IGF-I Signalling Controls the Hair Growth Cycle and the Differentiation of Hair Shafts

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Mesenchymal–epithelial signalling between the dermal papilla and the hair matrix regulates cell proliferation and differentiation in mature hair follicles. The molecular basis of these interactions is largely unexplored. According to its expression in the dermal papilla, IGF-I is likely involved in reciprocal signalling. To examine its biological function in pelage follicles further, we generated transgenic mice that express *Igf-I* in the inner root sheath and the medulla using an involucrin promoter fragment. We demonstrate that *Igf-I* affects follicular proliferation, tissue remodelling, and the hair growth cycle, as well as folliclular differentiation. Transgenic skin temporarily lacks visible adipose tissue in telogen. The onset of the second, aberrant growth phase is markedly retarded. Transgenic guard hairs are significantly elongated and a small fraction of hair follicles is severely disoriented. The microscopic appearance of most hair shafts is altered and, strikingly, *Igf-I* transgenic mice lack hairs with a zigzag shape due to the suppression of hair shaft bending. All transgenic effects are partially compensated by ectopic expression of *Igfbp3*. Finally, *Pdgfrα* was identified as the first molecular target that is affected in *Igf-I* transgenic mice. In summary, our data identify IGF-I signalling as an important mitogenic and morphogenetic regulator in hair follicle biology.

Key words: bending/cell proliferation/differentiation/hair cycle/hair shaft/*Igf-I*/skin/zigzag hair
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It has been known for a long time that the follicle's dermal papilla, which is of mesenchymal origin, plays an important role in the control of all aspects of hair follicle morphogenesis and life. Inductive processes between the embryonic mesenchyme and the overlying ectoderm are required to form an epithelial placode which, under the influence of condensed mesenchymal cells, the prospective dermal papilla, gives rise to the complex epithelial structure of the mature hair follicle (Hardy, 1992). Interestingly, hair follicle morphogenesis is induced in consecutive waves. They give rise to four different hair types, guard, awl, auchene, and zigzag hairs, which differ in length, structure, and shape. The distinct waves appear to have different requirements with respect to proteins such as EDA-A1 and its cognate receptor EDAR and the BMP inhibitor Noggin. While *Eda-A1* and *Edar* mutant mice lack the first wave of follicle induction, *Noggin* knockout mice do not initiate secondary hair follicles (Headon and Overbeek, 1999; Botchkarev *et al*, 2002; Laurikkala *et al*, 2002). Beyond morphogenesis, *Noggin* appears also to play a role in the maintenance of zigzag hair follicles (Guha *et al*, 2004). Nevertheless, the molecular mechanisms of establishing and maintaining different hair follicle types are still unknown.

Abbreviations: IGF-I, insulin-like growth factor-I; *Igfbp3*, insulin-like growth factor binding protein 3

Insulin-like growth factor-I (*Igf-I*) and some high-affinity IGF-binding proteins are expressed in the hair follicle, suggesting a role of the IGF signalling pathway in follicle biology. In the skin, *Igf-I* has been reported to be exclusively expressed by mesenchymal cells of the dermis and the dermal papilla (Tavakkol *et al*, 1992; Little *et al*, 1996). Its receptor, IGF-IR, is synthesized in mesenchymal as well as in epithelial cells. Gene expression has been demonstrated for the dermal papilla and cells of the hair matrix (Hodak *et al*, 1996; Rudman *et al*, 1997). In humans, IGF-binding protein 3 is mainly expressed in the dermal papilla of hair-producing follicles (Batch *et al*, 1996). Further reports reveal the capability of keratinocytes to produce insulin-like growth factor binding protein 3 (Igfbp3) (Wraight *et al*, 1994). In mouse, IGFBP3 protein has been localized to the zone of follicular apoptosis at the onset of the regression phase of the hair cycle, which has been interpreted as a direct effect of *p53* expression (Botchkarev *et al*, 2000; Botchkarev *et al*, 2001). Recent work on the murine hair cycle has confirmed gene activity in dermal papilla cells of hair-producing follicles. Nevertheless, the data also show that expression in dermal papilla cells dramatically increases during the regression and early resting phase of the hair growth cycle (Schlake *et al*, 2004). Strong *Igfbp4* expression is seen at the border between the dermal papilla and hair matrix in human hair follicles, whereas *Igfbp5* transcription is visible in dermal papilla cells of active follicles (Batch *et al*, 1996).

IGF-I is a potent mitogen supporting cell growth and survival (Stewart and Rotwein, 1996). It also plays a role in some differentiation processes (Musaro and Rosenthal, 1999; Hsieh *et al*, 2004). IGF-binding proteins can act either in an agonistic or in an antagonistic way depending on the experimental conditions (Clemmons, 1992). In addition, there is good evidence for an IGF-independent mode of action that can, for instance, induce apoptosis (Rajah *et al*, 1997; Hong *et al*, 2002). It is still unknown whether this capability of IGFBP is mediated by an as-yet unknown receptor or by a direct mechanism. The latter possibility is supported by the finding of an internalization of IGFBP3 via distinct endocytic pathways and of a nuclear localization of the protein (Lee *et al*, 2004).

In the skin, IGF-I appears to act as a mitogen; effects on differentiation processes have not yet been reported. In the absence of IGF-I protein, organ-cultured human hair follicles undergo a transformation that is reminiscent of the events at the beginning of the regression phase of the hair cycle that is characterized by the lack of cell proliferation (Tezuka *et al*, 1990; Philpott *et al*, 1994). Ectopic expression of *Igf-I* either in the epidermis or in the hair follicle causes epidermal hyperplasia and stimulation of vibrissa growth, respectively (Bol *et al*, 1997; Su *et al*, 1999). Whereas mice deficient for either *Igf-I* or *Igf-II* do not show any alterations in the epidermis and the hair follicle, double-deficient mice suffer from epidermal hypoplasia and a reduction of the number of hair follicles, which, in addition, are retarded in their development (DeChiara *et al*, 1990; Liu *et al*, 1993). A similar phenotype is seen in *Igf-Ir* knockout mice (Liu *et al*, 1993).

