A crucial role for Fgfr2-IIIb signalling in epidermal development and hair follicle patterning

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Summary

To understand the role Fgf signalling in skin and hair follicle development, we analysed the phenotype of mice deficient for Fgfr2-IIIb and its main ligand Fgf10. These studies showed that the severe epidermal hypoplasia found in mice null for Fgfr2-IIIb is caused by a lack of the basal cell proliferation that normally results in a stratified epidermis. Although at term the epidermis of Fgfr2-IIIb null mice is only two to three cells thick, it expresses the classical markers of epidermal differentiation and establishes a functional barrier. Mice deficient for Fgf10 display a similar but less severe epidermal hypoplasia. By contrast, *Fgfr2-IIIb*$^{-/-}$, but not *Fgf10*$^{-/-}$, mice produced significantly fewer hair follicles, and their follicles were developmentally retarded. Following transplantation onto *nude* mice, grafts of *Fgfr2-IIIb*$^{-/-}$ skin showed impaired hair formation, with a decrease in hair density and the production of abnormal pelage hairs. Expression of *Lef1*, *Shh* and *Bmp4* in the developing hair follicles of *Fgfr2-IIIb*$^{-/-}$ mice was similar to wild type. These results suggest that Fgf signalling positively regulates the number of keratinocytes needed to form a normal stratified epidermis and to initiate hair placode formation. In addition, Fgf signals are required for the growth and patterning of pelage hairs.

Key words: Fgfr2-IIIb, Skin, Epidermis, Hair follicle, Hair differentiation, Mouse

Introduction

The integument of the early mouse embryo comprises a single ectodermal cell layer. Subsequent reciprocal signalling between the ectoderm and the underlying mesenchyme results in the formation of the epidermis and dermis, respectively (reviewed by Fuchs, 1993; Watt, 2001). In addition, more localised inductive signals, thought to initiate in the mesenchyme, result in the formation of the vibrissae and pelage hair follicles. In females, induction and development of mammary glands also occurs in a similar fashion. A number of molecules have been implicated in the growth and differentiation of skin and its appendages. There is considerable evidence from gene targeting studies that signalling by fibroblast growth factors (Fgfs) plays important roles (Hebert et al., 1994; Celli et al., 1998; De Moerlooze et al., 2000; Ohuchi et al., 2000; Suzuki et al., 2000; Li et al., 2001; Revest et al., 2001).

Fgfs are a large family of intercellular signalling molecules that mediate their biological responses by binding and activating high affinity cell surface receptors (Fgfr) (reviewed by Johnson and Williams, 1993; Ornitz et al., 1996; McKeehan et al., 1998). There are four genes that encode Fgf receptors with intrinsic tyrosine kinase activity (*Fgfr1* to *Fgfr4*), but additional complexity is achieved by alternative splicing. *Fgfr2* can encode two receptor isoforms, Fgfr2-IIIb and Fgfr2-IIIc, that have different Fgf-binding specificities and are expressed on different cell lineages. Fgfr2-IIIb is located on the epithelia of ectodermal and endodermal organs, and is activated by four known ligands, Fgf1, Fgf3, Fgf7 and Fgf10. The latter two Fgfs are expressed predominantly in mesenchyme adjacent to epithelia expressing Fgfr2-IIIb (Peters et al., 1992; Orr-Urtreger et al., 1993; Mason et al., 1994; Finch et al., 1995; Ornitz et al., 1996; Yamasaki et al., 1996). To understand the function of Fgf signalling in epidermal and hair follicle development, we have examined in detail the phenotype of mice deficient for Fgf10 or Fgfr2-IIIb.

The generation and characterisation of mice deficient for *Fgfr2-IIIb* or *Fgf10* has been described previously (Revest et al., 2001; Min et al., 1998). The Fgfr2-IIIb isoform-specific null mice were generated by placing translational termination codons in three reading frames within exon IIIb, followed by an IRES-*lacZ* cassette. These changes result in a truncated form of Fgfr2-IIIb that lacks part of the ligand-binding domain, and the entire transmembrane and tyrosine kinase domains. Expression of the alternatively spliced isoform Fgfr2-IIIc remains intact. Previous studies have shown that Fgfr2-IIIb is highly expressed in the basal keratinocyte layer (Revest et al., 2001; Mailleux et al., 2002). By contrast, Fgf7 and Fgf10, the main ligands for this receptor, are expressed in the dermis, consistent with a role in mesenchymal-epithelial signalling during skin development (Mason et al., 1994; Beer et al., 1997; Mailleux et al., 2002). However, to date, the functional role of

Fgf/Fgfr2-IIIb signalling in epidermal and hair follicle development is not well understood.

We show that mice null for Fgfr2-IIIb have a severe epidermal hypoplasia resulting from a loss of keratinocyte proliferative capacity. Despite the extremely thin suprabasal layer, epidermal differentiation and establishment of barrier function appears to proceed normally. Mice deficient for Fgf10 show a similar but less severe phenotype. *Fgfr2-IIIb*$^{-/-}$ mice also show a reduced and abnormal development of hair follicles, a defect that is not recapitulated in the *Fgf10*$^{-/-}$ mice. Transplantation of *Fgfr2-IIIb*$^{-/-}$ skin also reveals abnormal hair type formation. A model to explain these finding is presented.

Materials and methods

Mouse strains and tissue collection

Fgfr2-IIIb$^{-/-}$ mice have been previously described (Revest et al., 2001). *Fgf10*$^{-/-}$ mice have been previously described (Min et al., 1998) and were kindly provided by D. Ornitz (Washington University Medical School, St Louis, MO). Pregnant mice obtained by timed matings were injected intraperitoneally with 5 mg of 5′-Bromo-deoxyuridine (BrdU) per 20g mouse. Embryos were harvested 2 hours later at the desired stages of development and genotyped by PCR using genomic DNA isolated from yolk sac. Back skin was dissected and either frozen in OCT compound (Sigma, UK) for cryostat sections (10 μm), or fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated and embedded in wax for cutting 5 μm sections. Alternatively, whole embryos were fixed in 95% ethanol/1% acetic acid overnight at 4°C and embedded in wax for saggital sections.

Histology, in situ hybridisation and immunohistochemistry

Histology and in situ hybridisation were carried out using standard procedures and as previously described (Revest et al., 2001). Probes used were *Bmp4*, *Shh* and *Lef1* (Revest et al., 2001; Mailleux et al., 2002).

