FGF signaling is required for initiation of feather placode development

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Summary

Morphogenesis of hairs and feathers is initiated by an as yet unknown dermal signal that induces placode formation in the overlying ectoderm. To determine whether FGF signals are required for this process we over-expressed soluble versions of FGFR1 or FGFR2 in the skin of chicken embryos. This produced a complete failure of feather formation prior to any morphological or molecular signs of placode development. We further show that *Fgf10* is expressed in the dermis of nascent feather primordia, and that anti-FGF10 antibodies block feather placode development in skin explants. In addition we show that FGF10 can induce expression of positive and negative regulators of feather development and can induce its own expression under conditions of low BMP signaling. Together these results demonstrate that FGF signaling is required for the initiation of feather placode development and implicate FGF10 as an early dermal signal involved in this process.

Supplemental data available online

Key words: FGF signaling, Feather development, FGFR1-Fc, FGFR2-Fc, Su5402, Fgf10, Placode induction

Introduction

The body of most vertebrates is covered by cutaneous appendages such as feathers, hairs or scales. During embryogenesis these organs form from the epidermis and the underlying dermis through conserved mechanisms involving a series of reciprocal inductive interactions between these two tissues. A first signal from the dermis initiates the formation of local thickenings in the overlying epithelium called placodes. Once formed, the placodes signal back to the dermis and induce the formation of dermal condensations. Reciprocal signaling between these two structures then controls further development of the appendage rudiments. Spacing of buds is thought to result from the interplay between a positively acting wave of induction, propagating through the developing tract, and negative signals from already established placodes, which prevent placode induction in the near vicinity. This mechanism thus promotes the formation of regularly spaced feathers, hairs and scales (reviewed by Millar, 2002; Sengel, 1976; Sengel, 1990).

Members of the BMP, Delta/Notch, SHH, WNT and FGF families of signaling molecules are expressed during cutaneous appendage development. Several of these have been shown to play important roles in the communication between epithelial and mesenchymal cells (reviewed by Millar, 2002). However, the identity of the first dermal signal still remains unknown. As a consequence the molecular mechanisms controlling the earliest stages of appendage development also remain poorly defined.

Fibroblast growth factors (FGFs) play essential roles in many aspects of embryogenesis and have previously been implicated as positive regulators of hair and feather development (reviewed by Ornitz and Itoh, 2001). Beads soaked in recombinant FGF protein were sufficient to induce feather buds in skin explants isolated from scaleless mutant chick embryos that lacked most feathers because of an ectodermal defect (Song et al., 1996). FGF-soaked beads also induced ectopic buds in explants from apteric regions of wild-type embryos and bud fusions in regions of ongoing bud development (Widelitz et al., 1996). Expression during epithelial appendage formation has so far only been analyzed for a subset of the 22 members of the FGF family. Previous studies have shown that *Fgf2* and *Fgf4* are expressed in the feather placodes (Jung et al., 1998; Song et al., 1996). Whether they are already expressed prior to placode formation is a matter of debate. Expression of *Fgf10* has recently been described in the mesenchyme of feather and whisker hair primordia, but no expression was detected prior to placode formation (Ohuchi et al., 2003; Tao et al., 2002). *Fgf5* and *Fgf7* are expressed during hair follicle morphogenesis but are not required for the initiation of hair development (Guo et al., 1996; Hebert et al., 1994). Based on these data it has been suggested that FGFs may function as secondary inducers that promote hair and feather development but are unlikely to be involved in the initial induction of the placodes.

We have examined the function of fibroblast growth factor (FGF) signaling in the initiation of feather development in the chick. For this purpose we have used replication-competent avian retroviruses to over-express secreted dominant negative versions of FGFR1 and FGFR2 in ovo. This caused a complete block of feather development prior to the initiation of feather

placode formation. We further show that *Fgf10* is expressed in the dermis of nascent feather buds and is required for placode induction in skin explants. In addition, we demonstrate that FGF10 can stimulate expression of positive and negative regulators of feather development in the overlying ectoderm and can induce its own expression under conditions of low BMP activity. Together these results demonstrate that FGF signaling is required for the initiation of feather placode development and point towards FGF10 as an early dermal signal involved in this process.

Materials and methods

Embryos

Pathogen free, fertilized White Leghorn eggs (SPAFAS, Charles River, Sulzfeld/Germany) were incubated at 37.5°C in a humidified incubator and embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Production of retroviruses carrying secreted forms of mFGFR1 (IIIc) and mFGFR2 (IIIb)

The RCAS-FGFR1-Fc retrovirus (referred to as RCAS-R1) was generated by fusing the extracellular domains of mouse FGFR1 [splice isoform IIIc; GenBank accession number NM_010206, amino acids 1-30 and 120-367 as described by Werner et al., (Werner et al., 1993)] to the Fc-fragment of mouse-IgG (GenBank accession number AB097849; aa228-463) including a Ser-Ser linker. Similarly, a secreted, Fc-tagged form of mouse FGF-receptor 2 (splice isoform IIIb; referred to as RCAS-R2) was constructed by PCR amplifying the extracellular domains of FGFR2 (GenBank accession number M63503; aa1-258) and the Fc fragment as described by Celli et al., (Celli et al., 1998). Both constructs were cloned into the Slax shuttle vector and then transferred into the RCASBP(A) retroviral vector (Hughes et al., 1987). Viral stocks of 1×10^9 infectious units/ml were purified according to the method of Morgan and Fekete (Morgan and Fekete, 1996). Viral infection and titers were monitored by detection of viral gag protein using the anti-gag antibody AMV3C2 developed by D. Boettiger and obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Science, Iowa City IA52242.

RCAS-R1 virus was initially injected into migrating neural crest cells adjacent to the midbrain at HH8-9, resulting in widespread infection throughout the embryos. These embryos were harvested after 14 days (HH38-40, n=35). In addition, both RCAS-FGFR types were injected into sub-ectodermal mesenchyme of the back at HH18-20. Some of these embryos were incubated until HH38 and assayed for morphological changes in feather development (n=10 each for RCAS-R1 and RCAS-R2). The majority were incubated until HH27-32 and analyzed by whole-mount in situ RNA detection for marker gene expression. For each marker and stage, at least three, but more typically 5-10 infected specimens were examined. Altogether, 15 RCAS-R1- and 17 RCAS-R2-infected embryos were analyzed prior to placode formation (HH27-28), and 116 RCAS-R1- and 52 RCAS-R2-injected embryos were analyzed at HH29-33. Except for 13 embryos in which the virus infection apparently had failed, all specimens analyzed after HH29 showed a large patch devoid of any sign of feather bud development in the spinal tract. No alterations of feather development were detected at any stage if control viruses encoding alkaline phosphatase, the Fc fragment alone, or GFP were injected (n=41) (Fekete and Cepko, 1993). A limited number of embryos was also injected subectodermally with RCAS-R2 or RCAS-AP at HH23-24 and HH26, respectively. These embryos were harvested at HH30 and analyzed by double whole-mount in situ RNA detection for *Bmp2* and *Fgfr2-Fc* expression (using an Fc-specific probe).