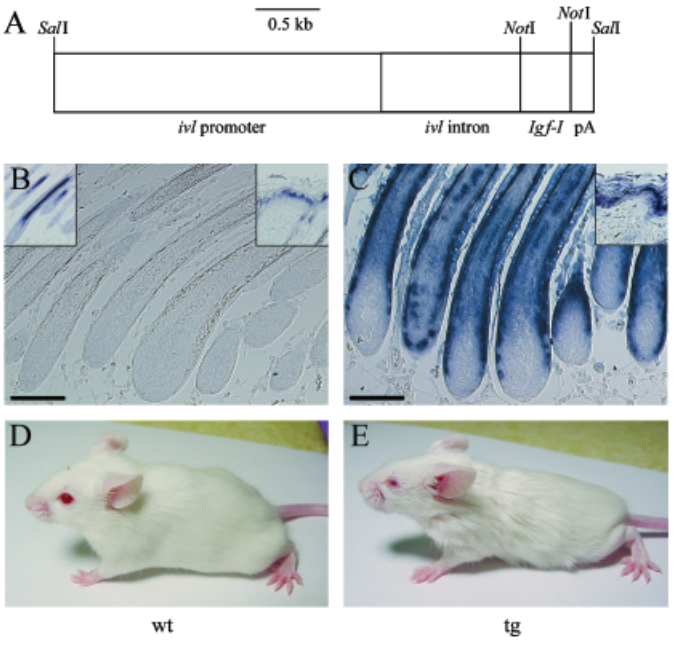

Figure 1
ivl::Igf-I (insulin-like growth factor-I) transgenic mice develop abnormal hair coats. (*A*) The transgenic construct for the generation of ivl::Igf-I transgenic mice is shown as a schematic. The involucrin (*ivl*) promoter and *ivl* intron belong to the *ivl* promoter fragment, which has been characterized previously (Carroll *et al*, 1993). pA represents the SV40 polyadenylation signal. *Not*I sites are used for cDNA cloning, whereas an *Sal*I restriction digest releases the transgenic DNA from the vector. (*B*, *C*) Non-radioactive *in situ* hybridizations demonstrate that transgenic mouse lines show strong ectopic expression of *Igf-I* in anagen hair follicles. The insets in (*B*) visualize the expression pattern of the endogenous *ivl* gene that is transcriptionally active in the granular layer of the epidermis and the medulla of the hair shaft. The *ivl* promoter fragment drives transgene expression in the granular layer of the epidermis (inset in (*C*)) and the medulla as well as the inner root sheath of the hair follicle. Expression from the endogenous and the transgenic *ivl* promoter is much stronger in the hair follicle than in the epidermis. (*D*, *E*) At the age of 4 wk, the hair coat of transgenic mice is characterized by a less smooth appearance as compared with wild-type littermates. *Scale bars* = 100 μm.

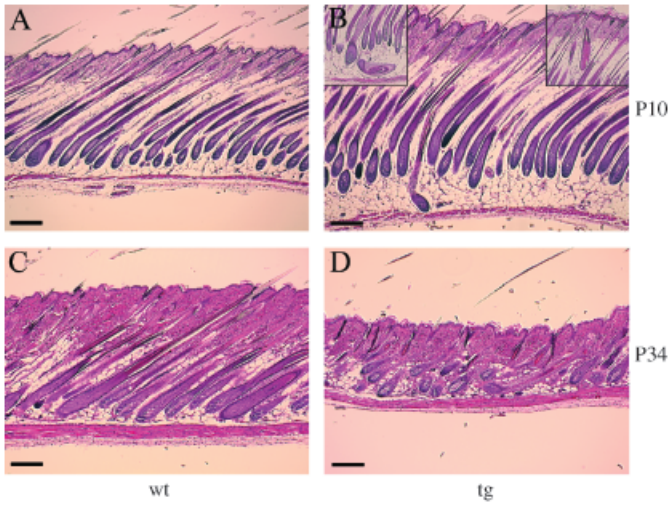

Figure 2
Abnormal morphology of ivl::Igf-I (insulin-like growth factor-I) transgenic skin. The histology of transgenic skin was analyzed by hematoxylin and eosin staining of murine skin sections. (*A*, *B*) During the first growth phase, transgenic skin is remarkably thicker than wild-type tissue. Although all transgenic follicles appear to be slightly longer, a few hair follicles are extraordinarily elongated. The distance between the majority of follicles and the muscle layer is significantly enlarged and the orientation of some follicles is completely disturbed in transgenic skin (*insets* in B). (*C*, *D*) In the second growth phase, transgenic skin is thinner than its wild-type counterpart. Follicles of transgenic mice appear to be less developed than in wild-type skin, and their disorientation is enhanced. Furthermore, ectopic *Igf-I* expression significantly increases the diversity of hair follicle lengths in the second growth phase as compared with the first anagen. *Scale bars* = 200 μm.

In this study, we demonstrate that transgenic expression of *Igf-I* in murine skin using an involucrin (*ivl*) promoter fragment has pleiotropic effects. Some follicles are extraordinarily elongated and guard hair shafts are longer than their wild-type counterparts. During the resting phase of the hair cycle the fat of the subcutis is temporarily eliminated. Furthermore, re-initiation of hair growth during the hair cycle is significantly retarded. The most obvious phenotype of *Igf-I* transgenic mice is a perturbed hair coat. Our data reveal that this effect is due to the absence of zigzag hairs that have adopted an alternative structure. Finally, we demon-

strate an aberration of hair shaft differentiation and identify *Pdgfrα* as a molecular target of transgene expression. Thus, we present evidence for a mitogenic and morphogenetic function of *Igf-I* in the pelage hair follicle.

Results

Ectopic expression of *Igf-I* in the hair follicle causes alterations of the hair coat IGF-I is synthesized and secreted by dermal cells of the skin and by dermal papillae of hair follicles. To examine the role of *Igf-I* in formation of pelage hairs comprehensively, we sought to overexpress it ectopically during the growth phase of the hair cycle. To achieve transgene expression in the hair follicle, the *ivl* promoter that is active in the inner root sheath and the medulla was used (Fig 1*A*) (Carroll *et al*, 1993). Three independent transgenic lines with significant ectopic *Igf-I* expression were investigated in more detail (Fig 1*B* and *C*). Transgene expression is maximal in the proximal inner root sheath and substantial in the proximal medulla of the hair shaft (Fig 1*C*). Low-level expression is detectable in the granular layer of the epidermis (Fig 1*C*, *inset*).

Development of mice of all transgenic lines was normal during embryogenesis and postnatally. They reached adulthood without any alterations in behavior and healthiness. At birth, their skin appeared to be slightly thicker and wrinkled as compared with wild-type littermates (data not shown). After the hair had emerged through the epidermis, discrimination of transgenic and wild-type mice became even easier. Whereas wild-type animals developed a very smooth hair coat, the fur of *Igf-I* transgenic mice appeared more disordered (Fig 1*D* and *E*). This alteration in hair coat appearance became more obvious during the first month of life, thereby facilitating the distinction of mice with different genotypes.

