FGF Induces New Feather Buds From Developing Avian Skin

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Induction of skin appendages involves a cascade of molecular events. The fibroblast growth factor (FGF) family of peptide growth factors is involved in cell proliferation and morphogenesis. We explored the role of the FGFs during skin appendage induction using developing chicken feather buds as a model. FGF-1, FGF-2, or FGF-4 was added directly to the culture medium or was released from pre-soaked Affigel blue beads. Near the midline, FGFs led to fusion of developing feather buds, representing FGFs' ability to expand feather bud domains in developing skin. In lateral regions of the explant where feather placodes have not formed, FGF treatment produces a zone of condensation and a region with an increased number of feather buds. In ventral epidermis that is normally apteric (without feathers), FGFs can also induce new feather buds. Like normal feather buds, the newly induced buds express Shh. The expression of Grb, Ras, Raf, and Erk, intracellular signaling molecules known to be downstream to tyrosine kinase receptors such as the FGF receptor, was enriched in feather bud domains. Genistein, an inhibitor of tyrosine kinase, suppressed feather bud formation and the effect of FGF. These results indicate that there are varied responses to FGFs depending on epithelial competence. All the phenotypic responses, however, show that FGFs facilitate the formation of skin appendage domains. ***Key words: hair/skin appendage morphogenesis/Shh/induction. J Invest Dermatol 107:797–803, 1996***

Molecules involved in the induction of skin appendages are of fundamental importance to skin morphogenesis. The ability to understand and modulate these factors may have great implications for the development, cycling, and regeneration of skin appendages. We have been searching for molecules involved in the early stage of skin appendage induction using developing chicken skin as a model (Ting-Berreth and Chuong, 1996b; reviewed in Widelitz *et al*, in press). Among these molecules, fibroblast growth factors (FGFs) have been shown to have remarkable effects on feather development.

The FGFs are a family of peptide growth factors that stimulate cell proliferation, morphogenesis, and repair. Currently, nine FGF isoforms have been identified. Abnormal expression of FGFs has been associated with cancer (Dickson and Fantl, 1994; Esteller *et al*, 1995; Fujimoto *et al*, 1995) and atherosclerosis (Hughes *et al*, 1993). So far, four types of FGF receptors (FGFRs) have been identified. All of these receptors belong to the immunoglobulin superfamily and act as tyrosine kinases to transmit their intracellular signals (Fernig and Gallagher, 1994). Although each of the nine FGF isoforms can bind to the four FGFRs with different binding affinities, there are no strict specificities between FGFs and FGFRs (Fernig and Gallagher, 1994). The FGFR expression patterns in developing chicken wings suggest, however, that they are developmentally regulated (Szebenyi *et al*, 1995). FGFs have been shown to be involved in the epithelial-mesenchymal interactions of developing limb buds and can indeed induce new limb buds from the developing chicken body wall (Cohn *et al*, 1995).

FGFs and their receptors appear to play a role in skin appendage formation during embryonic development. In embryonic chicken skin, FGFs and FGFRs are specifically expressed in feather germs (Noji *et al*, 1993). Transgenic mouse skin expressing FGF-7 from a keratin-14 promoter suppressed hair follicle formation (Guo *et al*, 1993). Expression of a dominant-negative FGF-7 in transgenic mice produced a wavy hair shaft resembling that in the rough mutant mouse (Guo *et al*, 1996). Transgenic mice expressing a dominant-negative FGFR from a keratin-10 promoter had disrupted and thickened epidermis (Werner *et al*, 1993). These data point out the importance of FGF in skin appendage morphogenesis. The roles of FGF, however, on the embryonic induction of skin appendages and on early stages of skin appendage development have not been explored.

FGF transcripts are also found in the hair follicles of adult mice (Moore *et al*, 1991; Sutton *et al*, 1991; reviewed in Widelitz *et al*, in press) and rats (Danilenko *et al*, 1995). Modulating FGF expression can have profound effects on hair formation. Treatment of newborn mouse skin with FGF-1 or FGF-2 delayed entry into the first and subsequent hair cycles (du Cros, 1993a). FGF-2 also affects the initiation and development of hair follicles (du Cros, 1993b). Additionally, FGF-2 is required for the growth of dermal papilla cells *in vitro* (Lichti *et al*, 1993). Another FGF family member, FGF-5, is implicated in regulating the length of the hair cycle (Hebert *et al*, 1994). FGF-7 induces hair growth in a concentration-dependent fashion (Danilenko *et al*, 1995). Both FGF-1 and FGF-7 can protect skin from alopecia induced by cytosine arabinoside treatment (Danilenko *et al*, 1995). Hence, in experimental systems,

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Abbreviation: FGFR, fibroblast growth factor receptor.

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the FGFs play important roles in regulating the growth and development of adult hair.

In this study, we show that in developing skin, FGF can increase mesenchymal condensation and can increase the number of skin appendages in feather-forming regions and in regions that normally do not form feather buds. These results suggest that FGFs play a key role in the induction of skin appendages.