Wax sections of back skin were dewaxed in xylene, rehydrated through graded alcohols, and used for detection of loricrin (BabCO, USA) or Ki67 (Novocastra, USA). Sections were boiled twice for 10 minutes in 0.01 M citrate buffer (pH 6) then blocked with swine serum (1:25). All dilutions were in PBS. Anti-loricrin (1:250) or anti-Ki67 (1:200) was applied for 40 minutes at room temperature. A biotinylated swine anti-rabbit antibody (DAKO, UK) was applied (1:500) for 40 minutes, followed by streptavidin-peroxidase for 40 minutes (1:500). A DAB substrate kit (Vector Labs, USA) was used for detection.

Saggital wax sections of embryos were dewaxed in xylene, rehydrated through graded alcohols, and used for co-detection of BrdU and keratin 14 (K14). Sections were hydrolysed for 8 minutes in 1M HCl at 60°C, then blocked with goat serum (1:25). Anti-BrdU (Abcam, USA) and anti-K14 (BabCO, USA) antibodies were applied (1:1000) for 35 minutes at room temperature. A peroxidase-conjugated goat anti-rat antibody (BrdU) (1:200) and an alkaline phosphatase-conjugated goat anti-rabbit antibody (K14) (1:25) were co-incubated for 35 minutes. A DAB substrate kit (Vector Labs, USA) was used for detection of BrdU and an alkaline phosphatase kit (Vector Labs, USA) was used for detection of K14.

For immunofluorescence, cryosections were fixed for 10 minutes in cold acetone and blocked with goat serum (1:25). Anti-keratin 10 (Chemicon, UK)(1:500), anti-K14 and anti-nidogen (Chemicon, UK) (1:500) antibodies were co-incubated for an hour followed by goat anti-IgG1-TRITC (K10) and goat anti-rabbit-FITC with goat anti-rat-TRITC (K14 and nidogen, respectively) (1:100). Cell nuclei were labelled by using Hoechst stain (1:500) with the secondary antibodies.

Quantitative histomorphometry

The percentage of BrdU-positive cells in the basal layer of the interfollicular epidermis was determined by counting three microscopic fields on two slides from at least three different embryos at each stage of development for both wild-type and *Fgfr2-IIIb*$^{-/-}$ mice. The thickness of the epidermis was determined by measuring the length and area on the same slides using NIH Image 1.61. The number of hair follicles per unit length of epidermis, together with their morphological stage, was determined on the basis of defined morphological criteria using Haematoxylin and Eosin stained wax sections of E17.5 embryos (Hardy, 1992; Paus et al., 1999). Every tenth section from each specimen was analysed, thus ensuring that the microscopic field contained new hair follicles. Five to 20 longitudinal sections per embryo were taken from the same anatomical site and analysed: five *Fgfr2-IIIb*$^{-/-}$ embryos were compared with eight wild-type littermates; *Fgf10* wild type n=4, *Fgf10*$^{-/-}$ n=4. All sections were analysed at 100× magnification, means and s.e.m. were calculated from pooled data. Differences were judged significant if P<0.05, as determined by Student's t-test.

Skin permeability assay

Embryos were dissected, incubated for 1-5 minutes in methanol, rinsed in PBS, and then incubated for 2 hours in 0.1% Toluidine Blue. Embryos were rinsed in PBS and photographed (Hardman et al., 1998).

Skin grafts

To perform skin grafts, a full-thickness skin disc (6 mm) was excised from male nude mice. A similar biopsy punch from the back skin of E18.5 knockout and wild-type littermate embryos was applied onto the recipient fascia. Steri-strips, circular plasters and gauze bandages (Southern Syringe Service, London, UK) were applied to secure the grafts in place. Dressings were removed after 7 days. Grafts were excised and bisected along the anterioposterior axis 21 days after grafting. Half of each graft was fixed in 4% paraformaldehyde overnight and processed for histological examination as above. Hairs were plucked from the other half of the graft for microscopic analysis.

Results

Impaired skin development in mice deficient for Fgfr2-IIIb

Fgfr2-IIIb$^{-/-}$ mice do not survive beyond birth. However, at term the skin of these mice is clearly abnormal with a semi-transparent appearance (Revest et al., 2001). From E13.5 onwards, the ectoderm of wild-type mice expands into an epidermis consisting of several distinct layers of differentiated cells. By contrast, *Fgfr2-IIIb*$^{-/-}$ foetuses show an absence of proper epidermal stratification and produce an epidermis of only one to three cells in depth (Fig. 1A-F). In addition, the basal cells are flatter and less densely packed than in the wild-type epidermis. Image analysis on multiple skin sections shows that the epidermis of E14.5 to E18.5 *Fgfr2-IIIb*$^{-/-}$ mice is consistently 2.5 to 3.5 times thinner than that of wild-type littermates (Fig. 1H). These findings are consistent with previous observations of the epidermis of mice with compromised Fgfr2-IIIb function (Celli et al., 1998; De Moerlooze et al., 2000; Li et al., 2001; Revest et al., 2001). To investigate the mechanisms that underlie the failure of skin stratification, we assessed the number of proliferating and apoptotic cells. Cell proliferation was quantified on skin sections from embryos at several stages of development by injecting pregnant females with BrdU 2 hours prior to sacrifice, and subsequently immunostaining for BrdU incorporation (Fig. 1A-F). To facilitate identification of the basal keratinocytes, the

sections were also immunostained for K14. A significant reduction in the number of BrdU-positive cells was observed in the basal layer of the interfollicular epidermis of *Fgfr2-IIIb*$^{-/-}$ mice from E14.5 to E16.5, a period that normally correlates with the development of stratification (Fig. 1A-D,G, and data not shown). At E18.5, the difference in the number of proliferating keratinocytes was no longer significant (Fig. 1E-G). There was no significant change in the amount of proliferation observed in the underlying dermis. To check whether the reduced thickness of *Fgfr2-IIIb*$^{-/-}$ mouse skin was exacerbated by an increase in apoptosis, we also analysed sections using an in situ oligo-ligation labelling assay. No significant changes in apoptotic cell numbers were detected in *Fgfr2-IIIb*$^{-/-}$ mice compared with their wild-type littermates (data not shown).

A major ligand of Fgfr2-IIIb is Fgf10, and mice deficient for this ligand show many of the phenotypic abnormalities associated with *Fgfr2-IIIb*$^{-/-}$ mice (Ohuchi et al., 2000). The epidermis of *Fgf10*$^{-/-}$ mice is thinner than that of wild-type mice, but the hypoplasia is relatively mild, resulting in a significantly thicker epidermis than that seen in *Fgfr2-IIIb*$^{-/-}$ mice (Fig. 2).