Cutaneous changes in ivl::Igf-I transgenic mice HK1::Igf-I transgenic mice developed epidermal hyperplasia due to strong ectopic gene expression in the epidermis (Bol *et al*, 1997). The involucrin promoter fragment also gives rise to some gene transcription in the epidermis, although to a much lesser extent than in the hair follicle. Thus, we investigated the morphology of the epidermis in our transgenic lines and could detect only a very slight increase in thickness (Fig 2*A* and *B*); we did not find any abnormalities of the epidermal differentiation program (data not shown). On the other hand, the overall thickness of the skin was significantly greater than in wild-type mice of the same sex and seemed to correlate with an increase in the intracutaneous length of hair follicles. A similar effect was described for HK1::Igf-I transgenic mice (Bol *et al*, 1997). Whereas most follicles are only slightly elongated in ivl::Igf-I transgenic mice, a few follicles are considerably lengthened. As a consequence, the distance between the proximal end of most hair follicles and the muscle layer of the *Panniculus carnosum* is clearly enlarged as compared with wild-type skin (Fig 2*A* and *B*). The number of hair follicles is not significantly affected by the expression of the *Igf-I* transgene; the density of follicle infundibuli and the number of follicles per section are comparable in wild-type and transgenic skin (Fig 2*A* and *B*). Furthermore, counting of the number of follicles per square millimeter also did not reveal any substantial difference among wild-type and transgenic mice (wt *vs* tg: 105 ± 5 *vs* 107 ± 4).

In wild-type skin, all hair follicles show a parallel orientation. This ordered arrangement is largely preserved in ivl::Igf-I transgenic mice. Nevertheless, some hair follicles are clearly disoriented. Whereas some kinks of the severely elongated follicles may be due to artificial mechanical stress during the preparation of the skin and sections, other follicles definitely show an altered angle of down-growth or have grown in parallel to the muscle layer over long distances (Fig 2*B*, *insets*).

After initial morphogenesis, the hair follicle traverses through cycles of growth (anagen), regression (catagen), and resting (telogen). Interestingly, the histological abnormalities in transgenic skin change and increase in severity in the second growth phase (Fig 2*C* and *D*). The disorientation of hair follicles becomes more obvious, since many follicles are affected. Furthermore, transgenic hair follicles are now significantly shortened as compared with their wild-type counterparts. This difference is probably responsible for the pronounced thinning of transgenic skin. The marked decrease in skin thickness at P34 is not due to a delay of the second growth phase, since, even at earlier or later time points during this period, we did not detect thicker skin (data not shown).

Ectopic *Igf-I* prolongs the resting phase of the hair cycle To elucidate how the thickened skin of ivl::Igf-I transgenic mice during the first growth phase is converted into the thin structure of the second anagen, we investigated the histological alterations in the skin during the first regression and resting phase of the hair cycle as well as the re-formation of the hair-producing follicle at the beginning of the second anagen. Concomitantly, we analyzed transgenic *Igf-I* expression by non-radioactive *in situ* hybridization. Clearly, the time point of catagen induction is indistinguishable in wild-type and transgenic mice (Fig 3*A* and *B*). At the onset of the regression phase, the transgene is still strongly expressed (Fig 3*C*). The progression of hair follicle regression is also not significantly affected in transgenic skin (Fig 3*D* and *E*). During the phase of club hair formation, ectopic *Igf-I* expression is still detectable in the inner root sheath (Fig 3*F*). Strikingly, catagen-associated reduction of the fat layer in wild-type skin is enhanced in transgenic mice. Finally, the adipose tissue is virtually absent from transgenic skin in early telogen (Fig 3*G* and *H*). During the resting phase, transgene expression is absent from the hair follicle (Fig 3*I*). In late telogen, the cutaneous fat is restored (data not shown).

In addition to the excessive loss of fat during the regression phase, the timing of the initiation of the second growth phase is severely altered in ivl::Igf-I mice. In wild-type animals, the second anagen starts with the outgrowth of new follicle buds about 3.5 wk after birth. At the age of 4 wk, the follicles acquire their final length and produce hairs for about the next 10 d (Fig 3*J* and *M*). In contrast to the wild-type situation, transgenic skin is still in the resting phase 4 wk postnatally (Fig 3*K*). Accordingly, hair follicles lack any transgene expression; nevertheless, a weak expression is evident in the epidermis

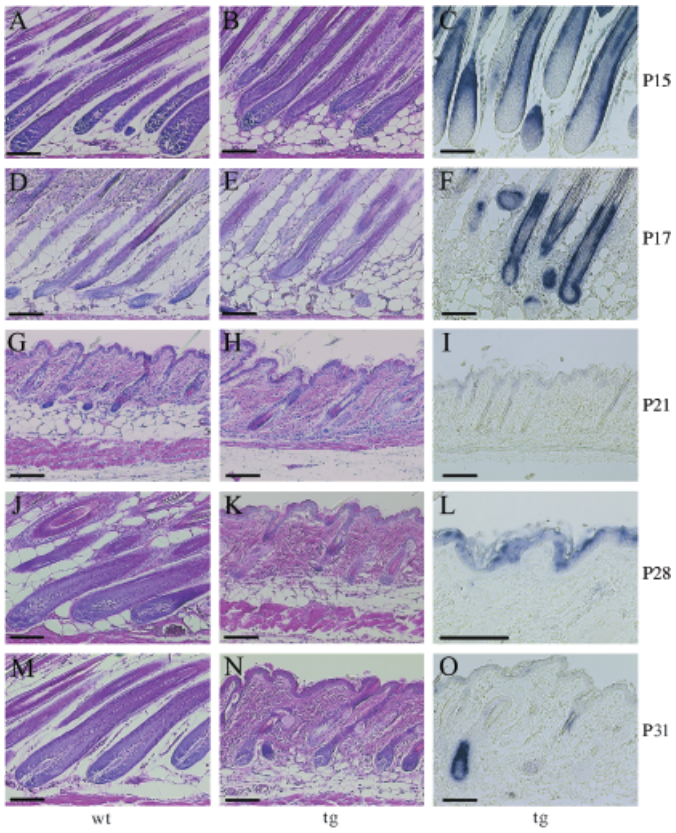

Figure 3
Ectopic expression of insulin-like growth factor-I (Igf-I) disturbs the resting phase of the hair growth cycle. The progression through the hair growth cycle in transgenic mice was analyzed by hematoxylin and eosin staining of murine skin sections of different ages, representing distinct stages of the cycle (*A*, *B*, *D*, *E*, *G*, *H*, *J*, *K*, *M*, *N*). Concomitantly, transgene expression was examined using non-radioactive *in situ* hybridization (*C*, *F*, *I*, *L*, *O*). (*A*, *B*) At P15, the proximal part of hair follicles has become thinner, indicating the onset of catagen. No differences between wild-type and transgenic skin are visible. (*C*) Transgenic *Igf-I* is expressed in inner root sheath (IRS) and medulla at the onset of catagen. (*D*, *E*) Progression through the regression phase is also unaffected by transgene expression. (*F*) During the formation of the club hair, transgenic *Igf-I* is still expressed in IRS. (*G*, *H*) At P21, wild-type and transgenic skin have entered the resting phase, but transgenic skin lacks any detectable fat layer. (*I*) During telogen, hair follicles lack any ectopic *Igf-I* expression. (*J*, *K*) At P28, a new anagen has been initiated in wild-type skin. In contrast to this, transgenic skin is still in telogen but the fat layer has been restored. (*L*) Extended staining demonstrates transgene expression in the epidermis in telogen. (*M*, *N*) In transgenic skin, the first signs of the outgrowth of new hair follicles are seen at P31. (*O*) Concomitantly, transgene expression in the hair follicle is restored. *Scale bars* = 100 μm.

