FGF signals specifically regulate the structure of hair shaft medulla via IGF-binding protein 5

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Accepted 19 April 2005

Development 132, 2981-2990
Published by The Company of Biologists 2005
doi:10.1242/dev.01873

Summary

Reciprocal interactions between the dermal papilla and the hair matrix control proliferation and differentiation in the mature hair follicle. Analysis of expression suggests an important role for FGF7 and FGF10, as well as their cognate receptor FGFR2-IIIb, in these processes. Transgenic mice that express a soluble dominant-negative version of this receptor in differentiating hair keratinocytes were generated to interfere with endogenous FGF signalling. Transgenic mice develop abnormally thin but otherwise normal hairs, characterised by single columns of medulla cells in all hair types. All structural defects and the accompanying changes of global gene expression patterns are restricted to the hair medulla. Forced transgenic expression of IGF-binding protein 5, whose expression level is elevated upon suppression of FGFR2-IIIb-mediated signalling largely phenocopies the defect of *dnFgfr2-IIIb*-expressing hairs. Thus, the results identify *Igfbp5*-mediated FGFR2-IIIb signals as a key regulator of the genetic program that controls the structure of the hair shaft medulla.

Key words: *Fgfr2*, *Igfbp5*, Hair follicle, Hair medulla, Hair shaft structure, Differentiation, Mouse

Introduction

During murine embryogenesis and early postnatal development, consecutive waves of induction give rise to different follicle types. They generate hairs differing in length, thickness and structure, and are designated guard, awl, auchene and zigzag hairs. The exact molecular mechanisms of the formation of different hair follicle types are unknown, but several intercellular signalling pathways have been shown to be involved in hair follicle morphogenesis. Whereas the genetic inactivation of *Eda* or *Edar* impairs the first wave of follicle morphogenesis (Headon and Overbeek, 1999; Laurikkala et al., 2002), ectopic overexpression of *Eda* in the epithelial compartment of the skin induces supernumerous hair follicles (Mustonen et al., 2003; Zhang et al., 2003). In the absence of Noggin, which stimulates appendage formation by neutralising BMPs (Botchkarev et al., 1999), mice show a significant retardation of follicle induction and fail to initiate the second wave of follicle morphogenesis (Botchkarev et al., 2002). Interestingly, many embryonic signalling pathways are re-used in the mature hair follicle. For example, the suppression of BMP signalling by ectopic expression of Noggin induces severe alopecia owing to abnormal differentiation of hair shafts (Kulessa et al., 2000).

Various fibroblast growth factors and their receptors FGFR1-FGFR4 show complex expression patterns within the mature hair follicle and, thus, appear to play an important role in follicle biology (Nakatake et al., 2001; Rosenquist and Martin, 1996; Suzuki et al., 2000). Genetic inactivation of *Fgf5* generates mice with significantly elongated hairs due to an extended growth period during the hair cycle (Hebert et al., 1994). The cognate receptor, FGFR1, is exclusively expressed by the dermal papilla, which is thought to play a pivotal role in all aspects of hair follicle biology. *Fgf7* (*Kgf*) and *Fgf10* are the only members of the fibroblast growth factor family that are known to be expressed in the dermal papilla (Rosenquist and Martin, 1996; Suzuki et al., 2000). Ablation of *Fgf7* leads to a matted and unkempt appearance of older mice, whereas *Fgf10*-deficient mice show no obvious hair phenotype (Guo et al., 1996; Suzuki et al., 2000).

FGF7 and FGF10 efficiently and specifically bind to FGFR2-IIIb, one of several diverse protein variants with distinct binding characteristics encoded by the *Fgfr2* gene (Igarashi et al., 1998; Ornitz et al., 1996). To interfere with FGFR2-IIIb signalling, various approaches have been used. Ectopic expression of a membrane-bound or soluble dominant-negative form of FGFR2-IIIb early during embryonic development causes reduced numbers of hair follicles (Celli et al., 1998; Werner et al., 1994). Mice deficient for *Fgfr2-IIIb* also suffer from reduced numbers of hair follicles. In addition, hair morphogenesis is retarded and follicles of transplanted mutant skin give rise to a single hair type characterised by a very thin hair shaft lacking any regular arrangement of air cells (Petiot et al., 2003).

By ways of experimental design, previous reports mostly dealt with the function of FGFs in morphogenesis and did not address their role in the mature hair follicle. To explore the role of signals from the dermal papilla in the mature hair follicle and for hair growth, I sought to interfere with the action of FGF7 and FGF10 by expressing a soluble dominant-negative form of FGFR2-IIIb in the mature follicle. I demonstrate that the hair coat of *Foxn1::dnFgfr2-IIIb* transgenic mice has a

silky appearance and the number of columns of medulla cells is reduced in a dose-dependent manner. All structural defects and the accompanying changes in global expression patterns are restricted to the hair medulla. I show that transgenic expression of insulin-like growth factor binding protein 5 in the hair follicle largely phenocopies the effects seen in *Foxn1:dnFgfr2-IIIb* transgenic mice. Our studies implicate signalling via FGFR2-IIIb in the control of the structure of the hair medulla via suppression of *Igfbp5* expression.

Materials and methods

Generation of transgenic mice

The soluble dominant-negative form of FGFR2 has been previously described (Celli et al., 1998). The coding region was released by digestion with *Bam*HI and ligated to *Not*I adapters. The coding regions of *dnFgfr1-IIIc* and *Igfbp5* were amplified by PCR of P10 cDNA from skin using the following primers, which contain *Not*I sites for subcloning: 5′-ACTGCGGCCGCCATGTGGGGCTGGAAGT-GCCTC-3′ and 5′-ACTGCGGCCGCTCAGTAGAGCGGTGAGGT-CATCAC-3′ for *Fgfr1-IIIc*; and 5′-ACTGCGGCCGCCATGGT-GATCAGCGTGGTCCTC-3′ and 5′-ACTGCGGCCGCTCACT-CAACGTTACTGCTGTC-3′ for *Igfbp5*. The Fgfr2-IIIb and Fgfr1-IIIc cDNAs were cloned into an expression vector containing a 30 kb promoter fragment of the *Foxn1* gene (further information is given below). The Igfbp5 cDNA was cloned into an *Ivl* expression vector that has been previously described (Carroll et al., 1993). The transgenes were released by digestion with *Sal*I and microinjected into fertilised eggs from FVB mice. Founder mice were bred to BALB/c mice.

Details of the *Foxn1* promoter construct

The 30 kb *Foxn1* promoter fragment that has been used in the present study covers all upstream sequences between the gene encoding a Na^+-dependent dicarboxylate transporter and the first coding exon of *Foxn1*, which is exon 2 (Schorpp et al., 1997). Thus, it contains both alternative promoters that are used for proper gene expression in thymic epithelium and skin (Schorpp et al., 1997). Extensive expression analyses did not reveal any difference in the temporal and spatial expression profiles of the *Foxn1* promoter fragment and the endogenous gene (Fig. 1D and inset therein; C. C. Bleul and T. Boehm, personal communication). A proper temporal expression pattern of the *Foxn1* promoter, which is important for the interpretation of some results of the present study is further demonstrated by transgenic lines that express *Dkk1* under the control of the *Foxn1* promoter (T.S., unpublished). In a previous study it has been shown that ectopic expression of *Dkk1* under the control of the cytokeratin 14 promoter abolishes any hair follicle induction (Andl et al., 2002). In contrast to these mice, *Foxn1::Dkk1* transgenic mice do not develop follicles but guard hair follicles (data not shown), clearly indicating that the *Foxn1* promoter fragment is activated at later stages during epithelial differentiation, as it is true for the endogenous promoter. As a consequence, no interference with guard hair follicle induction does occur.

