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RAPID COMMUNICATION

Shh-Bmp2 Signaling Module and the Evolutionary Origin and Diversification of Feathers

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ABSTRACT To examine the role of development in the origin of evolutionary novelties, we investigated the developmental mechanisms involved in the formation of a complex morphological novelty—branched feathers. We demonstrate that the anterior-posterior expression polarity of Sonic hedgehog (*Shh*) and Bone morphogenetic protein 2 (*Bmp2*) in the primordia of feathers, avian scales, and alligator scales is conserved and phylogenetically primitive to archosaurian integumentary appendages. In feather development, derived patterns of *Shh-Bmp2* signaling are associated with the development of evolutionarily novel feather structures. Longitudinal *Shh-Bmp2* expression domains in the marginal plate epithelium between barb ridges provide a prepattern of the barbs and rachis. Thus, control of *Shh-Bmp2* signaling is a fundamental component of the mechanism determining feather form (i.e., plumulaceous vs. pennaceous structure). We show that *Shh* signaling is necessary for the formation and proper differentiation of a barb ridge and that it is mediated by *Bmp* signaling. BMP signaling is necessary and sufficient to negatively regulate *Shh* expression within forming feather germs and this epistatic relationship is conserved in scale morphogenesis. Ectopic SHH and BMP2 signaling leads to opposing effects on proliferation and differentiation within the feather germ, suggesting that the integrative signaling between *Shh* and *Bmp2* is a means to regulate controlled growth and differentiation of forming skin appendages. We conclude that *Shh* and *Bmp* signaling is necessary for the formation of barb ridges in feathers and that *Shh* and *Bmp2* signaling constitutes a functionally conserved developmental signaling module in archosaur epidermal appendage development. We propose a model in which branched feather form evolved by repeated, evolutionary re-utilization of a *Shh-Bmp2* signaling module in new developmental contexts. Feather animation Quicktime movies can be viewed at http://fallon.anatomy.wisc.edu/feather.html. *J. Exp. Zool. (Mol. Dev. Evol.) 294:160–176, 2002.*

INTRODUCTION

Evolutionary novelties are derived features that are qualitatively different from, and not homologous with, antecedent structures (Müller and Wagner, '91; Raff, '96). The mechanistic basis of the origin of morphological novelties remains a central question in evolutionary biology. Variation in developmental processes is thought to be an important means of macroevolutionary change (Müller and Wagner, '91; Raff, '96; Wagner, 2000). Furthermore, the innovative use of pre-existing developmental processes or molecular signaling pathways has been hypothesized to be an important mechanism for the evolution of novel morphological structures (Raff, '96; Von Dassow and Munro, '99). However, in only a few instances have mechanistic changes in gene expression that are associated with the evolution of a morphological novelty been documented (Keys et al., '99; Wagner, 2001). The feather is a branched integumentary appendage that is an excellent example of a complex assemblage of morphological evolutionary novelties (Prum, '99). The feather has been hypothesized to have evolved from scutate scales of archosaurian ancestors (Lucas and Stettenheim, '72). The fundamental question is how novel structural diversity arose during the evolution of the integumental appendages of the archosaurs (the reptilian clade including birds, crocodilians, dinosaurs, and pterosaurs).

Based on a morphological analysis of feather morphogenesis, Prum ('99) recently hypothesized

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that feathers originated and diversified through a series of developmental processes that are hierarchically and historically contingent. This developmental model of the origin of feathers makes testable predictions about both the morphologies of primitive feathers and the role of developmental change in the origin of complex feather structure. Recent discoveries of fossil feathers from nonavian theropod dinosaurs have documented several plesiomorphic feather morphologies, including branched filamentous structures and central filaments hypothesized to be a rachis (Chen et al., '98; Ji et al., '98; Xu et al., '99; Ji et al., 2001; Xu et al., 2001; Norell et al., 2002). These findings demonstrate the phenotypic complexity of early integumental appendages during the early evolution of feather complexity (Prum, '99; Brush, 2000; Chuong et al., 2000), and they are congruent with predictions from the developmental model (Ji et al., 2001; Sues, 2001; Xu et al., 2001; Norell et al., 2002).

Epidermal appendages are initiated by signaling between an epithelium and subjacent mesenchyme leading to the formation of an epithelial placode. Recent studies of epidermal appendage morphogenesis have concentrated on the molecular mechanisms associated with placode induction and the feather bud as a morphological entity. Little to no attention has been paid to the molecular events that pattern and define the morphogenesis of the definitive feather structure. Here we present a comparative analysis of the development of feathers and scales in extant archosaurian lineages to test the hypothesis that changes in developmental mechanisms were critical to the evolution of feathers; specifically, to determine if there are common developmental and molecular mechanisms reutilized in the development of novel feather structures. This molecular developmental analysis provides a new context to understand the evolutionary origin of feathers from keratinized scales, and the diversification of complex feather morphologies, including the evolution of barbs, the rachis, and the planar pennaceous vane.