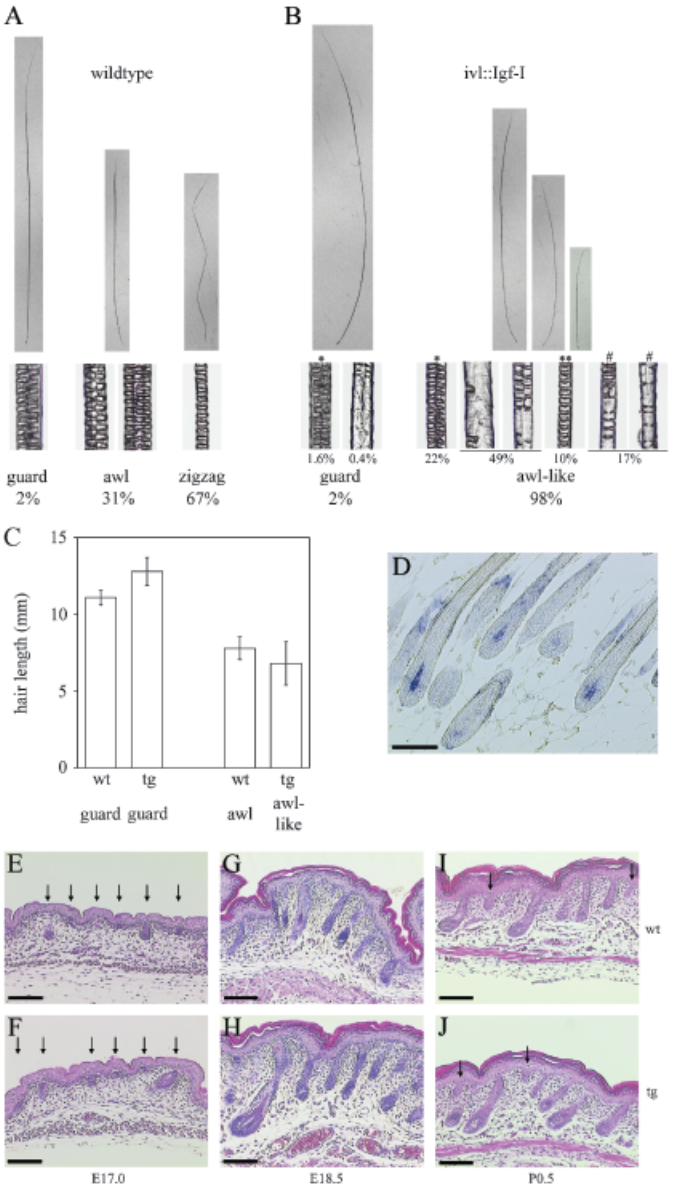

Figure 4
Hair alterations in ivl::Igf-I (insulin-like growth factor-I) transgenic mice. (*A*, *B*) Ectopic expression of *Igf-I* in the anagen hair follicle affects the structure of hair shafts. The overall length and shape of hairs is shown at the top, whereas the light microscopic appearance of the hair shaft structure is shown at the bottom. Most wild-type hairs reveal a regular pattern of air spaces, which is not visible in the majority of transgenic hair shafts. While in wild-type mice only awl hairs differ in their internal structure, i.e., they possess two or more columns of air spaces, the appearance of transgenic guard and awl-like hair shafts is highly heterogeneous. Moreover, transgenic mice completely lack zigzag hairs. The frequencies of each hair type and subpopulations thereof are indicated ($n \geqslant 46$). Asterisks indicate hair shafts with a normal light microscopic structure. # marks thin hair shafts with an abnormal structure. (*C*) Transgenic *Igf-I* expression has divergent effects on the length of hair shafts. While guard hairs are elongated ($p < 0.0001$), awl-like hairs are slightly shorter ($p = 0.009$) and more diverse in length than wild-type awl hairs. The mean hair length and the standard deviation are indicated ($n \geqslant 14$). (*D*) Non-radioactive *in situ* hybridization demonstrates that *Igfbp5* is expressed in the dermal papilla of some ivl::Igf-I transgenic hair follicles. (*E*–*J*) The time response of hair follicle induction is indistinguishable in wild-type and transgenic mice. Hair follicle morphogenesis was analyzed by hematoxylin and eosin staining of skin sections representing the period around zigzag hair follicle formation. At E17.0, guard and awl hair follicles are already induced, whereas zigzag follicles are still missing. The number of follicular buds, marked by arrows, is indistinguishable in wild-type and

Figure 4
(*caption continued*)
transgenic mice (E, F). At E18.5, guard and awl hair follicles are significantly elongated, whereas first zigzag follicles start to develop. Again, there is no difference between wild-type and transgenic skin (G, H). In newborn skin, zigzag follicle induction still continues. Recently induced follicles are clearly visible in wild-type and transgenic skin (arrows in (I) and (J)). Likewise, the follicle number and distribution of developmental stages is comparable in wild-type and transgenic skin (I, J). Scale bar = 100 μm.

during telogen as it is throughout all phases of the hair growth cycle (Fig 3*L* and data not shown). At postnatal day 28, the restored fat layer is clearly visible. The initiation of a new growth phase starts about 31 d after birth and mature follicles are visible from day 34 postnatally on (Fig2*D* and 3*N*); concomitantly, ectopic *Igf-I* expression in the hair follicle is restored (Fig 3*O*). Thus, transgenic hair follicles show a remarkable delay in the re-initiation of new anagen.

ivl::Igf-I transgenic mice show altered hair shafts Although some transgenic hair follicles are severely disoriented, their number is probably too small to explain the obvious disorder of the hair coat. The distortion of the hair cycle may contribute to the more pronounced effect in old mice. To elucidate the reason for the altered appearance of the fur of young transgenic mice, we next analyzed the composition of their pelage. In wild-type mice, it consists of four different hair types (guard, awl, auchene, zigzag), which can be distinguished according to their length, the number of medulla columns, and the presence and number of bends. Since IGF-I acts as a mitogen and an elongation of whiskers has been reported for *Igf-I* transgenic mice, we first sought to compare the length of wild-type and transgenic hair shafts (Su *et al*, 1999). Strikingly, we found only two hair types in *Igf-I* transgenic mice (Fig 4*B*). As in wild-type mouse, about 2% of all hairs are significantly longer than the rest. Interestingly, the average length of these transgenic hairs is increased as compared with wild-type guard hairs (Fig 4*A–C*). We noted that many transgenic guard hairs are longer than the longest guard hairs in wild-type mice. All other transgenic hairs are also straight; their average hair shaft length is, however, slightly reduced as compared with wild-type awl hairs and the variation in lengths is significantly enhanced (Fig 4*B* and *C*). Strikingly, no typical zigzag hairs could be found. Auchene hairs also seem to be absent from ivl::Igf-I transgenic mice, but due to their low frequency of about 0.1% it is difficult to arrive at a definite conclusion.