MATERIALS AND METHODS

Materials FGF-1 and FGF-2 were from R & D Systems (Minneapolis, MN). FGF-4 was from the Genetics Institute (Cambridge, MA). The polyclonal antibodies against Ras were from UBI (Lake Placid, NY). Antibodies against Raf-1, Erk-1, and Grb-2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to sonic hedgehog (Shh) were kindly provided by Dr. Andy McMahon (Bitgood and McMahon, 1995). Fertilized eggs were from SPAFAS (Preston, CT). Genistein was from Sigma Chemicals (St. Louis, MO). Genistein is a tyrosine kinase inhibitor that also has been reported to inhibit topoisomerase II and the production of reactive H_20_2 and to show weak estrogen activity (Koroma and de Juan, 1994).

Skin Explant Cultures The dorsal skin was removed from stage-31 or stage-34 chicken embryos and placed on culture inserts (0.4-μm pore size, Falcon, Franklin Lakes, NJ) in six-well culture plates containing Dulbecco's modified Eagle's medium with 2% fetal bovine serum so that the skin explant grew at the air/media interface. The explants were incubated in a humidified CO_2 incubator at 37°C.

FGFs were added directly to the culture media at the indicated concentrations, chosen from preliminary dose-response studies. Alternatively, Affigel blue beads (Hayamizu *et al*, 1991) soaked in FGF (850 ng per ml) for 1 h at 37°C were placed on top of the explants with fine forceps. Although the concentration of FGFs used to soak the beads is higher than the concentration placed in the culture media, the exact effective concentration at the skin surface is not known and is difficult to measure. For drug treatment, cultures were grown in the presence of 100 μM genistein for 4 d. The genistein-containing culture medium was refreshed every 2 d.

Immunohistochemistry Immuno-alkaline phosphatase staining was performed on Bouin's-fixed paraffin sections, as described (Chuong *et al*, 1990).

Measurements of Feather Diameter, Length, and Density To assess the effects of FGF on feather formation and growth, we analyzed the diameter, length, and density of feathers emerging from skin explants in the presence and absence of FGF. The effects were documented by photomicroscopy (Olympus, Lake Success, NY). All measurements were made from photographic enlargements. Images were digitized and analyzed using the Mocha image analysis software (Jandel Scientific, San Rafael, CA). Feather width was measured at the feather base to represent the feather diameter at the skin surface. Feather length represents the measurement from the feather base to the feather tip. (A schematic drawing is shown in **Fig 4*A***.) For feather density, a region with a specific response to FGF was selected, and the number of feather buds per unit area was calculated. Each experiment represents at least ten feather buds for each condition. The average $\pm$ SD was calculated. Feather density measurements are based on a combination of all four experiments.

RESULTS

Feather buds form from an immature, stage-31 embryonic chicken skin (Hamburger and Hamilton, 1951) explant grown in culture (Jiang and Chuong, 1992; Noveen *et al*, 1995a). A control explant prepared from a stage-31 embryo is shown in **Fig 1*A***. The explants were cultured for several days. At day 4, elongated feather buds had formed, as shown by Jiang and Chuong (1992) and in the periphery of **Fig 1*D***. Skin appendage formation was profoundly altered by exposure to the FGFs (FGF-1, FGF-2, and FGF-4). Because each of the FGFs produced similar effects in each of the experiments, only the results of a representative FGF family member are presented in detail.

FGF Causes the Formation of Stripes, Representing the Conversion of Interbud Domains to Bud Domains FGF-1 (500 ng per ml) or FGF-2 (500 ng per ml) added to the culture media caused adjacent feather buds growing near the midline to fuse, resulting in the appearance of stripes, which are inhibited in growth **(Fig 1*B,C*)**. At this developmental stage, the interbud domains were converted into feather bud domains. We further tested the responsiveness of stage-31 skin to FGF-4 by placing FGF-4–soaked beads on the center of the explants. In this experimental design, the rest of the skin explant serves as an internal control. FGF-4 caused the fusion of buds surrounding the bead in 4 d **(Fig 1*D*)**.

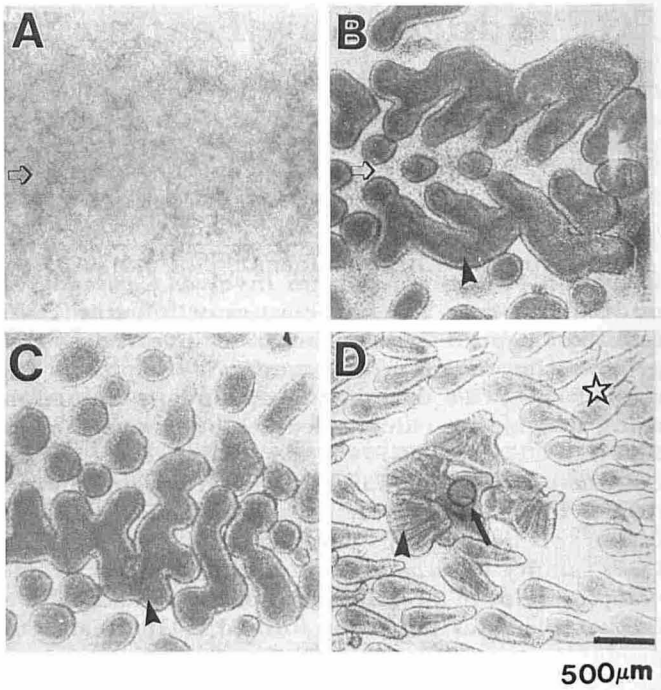

Figure 1. FGF causes fusion of buds by transforming interbud regions to bud regions. Skin explants prepared from stage-31 chicken embryos were prepared for growth in culture. *A*, control explant at day 0. *B–D*, explants were cultured for 4 d. Explants were treated with: *(B)* FGF-1 (500 ng per ml) or *(C)* FGF-2 (500 ng per ml) in the culture media, or with *(D)* FGF-4 (850 ng per ml)–coated beads (see *Materials and Methods*). The bead in *(D)* is indicated *(arrow)*. The feather buds at the periphery *(D)* develop normally and also serve as controls (☆). Fused feather buds *(B–D)* are marked *(arrowheads)*. *Open arrows* point to the midline. *Scale bar*, 500 μm.