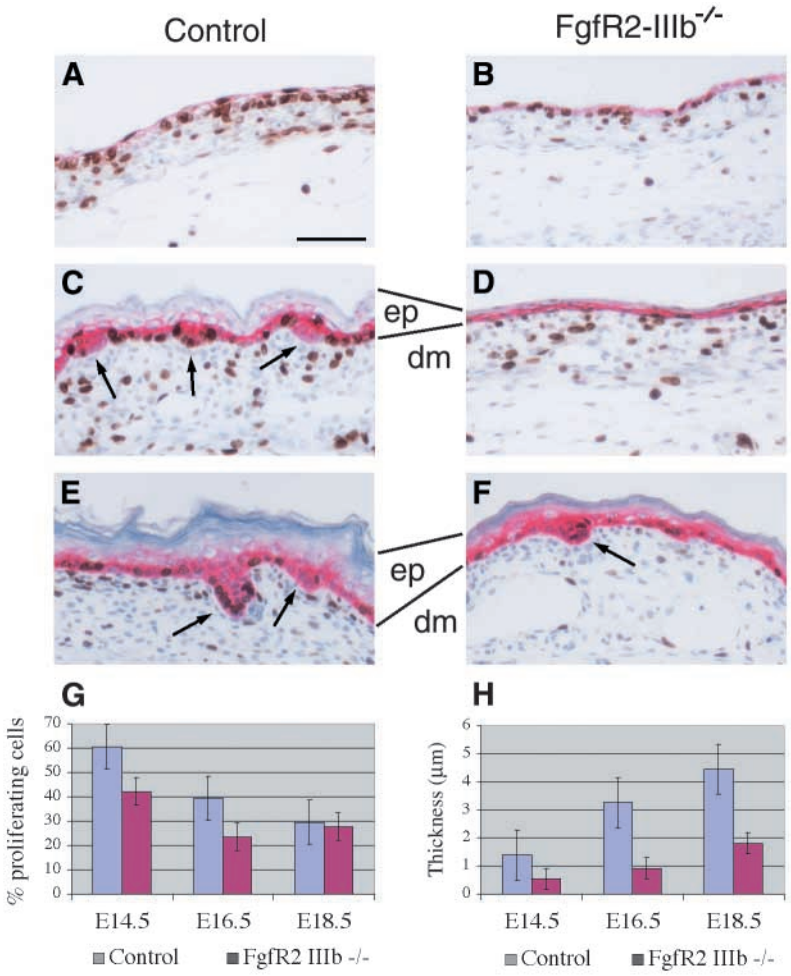

Fig. 1. The skin of *Fgfr2-IIIb*$^{-/-}$ mice is hypoplastic and shows a reduction in cell proliferation. (A,C,E) Wild-type embryos. (B,D,F) *Fgfr2-IIIb*$^{-/-}$ embryos. (A-F) Co-immunostaining for BrdU (brown) and K14 (red) on embryonic saggital sections through the back skin at E14.5 (A,B), E16.5 (C,D) and E18.5 (E,F), showing that the epidermis of the *Fgfr2-IIIb*$^{-/-}$ mice is severely hypoplastic. The arrows indicate hair placodes or follicles. (G) Quantitation of BrdU-positive cells in the basal layer of different embryo stages (bars indicate standard errors); cell proliferation is significantly decreased in the basal layer keratinocytes of *Fgfr2-IIIb*$^{-/-}$ embryos. (H) The interfollicular epidermis of wild-type skin is significantly thicker than that of *Fgfr2-IIIb*$^{-/-}$ littermates at all stages examined. dm, dermis; ep, epidermis. Scale bar: 5 µm.

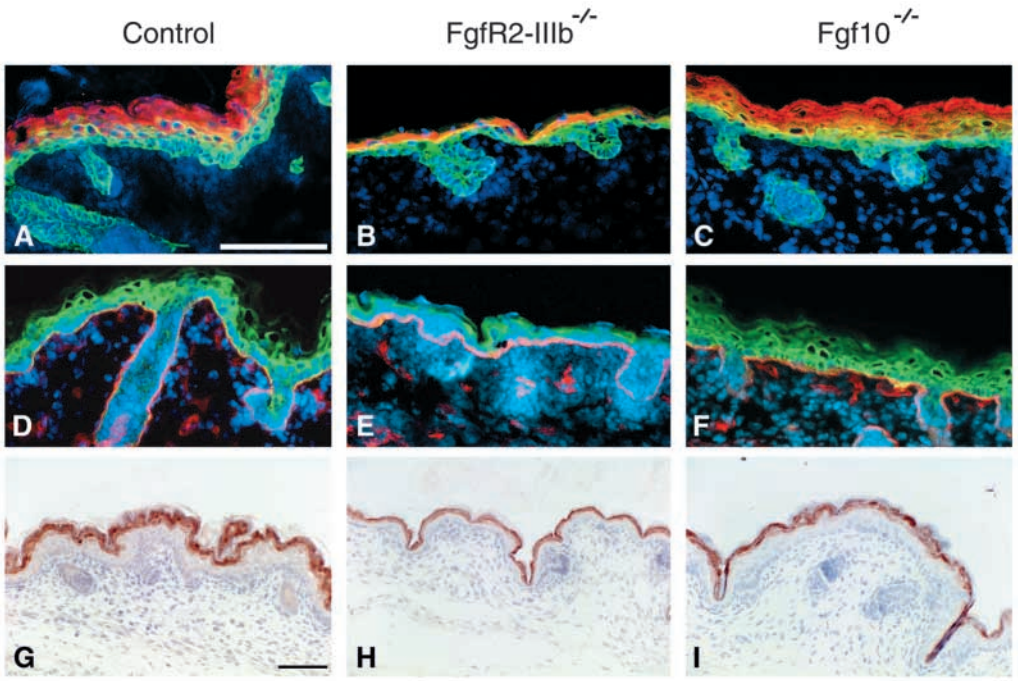

Fig. 2. Detection of differentiation markers in the epidermis of *Fgfr2-IIIb*$^{-/-}$, *Fgf10*$^{-/-}$ and control mice at E17.5. A,D,G and B,E,H are dorsal skin sections from control and *Fgfr2-IIIb*$^{-/-}$ littermate embryos, respectively. C,F,I are dorsal skin sections from *Fgf10*$^{-/-}$ mice. (A-C) Immunostaining for K14 (green) and K10 (red). (D-F) Immunostaining for nidogen (red) and K14 (green). (G-I) Skin sections were stained for loricrin (brown). Although the epidermis was much thinner in *Fgfr2-IIIb*$^{-/-}$ embryos compared with wild-type littermates and *Fgf10*$^{-/-}$ embryos, the markers of epidermal differentiation were present. Scale bar: 10 µm.