Details of promoter selection for expressing *Igfbp5*

Whereas *dnFgfr2-IIIb* is transgenically expressed in the hair cortex, it induces a dramatic increase in *Igfbp5* expression in the medulla. Although both proteins, dnFGFR2-IIIb and IGFBP5, are secreted and, thus, the location of their expression domains should be not that important, an ideal promoter to investigate the consequences of elevated levels of *Igfbp5* expression in *Foxn1::dnFgfr2-IIIb* transgenic mice would give rise to medium to strong expression in the hair shaft medulla. Unfortunately, no such promoter is available to date. Among characterised promoter fragments, the involucrin promoter is the best suited to express *Igfbp5* transgenically. Although it also gives rise to expression in the inner root sheath and differentiating layers of the epidermis, it is the only promoter fragment that drives expression in the medulla. Typically, the levels of transgene expression using the involucrin promoter are lower than that with the *Foxn1* promoter (see Fig. S1A,B in the supplementary material). Furthermore, the reduction in the number of medulla columns was also observed in a transgenic line with lower *Igfbp5* expression than in the other lines (see Fig. S1C,D in the supplementary material). Thus, a non-physiological effect in *Igfbp5* transgenic mice is unlikely. In addition, no promoter that will offer any advantage when compared with the involucrin promoter fragment is available.

Histology, immunohistochemistry, TUNEL assay and in situ hybridisation

Back skin representing the first (P10) or second (P31) growth phase of the hair cycle was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 6 μm for Hematoxylin and eosin staining, immunohistochemistry, TUNEL assay or in situ hybridisation. To address cell proliferation in hair follicles, skin sections were dewaxed, microwaved in 10 mM sodium citrate, and incubated with rat monoclonal anti-mouse Ki67 (DakoCytomation) as the primary antibody. Detection was performed with biotinylated goat anti-rat Ig (Pharmingen) and StreptABComplex/HRP (DakoCytomation). TUNEL assays were carried out using the In Situ Cell Death Detection, POD kit (Roche). Essentially, non-radioactive in situ hybridisations were performed as previously described (Bleul and Boehm, 2000). 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): arachidonate 8(S) lipoxygenase, nucleotides 2050-2069 and nucleotides 2573-2592 in Y14696 (543 bp); involucrin, nucleotides 121-140 and nucleotides 1303-1322 in NM_008412 (1202 bp); *K6hf*, nucleotides 1715-1734 and nucleotides 2424-2443 in NM_133357 (729 bp); *Sh3d19*, nucleotides 2542-2561 and nucleotides 3264-3283 in NM_012059 (742 bp); *Pai2*, nucleotides 550-569 and nucleotides 1240-1259 in X16490 (710 bp); *Foxq1*, nucleotides 900-919 and nucleotides 1663-1682 in NM_008239 (783 bp); *Dsc2*, nucleotides 2212-2231 and nucleotides 2703-2722 in NM_013505 (511 bp); *Hb4*, nucleotides 1744-1763 and 2424-2443 in AY028607 (700 bp); NM_183187, nucleotides 1928-1947 and nucleotides 2672-2691 (764 bp); and *Igfbp5*, nucleotides 2021-2040 and nucleotides 3175-3194 in NM_010518 (1174 bp). For *Fgfr2*, a fragment that comprises the coding region was used to generate gene-specific probes.

Microarray experiment and data analysis

To isolate single hair follicles, back skin of 10-day-old mice was cut into small pieces from which individual hair follicles were prepared using fine forceps. For each microarray hybridisation, about 300 hair follicles from three different mice were sampled and total RNA was isolated and converted to labelled cRNA. Mouse Genome 430 2.0 microarrays were hybridised with labelled probes as recommended by the manufacturer (Affymetrix). Further details are given below. The microarray data have been deposited in the Gene Expression Omnibus database (Accession Number GSE2463).

The major goal of the microarray analysis was to examine whether *dnFgfr2-IIIb* transgene expression mainly affects the medulla. From each mouse, about 100 hair follicles were isolated and divided into two pools of about 50 follicles each, and subjected to the preparation of total RNA using Trizol (Sigma). For each genotype (wild type and transgenic), three mice of independent litters were subjected to the procedure of hair follicle isolation at postnatal day 10. After separate RNA isolation steps, RNAs from mice of the same genotype were pooled and used to generate labelled cRNA according to the Affymetrix protocol. Each cRNA was hybridised to a single Mouse Genome 430 2.0 microarray. Data analysis was performed using the

Affymetrix GCOS software; for scaling, the target intensity (TGT) was set to 100.

By pooling RNA samples from 2×50 follicles each from three different mice, inter-individual variations as well as small differences between single follicles are expected to be eliminated (Kendziorski et al., 2005). In my experience, differences between individuals or samples are the major source of false-positive (and false-negative) results in microarray hybridisations. Indeed, the noise generated by chip-to-chip variations or by differences in probe preparations is very low (Wodicka et al., 1997). As the preparation of single hair follicles is extremely laborious and time-consuming, I deliberately accepted the possibility of a small number of false positive and false negative hybridisation results due to a single hybridisation, but according to previous publications and my own experience, mainly transcripts with signals next to the detection limit are affected (Wodicka et al., 1997). Because of their low level of expression, their independent verification via in situ hybridisation is very difficult and almost impossible. Thus, they would not aid to support the starting hypothesis. Nevertheless, the microarray results clearly demonstrate the reliability and validity of the data obtained through the chosen strategy. For none of the selected genes was a discrepancy between microarray data and independent verification approaches found (see Results).

To limit the number of extracted genes, I chose a fourfold change in expression as the threshold. Furthermore, I eliminated some seemingly upregulated genes from the final list, as the increased expression levels were a direct effect of the transgenic construct and not caused by transgene expression. These genes encode some immunoglobulins (which falsely appear to be increased in expression due to the use of a dnFGFR2-IIIb/IgG fusion construct) and a sodium-dependent dicarboxylate transporter (expressed at elevated levels because its very 3′-end is part of the *Foxn1* promoter fragment). As expected, the aberrant transcription of this intragenic region does not have any effect in transgenic mice, as indicated by various transgenic lines using the *Foxn1* promoter (data not shown). Finally, I chose a microarray signal intensity of 120 as a further threshold to eliminate the majority of false positive targets. This value is close to the actual and reliable detection limit and was empirically determined in a large set of microarray analyses (data not shown).

The decrease in the number of hair medulla columns in *Foxn1::dnFgfr2-IIIb* transgenic mice does not cause an artificial increase in the number of downregulated genes, when an at least a twofold change in expression is taken as the minimal threshold. This is demonstrated by a simple calculation: about 70% of all follicles contain only a single column of medulla cells. Most of the remaining hair follicles possess a medulla with two cell columns. Thus, the average number of medulla columns is 1.3: [(70×1) + (30×2)]/100. Taking into account that some hair follicles contain more than two columns of medulla cells, the correct value for the average number of medulla columns might be in the range of 1.4-1.5. Consequently, a general reduction of the number of medulla columns to 1 will produce only a 1.5-fold decrease in expression levels. As upregulated genes are affected by the same effect, genes that are only slightly increased in expression might be undetectable with the microarray approach. Thus, upregulated genes might be slightly under-represented among identified target genes. This effect is inherent to the specific biological situation of wild-type and *dnFgfr2-IIIb* transgenic mice and independent of microarray and data analysis.