Explant culture and bead implantation

Lumbar skin was isolated prior to placode formation at HH27-28 (referred to as ppf explants). Alternatively, upper thoracic skin was isolated after the first feather buds had appeared (HH29-30, referred to as eb explants, compare Fig. 3A and Fig. 4A) and cut along the midline to generate two similar halves. Skin explants were cultured in vitro for 16-48 hours as previously described (Neubüser et al., 1997). In different experiments the following reagents were added to the culture medium: 25 μM Su5402 (Calbiochem; >95% purity) and 0.5% DMSO, or 0.5% DMSO alone (16-hour cultures); 10-50 μg/ml FGFR1-Fc (IIIc) or 10-50 μg/ml FGFR2-Fc (IIIb); 5 μg/ml goat anti-FGF10 antibody (SC-7375; Santa Cruz Biotechnology, Inc) or control goat IgG (SC-2028; Santa Cruz Biotechnology, Inc). Bead implantations were performed as described previously (Neubüser et al., 1997; Vainio et al., 1993). Prior to implantation, heparin acrylic beads (Sigma) were soaked for 2 hours at 37°C in recombinant proteins (0.05, 0.1 or 0.5 mg/ml FGF10; 0.25 mg/ml Noggin-Fc or in 0.1 mg/ml FGF10 plus 0.25 mg/ml Noggin-Fc; all from R&D) or PBS. Beads soaked in 0.05-0.5 mg/ml FGF10 had similar effects. Protein-soaked beads were stored at 4°C for up to 3 weeks. Each experiment was performed at least twice, but more typically three to five times, and at least three, but more typically 5-10 specimens were examined for each condition.

RNA in situ hybridization and immunohistochemical analyses

Whole-mount RNA in situ hybridization was performed as previously described (Wilkinson and Nieto, 1993). The following plasmids were used to prepare antisense riboprobes: *Bmp2* (Francis et al., 1994), *β-catenin* (Hartmann and Tabin, 2000), *Fgf10* (Ohuchi et al., 1997), *Shh* (Riddle et al., 1993), *Fgfr1* and *Fgfr2* (Patstone et al., 1993), *Wnt6* (Schubert et al., 2002), *follistatin* (Patel et al., 1999). Chicken *Fgf3* was isolated from cDNA of HH29 chicken skin by PCR amplification using the following primers: 5′AAGTACCACCTGCAGATCCA3′ and 5′TACCAGAGTCTCTCCGCACT3′. The resulting PCR product was cloned into the TopoTA cloning vector (Invitrogen) and sequenced.

In situ hybridization was combined with immunohistochemical analysis of viral infection by embedding the stained embryos into Tissue Tek^R (Sakura) after in situ hybridization, followed by cryosectioning. The 10 μm sections were then processed for antibody staining using the mouse anti-gag antibody (1:1000 dilution, AMV3C2; Developmental studies Hybridoma Bank, University of Iowa) and the Vectastain ABC kit (Vector Labs) as described previously (Matise and Joyner, 1997).

Results

Forced expression of FGFR1-Fc and FGFR2-Fc blocks feather formation

To study the role of FGF signaling in early feather development, we generated secreted versions of FGFR1 or FGFR2 by fusing the extra-cellular ligand binding domains of the receptors to the Fc-fragment of mouse immunoglobulin (IgG) as previously described (Celli et al., 1998; Compagni et al., 2000; Peters et al., 1994). The ligand-binding region of FGF receptors consists of two immunoglobulin-like domains (IgII and IgIII). Ligand specificity is mostly determined by the carboxyl-terminal half of IgIII. This region is encoded by two alternative exons, IIIb and IIIc, which are subject to tissue-specific alternative splicing. IIIb isoforms of FGF receptors are predominantly found in epithelial lineages and preferentially bind to FGF1, 3, 7 and 10 in assays in vitro. IIIc isoforms are expressed in mesenchymal lineages and are high affinity

receptors for, for example, FGF1, 2, 4, 8 and 9 (reviewed by Ornitz and Itoh, 2001). During feather development *Fgfr1* is highly expressed in the dermis, whereas *Fgfr2* is expressed in the ectoderm (Noji et al., 1993). To sequester ligands that could bind to, and activate these receptors, we over-expressed secreted versions of FGFR1-IIIc or FGFR2-IIIb (referred to as FGFR1-Fc and FGFR2-Fc, respectively; Fig. 1A). Both constructs were introduced into RCASBP(A) replication-competent retroviral vectors and the purified virus (referred to as RCAS-R1 and RCAS-R2) was used to infect chicken embryos in ovo. Embryos were initially injected with the virus into migrating neural crest cells at Hamburger Hamilton stage (HH) 9 (Hamburger and Hamilton, 1951) to generate widespread infection throughout the body. For more localized infections, the virus was injected into subectodermal mesenchyme of the back at HH18-20. Antibody stainings against the viral gag protein revealed that both types of injections primarily resulted in infection of the mesenchyme and only occasionally included individual cells or small patches of ectoderm (Fig. 2G-J, Fig. 3I,J, and data not shown).

Feather development in embryos infected with control viruses (RCAS-GFP, RCAS-AP or RCAS-Fc) was indistinguishable from uninfected embryos (Fig. 1B and data not shown), indicating that virus infection itself does not disturb feather formation. In contrast, most embryos inoculated with a high titer RCAS-R1 or RCAS-R2 virus showed severe defects in feather development. Of 35 surviving embryos that had been injected with RCAS-R1 at HH9, 17% completely lacked feathers at HH38 (Fig. 1C), and 73% showed patches of skin lacking signs of feather development (in the following referred to as nude patches; data not shown). Only 11% of injected embryos did not show any defects, probably because of unsuccessful infection. Furthermore, in 20% of the embryos fusions between neighboring primordia gave rise to abnormally large and aberrantly shaped feathers (Fig. 1F, and data not shown). Subectodermal injection of RCAS-R1 into the back at HH18-20 and incubation to HH38 typically resulted in a nude patch on the back in the region of virus injection, whereas feather development in other parts of the embryo was unaffected (Fig. 1D, *n*=9/10). Embryos over-expressing FGFR2-Fc showed similar but more widespread defects. Out of ten surviving embryos injected at HH18-20, four were completely nude at HH38 (data not shown). The remaining six showed patches of normal feathers in a largely nude embryo (Fig. 1E). A comparison of the spreading of the viral infection revealed no significant differences between the two viruses (data not shown). Analysis of sections through the nude region of RCAS-R1-and RCAS-R2-infected embryos revealed a thinner epidermis and a less dense dermis when compared to control embryos (Fig. S1, http://dev.biologists.org/supplemental/). These results demonstrate that FGF signaling plays essential roles during skin and feather development.