To characterise the degree of epidermal differentiation in both *Fgfr2-IIIb*$^{-/-}$ and *Fgf10*$^{-/-}$ mice, the presence of proteins expressed in different layers of the stratified epidermis was determined by immunostaining. Nidogen was used to visualise the basement membrane (Fig. 2D-F), K14 as a marker of the basal layer of keratinocytes (Fig. 2A-F), K10 for the spinous layer (Fig. 2A-C), and loricrin for the granular and cornified layers (Fig. 2G-I). The results show that these differentiation-specific proteins were all expressed in the hypoplastic epidermis of both strains of mutant mice, demonstrating that there is little effect on the programme of epidermal differentiation.

A major function of the epidermis is to form an effective barrier against water loss and invasion by foreign bodies such as microorganisms. The barrier function is normally conferred by the stratum corneum, the terminally differentiated outer layer of epidermis. Although the epidermis of *Fgfr2-IIIb*$^{-/-}$ mice expresses loricrin, a marker of terminal differentiation, we also tested for acquisition of a functional barrier. As a measure of epidermal barrier function, a vital-dye exclusion assay was performed on foetuses at different stages of development (Fig. 3). Normal mouse embryos become impermeable to the vital dye by E18.5. Despite a severely hypoplastic epidermis, the *Fgfr2-IIIb*$^{-/-}$ mouse embryos established an effective barrier, as judged by dye exclusion. As anticipated, the less affected *Fgf10*$^{-/-}$ mouse embryos also established a barrier. Moreover, the spatial/temporal pattern for establishing the impermeable barrier was maintained (Fig. 3 and data not shown).

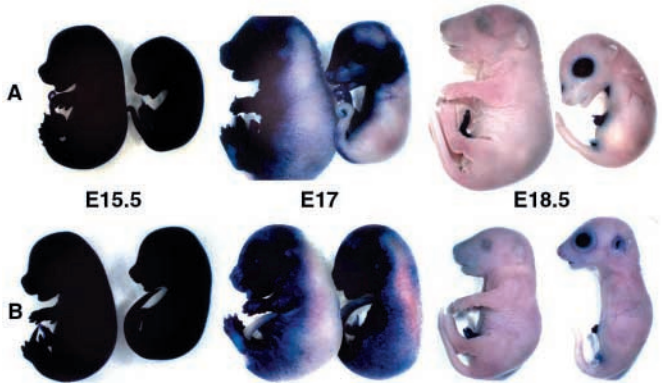

Fig. 3. Assessment of skin barrier function. (A) *Fgfr2-IIIb*$^{-/-}$ embryos paired with wild-type littermates. (B) *Fgf10*$^{-/-}$ embryos paired with wild-type littermates. Dye exclusion shows that the impermeable barrier is established normally in the mutant mice. Pairwise comparisons were photographed at the same magnification.

Hair follicle development in Fgfr2-IIIb$^{-/-}$ and Fgf10$^{-/-}$ mice

Hair follicles develop from placodes that appear as a dense patch of mesenchymal cells beneath a shallow invagination of epidermis (arrows in Fig. 1A-F). To obtain an initial measure of hair follicle distribution over the body of the embryo, we used whole-mount in situ hybridisation with a probe to the transcription factor *Lef1*, which is strongly expressed in the hair follicle placode. Similar results were obtained by staining whole embryos with Carmine Red, indicating that *Lef1* expression was not affected by Fgfr2-IIIb abrogation, and was therefore a suitable marker of hair placode and follicle development. At E13.5 and E14.5, induction of both vibrissal and pelage hair follicles was clearly visible on control embryos. By contrast, no pelage hair follicles were discernible on the surface of knockout embryos, although vibrissal follicles were present at both E13.5 and E14.5 (Fig. 4 and data not shown). However, the pattern of vibrissae on the muzzle was altered, with only three rows of vibrissae rather than the normal five. In addition, there were fewer vibrissae per row (Fig. 4C,D). The vibrissae located above the eye and on the cheek were present on *Fgfr2-IIIb*$^{-/-}$ embryos, but the follicle normally located just below the eye was not (black arrow Fig. 4C). Rather, there was an ectopic vibrissa on the side of the eye on *Fgfr2-IIIb*$^{-/-}$ embryos (Fig. 4D, arrow).

During early skin development, *Lef1* is expressed throughout the ectoderm, as well as in the developing pelage hair placodes (Fig. 4A, Fig. 5A,B). At later stages of foetal development, pelage hair placodes form on the dorsum of the *Fgfr2-IIIb*$^{-/-}$ foetus, but in reduced numbers. In situ hybridisation analyses using probes for *Lef1*, *Shh* and *Bmp4* showed that these genes are expressed in the hair follicles of *Fgfr2-IIIb*$^{-/-}$ mice in a similar pattern to those in wild-type mice (Fig. 5). *Lef1* expression was found initially in the placode, and then in the matrix and dermal papilla of the hair follicle, as previously described (Zhou et al., 1995; DasGupta and Fuchs, 1999) (Fig. 5A-D). *Shh* expression appeared in the hair follicle germ and, subsequently, in the hair matrix (Fig. 5E-H), whereas *Bmp4* was expressed in the dermal mesenchyme (Fig. 5I-L). These observations indicate that pelage

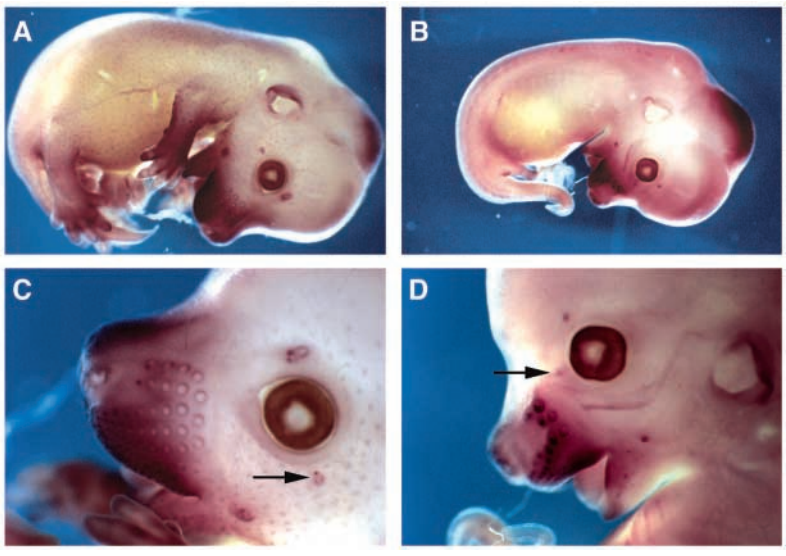

Fig. 4. Hair follicle induction as revealed by whole-mount in situ hibridisation for *Lef1*. Wild-type (A,C) and *Fgfr2-IIIb*$^{-/-}$ (B,D) littermate E14.5 embryos. No pelage hair placodes and a reduced number of vibrissae were visible on the skin of *Fgfr2-IIIb*$^{-/-}$ embryos. By contrast, wild-type embryos show both vibrissae and pelage hair placodes on the muzzle and body, respectively. Black arrows indicate the vibrissa located below the eye in wild-type embryos that is lost in the mutant embryos, and an ectopic vibrissa in *Fgfr2-IIIb*$^{-/-}$ littermate embryos.

hair follicles show delayed induction and are reduced in number, but morphogenesis appears to be relatively normal at these early stages.

