Quail-duck chimeras reveal spatiotemporal plasticity in molecular and histogenic programs of cranial feather development

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Summary

The avian feather complex represents a vivid example of how a developmental module composed of highly integrated molecular and histogenic programs can become rapidly elaborated during the course of evolution. Mechanisms that facilitate this evolutionary diversification may involve the maintenance of plasticity in developmental processes that underlie feather morphogenesis. Feathers arise as discrete buds of mesenchyme and epithelium, which are two embryonic tissues that respectively form dermis and epidermis of the integument. Epithelial-mesenchymal signaling interactions generate feather buds that are neatly arrayed in space and time. The dermis provides spatiotemporal patterning information to the epidermis but precise cellular and molecular mechanisms for generating species-specific differences in feather pattern remain obscure. In the present study, we exploit the quail-duck chimeric system to test the extent to which the dermis regulates the expression of genes required for feather development. Quail and duck have distinct feather patterns and divergent growth rates, and we exchange pre-migratory neural crest cells destined to form the craniofacial dermis between them. We find that donor dermis induces host epidermis to form feather buds according to the spatial pattern and timetable of the donor species by altering the expression of members and targets of the Bone Morphogenetic Protein, Sonic Hedgehog and Delta/Notch pathways. Overall, we demonstrate that there is a great deal of spatiotemporal plasticity inherent in the molecular and histogenic programs of feather development, a property that may have played a generative and regulatory role throughout the evolution of birds.

Key words: Feather morphogenesis, Quail-duck chimeras, Neural crest transplants, Epithelial-mesenchymal interactions, Modularity, Plasticity, Evolutionary developmental biology, *bmp4, bmp2*, *shh, delta*

Introduction

Feathers are a defining feature of birds. They are essential for flight, protection, thermoregulation, waterproofing and social displays, among other functions. Within individuals and across species there exists remarkable diversity in the size, shape, arrangement and color of feathers in association with evolutionary adaptations (Lucas and Stettenheim, 1972; Feduccia, 1996; Prum and Dyck, 2003). Despite considerable progress in understanding key events during feather morphogenesis, the precise cellular and molecular mechanisms for generating species-specific differences in feather pattern remain largely unknown. A principal mechanism facilitating feather evolution may be the maintenance of plasticity in developmental processes that control the temporal and spatial expression of genes required for feather morphogenesis. If such developmental processes are free to vary, then any associated changes in the domains and timing of gene expression could cause downstream modifications to feather pattern and morphology (Brush, 2000).

Arguably, the most crucial developmental process underlying feather morphogenesis is the series of reciprocal signaling interactions between the dermis and epidermis of the embryonic integument. Yet, identifying specific properties that would enable these signaling interactions to fluctuate in time and space, and thus, impart plasticity in the molecular and histogenic programs of feather development, has been elusive. One informative experimental approach might be to change the embryonic history of either the dermis or the epidermis in a manner that would alter the subsequent signaling interactions between these tissues and reveal inherent properties of the integumentary system such as the hierarchical levels of organization, inductive potentials or limits of competency. Fate-map studies have demonstrated that in the trunk and posterior portions of the head, the dermis is derived from mesodermal mesenchyme of dermomyotomal and somatopleural origin, while in the face and neck the dermis arises from neural crest mesenchyme (Noden, 1978; Noden, 1986; Couly et al., 1992; Olivera-Martinez et al., 2000; Fliniaux et al., 2004; Olivera-Martinez et al., 2004a). Neural crest mesenchyme is generated along the dorsal margins of the neural tube during neurulation and undergoes extensive migration throughout the craniofacial complex. These cells also differentiate into pigment-producing melanocytes, which become secondarily associated with the epidermis and are the source of color throughout the body (Cramer, 1991; Le Douarin and Dupin, 1993; Bronner-Fraser, 1994; Hirobe, 1995). The epidermis is a stratified epithelium of non-neural

ectodermal origin that produces the keratinized structural tissues characteristic of feathers (Couly and Douarin, 1988; Pera et al., 1999; Yu et al., 2004).

Heterotopic, heterochronic, heterospecific and heterogenetic tissue recombinations have demonstrated that the time of appearance, location, size, number and morphological identity of feathers are determined by the dermis (Cairns and Saunders, 1954; Saunders and Gasseling, 1957; Rawles, 1963; Wessells, 1965; Dhouailly, 1967; Dhouailly, 1970; Linsenmayer, 1972; Dhouailly, 1973; Dhouailly and Sawyer, 1984; Song and Sawyer, 1996; Prin and Dhouailly, 2004), but the specific cellular and molecular mechanisms through which this information is conveyed are unclear. Mesenchyme becomes competent to induce feathers at an early embryonic stage prior to any obvious morphological changes in either the mesenchyme itself or in the overlying epithelium (Widelitz et al., 1997). The first morphological indication of feather formation is the aggregation of mesenchyme into a thin, uniform layer of dense dermis beneath the epithelium (Wessells, 1965; Brotman, 1977; Mayerson and Fallon, 1985). The local epithelium then thickens into a specialized epidermal placode, the mesenchyme aggregates into a dermal condensation, the placode and mesenchyme rise above the integumentary surface, and both tissues undergo proliferation, cell movements and differentiation (Pispa and Thesleff, 2003; Olivera-Martinez et al., 2004b). Presumably, the dermis releases a primary signal, which instructs the epithelium to begin making a placode. This initial induction depends on the mesenchyme reaching a critical threshold of aggregation size and expressing higher levels of cell-adhesion molecules (Jiang et al., 1999). Although the identity of the first dermal signal is not known, likely candidates include molecules in the Bone Morphogenetic Protein (BMP) and Fibroblast Growth Factor (FGF) families (Tao et al., 2002; Pispa and Thesleff, 2003; Mandler and Neubuser, 2004). A decade of molecular research on feather morphogenesis suggests that a general hierarchical sequence of signaling events may be from the BMP and FGF pathways, to the Sonic Hedgehog (SHH) and Wnt pathways, to the Delta/Notch pathway, to numerous transcription factors and structural genes (Chuong et al., 2001; Song et al., 2004).