Early feather tract development is normal in embryos over-expressing FGFR1-Fc or FGFR2-Fc

In order to determine at which stage of feather development FGF signaling is required, we first analyzed whether regions competent to form feathers, the feather tracts, are specified normally in the presence of FGFR1-Fc or FGFR2-Fc. Tract formation is characterized by the formation of a dense dermis (Sengel, 1976). Between HH26 and HH28 several genes are up-regulated in diffuse stripes in the areas where the first feather buds will appear at HH29. Two such markers are *β-catenin* expression in the ectoderm and *Fgfr1* expression in the dermis (Noji et al., 1993; Noramly et al., 1999; Widelitz et al., 2000) (Fig. 2A,D).

In RCAS-R1- and RCAS-R2-infected embryos analyzed at HH27-28, the patterns of *Fgfr1* and *β-catenin* expression were indistinguishable from uninfected or control virus infected embryos (Fig. 2A-F). Successful infection was confirmed by subsequent sectioning of the stained embryos, followed by immunohistochemical detection of the viral gag protein. This,

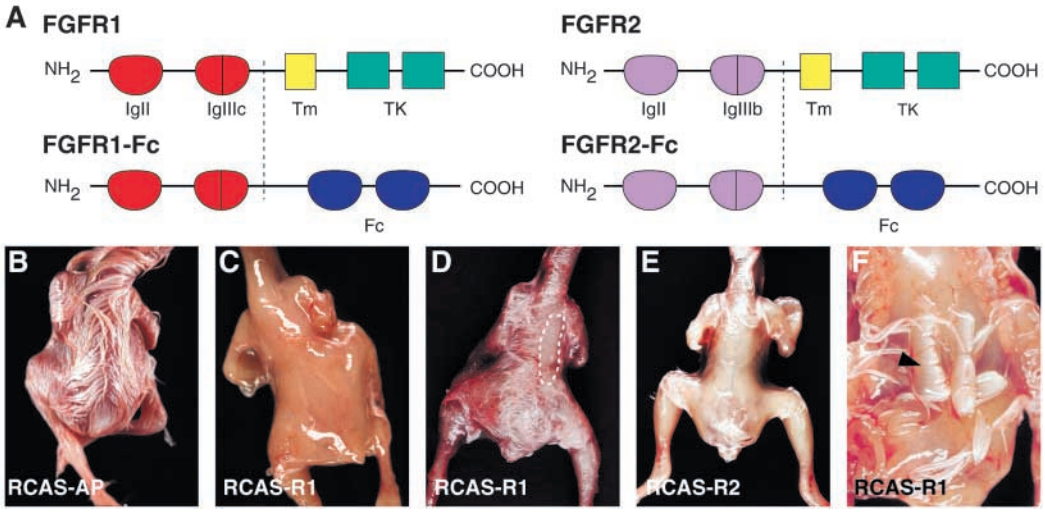

Fig. 1. Forced expression of secreted versions of FGFR1 and 2 blocks feather development. (A) The ligand binding regions of FGFR1-IIIc and FGFR2-IIIb (red) were fused to the Fc fragment of a mouse immunoglobulin (blue) to generated secreted receptor versions. Tm, transmembrane domain; TK, tyrosine kinase domains. (B-D) Feather phenotypes in embryos injected with RCAS-AP (B), RCAS-FGFR1-Fc (C,D,F), or RCAS-FGFR2-Fc (E). Embryos in B, C and F were injected with the retrovirus at HH9. The embryos in D and E were in injected into subectodermal mesenchyme of the back at HH18. All embryos were allowed to develop until HH38. The arrowhead in F indicates fusions between adjacent feather primordia.

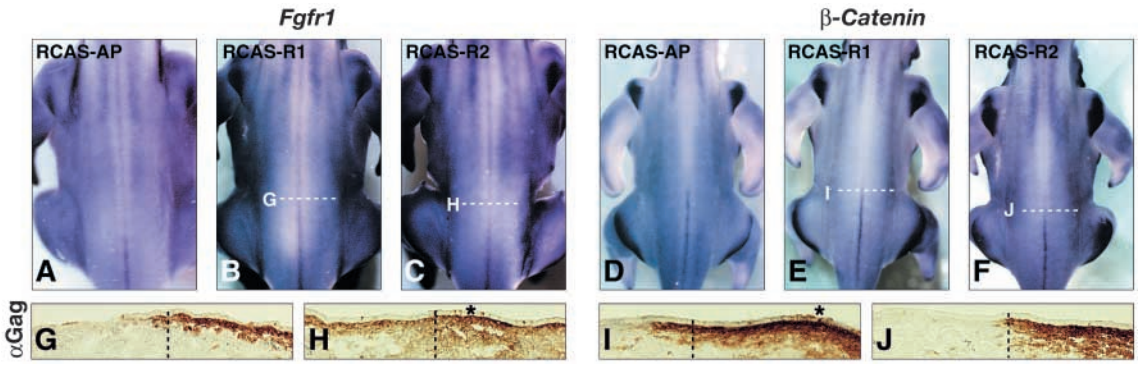

Fig. 2. Unchanged expression of *β-catenin* and *Fgfr1* in RCAS-FGFR1-Fc- and RCAS-FGFR2-Fc-infected embryos at HH27-28. (A-F) Embryos were virus-injected into the subectodermal mesenchyme of the back at HH18 and analyzed for *Fgfr1* and *β-catenin* expression by whole-mount RNA in situ hybridization at HH27-28. (G-J) Sections through the embryos shown in B, C, E and F stained with an antibody against the viral gag protein demonstrating widespread infection of the mesenchyme. Dashed lines indicate the position of the midline of the embryos, asterisks indicate regions with infected cells in the ectoderm.

together with the analysis of Hematoxylin and Eosin (HE)-stained sections of specimens infected with either RCAS-R1, -R2 or control virus revealed no obvious difference in the histological appearance and density of the dermis or the overlying ectoderm in the absence of FGF signaling (Fig. 2G-J, and data not shown). Expression of two other markers, the bHLH transcription factor *cDermo1* in the dermis (Scaal et al., 2001) and *Wnt6* in the epidermis (Chodankar et al., 2003), was also unchanged in FGFR1-Fc- and FGFR2-Fc-infected embryos at HH27-28 (data not shown). Together these results suggest that feather tract formation does not require FGF signaling.