To obtain a more quantitative and temporal analysis of hair follicle development in *Fgfr2-IIIb*$^{-/-}$ and *Fgf10*$^{-/-}$ mice, the first wave of hair follicles in wild-type and mutant littermates was counted and staged at E17.5. The analysis was restricted to the same anatomical area of dorsal skin for all groups analysed. *Fgfr2-IIIb*$^{-/-}$ mice (n=5) showed a highly significant decrease in the density of hair follicles compared with controls (Fig. 6A), whereas *Fgf10*$^{-/-}$ mice showed a smaller decrease that was not statistically significant (Fig. 6B) (Ohuchi et al., 2000). In addition to hair follicle density, the number of follicles at different stages of maturity was assessed using previously described morphological criteria (Hardy, 1992; Paus et al., 1999) (Fig. 6C-L). It was apparent from this analysis that not only was the number of hair follicles on *Fgfr2-IIIb*$^{-/-}$ embryos significantly reduced, but their development was also retarded, consistent with a delay in *Lef1* expression (Fig. 4, Fig. 6C). Although approximately 10% of the hair follicles on wild-type embryos had reached stage 4 of morphogenesis, no follicles of this stage were found in sections from *Fgfr2-IIIb*$^{-/-}$ embryos. The great majority of follicles on the mutant skin were at stages 1 or 2. Interestingly, although the follicles are delayed in their maturation and are reduced in number, they appear to contain a similar number of proliferating cells (Fig. 6E-K). In addition, some hair follicles in the mutant mice showed an abnormal appearance (Fig. 6L). By contrast, *Fgf10*$^{-/-}$ mice did not show any discernible difference in the density or staging of the pelage hair follicles compared with wild-type littermates (Fig. 6D).

Hair development in skin grafts of Fgfr2-IIIb$^{-/-}$ mice

As *Fgfr2-IIIb*$^{-/-}$ mice do not survive beyond birth, it was not possible to determine whether hair follicle development was only delayed or whether it was also abnormal. To address this issue, full-thickness skin grafts from E18.5 *Fgfr2-IIIb*$^{-/-}$ (n=4) and wild-type littermates (n=10) were transplanted onto the backs of nude mice, and hair development assessed 3 weeks after grafting. *Fgfr2-IIIb*$^{-/-}$ grafts displayed a reduced number of hair follicles, as expected, but histological examination of longitudinal sections showed that the follicles had lost their regular pattern of alignment (Fig. 7A-D). This lack of organised orientation was also detectable on early hair follicles at E18.5 (Fig. 6K,L). In addition, many hair follicles failed to penetrate the subcutis, and some appeared somewhat cystic in nature. However, at higher magnification the thickness of the interfollicular epidermis was similar to that of wild type, at 1 to 2 μm (Fig. 7A,B, insert). However, this represents a reduction in thickness of the normal wild-type epidermis rather than an increase in thickness of the mutant epidermis. The level of proliferation was similar in the epidermis and hair follicles of both wild-type and mutant grafts, as shown by Ki67 immunostaining (Fig. 7C,D and data not shown). A topical view of *Fgfr2-IIIb*$^{-/-}$ grafts clearly showed that the hair was more sparsely distributed as expected, but additionally, the hair shafts appeared to be finer (Fig. 7E,F). Light microscopy confirmed that the hair shafts were thin, and, moreover, guard, auchene, zig-zag and awl hairs were absent on the knockout grafts, whereas they were present on all wild-type grafts (Fig. 7G,H). Morphologically, the hairs from *Fgfr2-IIIb*$^{-/-}$ mice

