoclonal antibody directed against BrdU (Roche Biochemicals) and stained with the diaminobenzidine–peroxidase substrate kit (Vector Laboratories, Burlingame, CA).

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