Forced expression of FGFR1-Fc and FGFR2-Fc prevents feather placode development

We next examined the effects of forced expression of FGFR1-Fc and FGFR2-Fc on the initiation of feather bud development at HH29-30. During normal development, *β-catenin*, *Wnt6* and *Fgfr1* are expressed in diffuse stripes in the feather tracts prior to the initiation of bud development. Once buds start to form, they are locally up-regulated in the developing buds and down-regulated in interbud domains (Fig. 3A) (Chodankar et al., 2003; Noji et al., 1993; Noramly et al., 1999; Noramly and Morgan, 1998; Widelitz et al., 2000).

RCAS-R1- and RCAS-R2-infected embryos analyzed at HH29-30 typically showed a large patch devoid of any sign of feather bud development on the back, in the area into which the virus had been injected (Fig. 3). Although the injections were performed unilaterally, these nude patches usually extended across the midline onto the contra-lateral side and thus included the region were the first row of feathers should have formed. In these nude patches *Wnt6*, *β-catenin* and *Fgfr1* expression was usually still detectable in the diffuse patterns already observed prior to bud formation (Fig. 3B,C, and data not shown). Occasionally, several weak parallel stripes of expression of these markers were transiently detectable, in particular in RCAS-R2-infected embryos (Fig. 3C, and data not shown). This failure of *β-catenin*, *Wnt6* and *Fgfr1* to undergo a transition from broad, diffuse expression to up-regulated and localized expression in nascent buds indicates that bud formation is not initiated normally in regions infected with RCAS-R1 and RCAS-R2.

Bmp2 is the earliest marker of bud development identified so far and its localized expression in the ectoderm precedes placode formation (Jung et al., 1998; Noramly and Morgan, 1998). Slightly later, developing placodes also express *Shh* (Fig. 3D) (Morgan et al., 1998). In the nude patches of RCAS-R1- and RCAS-R2-infected embryos expression of the placode markers *Bmp2* and *Shh* was undetectable (Fig. 3E-H). Sectioning and subsequent virus detection of affected embryos after whole-mount in situ analysis and histological analysis, confirmed widespread infection in the mesenchyme of the nude patches and absence of any signs of feather placode formation (Fig. 3I,M′, and data not shown). Similar results were also obtained if embryos were injected at HH23-HH24, which resulted in smaller areas of infection restricted to one side of the embryo not including the first row (Fig. 3K,L). Embryos infected at HH26, in contrast, showed normal arrangements of *Bmp2*-expressing buds in the developing skin, including regions where *Fgfr2-Fc* transcripts were detectable (Fig. 3N,O, white circles). Likewise, in embryos infected at HH18-20 or HH23-24, virus infection was frequently also detectable in bud-containing regions adjacent to the nude patch (Fig. 3J, and data not shown). Cells in these regions are further away from the original site of virus injection than the cells inside the nude patch, and therefore most likely became infected at a later time point. Together these results show that FGF signaling is necessary for feather placode formation, the first step of feather bud development, in the first and subsequent rows. This requirement seems to be transient and at later stages feather bud development seems to be less sensitive to alterations in the level of FGF signaling. This conclusions is further supported by the analysis of additional markers such as *delta1*, *follistatin*, *Wnt7a* and *Wnt3a* and *Dermo1* (data not shown) (Crowe et al., 1998; Ohyama et al., 2001; Patel et al., 1999; Scaal et al., 2001; Viallet et al., 1998; Widelitz et al., 1999).

FGF signaling is required at the initiation stage of feather placode development

Over-expressing FGFR1-Fc or FGFR2-Fc blocks feather placode development. To better define the timing when FGF signaling is required, we took advantage of an in vitro culture

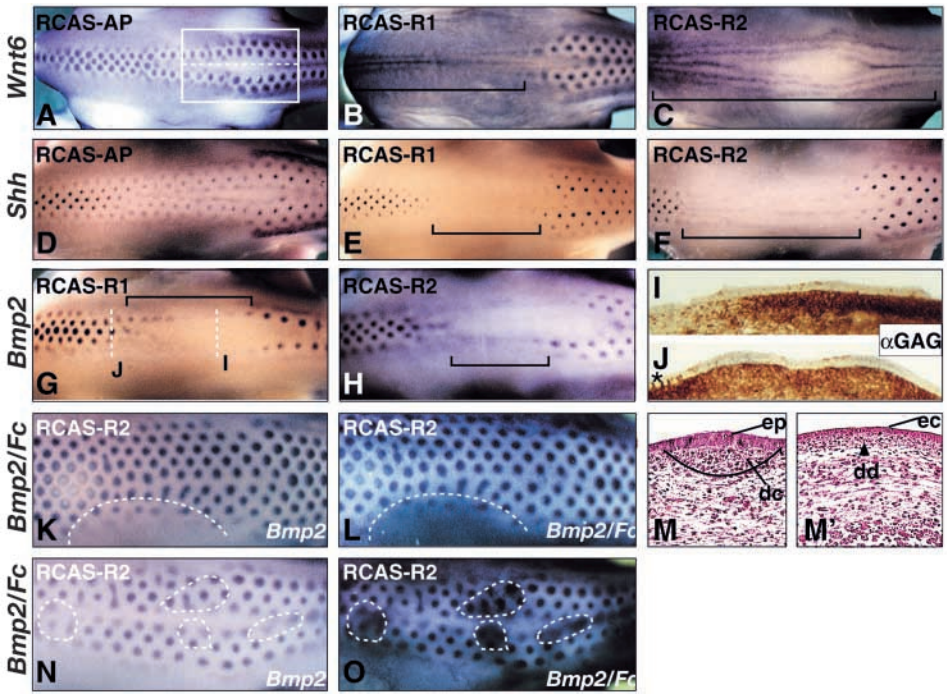

Fig. 3. Forced expression of FGFR1-Fc and FGFR2-Fc prevents feather placode development. (A-H) Embryos were injected with the virus into the subectodermal mesenchyme of the back at HH18 and analyzed for expression of *Wnt6*, *Shh* and *Bmp2* at HH29-30 by whole-mount RNA in situ hybridization. All embryos infected with RCAS-FGFR1-Fc or RCAS-FGFR2-Fc show a region devoid of any punctate expression (brackets). (I,J) Sections through the embryo shown in G stained with an antibody against the viral gag protein demonstrates viral infection in both the nude patch and adjacent bud containing regions. The asterisks in J indicate infected cells in the ectoderm. (M,M′) HE stained sections through a control (M) and nude region of an RCAS-FGFR1-Fc infected embryos (M') showing failure to form ectodermal placodes (ep) and dermal condensates (dc) in infected tissue. ec, ectoderm that has not formed a placode; dd, thickened dermis. (K,L) Embryos were injected subectodermally at HH23-24 and analyzed for *Bmp2* (K) and subsequently *Fc* expression (L, both in blue) by double whole-mount RNA in situ hybridization. Embryos showed loss of *Bmp2* expression and a failure to form buds in the infected area (indicated by white dotted line). (N,O) Embryos injected at HH26 show normal bud development and *Bmp2* expression in infected areas. Infection is monitored by subsequent *Fc* detection in *Bmp2/Fc* double stained embryos (indicated by white dotted circles in N and O).