A few targets with slightly enhanced expression levels in transgenic mice might be not detected because of the general reduction of the number of hair medulla columns. In addition, the analysis of hair follicles does not allow the identification of every gene that is expressed in more domains than the medulla but that is downregulated only in the latter compartment. This is demonstrated by a simple calculation. As already mentioned, the average number of medulla columns is about 1.4-1.5. Assuming that, for example, at least four cells of any inner root sheath (IRS) layer are needed to wrap 1.5 cells of the hair medulla (which is actually a very conservative assumption), wild-type expression in the medulla has to be 2.7-fold higher when compared with the IRS and expression in the medulla has to be completely lacking in the transgenic follicle to obtain at least a twofold change in expression in a microarray analysis (relative expression level in wild-type follicle: 4×1 + 1.5×2.7=8; relative expression level in transgenic follicle lacking expression in the medulla: 4×1 + 1.5×0=4). Thus, a significant difference in endogenous expression levels of distinct domains is essential to identify a decrease that is restricted to the medulla. Furthermore, the enhanced expression must occur in the medulla.

What can be expected if there is no significant difference in expression levels among distinct domains? For four IRS cells versus 1.5 medulla cells, one would obtain relative signal intensities of 4×1 + 1.5×1=5.5 for wild-type versus 4×1 + 1.5×0=4 for transgenic hair follicles that lack any medullary expression. Thus, the decrease in signal intensities would be in the range of 1.38-fold. A more realistic assumption of about 6 IRS cells that are needed to wrap 1.5 medulla cells would give a 1.25-fold decrease (6×1 + 1.5×1=7.5 versus 6×1 + 1.5×0=6).

For *K6hf* and *K17*, expression in the medulla might be enhanced when compared with the companion layer (Fig. 3E and data not shown). Nevertheless, the appearance of in situ hybridisation patterns such as in Fig. 3E can be misleading if interpreted simply by eye. Whereas the medulla is visible as a broad structure, the companion layer appears as a very thin band. In actual fact, the intensities of staining are not that different in both compartments. In addition, expression is far from being absent in the transgenic hair follicle. Thus, both effects, moderately enhanced expression in the medulla of wild-type follicles when compared with the companion layer and significant expression in transgenic medullae, might compensate each other. Actually, our microarray analysis revealed a 1.3-fold decrease in the expression of *K6hf* and *K17*. Likewise, quantitative RT-PCR did not show any significant difference in expression in wild-type and transgenic skin (transgenic versus wild type: –1× for *K6hf*; –1.5× for *K17*).

Analysis of hair and hair shaft structures

Hair was plucked from 4-week-old mice and single hair shafts were sorted to determine the composition of the hair coat. For the comparison of overall hair lengths and for the analysis of the internal structure of hair shafts, a Zeiss dissecting microscope and a Zeiss light microscope were used, respectively. Photographs were taken using a Sony and a Nikon digital camera, respectively.

Results

Ectopic expression of a soluble dominant negative form of FGFR2-IIIb causes defects in the hair coat

All four FGF receptors and several fibroblast growth factors are expressed in the mature hair follicle (Rosenquist and Martin, 1996; Suzuki et al., 2000). The dermal papilla, a major control unit, expresses FGF7 and FGF10, which specifically bind to the receptor variant FGFR2-IIIb (Igarashi et al., 1998; Ornitz et al., 1996). The *Fgfr2* gene is expressed in the hair matrix (Rosenquist and Martin, 1996).

To elucidate the role of FGF signals from the dermal papilla in mature hair follicles, I sought to interfere with endogenous FGFR2-IIIb signalling by expressing a dominant-negative form of this receptor (dnFGFR2-IIIb). As no strong promoter fragment for expression in the hair matrix or the dermal papilla is available, I chose the *Foxn1* promoter, which produces strong expression in the hair cortex (C. C. Bleul and T. Boehm, unpublished; details are given in Materials and methods). Furthermore, I used a soluble form of dnFGFR2-IIIb, which is

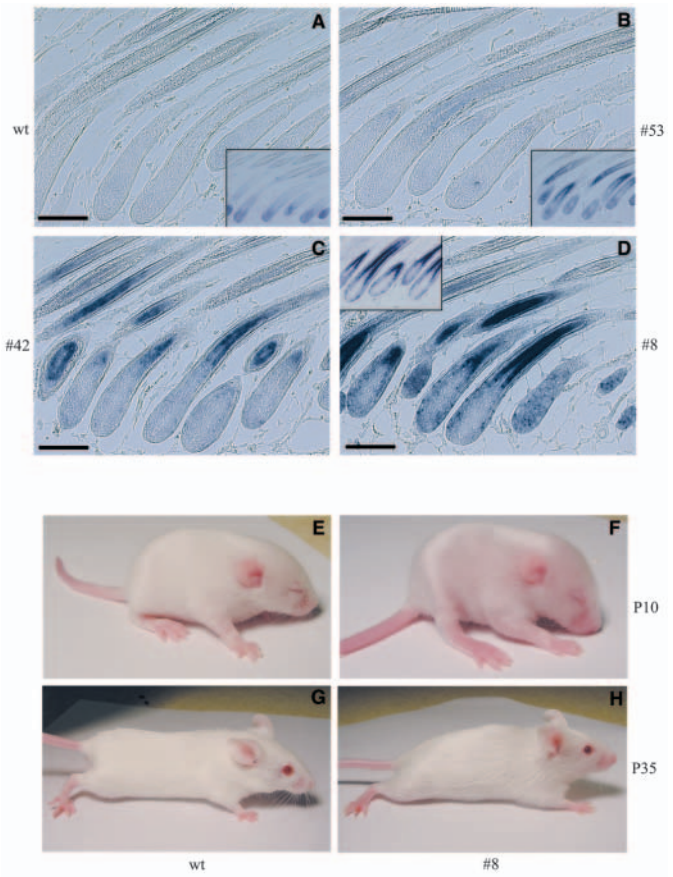

Fig. 1. *Foxn1::dnFgfr2-IIIb* transgenic mice develop abnormal hair coats. (A-D) All transgenic mouse lines show strong expression of *dnFgfr2-IIIb*, as demonstrated by non-radioactive in situ hybridisation. Nevertheless, transcript levels are significantly different, especially between line #53 and lines #42 and #8. The inset in B shows transgene expression in line #53 after extended staining whereas the inset in (A) shows endogenous gene expression of a corresponding wild-type section. The inset in D demonstrates the expression pattern of the endogenous *Foxn1* gene, which is identical to that of the *dnFgfr2-IIIb* transgene under the control of a 30 kb *Foxn1* promoter fragment (B-D). (E-H) Strong transgene expression causes a silky hair coat appearance. During early development of the pelage, it also appears to be less dense in transgenic mice when compared with their wild-type littermates; body skin is still visible at day 10 after birth (F). Scale bars: 100 μm.

more potent than the membrane-bound variant, to overcome the possible limitations of ectopic expression distant from the dermal papilla (Celli et al., 1998).