FGF Causes the Formation of More and Smaller New Buds From the Feather-Forming Region Because feather bud formation begins from the midline and moves toward the periphery, the feather buds found at the lateral edge of the skin are younger than those near the midline. Technically, stage-34 embryos allow us to prepare larger skin explants that cover a wider range of developmental stages. Each developmental stage has a different competence to respond to FGFs. We prepared explants from stage-34 embryos **(Fig 2*A*)** and grew them for 4 d **(Fig 2*B*)**. We then exposed these stage-34 skin explants to FGF-2 (500 ng per ml) for 4 d and observed three major changes in the skin morphology **(Fig 2*C,D*)**. First, a longitudinal dark stripe formed toward the lateral edge of the explant **(Fig 2*B–D*, *star*)**. With transillumination, as used here, darkness represents a higher cell density, so this probably represents a zone of mesenchymal condensation in which feather-forming activity is being "reset." Second, feather buds emerging beyond (or lateral to) this zone in the feather-forming region were dramatically smaller than those found in control cultures **(Fig 2*B–D*, *arrow*)**. Third, the feather buds closer to the midline became wider and shorter than control buds in response to FGF treatment **(Fig 2*C,D*, *open arrow*)**. In some explants, feather buds flanking the midline also fused into stripes, similar to those shown in **Fig 1**.

To verify that the FGF-induced small buds were indeed feather buds, we prepared sections of these explants **(Fig 3)**. Morphologically, the new buds were similar to short feather buds, whereas the

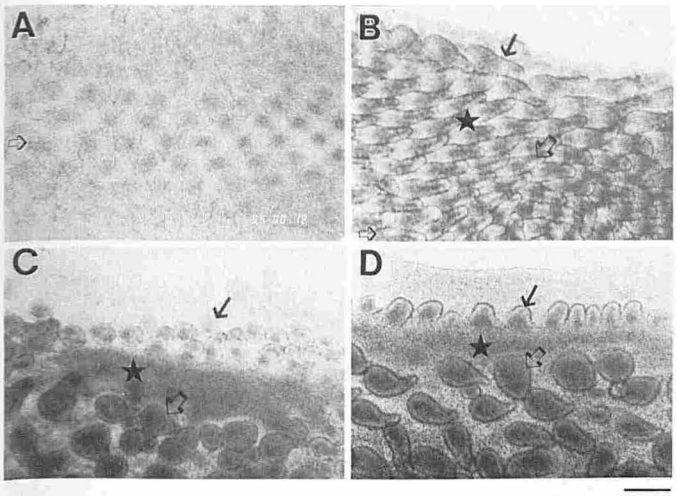

Figure 2. FGF increases the number of skin appendages in developing skin. Stage-34 embryonic chicken dorsal skin *(A)* was cultured for 4 d *(B)*. *(C,D)* two explants were cultured in the presence of FGF-2 (500 ng per ml) for 4 d. Because of the developmental sequence, feather buds are more mature near the midline *(small open arrows)*. The midline is in the middle of *panel A* and near the bottom of *panels B–D*. New feather buds are still forming near the lateral edge, which was originally a flanking area *(top, B–D)*. Older buds are indicated *(open arrows)*. Lateral to the older FGF-treated buds is an optically dense region (★). The equivalent region in control cultures is also indicated. Beyond this zone, at the lateral edge, is the zone of the youngest feather buds *(arrows)*. It is this zone that forms more new buds (compare *C,D* with *B*). *Scale bar*, 500 μm.