system for embryonic skin explants. Pieces of skin were isolated from the back either shortly before the formation of the first feather buds (HH28, referred to as prior to placode formation (ppf) explants), or after the first rows of buds had appeared (HH29-30, referred to as established buds (eb) explants). These explants were then cultured in the presence or absence of the following inhibitors of FGF signaling: the FGF receptor antagonist Su5402, recombinant FGFR1-Fc or FGFR2-Fc protein (Mohammadi et al., 1997). To allow a direct comparison between the number of buds at the onset of culture with the number present after culture, eb explants were cut in half along the midline as indicated in Fig. 3A and Fig. 4A. Of these, only one half was cultured; the contra-lateral side was immediately fixed and served as a reference.

Ppf explants cultured for 16 hours in the presence of Su5402 lacked all signs of feather bud development and did not express the placode markers *Shh* and *Bmp2* (Fig. 4B,D). Likewise, *β-catenin* and *Fgfr1* expression was not locally up-regulated in ppf explants cultured in the presence of the inhibitor (Fig. 4F,H). Instead both genes were expressed in patterns similar to the stage of explantation, a stripe in the case of *Fgfr1* and a diffuse low level expression in the case of *β-catenin* (compare with Fig. 2A,D). In contrast, explants cultured in control medium developed normal arrays of feather primordia expressing all four markers (Fig. 4A,C,E,G). Similar effects were observed if explants were cultured in the presence of 10-50 μg/ml recombinant FGFR1-Fc or FGFR2-Fc protein (data not shown). Whole-mount TUNEL analysis revealed no increase in the amount of cell death in SU5402-treated explants excluding impaired tissue survival in the presence of the inhibitor as the reason for the failure of feather bud development (data not shown).

In eb explant halves cultured in the presence of Su5402, expression of all four markers was detectable in previously formed buds (Fig. 4J,N, and data not shown), but no additional rows of buds had formed laterally compared to the reference halves fixed immediately after dissection (Fig. 4I,M, and data not shown). In contrast, additional rows of buds not present at the time of dissection had formed in explant halves cultured in the presence of DMSO (Fig. 4K,L). Importantly, even buds at the earliest stage of bud development, evident in the reference half by weak localized BMP2 expression (Fig. 4M, arrows), had continued to develop in the presence of Su5402 as indicated by increased *Bmp2* expression (Fig. 4N, arrows). Western blot analysis confirmed that the concentration of Su5402 used in these experiments was sufficient to block ERK phosphorylation in eb explants, arguing against an incomplete block of FGF signaling as the reason for the maintenance of

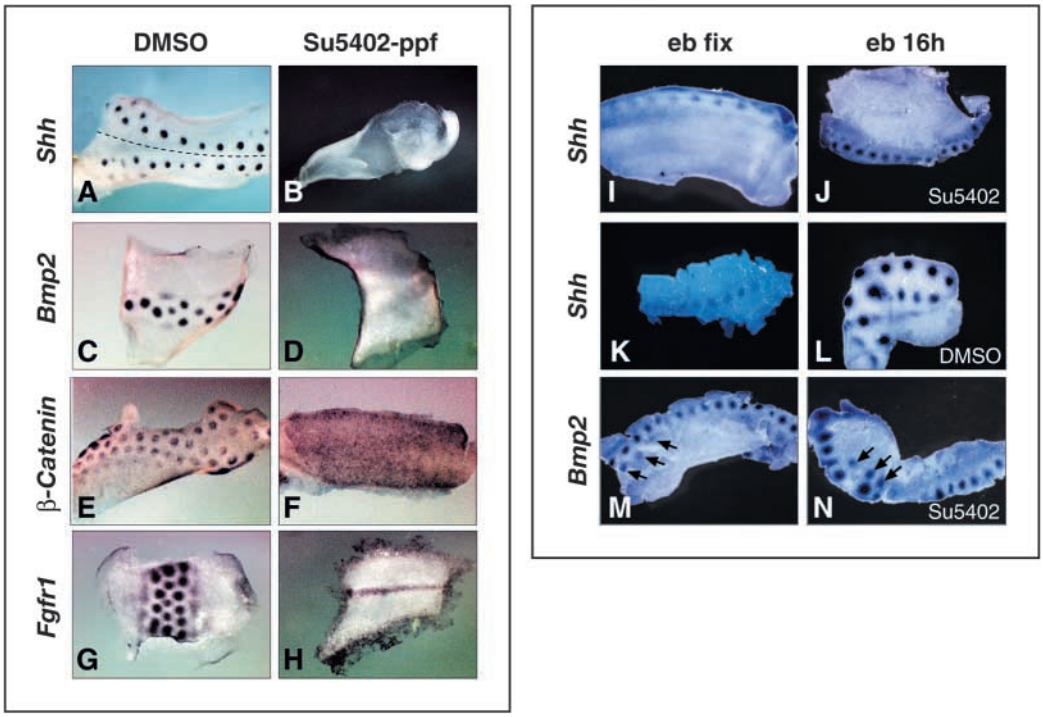

Fig. 4. Inhibition of FGF signaling in vitro prevents feather placode induction but not the maintenance of established feather buds. Skin explants isolated at HH28, prior to placode formation (ppf; A-H), were cultured in the presence of DMSO or Su5402 for 16 hours in vitro and analyzed by whole-mount in situ hybridization for *Shh*, *Bmp2*, *β-catenin* or *Fgfr1* expression. Explants isolated at HH29, when the first buds have been established (eb; I-N), were separated along the midline as indicated by the doted line in A, and halves were either fixed directly after isolation (I,K,M) or cultured for 16 hours in the presence of Su5402 (J,N) or DMSO (L), and expression of *Shh* (I-L) or *Bmp2* (M,N) was compared in the two halves.

established feather placodes (Fig. S2, http://dev.biologists.org/supplemental/). Together these results show that FGF signaling is required for feather placode development prior to the initiation of *Bmp2* expression, the earliest available placode marker, but is not required to maintain developing placodes during the culture period once *Bmp2* expression has been established.

