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Fibroblast growth factor 2 can replace ectodermal signaling for feather development

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ABSTRACT **The initiation and morphogenesis of cutaneous appendages depend on a series of reciprocal signaling events between the epithelium and mesenchyme of the embryonic skin. In the development of feather germs, early dermal signals induce the formation of epidermal placodes that in turn signal the mesoderm to form dermal condensations immediately beneath them. We find a spatially and temporally restricted pattern of transcription for the genes that encode fibroblast growth factor (FGF) 2 and FGF receptor (FGFR) 1 in developing feather germs of the chicken embryo. FGF-2 expression is restricted to the epidermal placodes, whereas FGFR-1 expression is limited to the dermal condensations. Transcription of these genes could not be detected in skins of scaleless (*sc/sc*) embryos that fail to develop feathers as a result of an ectodermal defect. Treatment of *sc/sc* skins with FGF-2 results in the formation of feathers at the site of application of the growth factor and the induced feathers express FGFR-1 in their dermal condensations. Thus, we have established FGF-2 as an epidermal signal in early feather germ formation. The observation that FGF-2 can rescue the mutant phenotype of *sc/sc* embryos suggests that FGF-2 either is, or is downstream from, the signal that the *sc/sc* mutant ectoderm fails to generate.**

Epithelial–mesenchymal interactions play an essential role in the morphogenesis of cutaneous appendages (1) (feathers, scales, and hairs), the limb (2–4), tooth (5), kidney (6), lung (7), and mammary gland (8). In the feather or scale forming regions of the skin of the chicken embryo, the epidermal placode is the first morphological predictor for the site of the formation of an appendage and it provides a signal to the dermis that results in the formation of dermal condensations immediately beneath the placode (1). The formation of epidermal placodes is determined by mesodermal signals as revealed from heterotypic and heterochronic recombinations of ectodermal and mesodermal components of embryonic skins. Dermal signals also determine whether scales or feathers will develop and, if feathers develop, determine their arrangement in a precise hexagonal pattern. Feathers appear in a specific spatiotemporal pattern within clearly identifiable feather tracts (9). Although a number of studies have suggested that cell adhesion molecules, extracellular matrix molecules, and transcription factors may be involved in skin morphogenesis (10, 11), no specific molecular mechanisms involved in the epithelial–mesenchymal interactions of early feather bud formation have been identified.

The scaleless (*sc/sc*) mutant (12), which lacks feathers and scales, is an ideal model system for studying early feather development. The skins of these mutant embryos do not develop ectodermal placodes (13) and the mutation affects the ectoderm in such a way that the normal sequence of tissue interactions is interrupted. It is not clear if an early dermal signal cannot be received by the ectoderm or if the ectoderm cannot respond to that signal. However, the mutant mesoderm is fully capable of participating in feather and scale development when recombined with genetically normal ectoderm (14–16). Signaling involving growth factors has been demonstrated in the early development of many developmental systems including some that, like the skin, depend on epithelial–mesenchymal interactions for their development (17). In the limb bud, for example, fibroblast growth factor (FGF) 2 and FGF-4 can substitute for the outgrowth inducing properties of the apical ectodermal ridge (2, 3). In the present study, we have examined the role of FGF signaling in the epithelial–mesenchymal interactions during the initiation of feather germ formation in genetically normal and *sc/sc* embryos.

MATERIALS AND METHODS

Scaleless embryos were obtained from fertile eggs produced by a scaleless flock maintained by the Department of Animal Genetics, The University of Connecticut (Storrs, CT). Normal embryos were obtained from fertile eggs purchased from SPAFAS farms (Norwich, CT). FGF-2 was purchased from R & D Systems. Heparin beads were obtained from Bio-Rad. The cDNA clone for alt-FGF-2 (18) was obtained from J. Lough (Medical College of Wisconsin, Milwaukee, WI). The cDNA for FGF receptor (FGFR) 1 (19) was obtained from E. Pasquale (The Burnham Institute, La Jolla, CA). All embryos were staged according to the normal stages of Hamburger and Hamilton (20).