A single column of air spaces is characteristic for zigzag hairs, whereas all other hair types contain two or more columns (Fig 4*A*). Interestingly, about 10% of transgenic hair shafts definitely contain a single column of air spaces (Fig 4*B*, *indicated by two asterisks*). Surprisingly, however, they do not possess any bends. Thus, these hairs are of the zigzag type based on the number of medulla columns but have the straight structure of awl hairs. According to their thickness, about 17% of transgenic hairs also belong to the class of straight zigzag hairs but lack a regular pattern of air spaces (Fig 4*B*, *hair shafts marked with #*). Therefore, the frequency of thin hair shafts (10% + 17%) is much lower than that of zigzag hairs in wild-type mice (67%).

In an independent study, we have demonstrated that expression of *Igfbp5* distinguishes among different hair follicle types (Schlake, 2005). Significant gene expression solely occurs in zigzag hair follicles. Thus, we analyzed the expression of *Igfbp5* in ivl::Igf-I transgenic skin to obtain molecular evidence for the presence of zigzag hair follicles. Interestingly, many transgenic hair follicles show significant *Igfbp5* expression (Fig 4*D*) (Schlake, 2005), supporting the presence of true zigzag follicles that have apparently lost their ability to produce normal zigzag hairs. To further corroborate a normal induction of zigzag hair follicles instead of formation of supernumerous awl hair follicles, we analyzed the temporal pattern of hair follicle morphogenesis. Whereas awl hair follicle buds are already formed at E17, zigzag hair follicles are induced around birth. Throughout this period, wild-type and transgenic skin do not differ from each other either by the number or by the distribution of developmental stages of hair follicles (Fig 4*E–J*), indicating the presence of normal waves of hair follicle induction. Thus, zigzag follicles of ivl::Igf-I mice give rise to transgenic hairs, some of which only have lost their bends (10% of transgenic hairs), whereas others have acquired a straight structure plus alterations of their light microscopic appearance (17% of transgenic hairs). Finally, the remainder of the zigzag hairs have been replaced by straight hairs with more than one column of medulla cells (about 50% of transgenic hairs), since the overall number of hair follicles is normal in ivl::Igf-I transgenic mice (see above).

To determine whether the slight elongation of guard hairs and/or the increase of hair shaft thickness are due to the mitogenic action of IGF-I, we addressed keratinocyte proliferation in the hair matrix by immunohistochemical staining for the proliferation marker Ki67. As already seen in our histological analyses, transgenic *Igf-I* expression did not alter the size and shape of the hair bulb as compared with wild-type littermates (Fig 2*A–D*). Likewise, we could not detect any significant alteration in the size of the proliferative compartment (Fig 5*A* and *B*). The number of Ki67-positive cells per section does not substantially differ among wild-type and transgenic mice (65 ± 5 *vs* 69 ± 7; $p = 0.1$), which is probably due to the overall mild effects in transgenic follicles.

We further addressed IGF-I-mediated signalling by immunohistochemical staining for phophorylated IGF receptor. Unfortunately, protein levels were too low to obtain any specific staining on skin sections. Since IGF-I, like other growth factors that are expressed in the hair follicle, mainly signals via the RAS/MAPK and PI3K/AKT pathways, we also analyzed signalling activity by immunohistochemical detection of phosphorylated ERK1/2 and AKT, respectively. For both proteins, we could not detect any differences in phosphorylation levels (data not shown), which stresses the fact that transgene expression in ivl::Igf-I mice leads to only moderate effects as already suggested by the overall phenotype. Thus, these analyses did not offer any hint as to the follicular cells that are directly affected by transgene expression.

In the light microscope, wild-type hairs reveal a regular pattern of air spaces within the medulla of the hair shaft (Fig 4*A*). Although about 30% of transgenic guard and awl-like hairs show such a ladder-like internal structure (Fig 4*B*, *hair shafts marked with asterisks*), about 70% of transgenic hair shafts appear completely different. Their thickness appears to be normal but no air spaces are visible in large parts of the hair shaft (Fig 4*B*, *hair shafts not marked with asterisks*). Nevertheless, sections clearly show that transgenic hair shafts still contain a medulla (data not shown). Thus, the abnormal appearance is most likely due to an aberrant differentiation program in either the cortex or the cuticle. Since

FOXO transcription factors appear to be involved in mediating IGF signals (Brunet *et al*, 1999; Nakae *et al*, 2000), we examined their expression in the hair follicle. Interestingly, only *Foxo3* is expressed in the follicle and gene transcription is restricted to the cortex (data not shown), suggesting that disturbances in this compartment might be responsible for the altered appearance of hair shafts. Consequently, to identify transgene-mediated molecular defects in the hair follicle, we mainly concentrated on the cortex and, to a lesser extent, on the cuticle. We also turned our attention to the hair matrix, where *Igf-Ir* is expressed (Hodak *et al*, 1996). For each epithelial compartment of the follicle, the expression of several differentiation markers was analyzed by *in situ* hybridization (Fig 5*C–J* and *data not shown*). For none of them a significant difference in expression levels was observed between wild-type and transgenic mice. Since *Igf-Ir* is also expressed by dermal papilla cells, we tested the activity of some papilla-specific genes. Among them, transcription of *Pdgfr*α appears to be slightly decreased in transgenic skin (Fig 5*K* and *L*), representing the first molecular aberration that we could identify in ivl::Igf-I hair follicles.

Ectopic expression of *Igfbp3* in the hair follicle partially restores the hair defect in ivl::Igf-I transgenic mice To test whether the observed effects are specific to the action of ectopic *Igf-I* as opposed to a general toxic effect of the transgene and, thus, whether they may be concentration dependent, we generated ivl::Igfbp3 transgenic mice to compensate transgenic *Igf-I* expression. Previous reports have demonstrated that IGFBP3 can act as an antagonist of IGF-I (Clemmons, 1992). We established four independent *Igfbp3* transgenic mouse lines, all of which show strong transgene expression (data not shown). In transgenic mice, the hair coat has a rough appearance and the length of hair shafts is significantly decreased due to a smaller proliferative compartment of the follicle as compared with wild-type littermates (Weger and Schlake, 2005).