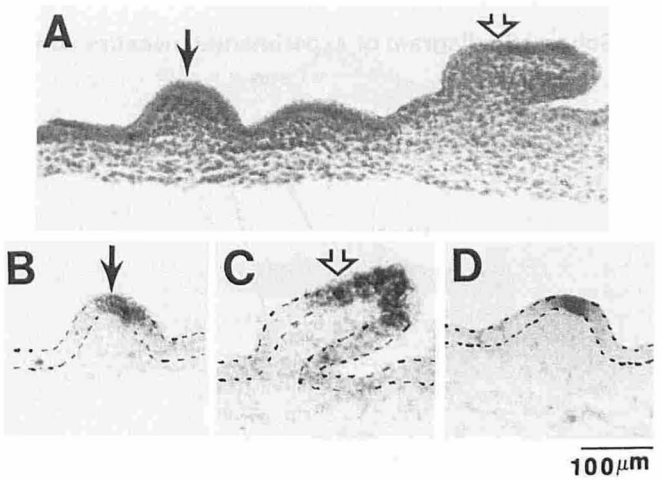

Figure 3. FGF-induced buds express characteristics of normal feather buds. *(A–C)* stage-34 skin explants were treated with FGF-2 (500 ng per ml) and cultured for 4 d. *(A)* explants were prepared as a paraffin block, sectioned, and stained with hematoxylin and eosin. The lateral side of the explant is toward the left edge of the panel. An FGF-induced new bud is indicated by the *solid arrows*, and the pre-existing older bud, which appears to be normal, is indicated by the *open arrows*. Compare this with **Fig 2*C,D*** (in **Fig 2**, the lateral side is toward the upper edge of the panel). *(B–D)* the sections are stained with antibodies to Shh using alkaline phosphatase–conjugated secondary antibodies. *(B)* an FGF-induced bud. *(C)* an old bud with a normal appearance in the same explant. *(D)* a control feather bud prepared from stage-35 skin. Note that all of these express Shh in the distal bud epithelia. *Scale bar*, 100 μm.

older feather buds had entered the long bud stage **(Fig 3*A*)** (for definition of short and long buds, see Widelitz *et al*, in press). We then stained them with antibodies to Shh, a signaling molecule recently shown to be expressed in the hair germ and feather bud (Bitgood and McMahon, 1995; Ting-Berreth and Chuong, 1996b). Shh was expressed in the distal epithelium of the induced buds **(Fig 3*B,C*)**. For comparison, a normal short bud from stage-35 skin that is of a similar developmental age as the FGF-induced bud is shown in **Fig 3*D***.

The results were analyzed quantitatively **(Fig 4)**. A schematic diagram describing how the measurements were made is shown **(Fig 4*A*)**. For these measurements, feather width is determined as the diameter at the feather base. Feather length was measured from the base to the tip of each feather. Feather density was determined as the number of full-length feathers within a unit area. The new buds were generally smaller and shorter than control buds **(Fig 4*B,C*)**. The buds also grew closer together, resulting in a higher feather density than that found in control cultures **(Fig 4*D*)**.

These findings demonstrate that FGF-1, -2, and -4 each can initiate or expand feather bud domains. Whether this leads to new or fused buds depends on the competency of the skin, which is determined by the developmental age. FGF-1, -2, and -4 have different potencies. FGF-4 is more potent in causing fusion in stage-31 skin, and FGF-2 is more potent in initiating new buds in stage-34 skin, whereas FGF-1 is slightly less efficient in causing these two phenomena.

FGF Also Induces the Formation of New Feather Buds From Apteric Regions of the Skin In the previous experiment, more feather buds were induced from the younger, lateral skin regions destined to form feather buds (part of the spinal tract). We wondered whether FGF could also induce feather buds from regions originally destined to be apteric. We used the two ventral, midabdominal apteric strips called the "pectoral apterium." Although sporadic buds can normally form in this region **(Fig 5*A–D*,** *arrow*), most of the area remains apteric **(Fig 5,** *star*). FGF was added in a localized fashion from Affigel blue beads (Hayamizu *et al*, 1991). FGF-4–soaked beads were placed on one strip **(Fig 5*A,B*)**, whereas the other was used as a control **(Fig 5*C,D*)**. Many new small feather buds formed around the FGF-coated beads **(Fig 5*B*,** *star*). Strips grown without beads or with control beads remained mostly apteric **(Fig 5*D*,** equivalent region). FGF-1 and FGF-2 had similar effects (data not shown), but FGF-4 gave more dramatic results. This result demonstrates that regions of stage-36 apteric epithelium are competent to form new skin appendages in response to FGF.

Intracellular Signaling Molecules Known to Mediate the FGF Pathway Are Enriched in Feather Bud Domains If the FGF pathway is physiologically involved in skin appendage formation, its molecular components should be present in the feather buds. FGFs and FGFRs are expressed in feather buds and hair germs (Moore *et al*, 1991; Sutton *et al*, 1991; Noji *et al*, 1993; reviewed in Widelitz *et al*, in press). In other systems, binding to the FGFR is transmitted intracellularly by signaling molecules. In these systems, Grb-2 acts in conjunction with Sos-1 as an adaptor between the tyrosine phosphorylated receptors and Ras. Downstream to Ras are Raf (MAPKKK), Erk (MAPK), and other molecules. We used immuno-alkaline phosphatase staining to examine whether these intracellular signaling molecules are present in the feather buds. At stage 31, these molecules were more evenly distributed in the epithelium and mesenchyme (not shown). At stage 34, higher levels of expression were maintained in the epithelium, whereas the molecules were redistributed in the mesenchyme **(Fig 6)**. In the mesenchyme, Grb-2 staining was light **(Fig 6*A*)**, whereas Ras was intensely stained all over **(Fig 6*B*)**. Raf and Erk staining was enriched in the anterior feather bud mesenchyme **(Fig 6*C,D*)**. The enrichment of these signaling molecules in the feather bud domains is consistent with the notion that the FGF signaling pathway is important in the formation of skin appendages. The heterogeneity of their distribution suggests that they may be involved in establishing anterior-posterior polarity by providing uneven growth kinetics to the developing feather bud.