FGF-2 was applied to dorsal *sc/sc* skins with heparin beads. The beads were soaked for 2 h in a solution of FGF-2 (1 mg/ml) made up in PBS and washed three times in PBS immediately before use. Heparin beads soaked in PBS were used as controls. The *sc/sc* skins were placed in Millipore filter chambers and transferred onto the chorioallantoic membrane of 10-day normal embryos and maintained in an incubator for 3 or 5 days (16).

Whole-mount *in situ* hybridizations were done using the digoxigenin method as described (4). A 445-bp antisense FGF-2 RNA probe was made using the full-length cDNA for alt-FGF-2 (18). The FGFR-1 antisense RNA probe was a 490-bp fragment encoding the 5′ end and the first immunoglobulin-like domain of FGFR-1 derived from a chicken cDNA clone (19). After photography of the stained embryos, some embryos were rehydrated, soaked in 30% sucrose solution overnight at 4°C, and embedded in O.C.T. Compound (Miles) and sectioned at 5 μm on a cryostat. Sections were examined with a Zeiss Axiophot.

RESULTS AND DISCUSSION

FGF-2 and FGFR-1 Transcripts Are Present in a Unique Spatial and Temporal Pattern in Normal Skin. We examined

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Abbreviations: FGF, fibroblast growth factor; FGFR, FGF receptor; E, embryonic day.

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the spatial and temporal pattern of transcription of the FGF-2 and the FGFR-1 genes by whole-mount *in situ* hybridization. In stage 33 [embryonic day (E) 8] skins of normal embryos, FGF-2 transcripts could be detected in all feather germs (Fig. 1*a*). The pattern of staining of early feather germs appears as filled circles arranged in a hexagonal pattern. Cross sections of stained embryos indicate that FGF-2 mRNA expression is restricted to the epidermal placodes of the feather germs (Fig. 1*b*). No FGF-2 mRNA could be detected in the interbud regions. FGF-2 expression in the epidermal placode precedes the formation of the dermal condensations. Once dermal condensations are formed below the placode, transcripts for FGFR-1 can be detected in these structures (Fig. 1 *c* and *d*). The pattern of transcription of FGFR-1 in the dermal condensation (Fig. 1*d*; ref. 21) was evident in all feather germs of all feather tracts examined. The restricted pattern of expression of FGF-2 and FGFR-1 mRNA in the ectoderm and mesoderm, respectively, indicate the specificity of the probes used.

***sc/sc* Skins Lack Transcripts for Both FGF-2 and FGFR-1.** Examination by whole-mount *in situ* hybridization of the skins of embryos that are homozygous for the recessive gene scaleless (*sc/sc*) of stage 33 (E8) failed to reveal any transcripts for FGF-2 (Fig. 2*a*) and for FGFR-1 (Fig. 2*b*). Positive signals for the two probes were detected only in tissues situated below the skin. The negative signal in the skins of the stage 33 embryos does not represent a developmental delay since no signal can be detected in *sc/sc* embryos up to stage 38 (E12).