Fgf3 and *Fgf10* are expressed in the dermis of developing feather buds and *Fgf10* expression in the future first row of buds precedes placode formation

We next searched for members of the FGF family that could function as the placode-inducing FGF signal identified by the previous experiments. Since tissue recombination experiments have shown that feather placode development is initiated by signals from the underlying dermis, we focused on members of the FGF family that function as mesenchyme to ectoderm signals in other developmental settings (Bei and Maas, 1998; Min et al., 1998; Sekine et al., 1999). By RT-PCR, we detected *Fgf3* and *Fgf10* transcripts in HH29 chick back skin (data not shown). *Fgf3* is expressed in the condensed mesenchyme underlying the ectodermal placodes of already formed buds at HH29 (Fig. 5B,I). No *Fgf3* expression, however, was detectable at HH28, prior to the formation of the first feather buds (Fig. 5A). In contrast, *Fgf10* was already expressed in the dermis at HH28. Expression was detected in a continuous stripe underlying the regions where the future first rows of buds will develop (Fig. 5D,G, arrowheads) and was subsequently up-regulated in the mesenchyme of nascent feather buds (Fig. 5E,H). In subsequent rows, no continuous stripe of expression was detected. Instead *Fgf10* was expressed in initially very weak and slightly diffuse spots (Fig. 5F unaffected area, and data not shown). Comparison of *Fgf10* expression in the dermis with *Bmp2* expression in the epidermis on adjacent sections suggested that the expression of both starts at about the same time (data not shown).

In agreement with previous observations, the early stripe of FGF10 expression was maintained in infected regions at HH28 in embryos injected with either RCAS-R1 or RCAS-R2 (data not shown). In nude patches of infected specimens analyzed at HH29-30, *Fgf3* and *Fgf10* expression was undetectable, similar to the other bud markers analyzed (Fig. 5C,F and data not shown). Expression of *Fgf10* was not restricted to the spinal tract. Similar patterns of *Fgf10* expression were also detected in other feather tracts including the scapular and pectoral tracts and the tail (Fig. 5J-L). Based on its temporal expression pattern, *Fgf10* could therefore serve as the placode-inducing FGF signal that we identified in the experiments described above.

FGF10 is required for feather placode formation

In order to directly test whether FGF10 is required for the initiation of feather bud development, we analyzed feather bud development in ppf explants cultured in the presence of FGF10-blocking antibodies. These antibodies have previously been used to inactivate FGF10 in tooth explants (Harada et al., 2002). They recognize recombinant FGF10 and a band of similar size in protein extracts from chicken skin, but show no cross-reactivity to FGF2, 3, 4, 5, 7, 8 or 18 in western blot

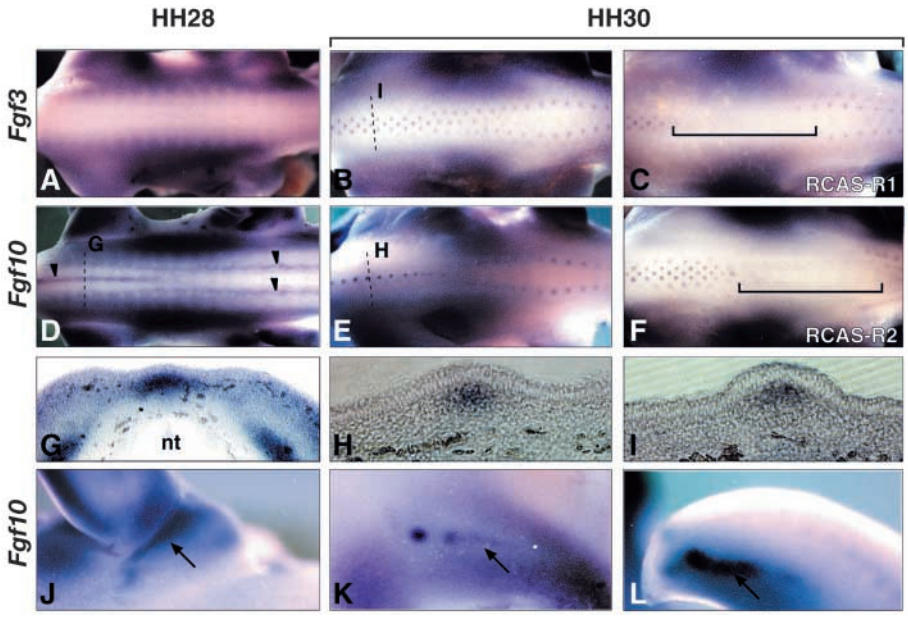

Fig. 5. Expression of *Fgf3* and *Fgf10* in early feather development. Detection of *Fgf3* (A-C) or *Fgf10* (D-F,J-L) expression by whole-mount RNA in situ hybridization of embryos at HH28, before the onset of feather placode development, and at HH30. (C,F) Expression of *Fgf3* and *Fgf10* in embryos infected with RCAS-FGFR1-Fc and RCAS-FGFR2-Fc at HH18. The nude region devoid of *Fgf3* and *Fgf10* expression is included with a bracket. (G-I) Vibratome sections, at the indicated positions, through the embryos shown in B, D and E, respectively. Arrowheads in D indicate the stripes of *Fgf10* expression in the region where bud development will be initiated. (J-L) Expression of *Fgf10* in the alar (J), pectoral (K) and tail tract (L). Arrows indicate the feather tract, delineated by *Fgf10* expression. nt, neural tube.

experiments (Fig. S3, http://dev.biologists.org/supplemental/). In ppf explants cultured for 16 hours in the presence of these antibodies, *Shh* and *Bmp2* expression was undetectable and no thickened feather placodes or dermal condensations formed (Fig. 6B,E). Previously formed *Bmp2*-positive buds were maintained in the presence of the anti-FGF10 antibodies, but no additional buds developed (Fig. 6F). In contrast, multiple *Shh*- and *Bmp2*-expressing feather buds formed in explants cultured in the presence of control IgG (Fig. 6A,D). Feather buds also developed in explants cultured in medium containing both the FGF10-blocking antibodies and recombinant FGF10 protein (Fig. 6C), but not in specimens cultured in the presence of the antibodies and FGF3, FGF4 or FGF7 protein (data not shown). Thus only FGF10 is able to titrate and neutralize the antibodies. Under the same conditions FGF3-blocking antibodies (Harada et al., 2002) had no effect on bud development in explants (data not shown). Together these results provide evidence that FGF10 is required for the initiation of feather bud development.