Three independent transgenic lines were established. Two lines (#8 and #42) carry very strong transgene expression in the hair cortex. Transcript levels in the third line (#53) are significantly lower, albeit still high compared with endogenous levels, which are at the threshold of detection (Fig. 1A-D).

Mice of the low expressing line #53 develop normally and their appearance is unaltered when compared with wild-type littermates. On the contrary, transgenic mice of lines #8 and #42 can be distinguished from their wild-type littermates as soon as the hair shafts emerge through the epidermis. Although these mice develop normally and the formation of the hair coat is neither accelerated nor retarded, the pelage has a bright and silky appearance and the skin is visible for a longer period during pelage formation (Fig. 1E-H).

To test whether the observed effect is specific for FGFR2-IIIb-mediated signalling, I generated transgenic mice that express a soluble dominant-negative form of FGFR1-IIIc as a control. FGF1 which is also expressed in the hair follicle binds with high affinity to FGFR1-IIIc as well as FGFR2-IIIb, while FGF7 and FGF10 exclusively bind to FGFR2-IIIb (Igarashi et al., 1998; Ornitz et al., 1996). Three independent transgenic lines expressed *dnFgfr1-IIIc* at high to very high levels. Nevertheless, none of them revealed a phenotype similar to *Foxn1::dnFgfr2-IIIb* transgenic mice (T. Schlake, unpublished). This shows that the phenotype observed in *Foxn1::dnFgfr2-IIIb* mice is specifically due to impaired FGFR2 signalling.

Foxn1::dnFgfr2-IIIb transgenic mice have reduced hair medullae

To clarify the reason for the altered hair coat appearance, I first investigated the composition of the pelage. In wild-type mice, four different hair types can be found with characteristic frequencies. Whereas about 65-70% of all hairs are of the zigzag type with three or four sharp bends within the hair shaft, 30% are straight hairs known as awl. The guard hairs are also straight but significantly longer than awl hairs and occur at a frequency of about 1-3%. Auchene hairs form only a minor fraction, with a frequency of about 0.1%, and have a single sharp bend within their hair shaft. Interestingly, *Foxn1::dnFgfr2-IIIb* transgenic mice possess all four hair types at normal frequencies (data not shown). Furthermore, the hair length is not affected (Fig. 2A-C).

The internal structure of the hair shaft is a further distinctive feature of different hair types. Zigzag hairs have a single column of medulla cells, whereas guard hairs contain two columns. Awl and auchene hairs have two or more columns of medulla cells, with two and three predominating. Under the light microscope, each column of medulla cells is visible as a ladder-like structure because of the regular arrangement of air spaces within the medulla. When I investigated the internal structure of transgenic hairs, I noted the complete absence of hairs with more than one column of medulla cells in lines #8 and #42 (Fig. 2B and data not shown). Furthermore, light microscopy of hair shafts revealed an altered differentiation of the medulla, as the regular spacing of air cells is disturbed or even missing. Interestingly, the structure of hair shafts is also affected in the low expressing line #53. In this line zigzag hairs have a normal appearance; however, guard hairs contain only one column of medulla cells and awl and auchene hairs possess one or two columns (Fig. 2C). This clearly indicates that the observed effect of transgene expression is dose dependent. In histological sections, the altered structure of hair medullae manifests itself in an abnormally dense packing of cells and the lack of vacuoles (Fig. 2D,E).

I next asked whether the absence of more than one column of medulla cells might be due to an apoptosis-driven reduction of medulla-forming cells. Detailed analysis of hair follicle sections failed to identify TUNEL-positive cells in the medulla (Fig. 2F,G), suggesting that the reduced medulla structure must be the result of reduced proliferation of progenitor cells. Unfortunately, analysis of cell proliferation specifically in the medulla is hampered by the fact that the medulla is only one

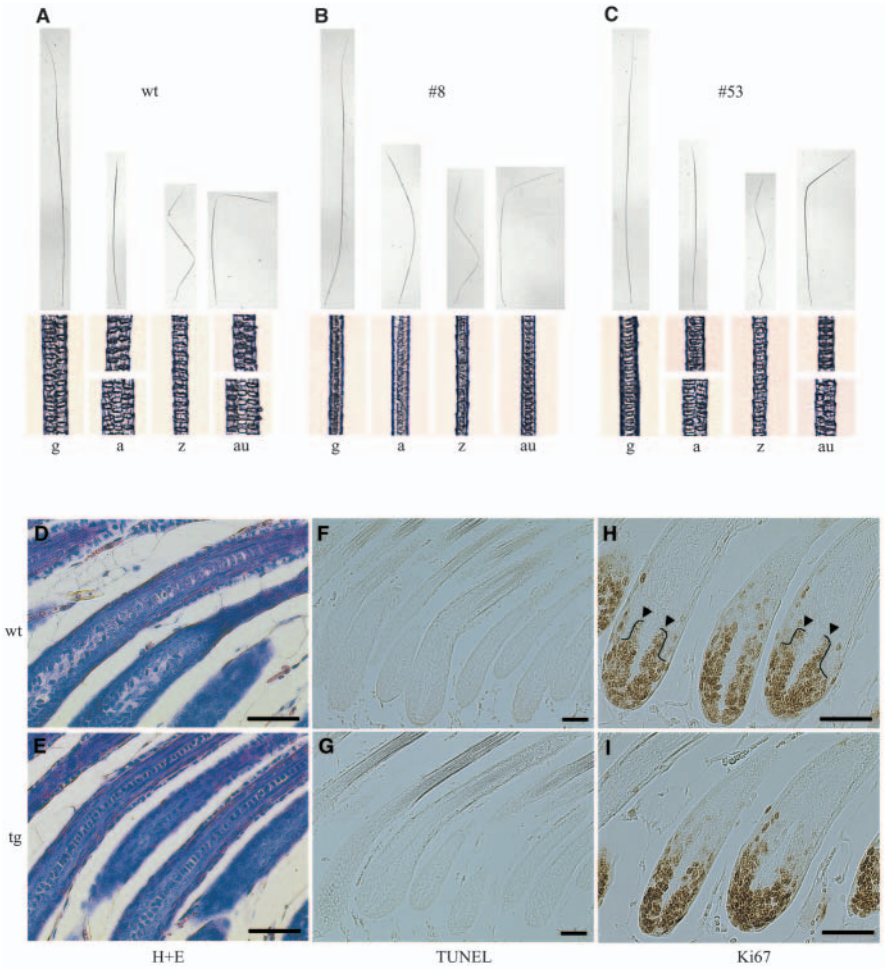

Fig. 2. The number of medulla columns is reduced in *Foxn1::dnFgfr2-IIIb* transgenic mice. (A-C) A comparison of hair coat composition demonstrates that transgenic mice still possess all four hair types present in wild-type animals. The overall length and shape of hairs is shown at the top, whereas the microscopic appearance of the hair shaft structure is shown at the bottom. The length of hair shafts is not affected. In wild-type mice, the number of medulla columns differs between single hairs of the awl and auchene type (A). In the strong expressing lines #8 and #42 the number of medulla columns is generally reduced to one (B). The number is reduced by one in guard, awl and auchene hairs of line #53, preserving the heterogeneity of hair shaft structures of awl and auchene hairs (C). a, awl hair; au, auchene hair; g, guard hair; z, zigzag hair. (D,E) Histological analyses of hair follicles reveal an abnormal structure of the medulla. (F,G) The matrix and medulla of wild-type and transgenic hair follicles do not contain TUNEL-positive cells. (H,I) The proliferative compartment of hair follicles is largely unaffected by transgene expression as demonstrated by Ki67 staining. In some wild-type follicles, the zone of proliferating cells next to the dermal papilla appears to extend more along the proximodistal axis than in more lateral parts of the proliferative compartment (arrowheads). Such an extension has never been observed in transgenic follicles. Scale bars: 50 μm.