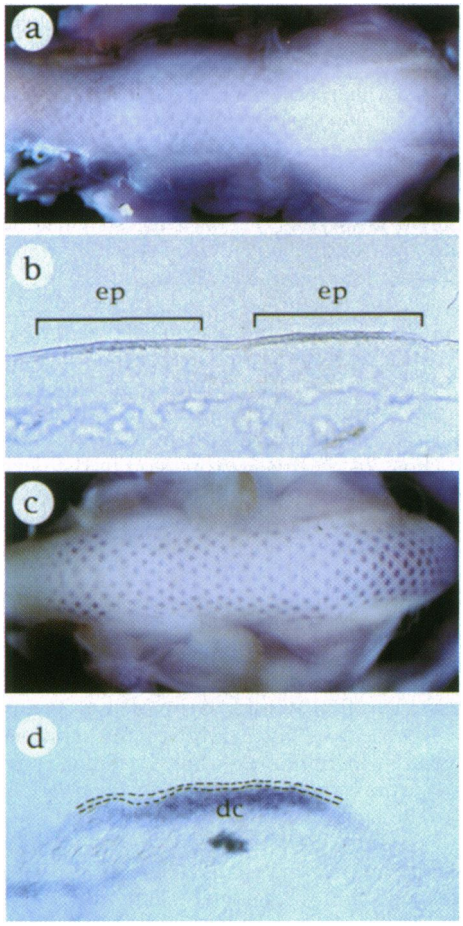

FIG. 1. Whole-mount *in situ* hybridization analysis for FGF-2 (*a* and *b*) and FGFR-1 (*c* and *d*) mRNA in skins of normal embryos. (*a* and *c*) Anterior is to the left of the photograph. The feather germs appear as full circles arranged in a hexagonal pattern. (*b*) Cross section of normal embryo at stage 33 (E8). FGF-2 mRNA is restricted to the epidermal placodes (ep). Two epidermal placodes are shown. (*c*) FGFR-1 mRNA is detected in the feather germs of stage 33 (E8) in normal embryo. (*d*) FGFR-1 mRNA is restricted to the dermal condensation (dc) immediately under the epidermal placode. The epidermis is outlined by the dotted lines.

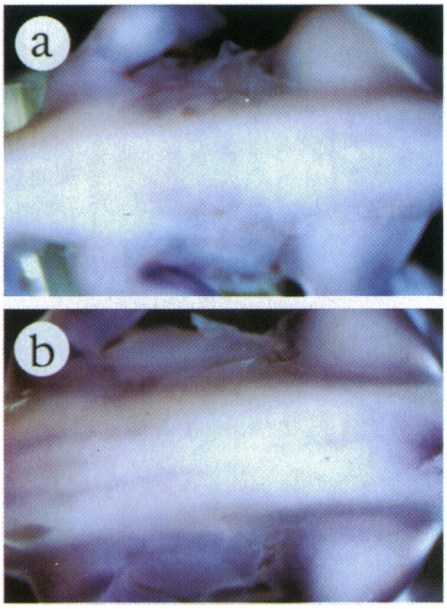

FIG. 2. Whole-mount *in situ* hybridization analysis for FGF-2 (*a*) and FGFR-1 (*b*) mRNA in skins of stage 33 (E8) *sc/sc* embryos. No FGF-2 (*a*) and FGFR-1 (*b*) transcripts can be detected. Positive signals for both probes are visible in tissues below the skin. Anterior is to the left of the photograph.

Exogenous FGF-2 Induces Feathers in *sc/sc* Dorsal Skins. Since FGF-2 transcripts are restricted to the epidermal placodes of normal feather germs and these structures and FGF-2 expression are absent from sc/sc skins, we hypothesized that the defective signaling in the mutant ectoderm may involve FGF-2. To test this hypothesis, heparin beads loaded with FGF-2 were applied to stage 33 (E8) *sc/sc* dorsal skins, placed on Millipore filter chambers, and cultured on the chorioallantoic membrane of 10-day chicken embryos for 3 or 5 days. Our results show that FGF-2 can induce the formation of feathers in *sc/sc* skins.

The results of FGF-2-treated *sc/sc* skins after 5 days of culture are illustrated in Fig. 3. PBS-soaked beads, used as control, did not stimulate any feather development in the *sc/sc* skins (Fig. 3*a*). In contrast, a dramatic induction of feathers is evident in the mutant skins that were treated with FGF-2-loaded beads (Fig. 3 *b–d*). The barb ridges and barbule cells (Fig. 3 *g* and *h*) seen in cross-sections of the FGF-2-treated *sc/sc* skins identify the induced appendages definitively as feathers. The FGF-2-treated *sc/sc* skins contained a number of fused feathers. An enlarged illustration of two such fused feathers is shown in Fig. 3 *e* and *f*. A cross-sectional view indicates that such appendages are outlined by one feather sheath but divided into two separate compartments by a septum (Fig. 3*h*). Most of the fused feathers observed at 5 days result from feather buds that were seen at 3 days to be located closely to each other.