We focus on the function and regulation of two intercellular signaling molecules— Sonic hedgehog (*Shh*) and Bone Morphogenetic Protein 2 (*Bmp2*)—during feather development. These genes are known to be involved in the development of many organ systems, including vertebrate integumentary appendages (Bitgood and McMahon, '95). In many developing systems, *Bmp*s, members of the TGFβ gene superfamily, have been shown to act in concert with *Shh,* or its invertebrate ortholog *hedgehog* (Basler and Struhl, '94; Borod and Heberlein, '98; Murtaugh et al., '99; Dahn and Fallon, 2000; Drossopoulou et al., 2000; Zhang et al., 2000; Zhang and Yang, 2001) and the two genes are expressed concomitantly in the development of nonfeather integumentary appendages of amniotes (Bitgood and McMahon, '95; St-Jaques et al., '98; Jung et al., '99).

We show that *Shh-Bmp2* signaling in the formation of integumentary appendages of birds constitutes a molecular signaling module[1], and that there is conservation of expression of this module early in the development of integumentary appendages of extant archosaurs (i.e., birds and crocodiles). Furthermore, the development of the novel tubular feather germ and feather branched structure is associated with the evolutionary reutilization of this signaling module in novel developmental contexts during the development of a feather. We demonstrate that this signaling module is necessary for the morphogenesis of barb ridges, and thus, is critical to the morphogenesis of the branched structure of the feather. The data suggest that the repeated evolutionary re-utilization of this modular molecular signaling unit within the epidermis of early scale development facilitated the origin of the novel epithelial structures that characterize feather morphology. In this paper, we give an introduction to feather structure and development. Then, we describe patterns of *Shh-Bmp2* expression in archosaurian feathers and scales, and document the association between variation in *Shh-Bmp2* expression and the growth of feather branched structure. Finally, we present experimental data documenting the modularity of *Shh-Bmp2* signaling and function of this module in feather morphogenesis.

METHODS

To investigate the molecular mechanisms underlying the development of feathers and scales, we analyzed the regulation of the expression of *Shh* and *Bmp2* by whole mount in situ hybridization (WMISH). To document downstream effects of these signaling molecules, we used WMISH to examine gene expression of the *Shh* receptor *Patched* (*Ptc*) and *Patched2 (Ptc2),* and the *Shh*-activated transcription factor *Gli1*. We assessed

[1]We use the term module after Gilbert and Boker (2001): A signaling module represents a developmental unit of integrated signaling systems that is shared between species and tissues, exhibiting conservation and descent with modification.

the function of *Shh* and *Bmp2* signaling in feather development by experimentally treating forming embryonic feather germs with ectopic SHH or BMP2 protein loaded on to beads, or by blocking the endogenous signaling of *Shh* and *Bmp2* by using pharmacological agents and viral vectors to overexpress molecular regulators of their intracellular signaling.

Observations and experiments were conducted on chick and duck natal down feathers, chick and duck scutate scales, and alligator scales to investigate the comparative patterns in gene expression and function among species, different appendages, and among morphologically distinct morphologies of the same appendage (e.g., plumulaceous vs. pennaceous natal down).

Specimen collection and preparation

Avian embryos of the Babcock strain of White Leghorn chickens (*Gallus gallus*; Madison WI), SPAFAS strains (Charles River Laboratories), and Pekin and Khaki Campbell ducks (*Anas platyrhynchus;* Metzger Farms) were incubated at 39°C until harvest. Alligator (*Alligator mississippiensis*) embryos were obtained from the Chenier National Refuge. Embryos were fixed overnight in 4% PFA and dehydrated in a methanol series and stored at −20°C until use. SPAFAS embryos were used solely for RCAS studies.