The order of events in chick feather development is well known based on studies in the trunk, and is described elsewhere in relation to the Hamburger and Hamilton (HH) staging system (Hamburger and Hamilton, 1951; Lucas and Stettenheim, 1972; Mayerson and Fallon, 1985; Widelitz et al., 1997; Yu et al., 2004). Feathers form as buds in consecutive rows that make up tracts or pterylae, and in the head these are termed ‘capital tracts’ (Lucas and Stettenheim, 1972). Individual rows are added sequentially so that a given tract can include feather buds at successive stages of development. The spacing between buds and between rows appears to be determined by lateral inhibition from preceding buds (Davidson, 1983; Jung et al., 1998; Noramly and Morgan, 1998). We hypothesize that the dermis of the capital tracts regulates the expression of genes known to play a role during feather morphogenesis, such as members and targets of the BMP, SHH and Delta/Notch pathways. As a functional test of our hypothesis, we use the quail-duck chimeric system (Schneider and Helms, 2003; Tucker and Lumsden, 2004), which is a potent experimental method for identifying molecular and cellular spatiotemporal patterning mechanisms. Japanese quail have cranial feathers that are relatively large, widely spaced and pigmented, whereas those of the white Pekin duck are smaller, closely arranged and un-pigmented (Lucas and Stettenheim, 1972). Moreover, quail and duck have highly divergent embryonic growth rates (17 versus 28 days to hatching; Fig. 1D). By exchanging premigratory cranial neural crest cells between quail and duck embryos, we challenge host epidermis to respond to species-specific variations in molecular signals that are promulgated by donor neural crest-derived dermis. We find that donor neural crest alters the spatial pattern and changes the time at which host cranial feathers form by regulating the expression of key molecular mediators. Such results demonstrate the essential role played by the dermis and the plasticity inherent in the overlying epidermis, and provide insight into developmental mechanisms that may have directed the variegated course of feather evolution.

Materials and methods

Generation of chimeric embryos

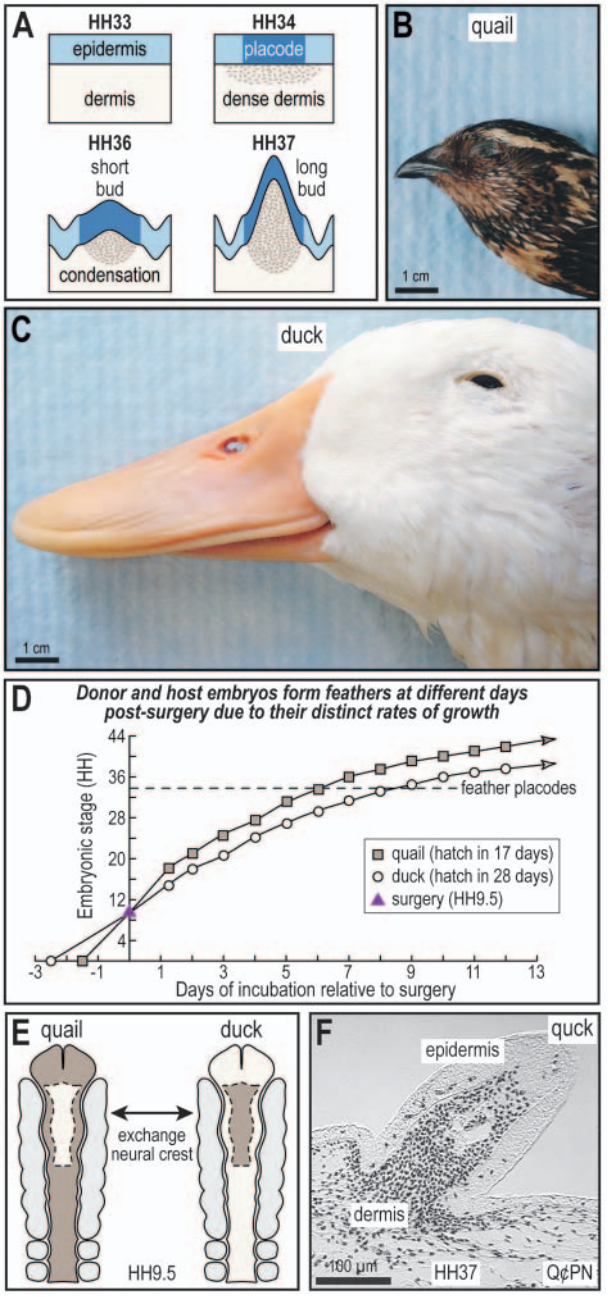
Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) and white Pekin duck (*Anas platyrhynchos*) were purchased from AA Labs (Westminster, CA) and incubated in a humidified chamber at 37°C. Embryos were matched at stage 9.5 using the Hamburger and Hamilton (HH) staging system for chicks (Hamburger and Hamilton, 1951), which can also be used for classifying quail (Le Douarin et al., 1996) and duck (Yamashita and Sohal, 1987; Schneider and Helms, 2003). Quail and duck eggs were windowed and embryos were visualized with Neutral Red (Sigma). At HH9.5, neural crest cells are found abundantly along the dorsal midline of the rostral neural tube (Tosney, 1982). Either unilateral or bilateral populations of neural crest cells from the level of the midbrain and rostral hindbrain were grafted orthotopically from quail to duck and duck to quail (Fig. 1E). Flame-sharpened tungsten needles and Spemann pipettes were used for all surgical operations (Schneider, 1999). Donor graft tissue was positioned and inserted into a host that had a comparable region of tissue removed. For controls, orthotopic grafts and sham operations were made within each species and were equivalent to those performed in previous studies (Noden, 1983; Schneider, 1999; Schneider et al., 2001; Schneider and Helms, 2003). Controls were incubated alongside chimeras, in order to ensure that the stages of grafted cells in the donor, host and chimeras were accurately assessed. After surgery, eggs were closed with tape and incubated until reaching stages appropriate for analysis. We used a combination of morphological characters, but emphasise post-cranial and extra-embryonic structures as these were unaltered by the surgery.

Histology and immunocytochemistry

Control and chimeric embryos from HH29 to HH42 were fixed in Serra’s (100% ethanol:37% formaldehyde:glacial acetic acid, 6:3:1) overnight at 4°C. To visualize the appearance of epidermal placodes and early feather buds, some embryos were stained with 0.02% ethidium bromide for 10 minutes at room temperature, rinsed in PBS and imaged under epifluorescent illumination. Embryos were dehydrated, paraffin embedded, cut into 7 or 10 μm sections, and mounted on glass slides. Representative sections were stained with Milligan’s Trichrome (Presnell and Schreibman, 1997) for histological visualization of the dermis and epidermis. To detect quail cells in chimeric embryos, representative sections were immunostained with the quail nuclei-specific Q¢PN antibody (Developmental Studies Hybridoma Bank, DSHB) following a previously published protocol (Schneider, 1999). This technique permanently labels quail cells by using a secondary antibody that is reacted with diaminobenzidine (DAB; Sigma). Sections were imaged using differential interference contrast microscopy (Fig. 1F).