The correlation between the site of bead deposition and feather germ formation was evaluated at 3 days to avoid any displacement of the beads as the feather filaments grow out during prolonged culturing. Feather buds are clearly evident after 3 days of culture, and these can be observed immediately next to the FGF-2-loaded beads (Fig. 4*a*). Thus, the exact correlation between the location of FGF-2-loaded beads and feather germ development in *sc/sc* skins mimics the situation in normal skins where the epidermal placode is a source of FGF-2 and the site of feather outgrowth.

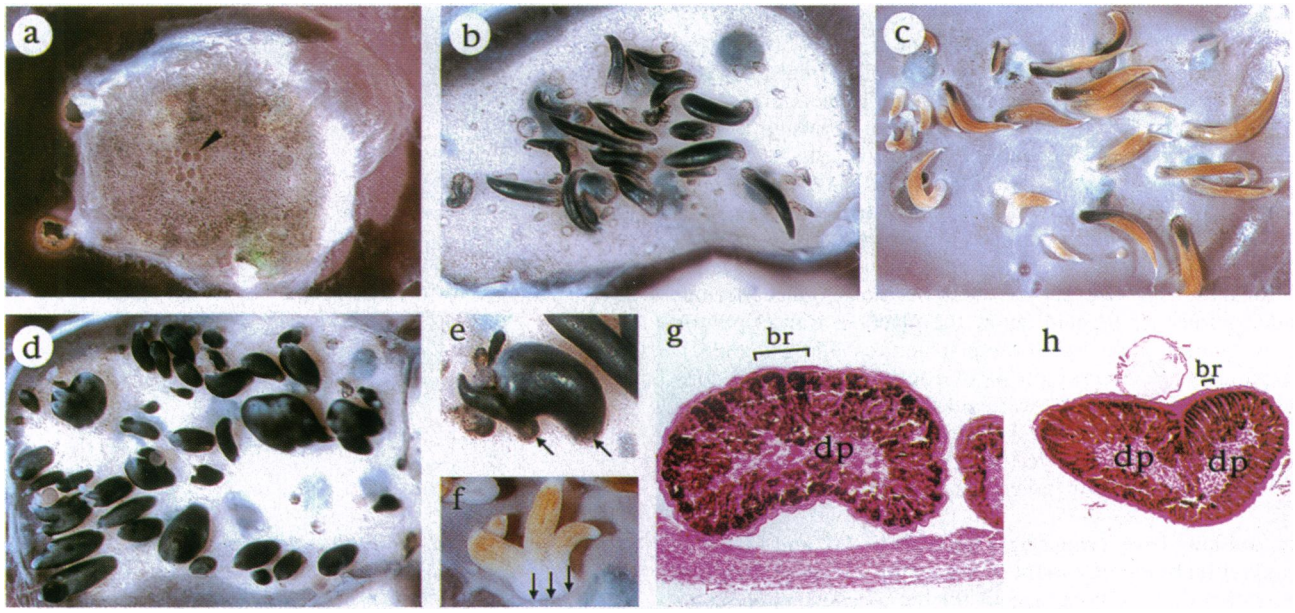

FIG. 3. Induction of feathers in dorsal skins of *sc/sc* embryo of stage 33 (E8) in response to FGF-2. FGF-2-loaded heparin beads were applied onto the surface of *sc/sc* skins that were cultured on the chorioallantoic membrane for 5 days. (*a*) *sc/sc* skin to which PBS-soaked beads were applied. The arrowhead indicates the deposited beads. No feathers developed. (*b–d*) Three skins treated with FGF-2-loaded beads. Although a few beads can still be seen on the skins, most were dislocated during processing of the specimen. The variation in pigmentation of the induced feathers between skins reflects the heterogeneity of the genetic background of the scaleless line. (*e* and *f*) Enlarged view of two fused feathers. The arrows mark the base of the fused feathers. (*g*) Cross section of a single feather. Even though the number of barb ridges (br) was abnormally large compared with the 9–13 barb ridges found in normal feathers, the barb ridges were normally arranged with a single dermal pulp (dp). (*h*) Cross section of fused feather. The feather was outlined by one feather sheath but a septum divides the inside into separate compartments.