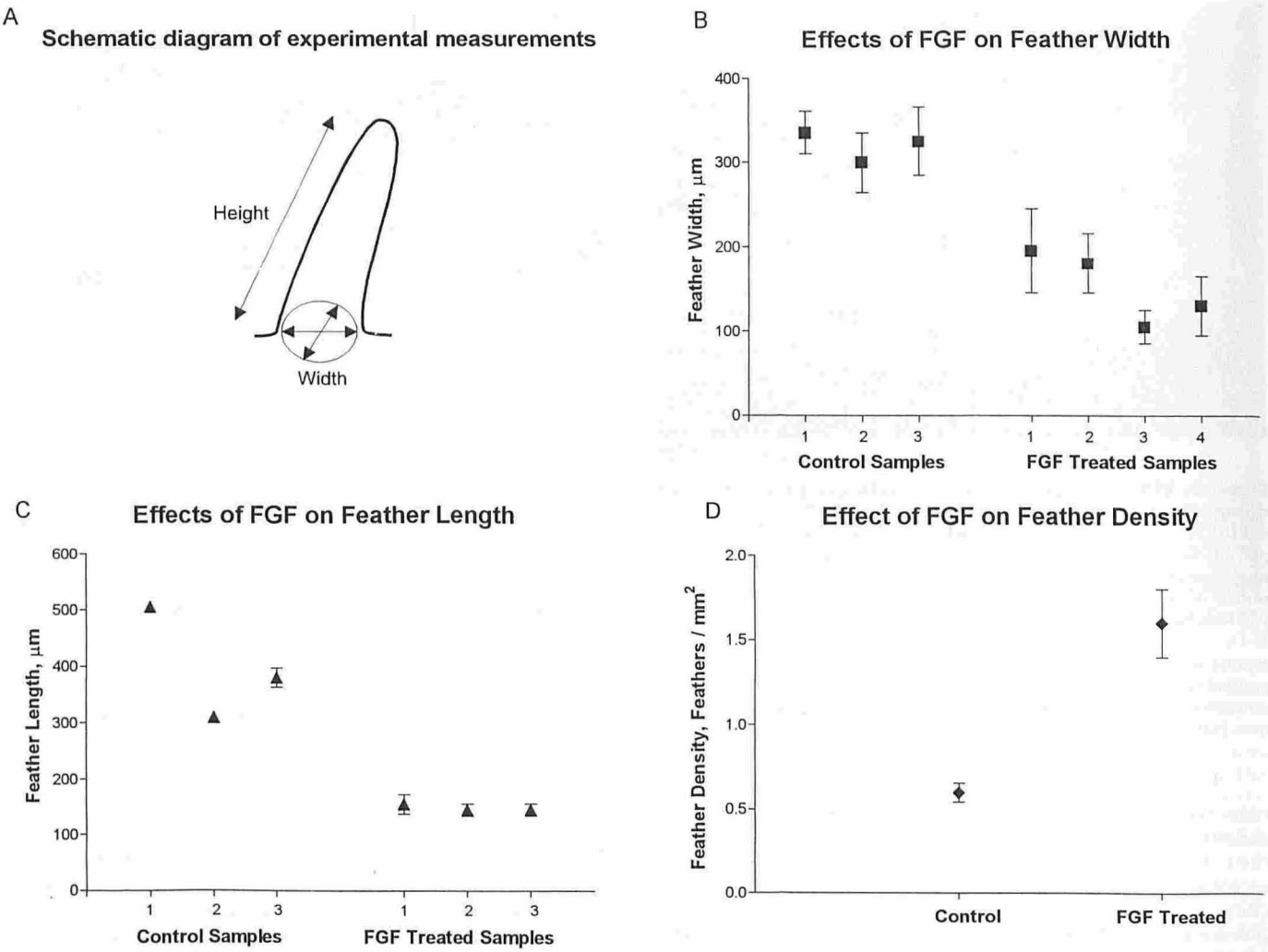

Figure 4. FGF causes feather buds in the lateral feather-forming region to be narrower and shorter and have a higher feather density. Control and FGF-2 (500 ng per ml)–treated skin explants prepared from stage-34 embryos were grown for 4 d. The explants were photographed, and the images were digitized and analyzed by image analysis software (Jandel, Mocha). *(A)* schematic diagram demonstrating how the measurements were made. Feather width was measured as the diameter at the feather base because the base of the feather bud is round (see **Fig 2**). Feather length was measured from the base to the tip of the feather bud. A minimum of ten feather buds was used to generate each data point. *(B)* results from three independent samples show the reduction of feather width. Two experimental points (FGF-treated samples 3 and 4) were obtained using feather buds from one control explant (control sample 3). *(C)* results from three independent samples show that the newly induced feather buds are shorter. *(D)* feather density was calculated as the number of feathers per unit area (within the selected region). Data were plotted from four replicate experiments. In the newly induced feather bud region, the density of feather buds increased and the buds were shorter and smaller. The mean ± SD is shown.

A Tyrosine Kinase Inhibitor, Genistein, Inhibits the Formation of Skin Appendages Growth factor receptors, such as FGF, exert their effects through tyrosine phosphorylation. To test whether inhibiting tyrosine phosphorylation can perturb skin appendage formation, we exposed skin explants to genistein, a specific tyrosine kinase inhibitor. A 2-d exposure of stage-31 skin explants to genistein (500 μM) blocked new feather bud formation. In addition, the feather buds that had already formed degenerated in response to genistein treatment and regressed to become small dermal condensations connected by condensation lines, representing remaining condensations (**Fig 7*A,B***, *open arrow*).