Whole mount in situ and immunohistochemistry

Whole mount in situ labeling was performed as described by (Nieto et al., '96) with the addition of 10% polyvinyl chloride to the color reaction and subsequent clearing in methanol. Whole mount immunohistochemistry followed the in situ protocol omitting the hybridization and proteinase steps. Overnight incubation of primary and secondary antibodies was used. Signal was detected using alkaline phosphatase conjugated antibodies and NBT/BCIP as a substrate. Probes used in this study were chick *Shh* (P. Beachy); *Bmp2* (E. Laufer); *Ptc2* (C. Tabin); *Ptc* (C. Tabin); and *Gli1* (C. Tabin). Interspecific hybridizations were performed at 65°C. Primary antibodies used in these studies were Shh 5E1 (gift of C. Chiang, 1:100), phosphorylated histone H-3 (ser10) (Upstate Biotechnology Institute, 1:100), and AMV-3C2 (Iowa developmental hybridoma bank developed by D. Boettiger). For detection of viral antigen in E16 feathers, feathers were removed and washed in PBS/0.1% Tween, 0.1% Triton-X to aid in antibody penetration into tissue. Anti-mouse-AP and anti-rabbit-AP secondary antibodies were used at 1:2,000 dilutions. Histological sections of tissue after immunohistochemical and in situ analysis were conducted using isopropanol as an antimedium for paraffin embedding.

Feather explants and chorio-allantoic membrane grafts

Skin explants from stage HH36 (Hamburger and Hamilton, '51) spinal tract were selected for size of the forming feather buds. Only tissue that had formed medium sized buds were chosen for further analysis in order to avoid affecting early formation of polarity and growth of the feather bud. Explants were folded dermal side in and cultured at 37°C in 2%FBS, 1% Pen-Strep, Dulbecco modified minimal essential medium (DMEM). After 24 hours the explants were treated with Noggin protein (5 μg/ml, gift from R. Harland). Similar preparation of tissue was performed for chorioallantoic membrane (CAM) grafts of chick tissue. CAM Explants were treated with cyclopamine (10 μg/ml, gift from W. Gaffield), in 1% DMSO/PBS, drop-wise on the surface of the explant four times daily until harvest.

Skin explants for bead implant studies were made from embryonic day 14 (E14) Pekin or Khaki Campbell duck rectrices (tail feathers), and E10 chick dorsal metatarsal skin and placed in PBS. Size selected heparin acrylic beads loaded with recombinant proteins were placed in a forming feather or forming scale and grafted on to the CAM of chick hosts until harvest. The beads were preincubated for 1 hour in recombinant human BMP2 protein (1mg/ml, gift from Genetics Institute), N-SHH protein (1mg/ml, gift from P. Beachy), and Noggin protein (300 μg/ml).

Viral mediated overexpression

RCAS virus constructs were grown and concentrated as described (Morgan and Fekete, '96). RCAS-caPKA was a kind gift of Drs. S. McKnight, Y. Brun, and B. Olwin; RCAS BMPR1b (K231R), described by (Zou and Nisewander, '96), was a gift from Dr. J. Lough. All virus infections were into the amnionic fluid of a stage HH31 SPAFAS chick to maximize expression only to the epidermal layers of feather follicles (Morgan et al., '98).

RESULTS

Feather structure and development

Feathers are branched integumentary appendages. A typical feather is characterized by a central rachis, a series of barbs connected to the rachis, and numerous barbules connected to the barbs (Fig. 1M). Feather and scutate scale development (reviewed in Lucas and Stettenheim, '72; Sawyer, '72; Prum, '99) begin with a thickened epidermal placode. In feathers, distal outgrowth of the placode leads to the formation of a nascent feather bud, which elongates into a tubular structure called the feather germ. Subsequently, feather barbs develop by longitudinal compartmentalization of the feather germ epithelium into barb ridges, that become the barbs, or primary branches, of the feather (Fig. 1M). In plumulaceous feathers (e.g., down feathers), barb ridges grow longitudinally within the feather germ and have a rudimentary rachis. In pennaceous feathers (i.e., feathers with a prominent rachis and a planar vane), the barb ridges grow helically around the tubular feather germ and fuse at the dorsal surface of the feather germ to form the rachis. Animations of both plumulaceous and pennaceous feather growth and development may be viewed at http://fallon.anatomy.wisc.edu/feather.html. The evolutionary innovations involved in the development of a feather from an archosaurian scale have been proposed to be hierarchically dependent (Prum, '99). Specifically, a tubular outgrowth necessarily precedes the subdivision of the tubular epidermis into barb ridges, and the formation of barb ridges necessarily