Gene expression analyses

In situ hybridization was performed as described (Albrecht et al., 1997). Sections adjacent to those used for histological and immunocytological analyses were hybridized with ^{35}S-labeled chicken riboprobes to genes expressed in integumentary mesenchyme and epithelia including members of: the Bone Morphogenetic Protein pathway, *bmp4* and *bmp2* (ligands), *follistatin* (antagonist) and *bmpr1a* (receptor); the Hedgehog pathway, *shh* (ligand) and *ptc* (receptor); and the Delta/Notch pathway, *delta1* (ligand) and *notch1* (receptor). Sections were counterstained with a fluorescent blue nuclear stain (Hoechst Stain; Sigma). Hybridization signals were detected using dark-field optics and the nuclear stain was visualized using epifluorescence. The spatiotemporal expression patterns and levels of these genes throughout integumentary mesenchyme and epithelia of chimeras were compared with that observed in control quail and duck at each stage analyzed.



Results

Spatiotemporal patterns of cranial feather buds are established by the neural crest

A comparative analysis of integumentary development reveals that the timing and sequence of events during cranial feather morphogenesis are equivalent between stage-matched quail and duck embryos but that species-specific differences in pattern can be observed from early stages onwards. Using epifluorescent illumination of whole-mount embryos stained with ethidium bromide, we find that quail feather placodes can be seen beginning at HH34, in one medial and two lateral rows along the cranial epidermis (Fig. 2A). By HH35, additional rows appear over the eyes, and by HH36 they span the entire dorsal surface of the cranial integument (Fig. 2B,C,I). These quail cranial feather buds are relatively large and widely spaced. Duck feather placodes also first appear at HH34 but they do so in multiple rows over each eye, lateral to the midline (Fig. 2D). Duck feather buds are relatively small and positioned close together, and by HH36 they are distributed across the cranial integument (Fig. 2E,F,J).

To test the extent to which the dermis regulates feather morphogenesis, we transplanted premigratory cranial neural crest cells destined to form the craniofacial mesenchyme from quail to duck embryos generating chimeric ‘quck’ embryos. We find that quail neural crest cells induce duck host epithelium to form feather buds on a quail-like timetable and spatial pattern. We collected quck chimeras at HH33, which is a stage when cranial feather buds are normally not present in control quail or duck embryos (Fig. 2G). Using whole-mount ethidium bromide assays, we observe epidermal placodes that form prematurely across the craniofacial region either individually, in small clusters or as entire rows amid regions of undifferentiated host epithelium (n=13; Fig. 2H). The extent to which these epidermal placodes have developed is equivalent to that observed on control quail embryos at HH36 (Fig. 2I). Generally, the placodes on these quck chimeras are widely spaced and large in size like those found on quail. In some locations along the cranial epidermis, rows of placodes are

Fig. 1. Quail-duck chimeric system to study cranial feather morphogenesis. (A) Cranial feather buds arise via interactions between the neural crest-derived dermis and the overlying epidermis. At HH33, there is little histological evidence for cranial feather development, but by HH34, epithelial placodes form in the epidermis and the mesenchyme aggregates into dense dermis. By HH36, the feather buds contain a discrete dermal condensation and they begin to rise above the level of the integument. Long buds are present after HH37. (B) Japanese quail and (C) white Pekin duck display considerable differences in the pattern, pigmentation and morphology of their head feathers. (D) Owing to their distinct maturation rates, quail and duck embryos that are stage-matched for surgery subsequently deviate in stage, which provides a potent experimental system with which to identify molecular signals that regulate feather morphogenesis. (E) Neural crest cells were cut either bilaterally (as shown) or unilaterally from the rostral neural tube and exchanged between quail and duck embryos stage-matched at HH9.5. Among other derivatives, these cells are destined to form much of the craniofacial dermis. (F) Chimeric ‘quck’ feather follicles contain duck host epidermis and quail-derived donor dermis stained black with an anti-quail antibody (Q¢PN). Individual quail-derived melanocytes associated with the duck host epidermis are present. Scale bar: 1 cm in B,C; 100 µm in F.

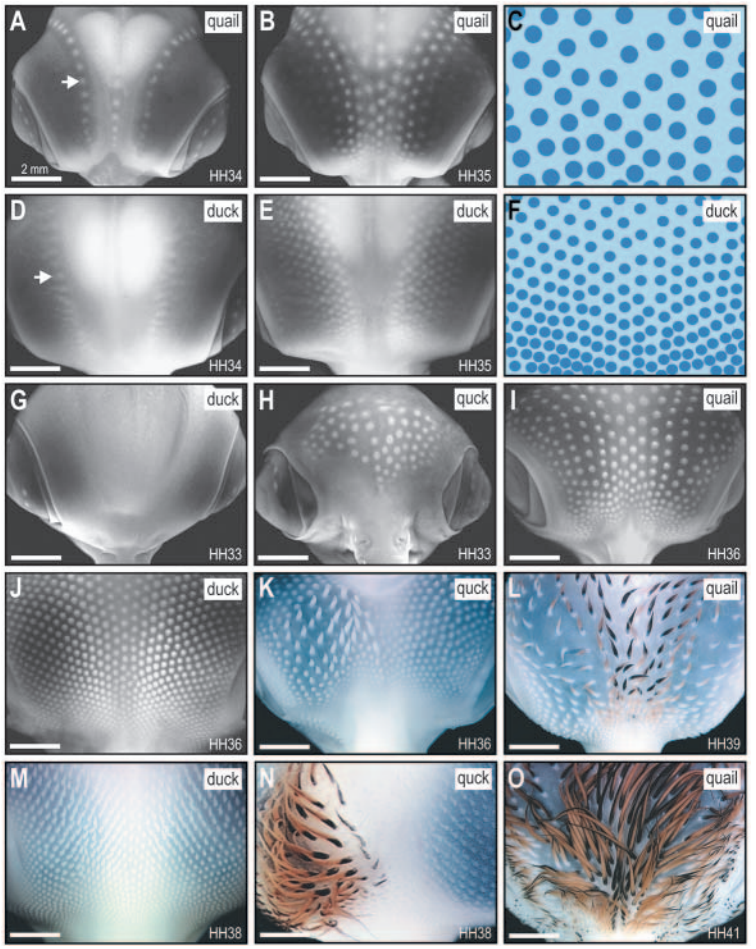


Fig. 2. The role of neural crest in the spatiotemporal patterning of cranial feather buds. (A) Starting at HH34, quail feather placodes can be seen in one medial and two lateral rows along cranial epidermis (arrow). (B) By HH35, additional rows appear over the eyes. (C) Quail feather buds are relatively large and widely spaced, shown schematically. (D) Duck feather placodes form at HH34 in multiple rows over the eyes, lateral to the midline (arrow). (E) Additional rows appear by HH35. (F) Compared with those of quail, duck feather buds are smaller and spaced closer together, as shown schematically. (G) At HH33, there are no cranial feather buds visible in either duck or quail (not shown). (H) However, HH33 chimeric 'quck' prematurely form feather placodes in duck host epidermis. The extent of differentiation, size and spacing of these quck feather buds are more like that observed in I. HH36 quail instead of duck, which is the host species. (J-L) When quck are at HH36, their feathers are like those found on HH39 quail. (M-O) Differences between host and donor feathers are more apparent by HH38, when quck feathers are like those of a quail at HH41. There is unilateral distribution of quail-derived pigment on the duck host, which is coincident with the type of neural crest transplant performed at HH9.5. Scale bar: 2 mm.

tightly aligned, whereas in other areas they appear asymmetric. Often, the distribution of epidermal placodes is closely correlated with the type of graft (bilateral or unilateral) used to generate the chimeric embryo (compare Fig. 2H with 2K). To ascertain if epidermal placodes can be induced at earlier stages, we also collected quck at HH29-HH32. We find that our transplants cause epidermal placodes to form as early as HH31 (n=6; Fig. 3Y,Z) and HH32 (n=4; data not shown). In quck chimeras collected prior to HH31, we observe no evidence of epidermal placodes (data not shown).