To test whether inhibition of tyrosine phosphorylation by genistein affects the FGF pathway, we used a combination of FGFs (see above): FGF-2 (500 ng per ml) in the media and FGF-4–coated beads (850 ng per ml) on top of the skin explant. The explant was cultured with genistein (500 μM) for 2 d. Genistein effectively inhibited the formation of skin appendages, even in the presence of exogenous FGF **(Fig 7*C*)**. These results are consistent with the notion that FGF induces skin appendage formation through a tyrosine phosphorylation signaling cascade.

DISCUSSION

FGF and the Molecular Cascade Involved in Feather Morphogenesis Our laboratory has been focusing on finding the molecular cascade involved in feather morphogenesis (Chuong *et al*, 1993). We started by working on adhesion molecules and have found that tenascin and neural cell adhesion molecule are involved in mediating the process of dermal condensations (Jiang and Chuong, 1992). We then looked for the upstream molecules that may regulate these adhesion molecules. One approach we took is to identify the intracellular signaling molecules involved in feather morphogenesis, with the rationale that any extracellular signaling molecules would function through cell surface receptors and exert their effects through intracellular signaling molecules. To this end, we found that protein kinase A is enriched in the feather germ mesenchyme, and protein kinase C is enriched in the inter-feather

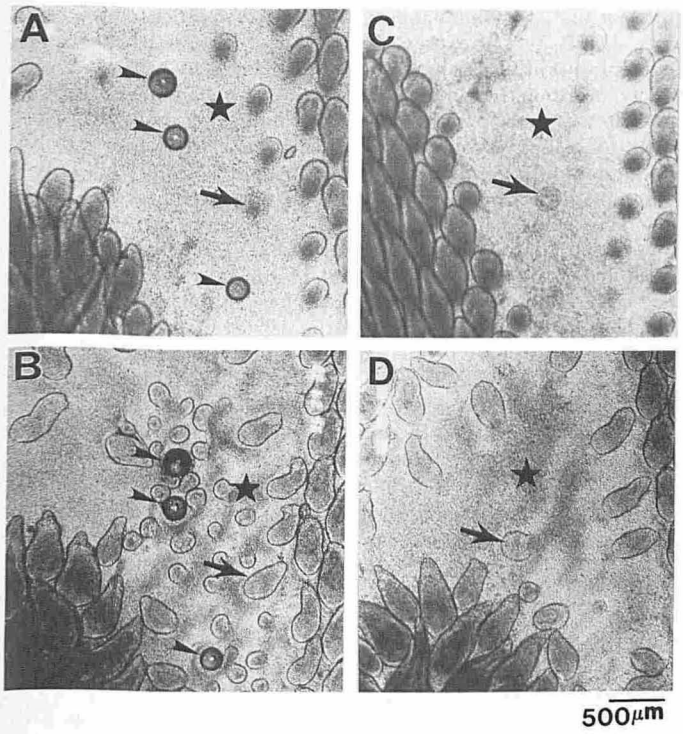

Figure 5. FGF induces new skin appendages from apteric epidermis. Stage-36 ventral embryonic chicken skin explants containing the apteric zone were cultured for 2 d in the presence *(A,B)* or absence *(C,D)* of FGF-4 (850 ng per ml)–coated beads. *(A,C)* skin at the beginning of culture. *(B,D)* 2 d later. The apteric region is marked (⋆). The feather buds that normally appear in the apteric region are indicated *(arrows)*. The beads are indicated by *arrowheads*. Note that in the apteric region, many new feather buds are induced by the FGF-4–coated beads. *Scale bar*, 500 μm.

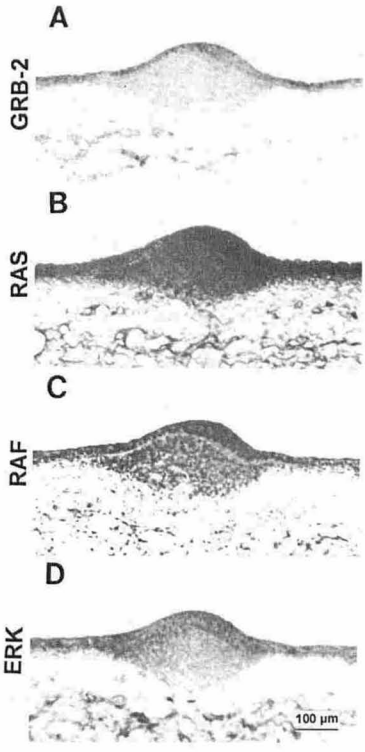

Figure 6. Signaling molecules known to be involved in the FGF pathway are specifically expressed in the feather bud domain. Longitudinal paraffin sections of developing stage-34 chicken dorsal skin were stained with antibodies using immuno-alkaline phosphatase methods. *(A)* Grb-2; *(B)* Ras; *(C)* Raf; *(D)* Erk. Note that these molecules, although with different distribution patterns, are all enriched in the feather bud domains. *Scale bar*, 100 μm.

germ mesenchyme. Protein kinase A agonists favor feather bud formation, whereas protein kinase C agonists favor the expansion of the interbud domain (Noveen *et al*, 1995a). What are the possible factors that regulate the spatial and temporal expression of these molecules in feather mesenchyme? We examined several growth factors and found that transforming growth factor–β can induce dermal condensations and suppress protein kinase C expression, but cannot induce phosphorylated cAMP response element binding protein expression (Ting-Berreth and Chuong, 1996a). To examine molecules further upstream, we found that Shh delivered with retroviral vectors can induce extra-large dermal condensations with a wide distribution of transforming growth factor–β expression (Ting-Berreth and Chuong, 1996b).