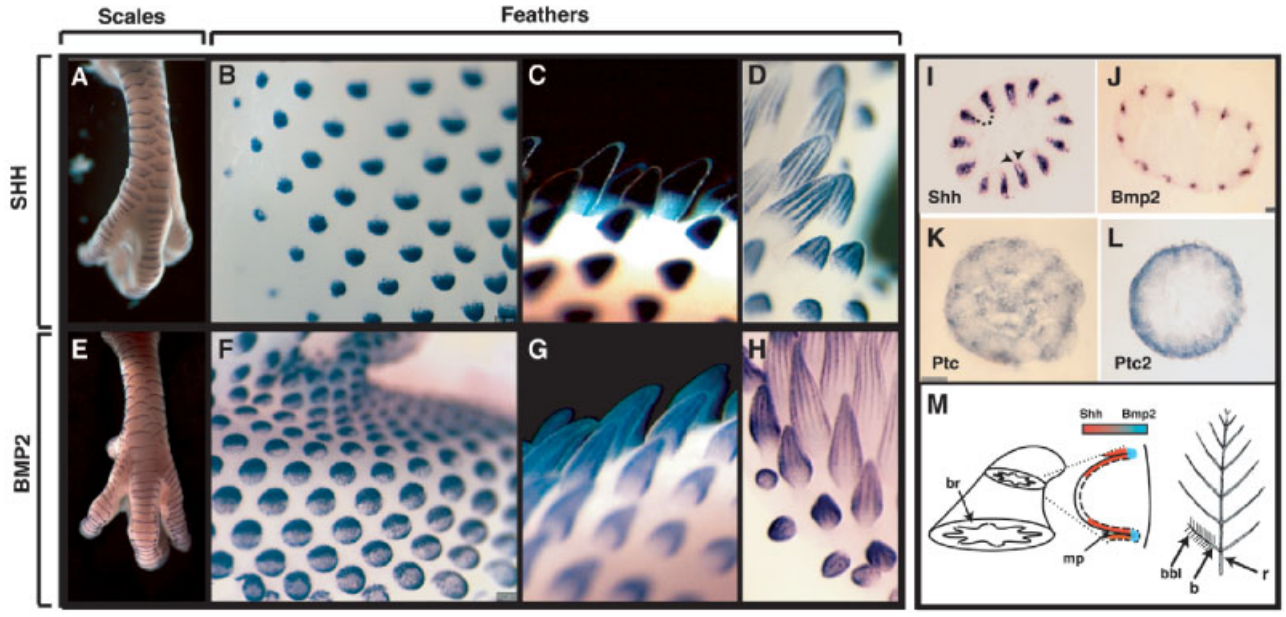

Fig. 1. Whole mount in situ hybridization (WMISH) of *Shh* and *Bmp2* in developing feathers and scales of the chick. Panels (A–B) and (E–F) are oriented with rostral at the top, and (C–D) and (G–H) are oriented with distal axis at the top. Expression of *Shh* and *Bmp2* is polarized within individual scale (A–E) and feather (B–F) placodes of an E11 chick shank and E10 pteryla, respectively. *Shh* is expressed in a posterior domain and *Bmp2* is expressed in an anterior domain within each placode. As feather buds grow distally, *Shh* (C) and *Bmp2* (G) expression is localized to the distal tip of the growing feather germs. Subsequently, *Shh* (D) and *Bmp2* (H) expression in feather germs becomes refined into longitudinal stripes along the proximal-distal axis of the feather. Histological sections through elongated feathers hybridized to *Shh* (I) and *Bmp2* (J) show that *Shh* is expressed throughout the folded marginal plate epithelium (arrowheads) of the forming barb ridge (outlined with dotted line), but that *Bmp2* expression is restricted to the peripheral folds. Histological sections through elongated feather germs hybridized to the *Shh* regulated genes *Ptc* (K) and *Ptc2* (L) show that genes regulated by *Shh* are expressed only in the peripheral sections of the marginal plate. (M), Schematic of the structure of a developing feather germ showing forming barb ridges (*br*) and expression domains of *Shh* and *Bmp2* within the marginal plate epithelium (*mp*) of a single barb ridge. The polarity of expression of *Shh* and *Bmp2* expression in the central to peripheral marginal plate epithelium is illustrated in a gradient model (bar). The barb ridges become the formed barbs (b) and barbules (bbl) of the feather. The rachis (r) forms through fusion of the barb ridges as they grow helically around the circumference of the feather (see Fig. 4F–J).

precedes the formation of the rachis by the fusion of barb ridges. Further innovation of barbules would lead to the formation of the closed, planar, pennaceous feather.

Shh and Bmp2 expression polarity in archosaur placodes

To investigate the molecular mechanisms involved in the morphogenesis and evolution of branched feather structure, we examined the development of integumentary appendages of extant archosaurian lineages represented by the chick (*Gallus gallus*), duck (*Anas platyrhynchus*), and alligator (*Alligator mississippiensis*). Through a comparative analysis of the molecular mechanisms of integumentary appendage development within the chick, other species of birds, or phylogenetically related archosaurs, we can begin to ask how change in developmental mechanisms have facilitated the formation of morphological complexity during feather evolution.