FGF10 is sufficient to induce genes essential for feather bud development

In order to test whether recombinant FGF10 protein would be sufficient to initiate feather bud development we applied beads soaked in FGF10 (referred to as FGF10 beads) to ppf explants and assayed for changes in gene expression normally associated with the onset of bud development. In explants cultured in the presence of FGF10 beads, expression of *Bmp2* was induced close to the bead (Fig. 7B). Likewise, FGF10 beads also induced an up-regulation of *Wnt6* expression (Fig. 7D), as normally observed in the ectoderm of nascent feather buds. A continuous stripe of *follistatin* expression normally precedes the localized expression of the earliest bud markers, labeling the regions where the next feather placodes will form. Subsequently, *follistatin* expression is transiently detected in the placodes, but is then maintained in a ring along the edge of each placode (Ohyama et al., 2001; Patel et al., 1999). In ppf explants cultured with FGF10 beads, upregulated expression of *follistatin* was detectable around the beads (Fig. 7E). PBS beads in contrast had no effects on the expression of any of the three markers (Fig. 7A,C,D). Thus, FGF10 is sufficient to induce changes in ectodermal gene expression characteristic of the initiation of bud development. Notably, while upregulating marker gene expression, FGF10 beads also blocked the formation of individual feather buds in their vicinity, similar to what has been described by Tao et al. (Tao et al., 2002). During development of the feather array, bud spacing is thought to be controlled through positive feedback

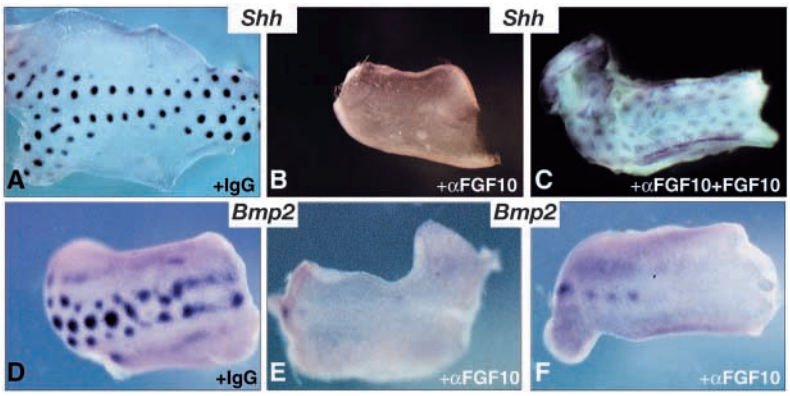

Fig. 6. FGF10 is required for feather development. (A-F) Skin explants (ppf: A-E; eb: F) were cultured for 16 hours in the presence control IgG or 5 μg/ml anti-FGF10 blocking antibodies, or in medium containing the same amount of anti-FGF10 antibody and recombinant FGF10 protein (C) and analyzed for expression of *Shh* or *Bmp2*.

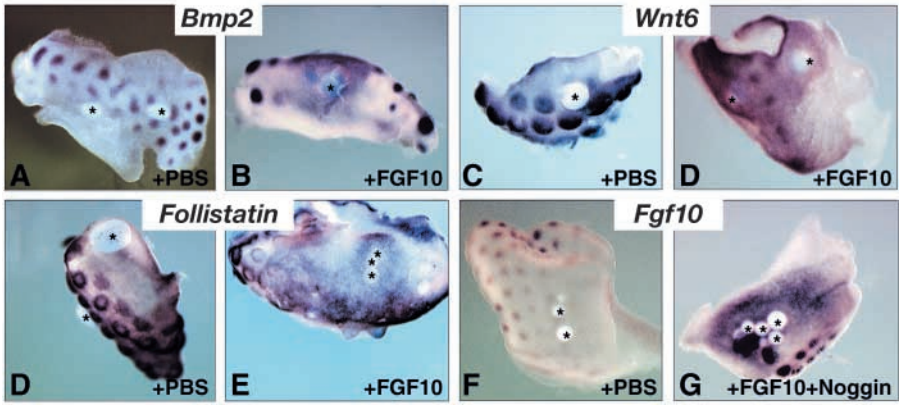

Fig. 7. FGF10 is sufficient for the induction of genes essential for feather bud development. (A-G) ppf-skin explants were cultured for 16 hours in the presence of beads soaked in recombinant FGF10, recombinant FGF10 plus Noggin-Fc, or PBS, and analyzed for the expression of *Bmp2*, *Wnt6*, *follistatin* or *Fgf10*. Asterisks indicate implanted beads.

loops and lateral inhibition. In order to test whether FGF10 might be involved in an autoregulatory loop, we analyzed whether FGF10 could induce its own expression. However, we observed no up-regulation of *Fgf10* expression around FGF10 beads applied to ppf explants (data not shown). Since FGF10 beads also induce strong expression of *Bmp2*, a negative regulator of feather development, induction of *Fgf10* expression could be suppressed around FGF10 beads because of excessive BMP2 signaling. In order to see whether this might be the case, we soaked beads in a mixture of recombinant FGF10 and recombinant Noggin-Fc protein to reduce the amount of BMP signaling in the vicinity of the beads. Application of FGF10/Noggin-Fc beads led to an induction of *Fgf10* expression in surrounding tissue (Fig. 7G), whereas beads soaked in Noggin-Fc alone or PBS did not result in any up-regulation of *Fgf10* expression (Fig. 7F, and data not shown). The effects of FGF10/Noggin-Fc beads on *Fgf10* expression varied depending on the position of the bead. Implantation of beads close to existing buds resulted in an increase in bud size close to the beads (n=5/8), while implantation at a greater distance from the prospective first row of buds also induced broad expression of *Fgf10* (n=3/8, Fig. 7G). Together these results imply that FGF10 protein is sufficient to induce *Fgf10* expression under conditions of low BMP signaling and thus identify a regulatory loop that may be involved in controlling *Fgf10* expression in the developing feather tract.

Discussion

Previous studies have implicated FGFs as positive regulators of feather development, but their precise roles and the extent to which FGF signaling is required for feather formation has been unclear. Here we show that FGF signaling is required for the initiation of feather placode development.

FGF2 and FGF4 can promote feather bud formation and can induce feathers in skin explants (Song et al., 1996; Widelitz et al., 1996). Both are expressed in the epidermal placodes and have been suggested to serve as locally acting promoters of placode development (Jung et al., 1998). Consistent with this suggested function our results show that FGF signaling is required for feather placode formation. Retroviral over-expression of soluble versions of FGFR1 or FGFR2 had no effect on the expression of early feather tract markers suggesting that FGF signaling is not required for feather tract specification. However, markers such as *Fgfr1*, *Wnt6* or *β-catenin* subsequently failed to undergo the transition from broad diffuse expression in the early tract to localized expression in the forming buds. Moreover, *Bmp2*, the earliest known marker of placode development (Jung et al., 1998; Noramly and Morgan, 1998), and *Shh*, a slightly later marker of feather placodes (Chuong et al., 2000; Morgan et al., 1998), were not detectable at all in the presence of FGFR1-Fc or FGFR2-Fc. The same effects were also observed if skin explants were dissected shortly before the onset of placode development and cultured in the presence of inhibitors of FGF signaling. Together these results point towards a requirement for FGF signaling just prior to or during the initiation of feather placode development.