Thus, our transplants of quail neural crest cells into duck hosts cause the timing of feather morphogenesis to advance by three embryonic stages. This shift reflects the developmental difference separating control quail and duck embryos that are stage-matched for surgery at HH9.5 and incubated for 6 days, which is when feather morphogenesis is initiated in quail (Fig. 1D). By HH34 and HH35, quck feather buds are similar to those found on control quail at HH37 (n=10) and HH38 (n=13), respectively (data not shown). By HH36, chimeric quck have patches of long feather buds similar to those found on HH39 quail (n=10; Fig. 2K,L). Moreover, Japanese quail are pigmented, whereas Pekin duck are white, and some of the HH36 quck chimeras already have pigmented quail-like feathers clustered among unpigmented and less developed feather buds derived from the duck host (n=6). The disparity in developmental stage between donor and host is even more apparent in quck cases collected at HH38, where patches of elongated well-developed brown and black quail-like feathers, which resemble those of control quail at HH41, are arranged among short white duck host feather buds (n=11; Fig. 2M-O).

Neural crest regulates histogenic programs of cranial feather morphogenesis

To elucidate the cellular nature of these transformations, we compared histological sections of stage-matched control and chimeric quck embryos at key time points during feather morphogenesis. In control quail and duck embryos, dense dermis and epidermal placodes have yet to form in the capital tracts at HH33 but appear from HH34 onwards (Fig. 1A; Fig. 3A). Dermal condensations can be detected by HH35, and by HH36 some feather buds have begun to rise above the level of the integument (Fig. 1A; Fig. 3D,E; Fig. 4B). By HH37, the height of these feather buds has become equal to or slightly longer than their width, and by HH39, some of the more mature quail feathers contain brown and black pigment at their distal tips (Fig. 2L; data not shown). Quck chimeras collected at HH33, however, have dermal condensations and elevated placodes that resemble those found on control quail at HH36 (n=4; Fig. 3B-D). Quck at HH32 have dermal condensations and placodes equivalent to those present in HH35 controls (n=4; data not shown), and quck analyzed at HH31 already have dense dermis and placodes like those observed in controls at HH34 (n=3; Fig. 3X-Z). Thus, in quck, the histogenic program of feather morphogenesis is shifted forward by three embryonic stages.

To assess if the premature development and quail-like patterns of quck feathers result from the presence of quail donor neural crest, we processed sections from chimeric embryos for the immunohistological detection of quail cells using the Q¢PN anti-quail antibody (Schneider, 1999). We find that in all chimeric quck cases with quail-like feather buds collected at HH31, HH32 and HH33, the dermis is derived primarily from quail donor neural crest, while the epidermis originates exclusively from the duck host (n=11; Fig. 3C,Y; data not shown). We also find that the extent to which quck