We centered our analysis on two patterning genes, *Shh* and *Bmp2*, that we found to be closely linked in epithelial appendage morphogenesis. Previous studies outlined the expression domains of *Shh* and *Bmp2* in early feather development (Nohno et al., '95; Ting-Berreth and Choung, '96; Noramly and Morgan, '98). We extended these analyses by demonstrating that the timing and polarity of expression of these two genes are conserved in development of placodes of feathers and scales in the chick, and that this initial pattern of expression is conserved in the placodes of the integumentary appendages of chick, duck, and alligator.

Initially, in the chick, *Bmp2* is expressed throughout the forming placode of feathers of the chick and its expression subsequently becomes polarized as *Shh* is expressed in the placode (data not shown) (Noramly and Morgan, '98). *Shh* has been shown to be expressed in a posterior domain within the thickened epithelial placode of avian scutate and scutellate scales (found on the tarsi and dorsal surface of the feet), and in the posterior domain of feather placodes (Figs. 1 and 2) (Nohno et al., '95; Ting-Berreth and Choung, '96). At the same stages of appendage development, *Bmp2* is expressed along the anterior border of the epithelial placode of both scales and feathers (Figs. 1 and 2) (Morgan et al., '98). Thus, the expression of *Shh* and *Bmp*2 in scale and feather placodes of the chick exhibits conservation of timing and anterior-posterior polarity of *Shh-Bmp2* expression within the epithelial placode (Fig. 1A,B,E, and F).

In order to see if these mechanisms are conserved among archosaurs, we analyzed the expression of *Shh* and *Bmp2* in the embryonic integumentary appendages of the duck and alligator. Analysis of *Shh* and *Bmp2* expression in forming duck scale and feather placodes showed similar expression polarity and timing as seen in chick embryos (Fig. 2). This suggests a conservation of the spatial and temporal regulation of *Shh* and *Bmp2* expression in early feather and scale development among birds. Alligators are members of the extant sister group of birds, and they have keratinized integumentary scales that are homologous with those of birds. Alligator scales exhibit a distinct scale ridge and morphological polarity similar to avian scutate scales (Ferguson, '85; Alibardi and Thompson, 2000). Inter-specific hybridization of chick antisense RNA probes for *Shh* and *Bmp2* showed specific hybridization to the scale placodes on the ventral surface of the tail of stage 21 alligator embryos. The expression of *Shh* and *Bmp2* exhibited the same anterior-posterior polarized pattern seen in both duck and chick (Fig. 2A,F).

Collectively, these data suggest that expression of *Shh* and *Bmp2* was regulated in a similar fashion in the scales of the common ancestor of birds and crocodilians, and that this plesiomorphic expression pattern has been maintained in the initial placode stages of feather bud morphogenesis (Fig. 2K).

Novel expression of Shh and Bmp2 during feather development

As feather development proceeds, the feather placode grows into a conical bud, and patterned subdivision of the conical epithelium of the feather germ forms the barb ridges that become the feather barbs and barbules. These morphogenetic processes and the structures formed are evolutionarily derived and unique to feathers (Prum, '99). The developmental transition from placode to the outward growth of the feather bud is correlated with co-expression of *Shh* and *Bmp2* in the distal feather bud epithelium as shown by sectioning (data not shown; Fig. 1C,G); co-expression is also seen in the early feather placode, and precedes formation of polarized expression of *Shh* and *Bmp2* (Morgan et al., '98). Subsequently, the expression of *Shh* and *Bmp2* becomes subdivided into longitudinal, or proximal-distal, stripes that