of several epithelial compartments in the hair follicle, most of which are unaffected by *dnFgfr2-IIIb* expression. Thus, proliferative compartments in wild-type and transgenic hair follicles are largely indistinguishable (Fig. 2H,I). However, in some follicles of wild-type mice proliferating cells located next to the dermal papilla, i.e. medulla representing progenitors, appear to extend more distally than all other precursor cells (Fig. 2H). This could not be found in transgenic follicles and, thus, might support the hypothesis of a reduced proliferation rate of medulla progenitors in *Foxn1::dnFgfr2-IIIb* hair bulbs.

Ectopic *dnFgfr2-IIIb* causes specific defects in the differentiation of the hair medulla

The above analyses indicated that the hair medulla is the only follicular compartment affected in *Foxn1::dnFgfr2-IIIb* transgenic mice. I wondered whether this result was also reflected at the molecular level. To address this question, I analysed the transcription levels of many genes that are expressed in distinct follicular compartments. As endogenous *Fgfr2* is predominantly expressed in the central matrix region, I mainly concentrated on the matrix, the medulla and the cortex (Rosenquist and Martin, 1996). This analysis revealed that expression in the hair matrix, hair cortex, hair cuticle, and inner and outer root sheaths are unaffected in transgenic hair follicles (see Table S1 in the supplementary material for list of genes tested). By contrast, the expression of several medulla specific genes is altered (see Table S1 in the supplementary material). Whereas expression of involucrin and *K6hf* (*Krtcap1* – Mouse Genome Informatics) is significantly decreased, transcripts of *Sh3d19* and plasminogen-activator inhibitor 2 (*Pai2*; *Serpinb2* – Mouse Genome Informatics) are almost absent (Fig. 3C-J). Interestingly, suppression of *K6hf* expression is restricted to the medulla; transcription in the companion layer is completely unaffected.

Transgene expression does not cause an early and general differentiation defect in the medulla, because, for example, arachidonate 8(S) lipoxygenase transcript levels are indistinguishable in wild-type and transgenic hair follicles (Fig. 3A,B). I also analysed the expression of the transcription factor *Foxq1*. It is expressed in the hair medulla and is of special interest because a mutation in the *Foxq1* gene is responsible for the satin mutation (Hong et al., 2001). Satin mice are characterised by a silky appearance and hairs that are thinner than normal, which is highly reminiscent of the *Foxn1::dnFgfr2-IIIb* phenotype. Nevertheless, gene expression studies revealed no difference between wild-type and transgenic hair follicles, again excluding a generally toxic effect of the transgene on gene transcription in the medulla (Fig. 3K,L).

Ectopic expression of *dnFgfr2-IIIb* exclusively affects the hair medulla

The above results suggested that the hair medulla is the only follicular compartment affected in *Foxn1::dnFgfr2-IIIb*

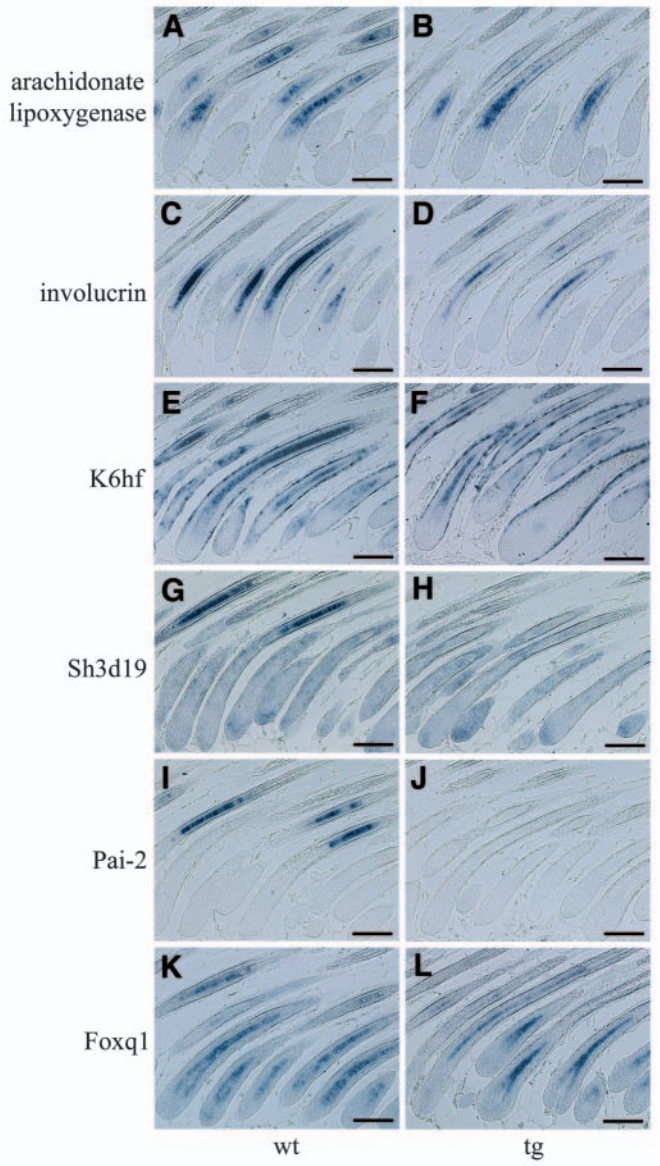

Fig. 3. Specific molecular defects in *Foxn1::dnFgfr2-IIIb* transgenic mice. Expression of markers of the hair shaft medulla was analysed by non-radioactive in situ hybridisation in wild-type (A,C,E,G,I,K) and transgenic (B,D,F,H,J,L) hair follicles during the growth phase. Transcription of involucrin (C,D), *K6hf* (E,F), *Sh3d19* (G,H) and *Pai2* (I,J) is severely reduced in transgenic hair follicles, while expression of arachidonate 8(S) lipoxygenase (A,B) and *Foxq1* (K,L) is normal. Scale bars: 100 μm.

transgenic mice. If this were so, the observed effects must be mediated by interference specifically with endogenous FGFR2 signalling, as FGFR2 is the only FGF receptor that is expressed in cells of the medulla or progenitors thereof (Rosenquist and Martin, 1996). Thus, to exclude a transgene effect on compartments other than the medulla and to elucidate the molecular basis of the observed phenotype in more detail, I sought to identify further genes affected on the transcriptional level in transgenic mice.