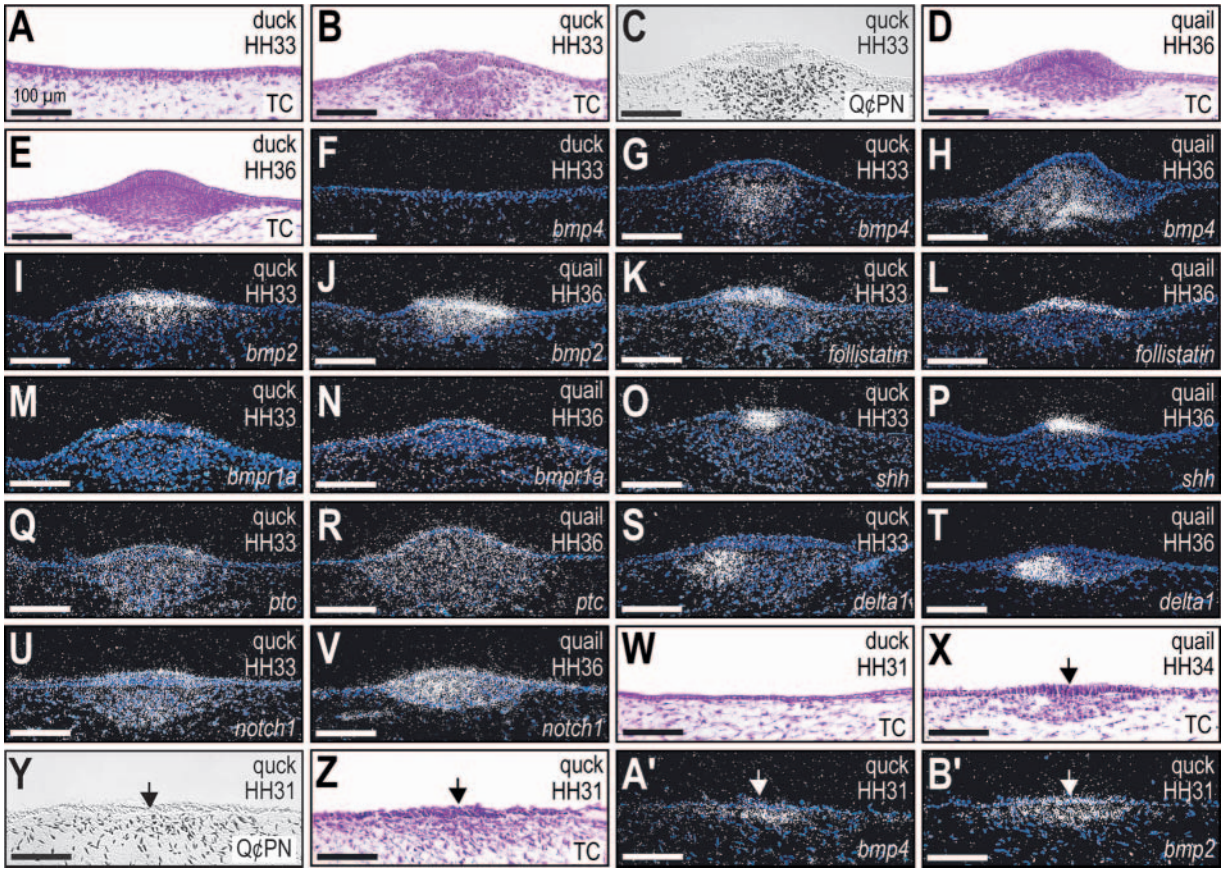


Fig. 3. Cranial neural crest regulates histogenic and molecular programs of feather morphogenesis. (A) At HH33, there are no cranial feather buds in either duck or quail (not shown) as stained histologically with trichrome (TC). (B,C) However, in chimeric 'quck' at HH33, the dermis is derived from quail donor neural crest (Q¢PN positive, black cells), which is on a faster timetable for development and induces premature formation of feather buds in duck host epidermis. The extent of differentiation, size and spacing of these quck feather buds are more like that observed in D, a HH36 control quail instead of an HH33 duck, which is the host species. (E) Control duck do not form short feather buds until HH36. (F) In situ hybridization analyses reveal that molecular markers of feather development such as *bmp4* are not expressed in the capital tracts of control quail and duck prior to HH34. (G,H) However, chimeric quck at HH33 express *bmp4* in cranial feather mesenchyme, which is equivalent to that observed for control quail at HH36. (I,J) HH33 quck express *bmp2* in the epithelium and mesenchyme like HH36 quail. (K,L) HH33 quck express *follistatin* in the epithelium and mesenchyme like HH36 quail. (M,N) HH33 quck express *bmpr1a* in the epithelium and mesenchyme like HH36 quail. (O,P) HH33 quck express *shh* in the epithelium like HH36 quail. (Q,R) HH33 quck express *ptc* in the epithelium and mesenchyme like HH36 quail. (S,T) HH33 quck express *delta1* in the mesenchyme like HH36 quail. (U,V) HH33 quck express *notch1* in the epithelium and mesenchyme like HH36 quail. (W) At HH31, there are no epidermal placodes or underlying dense dermis present in control duck or quail (not shown). (X) These do not appear in control quail and duck (not shown) until HH34 (arrow). (Y,Z) However, in chimeric quck at HH31, quail donor neural crest-derived mesenchyme has given rise to dense dermis (Q¢PN positive) and has induced epidermal placodes (arrows) of duck host origin (Q¢PN negative). (A′,B′) In situ hybridization analyses reveal that *bmp4* and *bmp2* are expressed in quail donor-derived mesenchyme prematurely at HH31, whereas normally they would not be expressed until HH34 (arrows). Other mesenchymal and epithelial molecular markers of feather morphogenesis are not yet detected except for *bmpr1a* and *notch1,* which are expressed continuously from at least HH29 in most tissues (data not shown). Scale bar: 100 μm.

feather buds are transformed in both size and placement correlates with the amount and distribution of quail donor neural crest cells in duck hosts. The more quail donor cells there are throughout the dermis, the more complete the transformation to a quail-like pattern. In less transformed cases we find fewer and/or more dispersed quail cells (data not shown). Where there is no quail donor mesenchyme (i.e. dermis derived from the duck host), feather buds have yet to form. This also holds true for those quck cases lacking any premature quail-like feather buds; here, the dermis is derived principally from the duck host (n=6; data not shown). In quck chimeras collected prior to HH31, we find no epidermal placodes or dense dermis, despite abundant quail-derived cells in the mesenchyme of the region of the presumptive capital tracts (n=6; data not shown). Quail donor neural crest cells also give rise to melanocytes that become secondarily associated with duck host epidermis and ultimately produce feather pigmentation (n=7; Fig. 1F).

Neural crest regulates expression of the BMP, SHH and Delta/Notch pathways

To test the extent to which the dermis regulates molecular programs for feather development, we performed in situ hybridization to assay for changes in the expression of

members and targets of the Bone Morphogenetic Protein, Sonic Hedgehog and Delta/Notch pathways. Our analyses conducted on control embryos collected from HH29 to HH38 demonstrate that the timing of expression for *bmp4*, *bmp2*, *follistatin*, *bmpr1a*, *shh*, *ptc*, *delta1* and *notch1* in the capital tracts is equivalent between stage-matched quail and duck. In sharp contrast, we find that in chimeric quck embryos the timing of gene expression is accelerated by three stages in both quail donor-derived dermis, and duck host-derived epidermis, which is consistent with our morphological and histological results. Prior to HH34, none of these genes is expressed in either the epidermis or dermis of control embryos (Fig. 3F; data not shown), except for *bmpr1a* and *notch1,* which are expressed continuously from at least HH29 in most tissues throughout the craniofacial region (data not shown). However, in chimeric quck collected at HH33, all of these feather markers are detected throughout the capital tracts in domains equivalent to those observed in control quail at HH36 (Fig. 3G-V). Specifically, in quck at HH33, we find *bmp4* and *delta1* expression restricted to the quail donor-derived dermal condensations of short feather buds (Fig. 3G,S), *shh* in host-derived epidermal placodes (Fig. 3O), and *bmp2*, *follistatin*, *bmpr1a*, *ptc* and *notch1* in both tissues (*n*=4; Fig. 3I,K,M,Q,U). These are the same expression patterns observed in control quail at HH36 (*n*=3; Fig. 3H,J,L,N,P,R,V). To determine how much earlier these genes could be experimentally induced, we collected chimeric quck at HH32, HH31, HH30 and HH29. We detect transcripts of *bmp4*, *bmp2*, *follistatin*, *bmpr1a*, *shh*, *ptc*, *delta1* and *notch1* in chimeric quck collected at HH32 (*n*=4), even though in control embryos *ptc*, *shh*, *delta1* and *follistatin* do not appear in developing feather buds prior to HH35 (*n*=4; data not shown). Similarly, *bmp4* and *bmp2* are expressed in nascent feather buds of chimeric quck collected at HH31 (*n*=3), whereas control embryos express these genes in the equivalent region no earlier than HH34 (*n*=4; Fig. 3A′,B′; data not shown). We do not detect any evidence of expression for these genes in the cranial integument prior to HH31 in chimeric quck, despite an abundance of quail donor-derived mesenchyme in the presumptive capital tracts (data not shown).

Donor neural crest can also delay the timing of feather morphogenesis

As another functional test of our hypothesis that the dermis regulates the expression of genes known to play a role during feather morphogenesis, we performed reciprocal transplants of premigratory cranial neural crest cells from duck into quail, generating chimeric ‘duail’ embryos. In general, we find that duck donor neural crest delays the molecular and histogenic programs of feather morphogenesis in quail hosts by three embryonic stages (*n*=8). Duail chimeras were collected at HH36, which is when stage-matched control quail embryos have consecutive rows of feather buds across the entire dorsal surface of the cranial epithelium (Fig. 2I). However, the capital tracts of these duail chimeras contain extensive epidermal regions that lack feather placodes (*n*=3; Fig. 4A). The absence of epidermal placodes is similar to that observed on duck donor controls prior to HH34 (Fig. 2G).