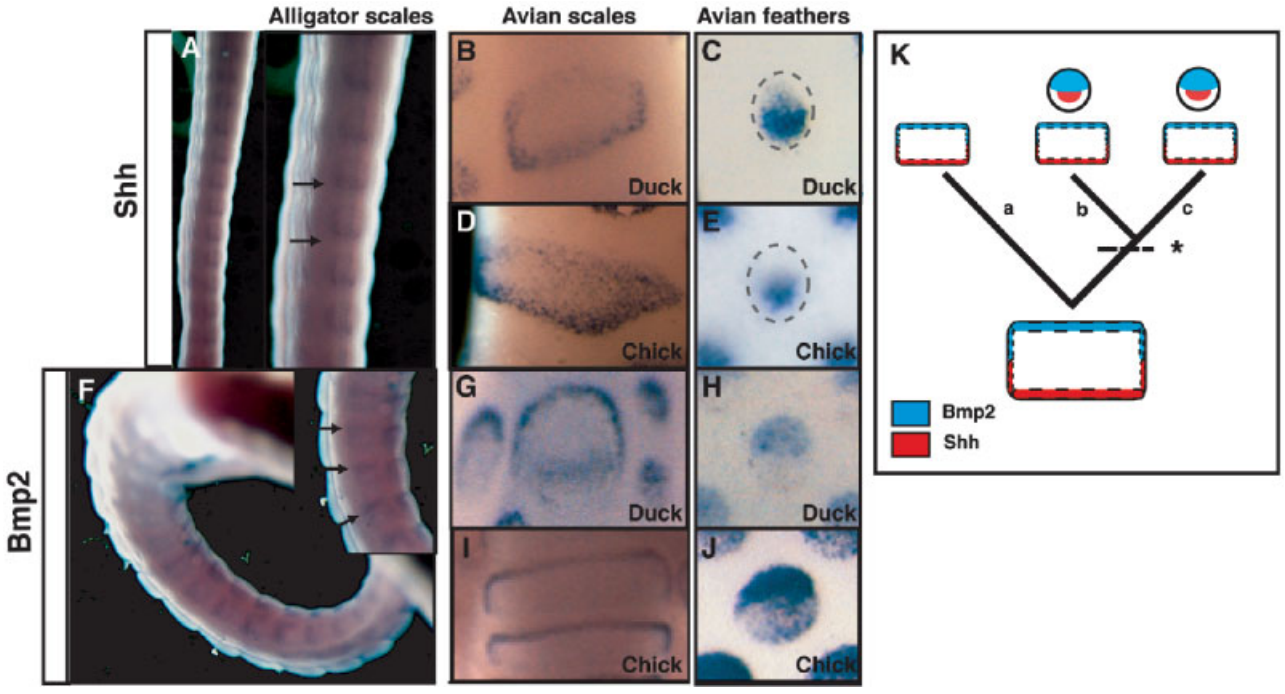

Fig. 2. Comparative phylogenetic analysis of *Shh* and *Bmp2* expression in placodes of integumentary appendages of archosaurs. (A, F), Expression of *Shh* and *Bmp2,* respectively, in the ventral tail epidermis of the embryonic alligator (*Alligator mississippiensis*). Gene expression is shown by WMISH using interspecific hybridization with chick probes for *Shh* and *Bmp2*. Picture insets in A and F are higher magnification images of the alligator tail shown. Arrows point to polarized expression of *Shh* or *Bmp2* in forming scale rudiments. The expression of *Shh* (B–E) and *Bmp2* (G–J) in: E15 Pekin duck (B, G) and E10 chick scutate scales (D, I); and from E15 duck (C, H), and E10 chick (E, J) feather pteryla. Dotted lines in (C) and (E) outline the forming feather placode containing *Shh* expression. (K), Phylogeny of the archosaurian orders (a) Crocodylia (alligator), (b) Anseriformes (duck), and (c) Galliformes (chick) showing the conservation of anterior-posterior expression polarity within the epidermal placodes of their integumentary appendages. The transcriptional regulation of both *Shh* and *Bmp2* from the ancestral archosaurian scale placode has been conserved in the derived feather placodes of birds. The * indicates the origin of feathers in an ancestor of the avian clade.

are parallel to the primary growth axis of the feather bud (Fig. 1D,H). These longitudinal domains of *Shh* and *Bmp2* expression mark the boundaries of the forming barb ridges as the tubular epithelium of the feather germ folds inward to compartmentalize portions of the epidermis into distinct barb ridges (Fig. 1M). The expression of *Shh* and *Bmp2* is identical in the embryonic natal down of chicken and ducks (data not shown), suggesting that the regulation of expression in this novel context is common to natal feathers.

Shh is expressed in the folded basal layer of the feather epidermis between barb ridges, which is called the marginal plate (Nohno et al., '95; Ting-Berreth and Choung, '96). *Shh* expression first appears peripherally and spreads into two stripes along the juxtaposed basal layers of neighboring barb ridges (arrowheads, Fig. 1I,M; and data not shown). The expression of *Bmp2*, however, is restricted to the peripheral folds of the marginal plate (Fig. 1J,M). The expression of the *Shh* receptors *Ptc, Ptc2* (Pearse et al., 2001) (Fig. 1K,L) and the down stream transcription factor, *Gli-1* (data not shown), which are transcriptionally regulated by *Shh* signaling, also exhibit a peripheral bias in their expression in the feather germ as the formation of the barb ridges proceeds.

Therefore, competence to respond to *Shh* signaling within the marginal plate epithelium is localized to the peripheral epidermis (Fig. 1M). A similar restriction in the competence to respond to *Shh* signaling is seen in the placode stage of feather development and may have a role in establishing axial polarity within the epithelium (Morgan et al., '98).

Barb ridges have an intrinsic polarity; the ramus, the core filament, is formed centrally and barbules differentiate towards the periphery of the

barb ridge. Polarized *Shh* and *Bmp2* expression in the marginal plate, which is the folded portion of the basal epithelium, may function in formation and maintenance of central-peripheral polarity within the barb ridges themselves.