To avoid complications in the interpretation of the results of global gene expression analysis in the hair follicle by contamination with cells from other compartments of the skin, pools of single hair follicles were used for microarray analyses (details are given in Materials and methods). The arrays contained oligonucleotide probe sets representing about 39,000 transcripts. Table 1 summarises the genes whose expression levels are most significantly affected in transgenic hair follicles. Involucrin and *Pai2*, which I had earlier shown to be downregulated in *Foxn1::dnFgfr2-IIIb* hair follicles (Fig. 3C,D,I,J), were also identified in this experiment. The failure to detect *K6hf* and *K17* is due to the fact that expression in transgenic medullae is still significant and that both are also expressed in the companion layer where their transcript levels are not changed, making the overall difference too small for reliable detection (Fig. 3E,F; see Materials and methods for a detailed explanation). *Sh3d19* is not represented on the microarray.

To confirm independently the identified differences in expression and to explore the expression domains of these genes, in situ hybridisation analyses were carried out for a random sample of the genes listed in Table 1. Reliable results were obtained for most of the selected genes, all proving a deregulation of transcript levels (Fig. 4). In addition, all changes are restricted to the hair medulla. Interestingly, three genes are upregulated in transgenic hair follicles, whereas the majority of affected genes has reduced expression levels. I also noticed that basic hair keratin 4 reveals strong expression in the medulla of wild-type follicles (Fig. 4C). This is remarkable in the light of results on human *Hb4* expression that exclusively occurs in filiform papillae of tongue but not in the hair (Langbein et al., 2001).

Ectopic expression of *Igfbp5* phenocopies *Foxn1::dnFgfr2-IIIb*-mediated reduction of medulla columns

I next asked how transgene expression might translate into the observed phenotype and sought to delineate a hierarchy of molecular events. In this context, *Igfbp5* appeared to be the most interesting and promising candidate for several reasons. First, *Igfbp5* is the only gene among those identified whose product is involved in intercellular signalling. Thus, it might transduce initial signals to remote regions. Second, IGFBP5 can act as an antagonist of IGF, which is known to have mitogenic functions, and might thereby be capable of reducing cell proliferation. Third, endogenous *Igfbp5* transcript levels differ between distinct types of follicles (Fig. 4G,I), indicating a possible relationship to the number of medulla columns. In contrast to the wild-type situation, all transgenic hair follicles show strong *Igfbp5* expression in the medulla that might be responsible for a general one-column medulla (Fig. 4H,J).

To address the role of *Igfbp5* in mediating the *Foxn1::dnFgfr2-IIIb* transgenic effect, transgenic mice that ectopically express *Igfbp5* were developed. For this, I employed an involucrin promoter fragment (a detailed discussion of promoter selection is presented in the Materials and methods). Among available promoters it is the only one that also drives gene expression in the medulla, which is the domain of increased *Igfbp5* expression in *dnFgfr2-IIIb* transgenic mice (Carroll et al., 1993). All four independent transgenic lines revealed moderate-to-strong transgene expression and an abnormal appearance of their hair coat (T.S., unpublished). Nevertheless, their pelage is neither bright nor

Table 1. Genes affected in the hair follicle by the expression of *dnFgfr2-IIIb*

Gene	Fold change	Description
Eif2s3	–208×	Eukaryotic translation initiation factor 2, subunit 3
Krt2-16	–20×	Basic hair keratin 4
NM_183187	17×	EST (homologous to human DRR1)
stefin A1	–16×	Cysteine proteinase inhibitor
stefin 2-like	–9.8×	Cysteine proteinase inhibitor
Sprrl1	–8.6×	Small proline rich-like 1
Sprr1b	–7.0×	Small proline-rich protein 1B
AK004611	6.5×	EST
Serpinb2	–5.7×	Plasmiogen activator inhibitor 2 (PAI-2)
Crisp1	–5.3×	Acidic epididymal glycoprotein 3
Snai3	–5.3×	Snail homolog 3
Aldh1a3	–4.9×	Retinaldehyde dehydrogenase 3
Ivl	–4.6×	Involucrin
Dsc2	–4.6×	Desmocollin 2
NM_133675	–4.6×	EST
Igfbp5	4.3×	Igf-binding protein 5
Sprr2h	–4.3×	Small proline-rich protein 2H
BC010814	–4.3×	Transmembrane 4 superfamily member 4
S100A9	–4.3×	Calcium binding protein A9 (calgranulin B)
BB414446	–4.3×	14-3-3 protein tau
AF365876	–4.3×	EST (ADMP)
C89521	–4.3×	Similar to stefin 3 (cysteine proteinase inhibitor)
BC025872	–4.0×	Similar to neurofilament, heavy polypeptide

To exclude unreliable results, only genes with either wild-type or transgenic gene expression signals above 120 were selected. Furthermore, genes with differences in expression levels of less than fourfold were excluded to limit the number of extracted genes (for further information, see the Materials and methods). Genes that have been tested and confirmed by in situ hybridisation are shown in bold. Genes that have been tested but could not be detected by in situ hybridisation are shown in italics. For none of the tested genes could a discrepancy between microarray data and in situ hybridisation results be detected.

silky. A detailed analysis of their hair coat demonstrated that *Igfbp5* transgenic mice possess all major hair types with normal frequencies. The hair shafts are significantly reduced in length and, most interestingly, contain only one column of medulla cells (Fig. 5). Within the medulla, the spacing of air cells is, however, very regular.

Discussion

Although several studies have suggested an important role for FGF signalling in hair follicle morphogenesis, its function in the mature follicle has not yet been well investigated. In the present work, I have demonstrated that signalling through FGFR2-IIIb specifically regulates the structure of the hair medulla via IGF-binding protein 5 (Fig. 6).

The known ligands of FGFR2-IIIb are FGF1, FGF3, FGF7 and FGF10 (Igarashi et al., 1998; Ornitz et al., 1996). Of these, all but FGF3 are expressed in the hair follicle (Rosenquist and Martin, 1996; Suzuki et al., 2000). Whereas FGF1 is expressed in the epithelial compartment of the hair follicle, FGF7 and FGF10 are secreted by cells of the dermal papilla. Ablation of *Fgf7* or *Fgf10* has only mild or no effects, respectively, on the hair coat, which is probably due to redundant functions and to