Detailed analyses of double transgenic mice demonstrated that ectopic expression of *Igfbp3* partially counteracted transgenic *Igf-I*. We could not detect a reduction of the fat layer during telogen (Fig 6*A* and *B*) and the histology of skin representing the second growth phase appeared quite normal in double transgenic mice (Fig 6*C* and *D*). Furthermore, the average and maximal length of guard hair shafts was comparable in wild-type and double transgenic mice (Fig 6*E*). Whereas in ivl::Igf-I transgenic mice six of 28 guard hairs lacked a visible pattern of air cells in the hair shaft, all of 21 double transgenic guard hairs analyzed revealed a normal hair shaft structure. The appearance of awl-like hairs was still heterogeneous. Most interestingly, a partial restoration of the zigzag phenotype could be observed. About 20% of all hairs now reveal a medulla with a single column of air spaces and possess three to four sharp bends (Fig 6*F*).

Discussion

In this study, we performed a comprehensive analysis of IGF-I's role in the skin and demonstrated that it has pleiotropic effects covering cell proliferation, tissue remodelling,

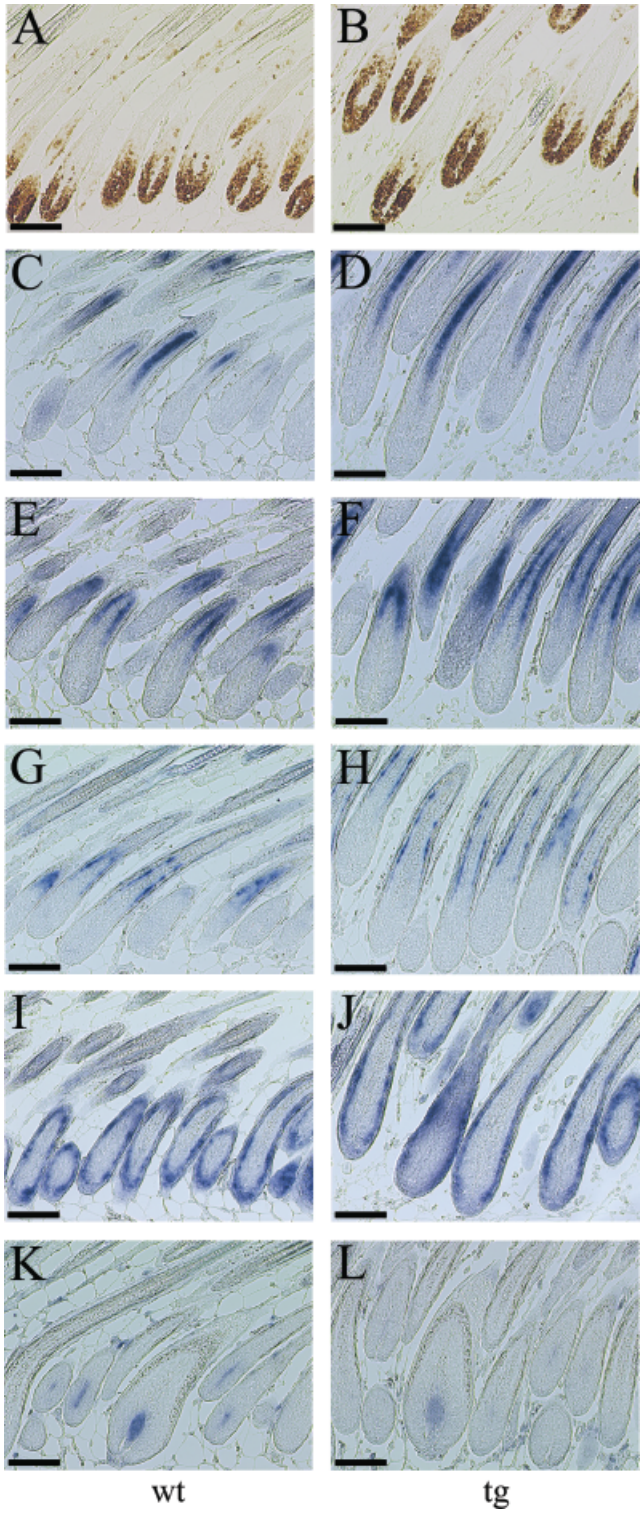

Figure 5
Proliferation and gene expression in ivl::Igf-I (insulin-like growth factor-I) transgenic hair follicles. (*A*, *B*) Immunohistochemical staining for the proliferation marker Ki67 indicates that the size of the proliferative compartment of hair follicles is almost unchanged in ivl::Igf-I transgenic mice. (*C–L*) Gene expression in distinct domains of the hair follicle was analyzed by non-radioactive *in situ* hybridization at the age of 10 d. For none of the epithelial compartments, a significant difference in gene expression could be detected. Results for involucrin (*C*, *D*), *Foxn1* (*E*, *F*), *Ha2* (*G*, *H*), and *Gata3* (*I*, *J*) are shown as representatives. In contrast to this, *Pdgfr*α expression appears to be slightly downregulated in the dermal papilla of transgenic mice (*K*, *L*). *Scale bar* = 100 μm.

and hair cycle control, as well as differentiation within the hair-producing follicle.

Effects of IGF-I signalling on pelage hair follicles In the skin, *Igf-I* is expressed by cells of the dermis and the dermal papilla of hair follicles (Tavakkol *et al*, 1992; Little *et al*, 1996). Previous reports have suggested a mitogenic role in the epidermis and whisker follicles (Bol *et al*, 1997; Su *et al*, 1999). Furthermore, IGF-I appeared to be essential to maintain *in vitro* cultured human hair follicles in the active, hair-producing state (Philpott *et al*, 1994). IGF-I-mediated effects on differentiation processes in the hair follicle have not yet been reported. To address *Igf-I's* role in pelage hair follicles, we have generated transgenic mice that ectopically express *Igf-I* in the mature hair follicle.

Our data suggest that the mitogenic response of different hair types to IGF-I is not uniform. Whereas transgenic guard hairs are slightly elongated, the average length of awl-like hairs is decreased as compared with wild-type awl hairs. In a significant fraction of follicles, *Igf-I* appears to have mitogenic effects that are restricted to precursors of the medulla. In wild-type mice, about 65%–70% of all hairs belong to the zigzag type, which contains a single column of medulla cells. The remaining hairs are thicker and have two or more columns. Whereas the number of hair follicles is unaffected in transgenic skin, the frequency of follicles that produce thin hairs is reduced more than two-fold. Thus, more than 50% of zigzag hairs have been replaced by a thicker hair type. We cannot fully exclude an aberration of hair follicle induction that alters the relative abundance of distinct follicle types, although the temporal pattern of hair follicle morphogenesis appears to be normal. But the frequency of *Igfbp5*-expressing follicles, which represent zigzag hair follicles (Schlake, 2005), is greater than 50%, indicating that at least part of the thicker hairs is of zigzag origin. Most likely, their increased thickness is a consequence of enhanced proliferation of medulla precursors.