Shh-Bmp2 prepattern and the development of branched feather morphology

Natal down of the chick exhibits stereotypical variations in barb branching (Watterson, '42), but the developmental basis for these morphological variations are not known. We found that the longitudinal domains of *Shh* and *Bmp2* expression along the feather germ show patterns that presage these variations in down morphology.

The longitudinal *Shh* expression domains demonstrate four distinct variations from simple linear propagation of the stripes at the base of the feather germ: bifurcation, termination, de novo initiation, and fusion (Fig. 3A–E). Because these linear domains of *Shh* expression mark the marginal plate epithelia that border neighboring barb ridges, the specific variations in *Shh* expression represent four distinct mechanisms of barb ridge morphogenesis: *Shh* expression domain bifurcation yields new barb ridge formation (Fig. 3A,F); expression domain termination leads to barb ridge fusion (two barbs connected to a single basal barb) (Fig. 3B,G); de novo domain initiation of *Shh* expression domain yields division of a barb ridge into two (two barbs connected by a single

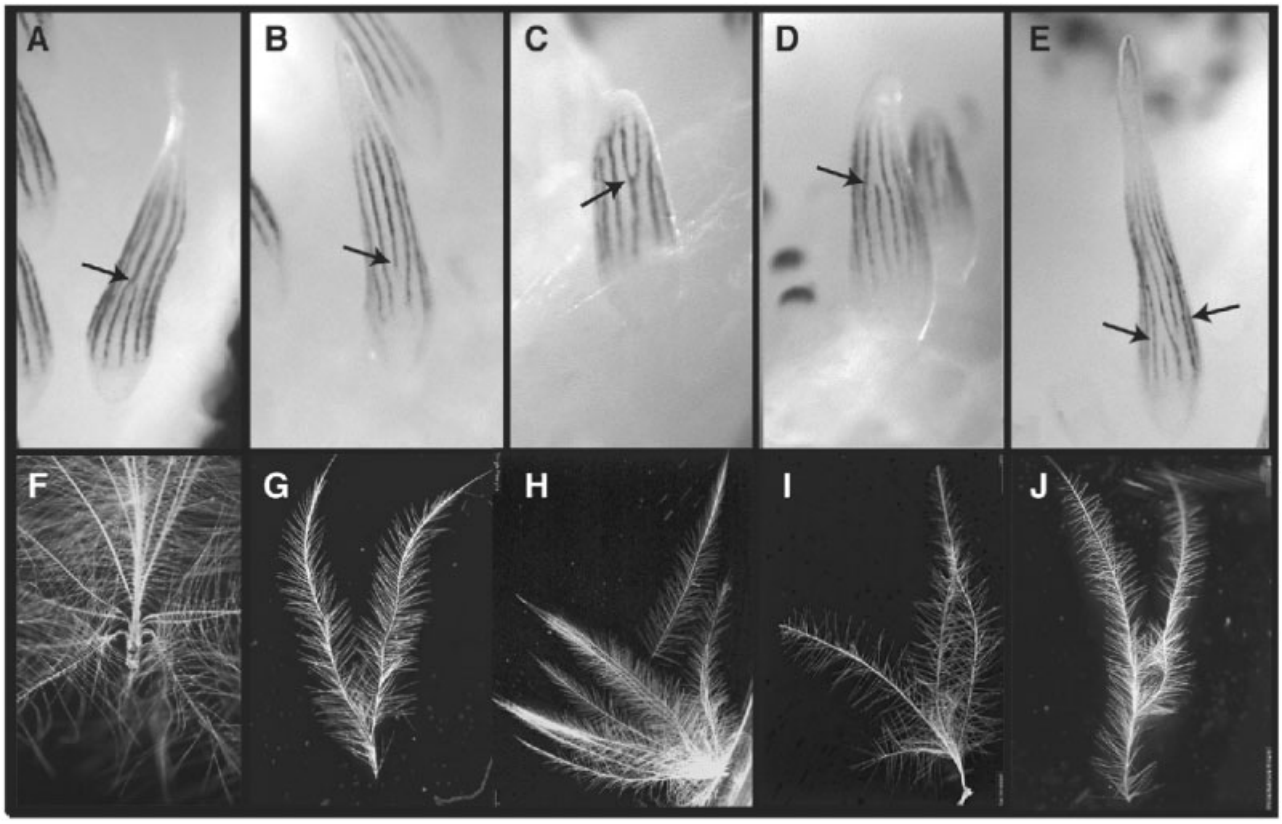

Fig. 3. Variation in patterns of *Shh* expression correlate with the phenotypic variation in natal down of chick. (A–E), Feather germs of comparable stage from the femoral tract of a stage HH35 White leghorn chick embryo illustrate four variations in the propagation of longitudinal *Shh* prepattern in feather germs within a single tract of an embryonic chick. (A) Bifurcation of *Shh* expression domain; (B) Termination of *Shh* expression domain; (C) Fusion of *Shh* expression domains; (D) *de novo* initiation of new *Shh* expression domain; and (E) Simultaneous cessation and initiation events. These longitudinal *Shh* expression domains indicate the folded marginal plate epithelium that differentiates neighboring barb ridges. (F–J), Each class of *Shh* expression pattern observed corresponds to an observed phenotype in day-old chick natal down. (F) New barb ridge formation resulting in addition of a barb; (G) Barb fusion in which two barbs are connected proximally to form a single branched barb; (H) Barb loss in which a free barb is unconnected to the rest of the feather; (I) Barb division in which a single barb splits proximally into two basal bars; and (J) Simultaneous barb fusion and barb division. Evidence of barb loss was found in ensheathed down feathers of newly hatched chicks. In G, I, and J, the barb shown has been removed from the down feather to illustrate the phenotype of interest.

distal tip) (Fig. 3D,I); and fusion of *Shh* expression domains leads to barb ridge loss (a free barb unconnected to the rest of the feather) (Fig. 3E,J). We found four classes of feather phenotypes in natal down from the femoral tract of newly hatched chicks that correlate precisely with the observed pattern variation of *Shh* expression in the feather germs of the embryonic femoral tract (Fig. 3F–J). The longitudinal domains of *Bmp2* expression in the feather germ show identical pattern variations to those seen with *Shh* (data not shown). The correlation of the observed *Shh* and *Bmp2* expression pattern and the branching phenotypes of chick natal down suggests that regulation of *Shh* and *Bmp2* expression and function is a critical component for the formation of the distinct and varied branching phenotypes of feathers. Experiments testing the necessity of *Shh* and *Bmp2* signaling in barb ridge formation support this hypothesis (see below).