In the prospective first row of buds we have detected FGF10 expression in the dermis prior to the localized expression of any of the known placode markers in the overlying epidermis. Such a stripe, however, was not detectable in subsequent rows. There, we first detected *Fgf10* expression in weak and slightly diffuse spots in the dermis at roughly the same time when *Bmp2* expression became detectable in the overlying epidermis. We have furthermore shown that blocking antibodies recognizing FGF10 mimic the effects of soluble FGF receptors and block the initiation of placode development in skin explants. Together these observations make FGF10 a candidate for the FGF signal necessary for feather placode development. Whether FGF10 serves to initiate placode development or whether it collaborates with other signals and functions to promote and stabilize placode development after an initial induction by a different signal is still unclear.

Secreted versions of FGFR2-IIIb or FGFR1-IIIc are expected to preferentially bind to and sequester FGF10, FGF7 and FGF3, or FGF2, FGF4 and related FGFs, respectively (reviewed by Ornitz and Itoh, 2001). Both types of receptors also bind other members of the FGF family, but with lower affinity (Ornitz et al., 1996; Orr-Urtreger et al., 1993) (M.M. and A.N., unpublished). In our experiments, retroviral over-expression of either of the two receptor isoforms blocked feather development. Except for a more widespread effect of FGFR2-IIIb-Fc, the resulting phenotypes were highly similar, most likely because both receptor isoforms block feather development by sequestering FGF10 and/or a related FGF. The more widespread effect of FGFR2-IIIb-Fc would then be explained by the higher affinity of this receptor isoform to members of this subfamily of FGFs. Alternatively, FGFR1-Fc

could block feather development by sequestering FGF2 and FGF4 produced in feather placodes, a block of feather bud development after initiation of placode development, followed by bud regression. However, this appears less likely since culture of skin explants in the presence of FGF signaling inhibitors after the onset of placode development did not result in bud regression.

In skin explants, FGF10 was sufficient to up-regulate ectodermal expression of *Wnt6*, *Bmp2* and *follistatin*, molecules that have been implicated as positive and negative regulators of feather development. Several previous studies have shown that canonical WNT signaling functions as an essential, positively acting signal during early epithelial appendage development (Andl et al., 2002; Huelsken et al., 2001; Kratochwil et al., 1996; Noramly et al., 1999; Widelitz et al., 2000). However, it is so far not clear whether for placode development WNT signaling is required in the ectoderm or the dermis. Furthermore, it is unclear which member of the Wnt family activates the pathway in either of the tissues. Keratin-14 (K14)-promoter driven over-expression of Dkk1, a soluble Wnt antagonist, in the epidermis of transgenic mice lead to a complete failure of placode formation and the subsequent absence of all hairs, a phenotype similar to the one we observed in the chick after over-expression of FGFR1-Fc or FGFR2-Fc (Andl et al., 2002). Given these similarities, it is tempting to speculate that Wnt/β-catenin and FGF signaling could cooperate in a common pathway in initiating feather placode development. Such a situation has recently been shown for the initiation of limb development (Barrow et al., 2003; Kawakami et al., 2001). There, Wnt/β-catenin in the early limb mesenchyme appears to be required to initiate *Fgf10* expression in the same tissue. Mesenchymally derived FGF10 then regulates expression of *Wnt3a* in the overlying ectodermal cells, which is required to activate expression of *Fgf8* in the same cells. In analogy, Wnt signaling in the dermis could act upstream of dermal *Fgf10* expression during feather development. FGF10-mediated up-regulation of expression of *Wnt6*, and maybe other members of the Wnt family, in the ectoderm could then activate the pathway in the epidermis and promote placode formation.

FGF10 also induces expression of *Bmp2*, a negative regulator of feather development. According to the current model, BMP2 controls bud spacing and bud size through lateral inhibition (Jiang et al., 1999; Jung et al., 1998; Noramly and Morgan, 1998). The buds themselves, expressing high levels of *Bmp2*, are thought to be protected from the inhibitory effect of BMP signaling, e.g. through the production of BMP antagonists. We have found that FGF10 also upregulates expression of the secreted BMP antagonist Follistatin. A uniform stripe of *follistatin* expression normally precedes feather placode formation and has been suggested to label a region competent to form feather buds (Patel et al., 1999). Feather placodes therefore always develop within a region of high *follistatin* expression in which BMP activity might be low. Our finding that FGF10 protein can induce *Fgf10* expression, but only in the context of low BMP activity, achieved through application of beads soaked in a mixture of FGF10 and Noggin-Fc suggests that the level of BMP signaling is critical in determining the response to FGF10. The high level of *Bmp2* expression induced in response to beads soaked in FGF10 protein alone could result in increased BMP2 signaling, in spite of the simultaneous induction of *follistatin*, and in inhibition of further bud development. A recent study by Tao et al. (Tao et al., 2002) supports this idea. These authors used a retroviral construct to broadly over-express FGF10 in chicken skin and found a complete failure of feather development, associated with widespread induction of *Bmp2*, and a loss of all periodic gene expression. Thus, in spite of its requirement for placode development and its dermal expression, from current knowledge, FGF10 lacks important properties that would be expected of the primary inductive signal from the dermis that initiates feather development.

Tissue recombination between mouse and chick tissues have shown that the inductive signals exchanged between dermis and epidermis that initiate cutaneous appendage formation are conserved between birds and mammals. We would therefore predict that FGF signaling also functions in the initiation of hair follicle development. Indeed, Celli et al. (Celli et al., 1998) have described a complete absence of hair follicles in transgenic mice over-expressing FGFR2-IIIb-Fc under the control of a metallothionein promoter in 50% of the founder animals but did not characterize these defects further. Nevertheless, this study supports the idea that ligands capable of binding to FGFR2-IIIb are also involved in the initiation of hair follicle formation in the mouse. Knockouts of individual members of the FGF family and individual FGFR isoforms have so far failed to clearly reveal this requirement, most probably because of redundancy (Arman et al., 1999; De Moerlooze et al., 2000; Guo et al., 1996; Hebert et al., 1994; Min et al., 1998; Petiot et al., 2003; Sekine et al., 1999; Suzuki et al., 2000). Ultimately, the generation of mice deficient in several members of the FGF or FGF receptor family will therefore be necessary to determine which members of the FGF family collaborate in the initiation of hair follicle development.

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