FGF-2 Signaling Is Not Sufficient to Establish the Anteroposterior Orientation of the Feathers. The anteroposterior orientation of feathers in normal skins is determined by the ectoderm (22). The examination of the *sc/sc* skins treated with FGF-2 after 5 days (Fig. 3 *b–d*) suggests that the induced feathers do not assume an anteroposterior orientation. To examine this in more detail, we analyzed FGF-2-treated *sc/sc* skins after 3 days of culture to see if these skins expressed FGFR-1 mRNA in their mesoderm, and if they did, if the pattern of expression was asymmetric as it is in normal feather germs (Fig. 4*c*). FGFR-1 transcripts are present in the dermis of the feather germs induced in the *sc/sc* skins treated with FGF-2 (Fig. 4*b*) in sharp contrast to their absence in intact *sc/sc* skin (Fig. 2*b*). FGFR-1 expression in the dermis of the treated mutant skins does not display the orderly asymmetric pattern of expression that predicts the anteroposterior orientation of feather germs in normal skins. The abnormal pattern is consistent with the failure of the induced *sc/sc* feathers to orient themselves in an anteroposterior direction. The results suggest that FGF-2 signaling is not sufficient for the establishment of the well-organized anteroposterior orientation of feathers.

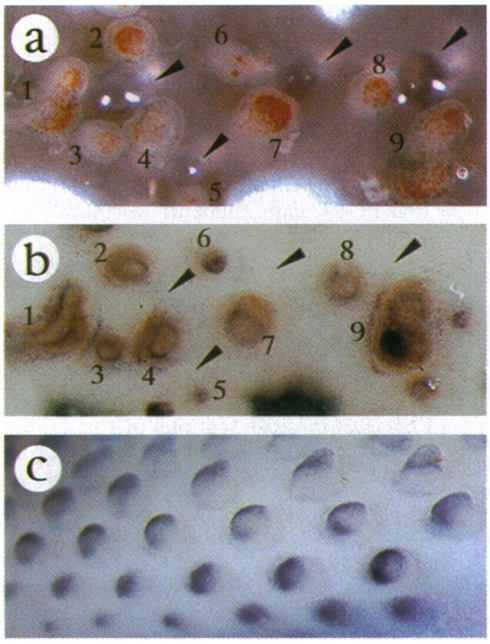

FIG. 4. FGFR-1 expression in the feather buds induced by FGF-2 beads in *sc/sc* skins and in intact normal skin. The photographs shown in *a* and *b* represent the same skin that was cultured for 3 days on the chorioallantoic membrane. In *a*, the arrowheads point to four FGF-2-loaded beads. The numbers 1–9 identify feather germs that surround the beads. In *b*, the skin was processed for *in situ* hybridization with the FGFR-1 RNA probe. Transcripts for FGFR-1 are present in the dermis of the feathers but fail to display the asymmetric pattern of expression that predicts the anteroposterior orientation in outgrowing feather germs of normal embryos as shown in *c*. In *c*, anterior is to the left of the photograph. Beginning at the bottom of the photograph each row represents developmentally more advanced feather germs.

The presence of FGFR-1 in the treated *sc/sc* skins indicates that the response to FGF-2 involves the activation of the FGFR-1 gene in the mutant dermis. Preliminary results suggest that the expression of the receptor may result from a direct response to the ligand as has been reported for the induction of activin receptors cActR-IIB (23) and cActR-IIa (24) by exogenous activin. We have detected FGFR-1 expression in denuded stage 33 (E8) *sc/sc* dorsal dermis after treatment with FGF-2. This mutant dermis has not been influenced by ectodermal signals but is fully competent to participate in feather formation when recombined with normal ectoderm (14–16).