To assess if the delay in duail feather development results from duck donor neural crest-mediated changes in gene expression, we processed sections histologically, with the Q¢PN anti-quail antibody, and for in situ hybridization. We find that in chimeric duail cases collected at HH36, those cranial regions lacking feather buds contain dermis derived from duck donor neural crest, while the epidermis originates from the quail host (*n*=3). Conversely, where there is no duck donor mesenchyme (i.e. Q¢PN-positive dermis derived from the quail host), feather buds are present (Fig. 4B-E). Molecular analysis of these duail chimeras at HH36 reveals that *bmp4*, *bmp2*, *follistatin*, *shh*, *ptc* and *delta1* are expressed in feather buds derived from quail host tissues, but not in regions where the dermis is derived from duck donor neural crest (*n*=3; Fig. 4F-K; data not shown). After HH37, duck-derived dermis along with quail host-derived epidermis form well developed feather placodes like those found on duck controls subsequent to HH34 (*n*=4; Fig. 4L-N). We also find that some pigmented feather buds in duail embryos older than HH38 are derived primarily from duck dermis, which is a source of normally non-pigment-producing melanocytes (Fig. 4M).

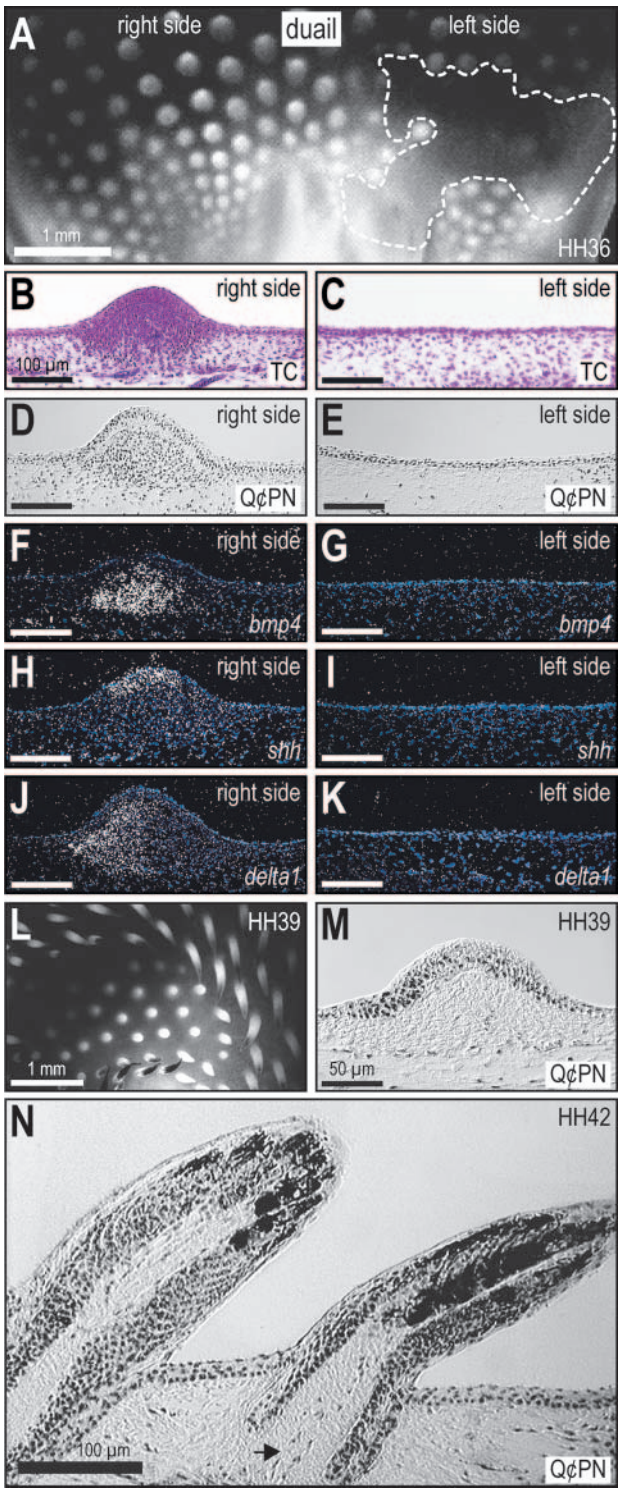
Discussion

Neural crest plays a central regulatory role during cranial feather morphogenesis

Our study focuses on feather formation in cranial integument and employs the quail-duck chimeric system (Schneider and Helms, 2003; Tucker and Lumsden, 2004) in order to exploit species-specific differences in morphology and rates of maturation. We generated chimeras by exchanging premigratory neural crest cells from the midbrain and rostral hindbrain between quail and duck embryos. Chimeric ‘quck’ contain quail donor dermis, which normally would follow a relatively faster developmental timetable, juxtaposed with duck host epidermis, which usually would undergo a relatively slower ontogeny. By contrast, chimeric ‘duail’ have relatively delayed duck donor dermis beneath quail host epidermis that ordinarily would develop more rapidly. In both types of chimeras, the morphology and timing of development of the dermis and epidermis were transformed in accordance with the identity of the donor species. Thus, our transplant experiments validate the essential function of the dermis as a primary source of spatiotemporal patterning information in the capital tracts.

Our quail-duck experimental approach augments results from previous inter-specific feather studies in several important ways and as a consequence reveals novel mechanisms of integumentary development. The fact that certain species-specific differences in feather morphology are conveyed by the dermis is well established based upon other recombinations such as those using chick and duck tissues (Dhouailly, 1967; Dhouailly, 1970). But in order to understand developmental mechanisms through which the dermis exerts its influence on the epidermis, we use quail instead of chick. This takes advantage of the substantial difference in maturation rates between quail and duck, and capitalizes on the ubiquitous quail nuclear marker not present in chick or duck, which allows us to distinguish between donor and host tissues. Our experiments also entail in ovo transplants of premigratory neural crest cells destined to form the craniofacial mesenchyme rather than in vitro recombinations of stage-matched dermis and epidermis that had already become components of the integument. This permits progressively asynchronous donor mesenchyme and host epithelium to interact with one another continuously from

the moment they first meet, and allows us to observe resultant neural crest-mediated changes to molecular and histogenic programs underlying feather development. Thus, by design, our experiments illuminate the overriding regulatory capabilities of the mesenchyme, as well as the plasticity inherent in the overlying epithelium during cranial integumentary development. Such results can probably be extrapolated to include integument throughout the body.