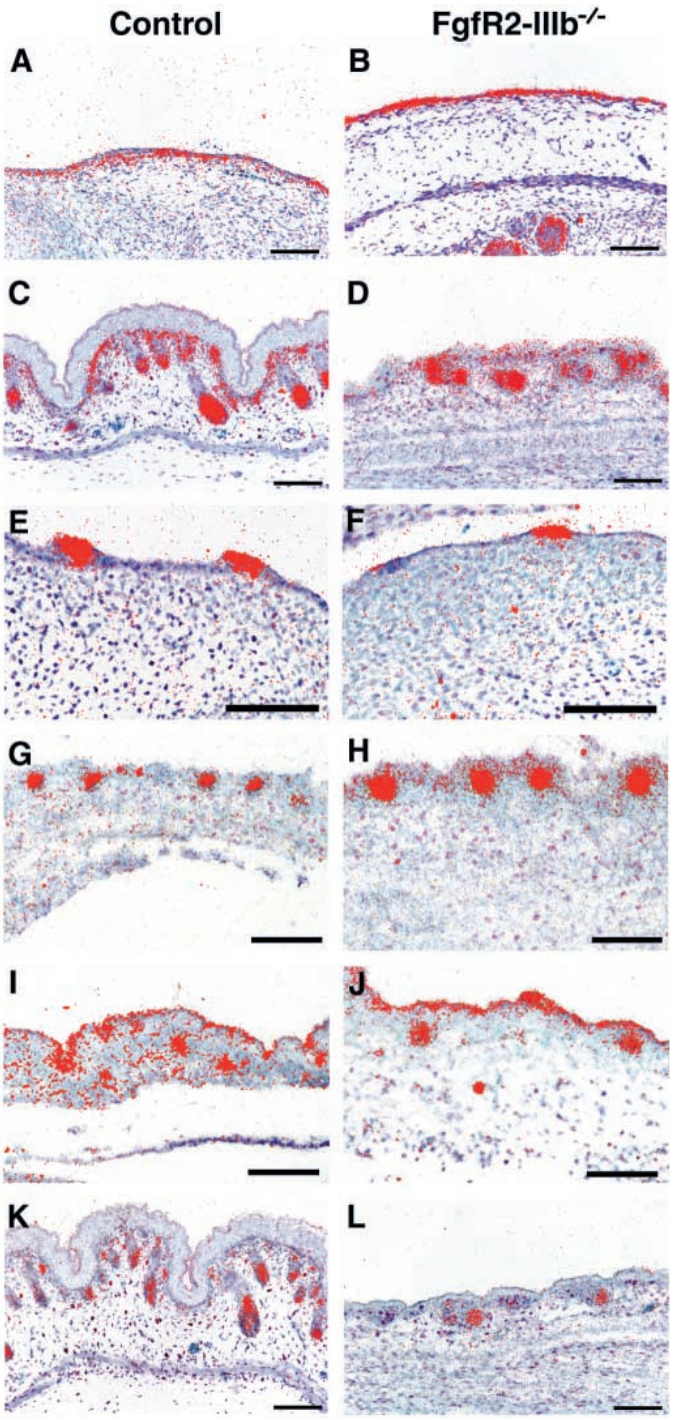

Fig. 5. *Lef1*, *Shh* and *Bmp4* expression in the developing hair follicles. In situ hybridisation analysis of tissue sections. Panels A,C,E,G,I,K are wild-type and B,D,F,H,J,L are *Fgfr2-IIIb*$^{-/-}$ littermate embryos. (A,B) At E13.5, *Lef1* is expressed throughout the ectoderm and forming hair placodes. (C,D) *Lef1* expression at E19.5 in the matrix and dermal papilla of mature hair follicles. (E,F) *Shh* expression at E13.5. (G,H) At E17.5, *Shh* is expressed in the hair matrix. At E16.5 (I,J) and at E19.5 (K,L), *Bmp4* is expressed in the dermal mesenchyme and hair matrix. The silver grains in the dark-field images were selected, coloured red with Adobe Photoshop 5.0, then superimposed onto bright-field images. Scale bars: 100 μm.

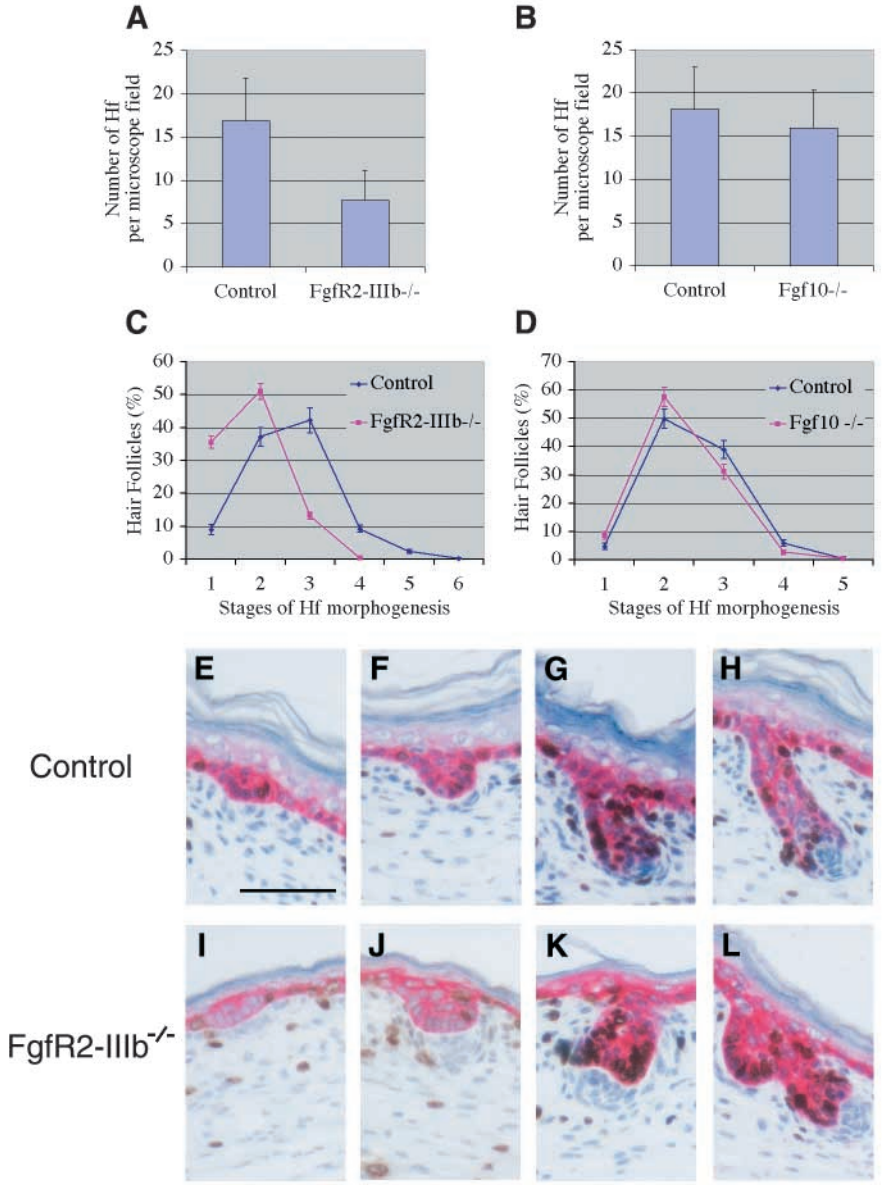

Fig. 6. Hair follicle morphogenesis is retarded in *Fgfr2-IIIb*$^{-/-}$ but not in *Fgf10*$^{-/-}$ mice at E17.5. (A,B) Numbers of hair follicles per microscopic field in section of back skin of *Fgfr2-IIIb*$^{-/-}$ and *Fgf10*$^{-/-}$ embryos, compared with wild-type and heterozygote littermates. A dramatic decline in the number of induced hair follicles was found in *Fgfr2-IIIb*$^{-/-}$ embryos compared with wild-type littermates (mean±s.e.m., $P<0.001$), whereas *Fgf10*$^{-/-}$ embryos showed no significant decrease. (C,D) Percentage of hair follicles at distinct stages of morphogenesis. Morphogenesis is retarded in *Fgfr2-IIIb*$^{-/-}$ embryos compared with wild-type littermates ($P<0.0005$ for all points), whereas *Fgf10*$^{-/-}$ embryos do not show this difference ($P\geq0.03$ for all points). Because of slight strain differences, each knockout strain was compared with littermate controls. (E-L) Immunostaining for BrdU (brown) and K14 (red) through the back skin of E18.5 embryos. (E-H) Control and (I-L) *Fgfr2-IIIb*$^{-/-}$ hair placodes/follicles at stage 1 (E,I), 2 (F,J), 3 (G,K) and 4(H). L shows an abnormal (stage 3) hair follicle. Scale bar: 5 μm.

were much finer than normal pelage hairs, and lacked any regular arrangement of air cells. Thus, none of the hairs from *Fgfr2-IIIb*$^{-/-}$ grafts resembled any of the known types of pelage hair, suggesting that Fgf signalling is also necessary for normal hair morphogenesis.