The Induction of Feathers in *sc/sc* Skin by Exogenous FGF-2 Is Temporally Restricted. Feathers could be induced by FGF-2 in *sc/sc* skins from 7- and 8-day embryos (Fig. 5*a*) but not from 11-day embryos (Fig. 5*b*). Thus the response to the growth factor is temporally limited. This change in the response to FGF-2 is similar to the results obtained from tissue

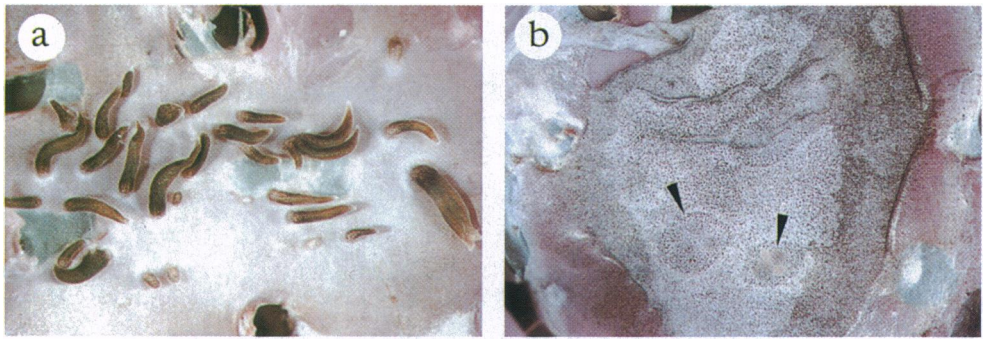

FIG. 5. Developmental stage specific competence of *sc/sc* skin response to FGF-2. FGF-2-loaded beads were applied to stage 31 (E7) and stage 37 (E11) *sc/sc* skins and cultured for 5 days. (*a*) Stage 31 (E7) *sc/sc* skin developed feathers under the influence of FGF-2 beads. (*b*) Stage 37 (E11) *sc/sc* skin did not respond to the FGF-2 stimulus. The arrowheads identify the sites where the beads were applied.

recombination experiments in which *sc/sc* dermis of different stages was recombined with normal 11-day foot ectoderm (16). Feathers develop when such foot ectoderm is recombined with 7- to 8-day dorsal mesoderm (25). Eight-day *sc/sc* dermis can participate with normal ectoderm in feather formation, but this ability is gradually lost from mutant mesoderm of increasing developmental ages (16). Thus, there is a developmental window in which the mutant mesoderm can respond to both FGF-2 signaling and to cues from normal ectoderm.

Our results indicate that epidermal placode derived FGF-2 is an important signal in early feather development of normal embryos and that this signal is absent from the skin of the *sc/sc* mutant that lacks epidermal placodes. Furthermore, providing exogenous FGF-2 to *sc/sc* skins is sufficient to result in the formation of complex structures such as a feathers. The FGF-2 signaling by the epidermal placode of the feather germ reported herein is analogous to the FGF signaling by the apical ectodermal ridge of the outgrowing limb bud. This specialized ectodermal structure is essential for distal outgrowth of skeletal elements of the limb (26). The apical ectodermal ridge of the limb bud, however, expresses FGF-2, -4, and -8, and all three FGF family members can perform the function of the apical ectodermal ridge (2, 3, 27, 28). Whether similar redundant signaling is generated by the epidermal placode of the feather germ is not known at this time.

The limb and the skin differ, however, in that in the former the ectodermally derived FGF results in the formation of a mesodermally derived skeleton whereas in the latter the appendages obtained are derived from the ectodermal cells that were the source of the FGF. The events that take place in the early morphogenesis of the skin may be more analogous those of the tooth (29) or kidney (30, 31) where the structures such as the tooth cusp and the tubules and collecting ducts of the pro- and mesonephros are derived from the epithelial structures that were the source of FGF signals early in their development.

The results obtained with the *sc/sc* skins indicate that this mutant is an excellent system to elucidate signaling pathways by FGF and other signaling molecules in the morphogenesis of cutaneous appendages. Mechanisms identified from this system may be applicable to the understanding of developmental mechanisms in other organs in which epithelial–mesenchymal interactions play a role.

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