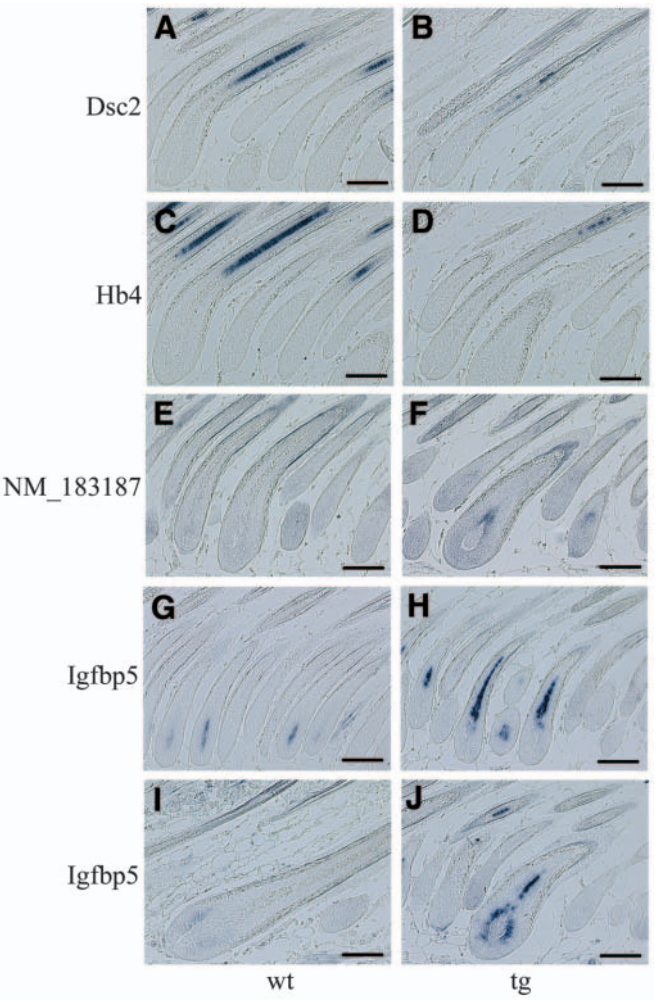

Fig. 4. Molecular abnormalities in *Foxn1::dnFgfr2-IIIb* transgenic mice are restricted to the medulla. Differential gene expression in wild-type (A,C,E,G,I) and transgenic (B,D,F,H,J) hair follicles that has been identified by microarray analyses was confirmed by non-radioactive in situ hybridisation. Gene expression is either strongly downregulated (A-D) or significantly upregulated (E-J) in the medulla of transgenic follicles. Whereas *Igfbp5* is markedly expressed in small follicles of wild-type skin (G), large guard hair follicles show only weak signals (I). Scale bars: 100 μm.

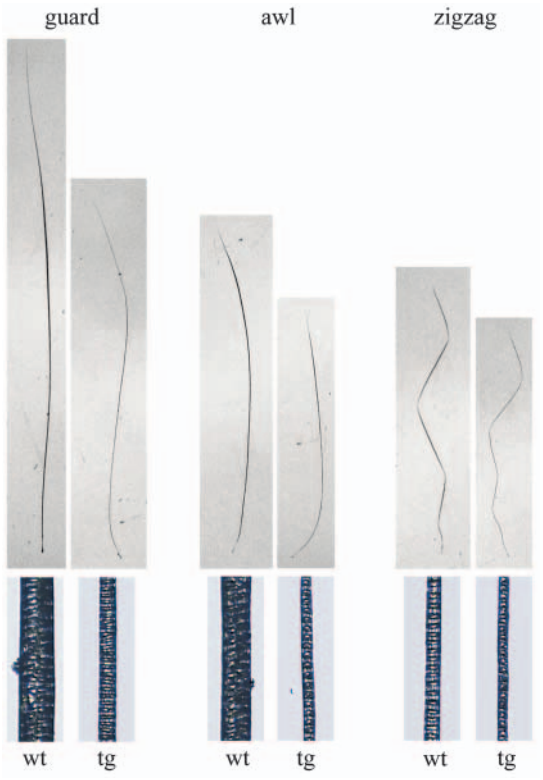

Fig. 5. Expression of *Igfbp5* in the hair follicle reduces the number of medulla columns. Transgenic expression of *Igfbp5* in hair follicles during the growth phase using the involucrin promoter decreases the length of hair shafts (top) and reduces the number of air space columns to one (bottom). The extraordinary thinness of hair shafts severely hampered the isolation of intact hairs. Thus, the presence of auchene hairs could not unequivocally be confirmed.

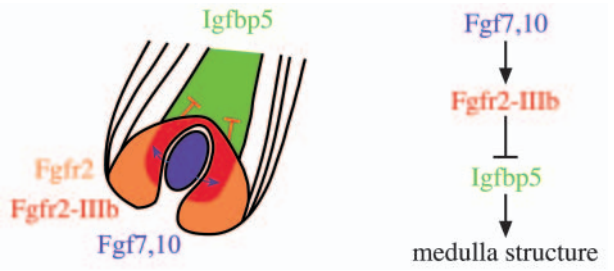

Fig. 6. Model for the effect of FGF signals on the follicular expression of *Igfbp5* and on the structure of the hair shaft medulla. Whereas the expression of *Fgfr2* in the hair matrix has been previously demonstrated, the assumption of *Fgfr2-IIIb* expression in the precursors of the hair medulla is based on the presented data.

their co-expression (Guo et al., 1996; Suzuki et al., 2000). Mice deficient for *Fgfr2-IIIb* or ectopically expressing dominant-negative forms of the FGFR2-IIIb protein prior to follicle induction produce significantly fewer hair follicles than wild-type littermates, indicating a role of FGF signalling in follicle morphogenesis (Celli et al., 1998; Petiot et al., 2003; Werner et al., 1994). Unfortunately, this early effect hampers the analysis and interpretation of the role of *Fgfr2-IIIb* in the mature hair follicle. The use of the *Foxn1* promoter that is active in the differentiating hair cortex limits transgene expression to the time after follicular induction and the first morphogenetic steps (for further details on the *Foxn1* promoter, see Materials and methods). Obviously, transgene expression in primary hair follicles does not interfere with later inductive waves, as the number of hair follicles is normal (see hair follicle densities in Figs 1-4 and data not shown) and all hair types are present.

A potential limitation of the use of dominant-negative receptor variants is the promiscuity of receptor-ligand interactions. Potential aberrations may also be due to competition for ligands among distinct receptors. Using transgenic mice expressing *Fgfr1-IIIc*, I excluded the possibility that FGF1-mediated signalling is responsible for the observed phenotype. Furthermore, the lack of any hair phenotype in *Foxn1::Fgfr1-IIIc* transgenic mice demonstrates that the observed effects in *Foxn1::dnFgfr2-IIIb* transgenic mice are not due to interference with FGF1-mediated signalling via other receptors. A *Fgfr2-IIIb*-specific effect is further supported by the fact that only the medulla is affected in transgenic hair follicles. According to previous expression studies, only *Fgfr2* is expressed in medulla precursor cells (Rosenquist and Martin, 1996). Together, our data suggest that the observed effects are specifically due to impaired FGFR2-IIIb signalling, which is most likely to be mediated by FGF7 and FGF10.

Interestingly, interference with endogenous FGFR2-IIIb signalling does not switch between different states of hair type-specific features (e.g. multi-column medulla versus single-column medulla) but affects the hair structure in a dose-dependent manner, thereby causing continuous transitions. Furthermore, hairs adopt only some features of different hair types, i.e. they produce reduced numbers of medulla columns, whereas other characteristics such as the shape are unchanged. Clearly, our *Foxn1::dnFgfr2-IIIb* transgenic mice demonstrate that FGF signals are necessary to develop hair shafts with more than one medulla column in an otherwise normal context.

The pelage of mice consists of four distinct hair types that are induced in consecutive waves. Very little is known about the establishment and maintenance of different hair follicles. EDAR signalling is somehow involved in the generation of zigzag hairs, as mice with a disruption of this pathway, as well as transgenic animals overexpressing EDA1, lack zigzag hairs while having about normal numbers of follicles (Cui et al., 2003; Mustonen et al., 2003; Sundberg, 1994a; Sundberg, 1994b; Zhang et al., 2003). It is unknown whether this transformation occurs during follicle induction or in the mature follicle. Our data unequivocally show that at least some distinctive features of hair follicle types are determined in the mature hair follicle. As the activity of the *Foxn1* promoter truly reflects the spatial and temporal expression pattern of the endogenous locus and, thus, starts rather late in follicular morphogenesis (for details see Materials and methods), transgene expression cannot have an effect on the induction of at least guard hair follicles. Nevertheless, *Foxn1::dnFgfr2-IIIb* transgenic mice develop guard hairs with only one column of medulla cells instead of two.