Discussion

Fgfr2-IIIb signalling in the epidermis

Previous studies have shown that normal skin development requires Fgf signalling from the dermis to Fgfr2-IIIb in the epidermis (Celli et al., 1998; De Moerlooze et al., 2000; Li et al., 2001; Revest et al., 2001). We have extended these studies to show that the abnormally thin epidermis seen in the absence of functional Fgfr2-IIIb signalling is caused by a reduction in keratinocyte proliferation. Although the basal layer of K14-positive keratinocytes proliferates at a rate compatible with foetal growth, the increase in proliferation that is required for building a stratified multi-layered epidermis does not occur. This might suggest that two levels of proliferation control exist, one that is established early and allows normal ectodermal and subsequent basal cell growth, and a second level of control that facilitates vertical expansion of the epidermis and may correlate with the appearance of the transit amplifying cells (Watt, 2001). Despite the loss of proliferative potential, the keratinocytes that form a one- to two-cell layer above the basal layer are able to express the proteins of early (K10) and late (loricrin) terminal differentiation.

Dermally produced Fgf10 is a major ligand for epidermal Fgfr2-IIIb, and mice deficient for Fgf10 also have a hypoplastic epidermis, albeit much less severe than that seen in the *Fgfr2-IIIb*$^{-/-}$ mice (Ohuchi et al., 2000; Suzuki et al., 2000; Tao et al., 2002). This suggests that some other members of the Fgf family may partially compensate for the loss of Fgf10. The best candidate would be Fgf7, which is also expressed in the dermis and signals through Fgfr2-IIIb (Werner et al., 1992; Mason et

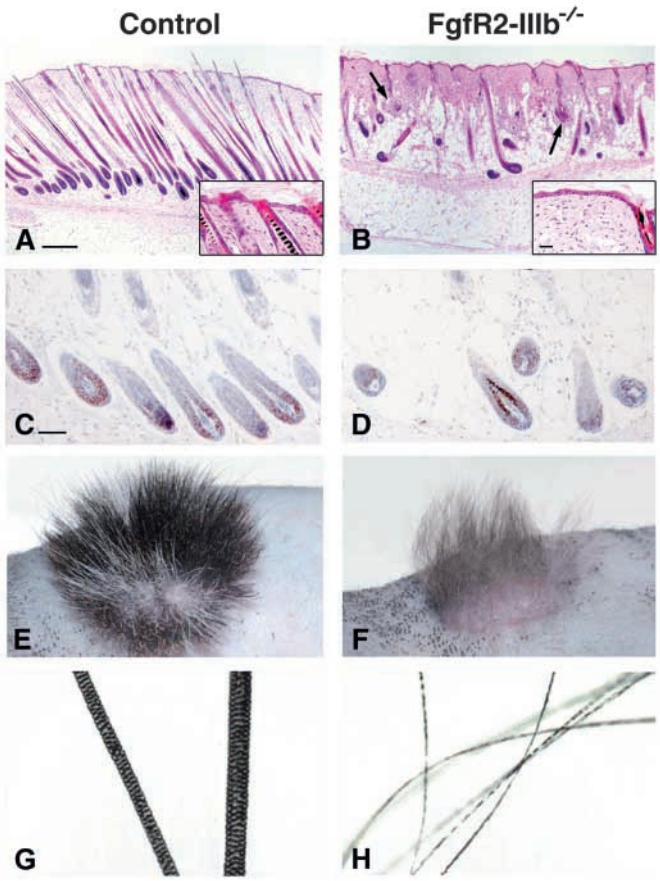

Fig. 7. Hair development in skin grafts from *Fgfr2-IIIb*$^{-/-}$ and wild-type E18.5 embryos three weeks post grafting. A,C,E,G and B,D,F,H show skin grafts from wild-type and *Fgfr2-IIIb*$^{-/-}$ littermate embryos, respectively. (A,B) Haematoxylin and Eosin stained longitudinal sections reveal a reduced number of hair follicles, a loss of follicle alignment and some follicles associated with cysts (arrows) in *Fgfr2-IIIb*$^{-/-}$ grafts. Insert shows higher magnification of the interfollicular epidermis. (C,D) Proliferation in hair follicles detected by Ki67 immunostaining (brown). (E,F) Grafted skin on the back of nude mice. Note the sparse thin hairs growing on the *Fgfr2-IIIb*$^{-/-}$ grafts. (G,H) Light microscopy showing abnormal hair that lacks any regular arrangement of air cells from the *Fgfr2-IIIb*$^{-/-}$ graft (H). Scale bars: in A,B, 100 μm; in insert, 1 μm; in C,D 5μm.

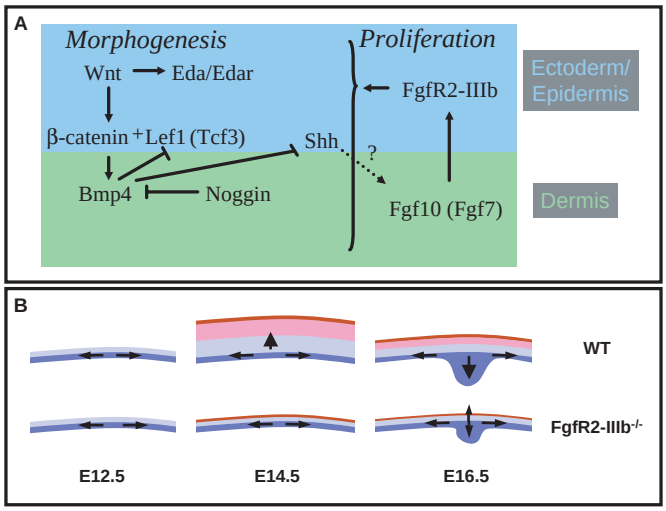

Fig. 8. Model depicting the signalling pathways implicated in hair follicle morphogenesis. (A) Hair placode initiation requires activation of the Wnt/β-catenin and Eda/Edar signalling pathways. Loss of Lef1 impairs pelage hair formation and is essential for whisker induction by activating Bmp4. However, noggin has a negative regulatory effect on Bmp4, which in turn appears to negatively regulate Lef1 expression, although there is some evidence that Bmp4 can positively regulate Lef1 in whisker formation. Shh is not apparently required for placode induction but rather for maturation of follicles. Shh may also act positively on *Fgf10* expression to maintain follicle growth. In addition, the absence of Fgfr2-IIIb signalling has a more profound effect on hair follicle number, and after grafting the follicles generate abnormal hairs suggesting a late effect on hair morphology. (B) The absence of Fgf/Fgfr2-IIIb signalling limits the number of epidermal precursor cells required for epidermal stratification, forcing proliferation of basal keratinocytes to be primarily channelled into lateral expansion. The lack of suprabasal cells, and the reduced proliferation rate in the mutant epidermis, causes more basal keratinocytes to be directed towards maintenance of the epidermis, thereby reducing the number available for the development of hair follicles.

al., 1994; Werner et al., 1994). Surprisingly, mice deficient for Fgf7 show no developmental skin abnormalities, nor any significant delay in wound repair, despite a large increase in Fgf7 transcription that is found in normal skin following wounding (Werner et al., 1992; Werner et al., 1994; Guo et al., 1996). More recently, Fgf22 has been shown to be expressed in the epidermis and hair follicles (Nakatake et al., 2001; Beyer et al., 2003). Its strong structural similarity to Fgf7 and Fgf10 make it another potential ligand for Fgfr2-IIIb that might act redundantly with Fgf7 and/or Fgf10.