To sort out the order of these and other molecules involved in feather morphogenesis, we cultured skin explant cultures in which the epithelium and mesenchyme were recombined. In this condition, the old feather buds disappear and new placodes are induced by the feather bud mesenchyme. During this regenerative process, we followed the reappearance of molecules using whole-mount *in situ* hybridization. We found that the order of appearance is (1) FGF-4, bone morphogenetic protein–2, bone morphogenetic protein–4, (2) Shh, Wnt-7a, (3) Msx-1, Msx-2, and (4) NCAM, Hox C6 (Chuong *et al*, 1996). This suggests that FGF and bone morphogenetic protein are very early events in the molecular cascade of feather morphogenesis and may be involved in the initiation of skin appendages. In this report, we focused on the roles of FGFs in feather bud initiation and development in cultured avian skin explants.

Competence of the Epithelia to Form Skin Appendages: Developing *Versus* Mature Skin Exogenous FGF treatment induces two kinds of responses in feathers. On pre-existing short feather buds, FGF promotes the fusion of adjacent buds that are inhibited in growth **(Fig 1)**. On regions where feather placodes have not formed, FGF initiates many small feather buds that are more densely packed than in normal control cultures **(Fig 2)**. Both responses can be explained by the fact that FGF enhances the formation of feather bud domains and reduces the formation of inter-bud domains. Why do many small buds form in one case and fused stripes form in another case? We postulate that this is due to the competence of the responding skin or to the difference in the molecular context. In the regions where feather buds are still forming, the molecular machinery for lateral inhibition still exists and probably allows the inhibitory signals to interact with FGFs, leading to discrete feather placodes, albeit with a much smaller interbud space. In the regions where short feather buds already exist, the inhibitory signals may not be organized sufficiently to counteract the effect of FGF, leading to the merger of feather bud domains. FGFs can also induce skin appendages from epithelia originally destined to become apteric **(Fig 5)**. This suggests that these epithelia are competent to respond to appropriate inducers to form new skin appendages. In apteric skin, epithelia have not formed feathers simply because the initiation signals, such as FGF, may be lacking.

Competence to respond to appropriate growth factors also plays a role in other developmental systems. Recently, FGFs (FGF-1, -2, -4, and -8) were shown to have striking effects on the induction of new limb buds from chicken embryos (Cohn *et al*, 1995; Crossley *et al*, 1996). This dramatic FGF effect is only possible when acting on early embryonic chicken epithelia. New limb buds cannot be induced from adult tissue, and it is not easy to induce new hair from adult skin. The induction of skin appendages by dermal papilla from amputated whisker follicles (Jahoda *et al*, 1984; Horne *et al*, 1986)

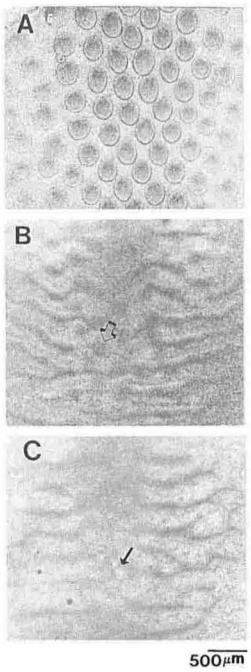

Figure 7. Genistein inhibits the formation of skin appendages, and this effect cannot be rescued by FGF-4. Stage-31 skin explants were cultured in the absence *(A)* or presence *(B,C)* of genistein (500 μM) for 2 d. For time 0, see **Fig 1A**. Note the suppression of feather bud formation *(A,B)*. In *(C)*, in addition to genistein in the media, FGF-2 (500 ng per ml) was added to the media and FGF-4 (850 ng per ml)–soaked beads *(arrow)* were placed on top of the explants. The suppression was not reversed. Dermal condensations are indicated *(open arrow)*. The stripes probably represent dispersing mesenchymal cells (see *Discussion*). *Scale bar*, 500 μm.

or glabrous skin (Jahoda *et al*, 1993), however, suggests that hair growth in older skin can occur if competent epithelial cells are activated.