Interestingly, ectopic expression of *Igf-I* in the mature hair follicle has striking effects on the hair growth cycle. Whereas the onset and progression of catagen are unaffected, reduction of the fat layer is enhanced. Actually, the fat cells virtually disappear in mid telogen. Afterward, the fat of the subcutis is surprisingly restored and a new growth phase is initiated. Based on the fact that transgene expression is absent from late catagen and telogen hair follicles, the temporary disappearance of adipose tissue in telogen skin is unexpected. During this phase of the hair cycle, a weak ectopic expression of *Igf-I* is only evident in the epidermis. Since adipose tissue is restored during telogen, the enhanced reduction of the fat layer is most likely due to residual IGF-I that has been produced during anagen and/or early catagen. Alternatively, enhanced levels of IGF-I during early catagen might initiate some unknown processes, whose consequences are not observed before telogen. Clearly, all data argue against a systemic effect on the status of the fat layer due to transgene expression in other tissues than the skin.

The onset of a new anagen is significantly delayed as compared with wild-type skin, which is probably caused by a need for fat restoration before hair production can be reinitiated. A possible link between the presence of subcutaneous fat and hair production is suggested by APOC1 transgenic mice that show loss of hair in combination with a complete deficiency of adipose tissue in the skin (Jong *et al*, 1998). The size of the subcutaneous adipose layer might be an indicator for the nutritional state, which appears to affect hair growth in mice and rats (Ibrahim and Wright, 1975; Mathur and Doe, 1976). Whether nutrition can also influence the hair growth cycle is unknown. Clearly, the mechanism of modulating hair growth cycling differs among ivl::Igf-I transgenic mice and other animal models such as *Fgf5* and *msx2* mutant mice (Hebert *et al*, 1994; Satokata *et al*, 2000). It is also different from the estrogen-mediated arrest in telogen, which does not affect the adipose tissue (Oh and Smart, 1996). Thus, the molecular basis of an IGF-I-mediated reduction of the fat layer and of the concomitant extension of telogen requires further study. Of note, the observed effect of transgenic IGF-I on the hair growth cycle may point to an unexpected role of IGFBP3 in the hair follicle. Strong upregulation of *Igfbp3* expression in dermal papillae during catagen and early telogen may be necessary to prevent excessive tissue remodelling, thereby guaranteeing a proper execution of the hair growth cycle (Schlake *et al*, 2004).

Control of hair shaft differentiation Murine hairs are characterized by a ladder-like internal structure that is due to a regular pattern of air spaces. Close inspection of ivl::Igf-I transgenic hair shafts revealed that this is missing in large parts of most hairs. Obviously, the altered structure has no effect on hair shaft thickness. In addition, the majority of hairs contain at least very small areas where air spaces are visible. The presence of medulla cell columns within the hair shaft is also supported by histological sections. A concentration-dependent effect of IGF-I on hair shaft differentiation is indicated by the partial reversal of the abnormal structure by the simultaneous presence of IGFBP3.

Analysis of signalling pathways downstream of IGF-I did not offer any clue as to which cells are directly affected by *Igf-I* transgene expression. Likewise, gene expression analyses for the epithelial compartments of the hair follicle did not reveal any difference between wild-type and transgenic mice. Thus, the molecular basis for the altered differentiation of the hair shaft is still elusive. Since, based on the expression pattern of *Igf-Ir*, the dermal papilla is a potential target of IGF signalling, we also addressed gene activity in this follicular compartment. Interestingly, *Pdgfr*α expression appears to be slightly decreased in transgenic follicles. Of note, an IGF-mediated downregulation of *Pdgfr*α expression was previously demonstrated for chick limb bud mesoderm (Carrington and Eierle, 1995). Nevertheless, it is still unclear whether and how the observed effect in the dermal papilla translates into one or more of the phenotypic alterations in our transgenic mice.

The murine fur consists of four different hair types. Our data on ivl::Igf-I transgenic mice strongly suggest that *Igf-I* might control the formation of specialized hair shaft structures, i.e., bends, that are characteristic for zigzag and auchene hairs. Thus, IGF-I might be among the elusive factors that determine the identity of hair follicles and hair shafts. As mentioned above, the ectopic expression of *Igf-I* increases the thickness of hair shafts. Is the lack of bends an indirect