Neural crest regulates expression of genes essential to feather morphogenesis

To determine the extent to which individual signaling pathways known to play a role in feather morphogenesis are regulated by the neural crest, we compared control and chimeric embryos at successive stages using in situ hybridization to assay for temporal changes in the expression of members of the BMP, SHH and Delta/Notch signaling pathways. As feather formation requires precisely timed dermal-epidermal signaling interactions, we hypothesized that expression of genes mediating these interactions would be altered in chimeric embryos because of intrinsic differences in growth rates between donor and host cells. For each signaling pathway that we examined, we observed a significant change in the timing of expression coincident with the embryonic stage of the donor neural crest-derived dermis (Fig. 5).

Members of the BMP, SHH and Delta/Notch signaling pathways play essential roles as both promoters and inhibitors of chick feather development (Ting-Berreth and Chuong, 1996; Crowe et al., 1998; Morgan et al., 1998; Patel et al., 1999; Chuong et al., 2000; Ashique et al., 2002; Yu et al., 2002; Pispa and Thesleff, 2003). In chicks, *bmp2* is expressed in emerging epidermal placodes and in dermal condensations, whereas *bmp4* is detected exclusively in the dermis during initial stages of mesenchymal aggregation and thereafter (Nohno et al., 1995; Chuong et al., 1996; Widelitz et al., 1997; Jung et al., 1998; Noramly and Morgan, 1998; Scaal et al., 2002). The BMP antagonist *follistatin*, is expressed first in presumptive placodal epithelium and then also in the mesenchyme (Ohyama et al., 2001). *Sonic hedgehog* is expressed in nascent epidermal placodes (Nohno et al., 1995; Widelitz et al., 1997) and its receptor *ptc* is first detected in dermis beneath the source of *shh*, and then also in the epithelium posterior to cells that express *shh* (Jung et al., 1998; Morgan et al., 1998). *Delta1*

Fig. 4. Donor neural crest can also delay molecular and histogenic programs of cranial feather development. (A) Duck cranial neural crest cells follow their own timetable for differentiation when transplanted into quail hosts. Resultant duail chimeras collected at HH36 contain feather buds as well as epidermal regions that lack placodes (broken outline). The absence of epidermal placodes is equivalent to that observed on control embryos at HH33 (compare with Fig. 2G). (B,C) The presence and absence of cranial feather buds can be seen in sections stained histologically with trichrome (TC). (D) Immunohistochemical analyses using an anti-quail antibody confirm that wherever placodes are present, the dermis is derived from quail host neural crest (Q¢PN positive, black cells). (E) By contrast, regions that lack placodes contain dermis derived from the duck donor (Q¢PN negative). (F,G) *Bmp4* is expressed in mesenchyme derived from the quail host but is not yet detected in mesenchyme of duck donor origin. (H,I) *Shh* is expressed in the epithelium overlying dermis from the quail host but not over dermis derived from the duck donor. (J,K) *Delta1* is detected in quail host dermis but not in duck donor-derived dermis. (L) Duail chimeras collected at HH39 have normal long feather buds alongside areas containing short feather buds like those observed on HH36 controls. (M) Immunohistochemical analyses confirm that the short feather buds, which are like those of a HH36 duck instead of a HH39 quail, are derived from duck donor neural crest (Q¢PN-negative). (N) Long feather buds composed of quail host epidermis (Q¢PN-positive) and duck donor dermis (Q¢PN-negative; arrow) are present in duail chimeras at HH42. Scale bar: 1 mm in A; 100 µm in B-K; 1 mm in L; 50 µm in M; 100 µm in N.

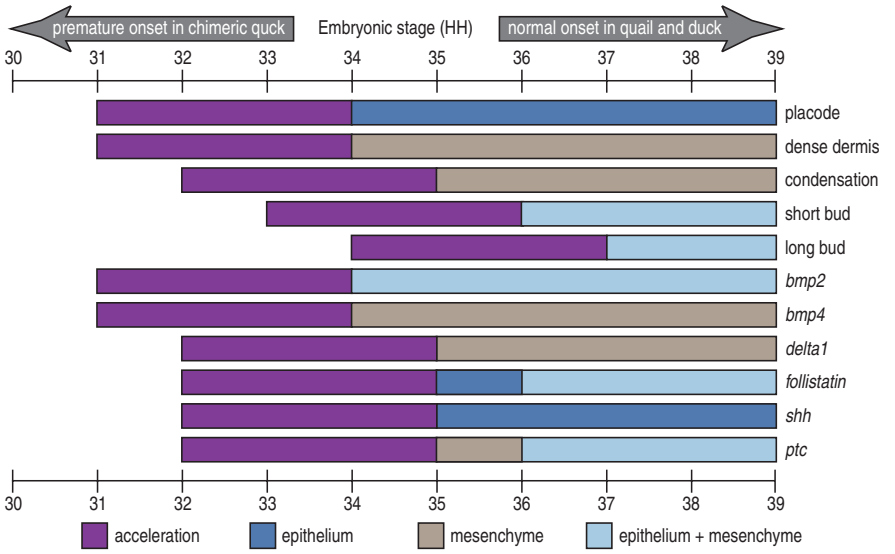


Fig. 5. Quail-duck chimeras reveal plasticity in cranial feather development. Bars represent stages when histogenic and molecular events are initiated in controls versus chimeras.

expression is restricted to the dermis, and *notch1* is present in both the dermis and epidermis (Chen et al., 1997; Crowe et al., 1998; Viallet et al., 1998). Our in situ hybridization analyses reveal expression patterns in the developing capital tracts of control quail and duck that are equivalent to those reported for the trunk tracts of chick. Based on our analysis of chimeras, we conclude that signaling by the BMP, SHH and Delta/Notch pathways is regulated by the dermis. Moreover, *bmp4* and *bmp2* are detected in feather primordia of controls and chimeras one stage earlier than any other ligand examined, suggesting that BMP signaling mediates the initial induction of feather buds by the neural crest-derived dermis (Fig. 5).

Thus, our molecular analyses demonstrate that neural crest cells function as the dominant source of spatial and temporal patterning information via the regulation of genes essential to cranial feather morphogenesis. Such results are consistent with those from previous quail-duck transplants, where quail donor neural crest cells were shown to govern beak morphology by executing autonomous molecular programs and by regulating gene expression in the mesenchyme and epithelia of the developing facial primordia (Schneider and Helms, 2003). Beyond the molecules examined here, we predict that other genes, including members and targets of the FGF, Epidermal Growth Factor and Wnt pathways, which play a role during feather morphogenesis (Noji et al., 1993; Tanda et al., 1995; Song et al., 1996; Widelitz et al., 1996; Noramly et al., 1999; Widelitz et al., 1999; Olivera-Martinez et al., 2001; Tao et al., 2002; Atit et al., 2003; Chodankar et al., 2003; Chang et al., 2004; Mandler and Neubuser, 2004; Rouzankina et al., 2004; Song et al., 2004), would be differentially regulated by donor neural crest. In the future, combining our chimeric approach with more quantitative and comprehensive methods of gene expression analysis, such as microarrays, could yield new candidate molecules that underlie feather morphogenesis.