The apparently normal expression of proteins associated with keratinocyte terminal differentiation is reflected by the attainment of a functional barrier, as measured by dye exclusion. The barrier starts to form from E16 at specific epidermal sites, and then spreads around the embryo as a moving front with the eyelids, ear tips and ventral neck region forming last at E17.5 (Hardman et al., 1998). Despite the lack of significant epidermal stratification, the barrier function in *Fgfr2-IIIb*$^{-/-}$ mice is achieved at the same stage as in wild-type mice (Fig. 3).

Fgfr2-IIIb signalling in hair follicle development

The analysis of hair follicle development showed that mice deficient for Fgfr2-IIIb exhibit defects both in the number and distribution of hair follicles (Fig. 6), as well as producing abnormal hairs (Fig. 7). A reduced number of hair follicles was apparent for both vibrissal and pelage hair. Interestingly, mice deficient for Fgf10 do not show a noticeable pelage hair phenotype (Suzuki et al., 2000), although some disruption of vibrissae similar to in the Fgfr2-IIIb nulls has been reported (Ohuchi et al., 2003). Following transplantation of *Fgfr2-IIIb*$^{-/-}$ skin onto nude mice, the hairs that grow are abnormally thin and show none of the patterning of air cells characteristic of normal pelage hairs.

An early marker of hair placode initiation is expression of the transcription factor Lef1 (Fig. 4). Gene ablation studies have shown that Lef1 is necessary for the proper development of mammary glands, vibrissae and pelage hairs (van Genderen

et al., 1994). Mice lacking this gene showed a reduced hair follicle number (van Genderen et al., 1994). However, the *Fgfr2-IIIb*$^{-/-}$ phenotype is not the same as that found in *Lef1*$^{-/-}$ mice, as the latter have no vibrissae and the majority of pelage hairs fail to form. This suggests that Lef1 is more likely to play a role in hair follicle induction/morphogenesis, whereas Fgfr2-IIIb is more likely to regulate the numbers of hair placodes induced. Several lines of evidence support this view (Fig. 8).

Recent studies have shown that Wnt signalling and the expression of catenin beta are essential for hair follicle induction (Gat et al., 1998; Huelsken et al., 2001; Andl et al., 2002). This would be consistent with signalling through the canonical Wnt pathway, where the transduced Wnt signal leads to the nuclear accumulation of Lef1 complexed with β-catenin, which forms part of a transcriptional complex. The small number of hair follicles formed in *Lef1*$^{-/-}$ mice might reflect the presence of another Lef1 family member, such as Tcf3, that could substitute for Lef1; Tcf3 also forms complexes with β-catenin (Zhou et al., 1995; Gat et al., 1998; DasGupta and Fuchs, 1999). There is also evidence to suggest that mesenchymally expressed *Bmp4* regulates *Lef1* activity, and that noggin negatively regulates *Bmp4*, providing a signalling network that modulates the patterning and morphogenesis of hair follicles (Fig. 8) (Kratochwil et al., 1996; Botchkarev et al., 1999). However, signalling through ectodysplasin (Eda) and its receptor (Edar) is also essential for follicle induction and appears to function upstream of Lef1, but it is not clear how Eda signalling relates to that of the Wnt/Lef1 pathway (Laurikkala et al., 2002). Previous studies have shown that although *Shh* is not essential for hair follicle induction, it is necessary for their subsequent maturation, indicative of a role in follicle morphogenesis (St-Jacques et al., 1998). By contrast, defective Fgf/Fgfr2-IIIb signalling has profound effects on the number and distribution of hair follicles. In addition, it is also clear that hair morphology is affected, thereby indicating a late effect on hair growth.

We propose that these effects are most easily explained by a regulatory effect of Fgfr2-IIIb signalling on the number of precursor cells needed for hair follicle formation. Hence, once a follicle placode is initiated the temporal expression of *Lef1*, *Shh* and *Bmp4* appear relatively normal. As hair follicle development in *Shh* deficient mice appears to arrest at an early stage, it is possible that *Shh* acts positively on *Fgf10* expression to maintain follicle growth (Fig. 8A). This would be analogous to late limb bud outgrowth, where *Shh* acts positively to maintain *Fgf10* in the limb bud mesenchyme (Ohuchi et al., 2000).

The abnormal hair morphology associated with grafts derived from *Fgfr2-IIIb*$^{-/-}$ embryos might result indirectly from the reduced proliferative potential of the epithelial cells, causing too few cells to be available for producing the first wave of hair follicles and resulting in many follicles failing to fully penetrate the subcutis. However, once established, grafted mutant hair follicles show similar levels of proliferation (Fig. 7C,D). If other signalling elements were functioning properly, attempts to drive the cells along the usual differentiation pathway would lead to different types of hair. Instead, only one abnormal hair type was found on grafts from the mutant mice, suggesting that there may also be a direct requirement for Fgf signalling for the patterning and morphology of the different hair types.

Taken together, a common theme appears to be emerging, where the loss of epidermal stratification and compromised hair development derive from a common Fgf-mediated function. We would suggest that proliferation of the ectoderm and the subsequent basal cells is maintained at a sufficient rate to keep pace with the growing embryo, but that the proliferation required to allow the build-up of a multi-layered stratified epithelium is severely restricted. This implies that there may be two pathways regulating cell numbers in the epidermis. One that controls ectoderm and stem cell division on the basement membrane and is Fgf/Fgfr2-IIIb independent, and another that is mediated through Fgf/Fgfr2-IIIb signalling, and that is important for epidermal stratification and normal hair follicle development. The suprabasal cells of the epidermis provide a reservoir to meet the major needs of the developing epidermis, allowing proliferation of basal keratinocytes to be channelled into lateral expansion and the developing hair follicle placodes. The dearth of suprabasal cells in the mutant could require more basal cells to contribute to the maintenance of the epidermis, thereby reducing the number available for lateral expansion (Fig. 8B). This explains the reduction in basal cell density in the mutant and the subsequent reduction in the number of hair follicles.

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