Interestingly, transgenic expression of dnFGFR2-IIIb and genetic inactivation of the transcription factor FOXQ1 cause very similar hair phenotypes (Hong et al., 2001), suggesting the possibility of a direct link. Expression studies in

Foxn1::dnFgfr2-IIIb transgenic mice, however, revealed no differences in *Foxq1* transcript levels. It is still possible that FGFR2-IIIb signalling affects FOXQ1-mediated transcription on the translational or post-translational level. At least some members of the large family of forkhead box (FOX) proteins are post-translationally modified (Nakae et al., 1999). Unfortunately, no specific antibody for FOXQ1 is yet available. As expression of *Fgfr2* in the follicle starts earlier, i.e. more proximal than that of *Foxq1*, and the domains of transcriptional activity only overlap very slightly, a regulation of *Fgfr2* expression by FOXQ1 can be excluded.

All genes that are affected in *Foxn1::dnFgfr2-IIIb* transgenic mice are expressed in the hair medulla, suggesting that the identified target genes lie in a genetic pathway directly controlled by *Fgfr2-IIIb*. Several structural genes are reduced in expression in *Foxn1::dnFgfr2-IIIb* transgenic mice. Among them, basic hair keratin 4 (HB4; KRT2-16 – Mouse Genome Informatics) is a highly interesting candidate. HB4 is the first hard keratin for which an expression in the hair medulla is demonstrated. In humans, *HB4* is expressed in filiform papillae of tongue but is completely absent from the hair follicle (Langbein et al., 2001). Furthermore, human hair shafts lack any air spaces although they usually contain a medulla. Thus, *Hb4* may be one of the genes that are responsible for the characteristic structure of murine hair shafts, i.e. a regular pattern of air spaces, which is missing in *Foxn1::dnFgfr2-IIIb* transgenic mice. ALDH1A3 catalyses the last step in retinoic acid synthesis. Previous reports have demonstrated specific expression in the hair follicle (Everts et al., 2004; Niederreither et al., 2002). Reduced levels of retinoic acid caused by the lack of ALDH1A3 may account for some effects in *Foxn1::dnFgfr2-IIIb* transgenic hair follicles. *Snai3* encodes a member of the Snail protein family (Kataoka et al., 2000). As a transcriptional regulator, SNAI3 might mediate some consequences of *dnFgfr2-IIIb* transgene expression. Interestingly, a previous report has suggested a link between FGF-signalling and the action of Snail proteins (Savagner et al., 1997).

Among target genes, *Igfbp5* is the most interesting one, as IGFBPs act as stimulators or inhibitors of the mitogen IGF depending on environmental conditions (Clemmons, 1992). An antagonistic effect of IGFBP5 on endogenous IGF signalling would be compatible with the increased *Igfbp5* expression and the reduced number of medulla columns in *Foxn1::dnFgfr2-IIIb* transgenic mice. The strong expression of *Igfbp5* in the medulla of transgenic hair follicles when compared with wild-type tissue indicates that, for unknown reasons, the innermost cells of the hair shaft are prone to high *Igfbp5* gene activity. Obviously, signalling via FGFR2-IIIb is needed to suppress this default expression pattern.

Using *Ivl::Igfbp5* transgenic mice, I could demonstrate that elevated levels of *Igfbp5* expression are sufficient to reduce the number of medulla columns. This strongly supports the idea that the inhibition of *Igfbp5* gene expression is causally linked to the production of hair shafts with more than one medulla column in wild-type hair follicles. The present data do not exclude the possibility that genes other than *Igfbp5* might contribute to the reduction of medulla column numbers in *Foxn1::dnFgfr2-IIIb* transgenic mice. As *Ivl::Igfbp5* transgenic hair shafts still possess a regular pattern of air spaces and the transgenic hair coat has no silky appearance, *Ivl::Igfbp5* transgenic mice are only a partial phenocopy of *Foxn1::dnFgfr2-IIIb* transgenic animals. Expression levels in *Ivl::Igfbp5* transgenic mice suggest that this is not due to insufficient *Igfbp5* expression, indicating that the control of the number of medulla columns and the regulation of medulla differentiation can be genetically separated.

I am grateful to Stefanie Sick and Brigitte Hammerschmidt for their excellent technical help. I thank Benoit Kanzler, Elsa Huber and Laurent Morawiec for producing transgenic mice. I also thank Lorne Taichman for the *Ivl* promoter plasmid, Glenn Merlino for the dnFGFR2-IIIb cDNA, and Conrad Bleul and Thomas Boehm for the *Foxn1* promoter construct. I thank Thomas Boehm for helpful discussions and comments on the manuscript. The work was supported in part by the Deutsche Forschungsgemeinschaft (grants Schl 565/1-1 and Schl 565/1-2).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/13/2981/DC1

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Table S1. Genes analysed for their expression in wild-type and *Foxn1::dnFgfr2-IIIb* transgenic mice

Gene	Expression domain	Description
BC019978	Mid-medulla	Mitochondrial Ca-dependent solute carrier
K6hf	Medulla, companion layer	Cytokeratin 6hf (hair follicle)
K16	Medulla, ORS	Cytokeratin 16
K17	Medulla, companion layer	Cytokeratin 17
Ha1	Proximal and mid-cortex	Acidic hair keratin 1
Ha2	Proximal cuticle	Acidic hair keratin 2
Ha5	Pre-cortex	Acidic hair keratin 5
Hb6	Mid- and distal cortex	Basic hair keratin 6
Ivl	Medulla	Involucrin
Alox8	Medulla	Arachidonate 8(S) lipoxygenase
epithin	Pre- and proximal cortex	Serine protease
Lpin3	Proximal cortex	Lipin 3
Cdh3	Matrix, proximal cortex	P-cadherin
Krtap5-1	Mid- and distal cortex	Keratin associated protein 5-1
Sh3d19	Mid- and distal medulla	SH3 domain containing protein D19
Pai2	Mid- and distal medulla	Plasminogen activator inhibitor 2
Efnb1	Matrix	Ephrin B1
Efnb2	Pre- and proximal cortex	Ephrin B2
Pac-1	Proximal IRS	Protein tyrosine phosphatase
Ttk	Matrix	Ttk protein kinase, esk kinase
Tnfr2	Matrix	Tumor necrosis factor receptor 2
Tnfrsf19	Matrix	Tumor necrosis factor receptor Troy
Gata-3	Pre- and proximal IRS	GATA-binding protein 3
Gtf2ird2	Pre- and proximal cortex	General transcription factor II i repeat domain 2
Hrs	Matrix, proximal medulla, ORS	Nuclear receptor co-repressor hairless
Foxq1	Pre- and proximal medulla	Forkhead box transcription factor Q1

Gene expression in the hair follicle was analysed by non-radioactive in situ hybridisation on sections of wild-type and transgenic skin. Genes for which reliable differences in expression between wild-type and transgenic hair follicles were detected are shown in bold. Expression in the matrical precursor cells of a specific compartment is indicated by the prefix ‘Pre-’.