The mesenchyme controls signaling interactions with the epithelium

Epithelial-mesenchymal signaling interactions drive the development of numerous vertebrate structures from the level of entire organ systems, such as the case for the limbs and facial primordia (Saunders and Gasseling, 1968; Wedden, 1987; Richman and Tickle, 1992; Francis-West et al., 1998; Schneider et al., 1999; Shigetani et al., 2000; Schneider et al., 2001; Hu et al., 2003), to the level of individual tissues, such as in relation to hair, glands, teeth and bone (Salaun et al., 1986; Fisher, 1987; Lumsden, 1988; Sharpe and Ferguson, 1988; Dunlop and Hall, 1995; Mitsiadis et al., 1998; Pispa and Thesleff, 2003). During their interactions, the epithelium and mesenchyme presumably function by providing instructive information or by creating a permissive environment that enables morphogenesis to proceed. The most common approach employed to define the role of either the epithelium or the mesenchyme at each step of the process has involved recombination of dissected tissues in vitro. In this context, cranial osteogenesis has been shown to be regulated by stage-specific interactions between neural crest mesenchyme and adjacent epithelia (Hall, 1978; Hall and Tremaine, 1979; Bee and Thorogood, 1980; Bradamante and Hall, 1980; Tyler and McCobb, 1980; Hall, 1982; Hall and Coffin-Collins, 1990; Mina et al., 1994; Dunlop and Hall, 1995; Vaglia and Hall, 1999; Couly et al., 2002). Although the epithelium is required in this process, its role appears to be permissive rather than instructive. The same appears to hold true for feather bud morphogenesis. For example, recombinations of differently staged dermis and epidermis from wild-type and featherless mutants demonstrate that, early on, the dermis is endowed with the ability to induce epidermal placodes, but this propensity is rapidly lost in the absence of proper epidermal interactions (Viallet et al., 1998). In other situations, however, the epithelium can serve an instructive role during pattern formation, dictating where teeth form (Thesleff and Sharpe, 1997; Tucker et al., 1998; Wang et al., 1998) and whether epidermis generates scales or feathers (Widelitz et al., 2000; Prin and Dhouailly, 2004).

Our chimeric data support the notion that integumentary epithelium behaves permissively, while the dermis acts instructively to establish the timing and spacing of feather bud development. However, this does not rule out the possibility that the epithelium can provide instructive information during later stages of differentiation, particularly when branching patterns may be transmitted from the epidermis in a species-specific manner (Harris et al., 2002; Yu et al., 2002). We presume that in our chimeras, host epidermis provides a developmental context that is equivalent in many ways to what the donor dermis would normally encounter in its native environment. This may be accomplished as a consequence of earlier programmatic events and signaling interactions whereby donor neural crest mesenchyme transmutes host epithelium to reflect the morphogenetic identity of the donor. Alternatively, the embryonic milieu of the host may remain permissively naïve in a way that encourages the donor cells to carry out autonomous programs. In either case, the donor

neural crest functions by elaborating a molecular set of instructions intrinsic to its own genome and by inducing a donor-specific program of gene expression and histogenesis, which overrides that of the host. The result is that instructive donor dermis can only make feather buds like those of the donor, whereas permissive host epidermis can make feather buds like those of either the host or the donor.

Developmental modules and plasticity may facilitate feather evolution

Traditionally, development or ontogeny has been characterized as a series of embryonic events ordered into a discrete chronological sequence. The relation of one event to the next can be merely temporal where each event is arranged after the other without any underlying mechanistic connection, or can be causal, where each event is a prerequisite for a subsequent event via processes like induction (Alberch, 1985). While defining events as either temporally or causally related may underestimate the dynamic, continuous and integrative nature of developmental systems, such a method is useful for distinguishing phenomenological associations from morphogenetic programs that function as modules during the course of ontogeny and phylogeny. Because modules consist of causally coupled processes, they may be more likely to undergo rapid and dramatic transformations that are due to changes in the timing and rates of developmental events such as those associated with heterochrony (Raff and Kaufman, 1983; Hall, 1984; Smith, 2003).

Feather formation is an especially good example of an iterative module comprising causally linked developmental events, yet fundamental parameters that define this module have not been sufficiently understood. We have shown that the dermis establishes where and when molecular and histogenic programs of cranial feather development begin to operate and we have gauged the degrees of plasticity inherent in the overall system. Once these programs are initiated, the entire sequence of events seems to unfold automatically, albeit shifted in time and space. In the case of the quck, epidermal differentiation can be induced by the dermis three stages earlier than normal, while in the duail, epidermal differentiation can be delayed three stages. By extension then, our results indicate that the molecular and histogenic programs underlying feather bud formation can be shifted through a total window of at least six developmental stages, which for each species represents almost 15% of their total incubation period. Yet, what we do not know from our studies are the absolute limits of the plasticity of the system. For example, cranial epidermal placodes do not form prior to HH31 in quck chimeras. This could be due to an epithelium that is either incompetent to respond to dermal signals or unable to create a permissive environment any earlier. Alternatively, the dermis may not yet be capable of instructing the epidermis until HH31. A further possibility has nothing to do with the tissues themselves but rather constraints imposed by the quail-duck chimeric system, which is limited by innate differences in the maturation rates between these birds. Using other avian species that have either relatively shorter or longer incubation periods could circumvent this restriction and reveal further the extent to which the developmental module underlying feather morphogenesis is free to vary.

Defining the limits of flexibility or plasticity inherent in developmental systems such as the feather module is a necessary step for identifying molecular and cellular mechanisms that may have played a generative and regulatory role during the course of morphological evolution. Plasticity is a measure of the capacity of ontogenetic programs to respond to internal and external perturbations and produce an integrated and sustainable phenotype. By combining plasticity with modularity, organisms have the remarkable potential to react spontaneously to new conditions and new gene functions, and generate new phenotypes (West-Eberhard, 2003; Schlosser and Wagner, 2004). Our transplants reveal that host epidermis has a rather seamless ability to accommodate and integrate morphogenetic modifications introduced by an internal stimulus, which in this instance involves neural crest-mediated changes to the spacing and timing of molecular and histogenic events. These properties of modularity and plasticity, which allow our chimeras to ‘adapt’ to an experimentally induced process, are likely to be the same features that enable organisms to evolve when variations are introduced by more natural means.

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