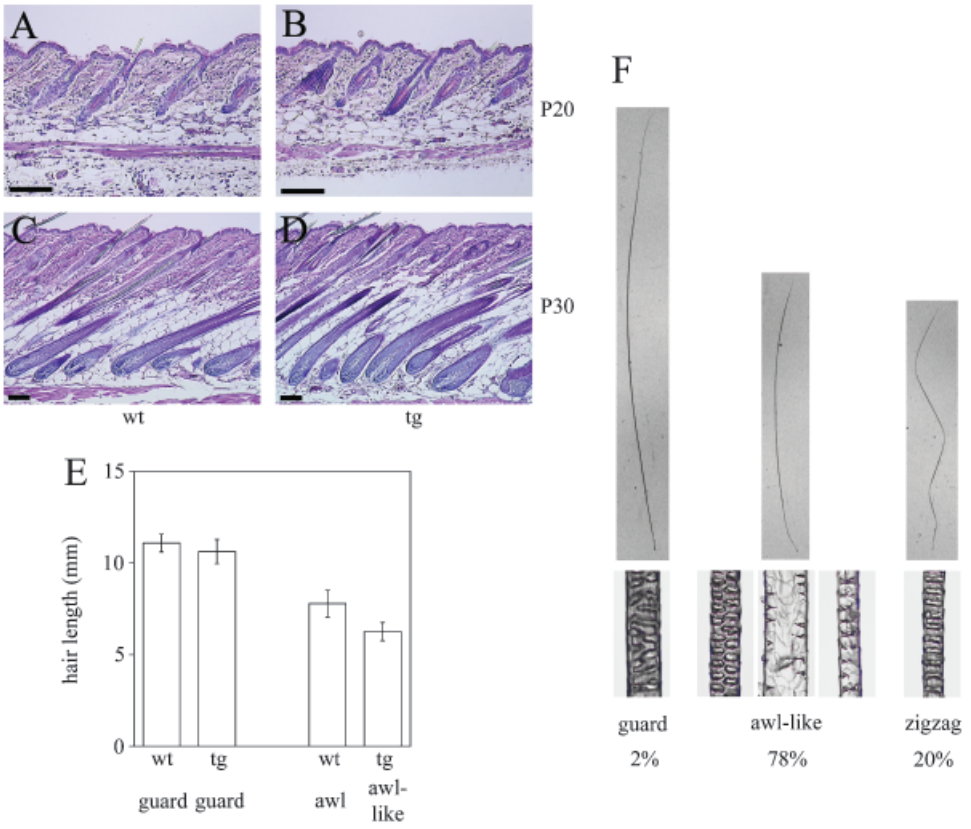

Figure 6
Ectopic expression of Igfbp3 partially compensates the effects in ivl::Igf-I (insulin-like growth factor-I) transgenic mice. (*A*, *B*) Double transgenic and wild-type skin are indistinguishable in telogen. The severe reduction of the fat layer in *Igf-I* transgenic skin does not occur in the presence of ectopic insulin-like growth factor binding protein 3 (*Igfbp3*) expression. (*C*, *D*) The development of mature, hair-producing follicles in the second growth phase is normal in double transgenic mice. (*E*) Double transgenic guard hairs might be slightly shorter than wild-type hair shafts ($p = 0.03$). Awl-like hairs are significantly shortened as compared with wild-type awl hairs ($p < 0.0001$), but the variation in length is reduced to wild-type levels. The mean hair length and the standard deviation are indicated ($n \geqslant 12$). (*F*) Transgenic *Igfbp3* partially restores the growth of zigzag hairs. The shape and relative lengths of hair shafts (top) as well as a detailed view of the hair shaft structures (bottom) are shown. The structure of awl-like hair shafts is still highly diverse, whereas the light microscopic appearance of guard and zigzag hairs appears to be homogeneous. The frequencies of each hair type are indicated ($n = 230$). *Scale bars* = 100 μm.

consequence of thickened hair shafts that may make bending impossible? Two lines of evidence argue against this assumption. Firstly, auchene hairs, which are similar to awl hairs, contain a single bend within their hair shaft. Thus, bending is still possible in the presence of two to three columns of medulla cells. Secondly, we have unequivocally identified straight hairs with a single column of air spaces in ivl::Igf-I transgenic mice. Usually, zigzag hairs are the only hair type with one medulla column. In wild-type mice, substantial expression of *Igfbp5* is restricted to zigzag hair follicles (Schlake, 2005). The finding of Igfbp5-positive papillae and medullae in ivl::Igf-I transgenic mice strongly supports the zigzag origin of thin hairs in transgenic mice.

Does the suppression of bent hair shaft structures occur in the mature follicle or does it already occur during morphogenesis of zigzag hair follicles? The involucrin promoter is predominantly active in the inner root sheath and the medulla of hair follicles. In addition, it also drives weak gene expression in differentiating layers of the epidermis (Carroll *et al*, 1993). Since epidermal differentiation and induction of guard hair follicles precede the initiation of zigzag follicles, it is possible that prospective zigzag follicles adopt an alternative fate. Nevertheless, the presence of differently affected, presumptive zigzag hairs in ivl::Igf-I transgenic mice may suggest that IGF-I acts in the mature follicle. In mature skin, vascularization and the presence of different follicle types may give rise to distinct microenvironments that slightly differ in the available amount of IGF-I, leading to different abnormalities of hair shafts in ivl::Igf-I transgenic mice. A concentration-dependent effect of IGF-I on hair shaft phenotypes is also indicated by the partial restoration of zigzag hair-producing follicles in ivl::Igf-I, ivl::Igfbp3 double transgenic mice. The unaffected time response of hair follicle induction further supports our view that transgenic IGF-I mainly acts in and on the mature hair follicle.

Materials and Methods

Generation of transgenic mice The coding regions of *Igf-I* and *Igfbp3* were amplified by PCR of P10 cDNA from the skin using the following primers, which contain *Not*I sites for subcloning: 5′-ATGTGCGGCCGCCATGTCGTCTTCACACCTC-3′ and 5′-ATGTGCGGCCGCCCTACATTCTGTAGGTCTTG-3′ for *Igf-I*; and 5′-ATGTGCGGCCGCCATGCATCCCGCGCGCCCCG-3′ and 5′-ATGTGCGGCCGCCCTACTGGCTCTGCACGCTGAG-3′ for *Igfbp3*. The cDNAs were cloned into the *Not*I site of an *ivl* expression vector that has been previously described (Fig 1*A*) (Carroll *et al*, 1993). The transgenes comprising the *ivl* promoter and intron, a cDNA, and a polyadenylation signal were released from the vector by digestion with *Sal*I, gel-purified, and microinjected into fertilized eggs from FVB mice. Founder mice were bred to Balb/c mice. All experiments comprising the generation and analysis of transgenic mice were approved by the MPI of Immunobiology.

Histology, immunohistochemistry Back skin from mice of various postnatal ages was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 6 μm for hematoxylin and eosin staining or immunohistochemistry. For immunohistochemical analyses, skin sections were dewaxed, microwaved in 10 mM sodium citrate, and incubated with the primary antibody. To address cell proliferation in hair follicles, rat monoclonal anti-mouse Ki67 (DakoCytomation, Hamburg, Germany) was applied. Activation of signalling pathways was analyzed using anti-rabbit Phospho-IGF-IR, anti-rabbit Phospho-p44/42 MAP Kinase, and anti-rabbit

Phospho-Akt (New England Biolabs, Frankfurt, Germany). Detection was performed with biotinylated goat anti-rat Ig and biotinylated goat anti-rabbit Ig (Pharmingen, Heidelberg, Germany), respectively, and StreptABComplex/HRP (DakoCytomation).

***In situ* hybridization** Back skin from mice of various postnatal ages was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 6 μm. Essentially, non-radioactive *in situ* hybridizations were performed as described previously (Bleul and Boehm, 2000). Briefly, sections were dewaxed, rehydrated, and subjected to a proteinase K digest for 20 min. Hybridization with digoxigenin labelled probes was performed overnight. After washing, sections were incubated overnight with a digoxigenin-specific antibody that is linked to alkaline phosphatase (Roche, Mannheim, Germany). For the staining reaction, BM Purple AP substrate (Roche) was applied. Both sense and antisense strands of gene-specific fragments were used as probes. These fragments were generated by PCR using the following gene-specific primers (fragment size is indicated): *Igf-I*: nt. 152–170 and nt. 518–537 in X04480 (386 bp); *Igfbp5*: nt. 2021–2040 and nt. 3175–3194 in NM_010518 (1174 bp); involucrin: nt. 121–140 and nt. 1303–1322 in NM_008412 (1202 bp); *Foxn1*: nt. 1359–1378 and nt. 1916–1935 in X81593 (577 bp); *Ha2:* nt. 1261–1282 and nt. 1439–1460 in X75649 (200 bp). *Gata3*: nt. 1394–1413 and nt. 1886–1905 in X55123 (512 bp); and *Pdgfrα*: nt. 2978–2997 and nt. 3864–3883 in NM_011058 (906 bp).

Analysis of hair, hair length, and hair shaft structures Hair was plucked from 4-wk-old mice and single hair shafts were sorted using fine forceps to determine the composition of the pelage. For measurements of overall hair lengths and for the analysis of the internal structure of hair shafts, a Zeiss dissecting microscope and a Zeiss light microscope (Zeiss, Oberkochen, Germany) were used, respectively. Photographs were taken using a Sony and a Nikon digital camera (Köln and Düsseldorf, Germany, respectively), respectively. Statistical significance of measured differences was addressed using Student's *t* test.

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