What is the molecular nature of the competence to form skin appendages? It probably represents a combination of available molecular factors that can be activated in concert by appropriate inducers, such as FGF. Together, these signals can cause a group of cells to grow and differentiate into skin appendages. Among the molecules expressed in the early epithelial placode are Msx-1 and Msx-2, which are associated with feather growth (Noveen *et al*, 1995b). Shh, a newly identified, secreted signaling molecule with powerful morphogenetic function (Echelard *et al*, 1993; Krauss *et al*, 1993; Riddle *et al*, 1993; Ericson *et al*, 1995; Marti *et al*, 1995), is also present in early skin appendage placodes (Bitgood and McMahon, 1995; Iseki *et al*, 1996; Noveen *et al*, 1996) and is involved in the formation of feather buds (Ting-Berreth and Chuong, 1996b). These molecules may be components of the competence cascade that can be stimulated by FGF activation. The expression sequence data suggest that FGF is upstream to Shh and Msx (Chuong *et al*, 1996), and FGF-4 was shown to induce Shh and Msx in limb buds (Laufer *et al*, 1994; Niswander *et al*, 1994). The finding that FGF-induced new buds express Shh in the placode is consistent with this FGF-Shh relationship in skin appendages.

FGF Signaling Pathway in Skin Appendage Formation

FGFs and their receptors have been shown to be expressed in skin. FGF-4 was found diffusely distributed through the epithelium of feathers at the short-bud, long-bud, and feather follicle stage (Nohno *et al*, 1995; for bud terminology, see Widelitz *et al*, in press). FGFR-1 transcripts were present in the mesenchyme of feather placodes. FGFR-2 was expressed in the feather placode epithelium. By the short feather bud stage, the FGFR-1 transcripts were in the anterior mesenchyme. FGFR-2 transcripts were in the posterior bud epithelium and in the mesenchyme between feather buds. FGFR-3 was expressed throughout the mesenchyme of developing skin in chickens (Noji *et al*, 1993). In hair, FGF was widely distributed in the epithelium. At the hair plug stage, it concentrated in the periphery of the involuting hair plug (Moore *et al*, 1991). FGF-2 was also concentrated at the epithelial-mesenchymal border in sheep skin hair placodes (Moore *et al*, 1991). FGFR-1 transcripts as determined by *in situ* hybridization were present in the mesenchyme of hair placodes; FGFR-2 was expressed in the placode epithelium (Orr-Urtreger *et al*, 1991) and in the hair matrix of the hair follicle (Rosenquist and Martin, 1996). FGFR-3 was seen in the pre-cuticle cells at the periphery of the hair bulb in the skin of mice (Rosenquist and Martin, 1996). FGFR-4 transcripts were in the periphery of the hair bulb and in the inner and outer root sheaths (Rosenquist and Martin, 1996). Transcripts for FGFR-5 were in the outer root sheath (Hebert *et al*, 1994). These complex patterns suggest the multiple roles of FGF in skin appendage formation. Because biochemically there is no one-to-one correspondence between the nine FGF ligands and the four FGFRs (Fernig and Gallagher, 1994), it is not surprising that each of the FGFs tested here appeared to have similar effects with different efficiencies.

The effect of FGFR activation is mediated through tyrosine phosphorylation (Fernig and Gallagher, 1994). After activation, as shown in many tyrosine kinase–type receptors, Grb works as an adaptor with Sos-1 to activate Ras, which in turn activates Raf (MAPKKK), Mek (MAPKK), and Erk (MAPK) (Roberts, 1992; McCormick, 1993; Marshall, 1995). The demonstration that Grb, Ras, Raf, and Erk-1 are all enriched in the developing mesenchymal skin appendage domains, but not in the interbud domains **(Fig 6)**, supports the possibility that FGF induces skin appendages through activation of this intracellular signaling cascade. It is possible, however, that these molecules are also stimulated by different tyrosine kinase receptors or that FGF may function through other pathways. Because we showed that CREB is phosphorylated specifically in the feather bud domain when feather buds are induced (Noveen *et al*, 1995a), it would be interesting to examine whether there is a direct relationship between FGF and cAMP response element binding protein in the future.

This work also suggests that it may be possible to alter skin appendage formation by modulating the signaling molecules downstream to FGF for specific applications. We tested the ability of genistein, a specific inhibitor of tyrosine kinase activity, to inhibit the normal induction of skin appendages and to block the ectopic effect of FGFs **(Fig 7)**. That genistein can do both further supports the possibility that the FGF pathway is normally involved in the induction of skin appendages. It also opens the possibility of altering skin appendage formation through pharmacologic modulation of signaling molecules.

One interesting observation is that the genistein-suppressed explant formed diagonal stripes connecting small dermal condensations **(Fig 7)**. In FGF-stimulated skin, the inter-bud regions were converted into bud regions. When fusion occurs between buds, they form diagonal stripes rather than a radial distribution from the center of individual buds **(Fig 1)**. This pattern is reminiscent of the hexagonally arranged fibrous lattice between dermal condensations originally observed by birefringence (Stuart *et al*, 1967). These investigators have proposed that mesenchymal cells generated outside of the dermal condensations orient and migrate along these collagen fibrous lattices toward the dermal condensations (Stuart *et al*, 1967). What we observed may be the increased "cellular traffic" caused by FGF on these diagonally arranged "cellular highways" and the dispersion of mesenchymal cells caused by inhibition of FGF signaling.

In summary, we showed that FGFs can act on developing skin to initiate new feather buds or expand the skin appendage domains, depending on the competence of the responding skin. This competence is dependent on the developmental stage and is mostly lost in the adult. A series of studies using the feather model from this laboratory have identified some of the relevant molecules. If we can

learn more about the molecular machinery of this competence, we may in the future be better able to manage wounded tissues that require skin appendage regeneration or disease conditions that involve alopecia.

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