In the duck, most of the first embryonic feathers to grow are pennaceous (i.e., they have a prominent rachis and planar vane), whereas embryonic feathers in a chick are plumulaceous [i.e., they have a minimal rachis and no planar vane (Lucas and Stettenheim, '72)]. The natal down feathers from chick and duck, which have these major structural differences in branching morphology, also exhibit the specific, predicted variations in *Shh* expression. Serial histological sections through an E20 duck rectrix (tail feather), which has a prominent rachis, document that the rachis forms by the fusion of barb ridges, beginning at the periphery and proceeding towards the interior, as they grow helically towards the dorsal surface (Fig. 4F–J). The longitudinal *Shh* expression domains along the dorsal surface of the feather germ of E16 duck rectrices show a gradual decrease and cessation of the *Shh* expression stripes which precede the initial barb ridge fusion event that forms the rachis ridge and the subsequent fusions of adjacent barb ridges to the rachis ridge (Fig. 4K). In serial histological sections, these fusion events are correlated with a decrease in *Shh* expression that proceeds in a peripheral to central manner (Fig. 4L–N) as in barb ridge formation (Fig. 4B–E). These results suggest that the longitudinal *Shh* expression domains constitute a molecular prepattern for the development of the barb ridge branching pattern in feathers.

Helical growth of barb ridges, the formation of a rachis, and the pennaceous vane are all correlated with the formation of a ventral locus of new barb ridge formation (Lucas and Stettenheim, '72). As predicted by variations in longitudinal *Shh* expression domains in plumulaceous chick down, the ventral locus of new barb formation is identifiable early in the pennaceous duck rectrix by the repeated, localized bifurcation of *Shh* expression domains on the ventral surface of the feather follicle (Fig. 4P). An analysis of cell division in embryonic duck down shows a localized focus of cell division (as detected by staining for the mitotic epitope, phosphorylated histone H3 (p-H3) (Hendzel et al., '97) on the ventral surface of feather germs at the site of new barb formation (Fig. 4S).

Interestingly, SHH protein immunoreactivity is also localized to the ventral region of new barb ridge formation, suggesting an additional function for *Shh* signaling in initiation of helical growth, which is a signaling event independent of expression in barb ridges (Fig. 4T). The dorsal-ventral polarized organization of embryonic duck feathers contrasts with chick natal down in which barb addition is random around the circumference of the feather germ after the origin of the first complement of barb ridges (data not shown; Watterson, '42). Chick down also has localized foci of cell division and SHH protein expression, but notably without a ventral bias (Fig. 4U–V).

In summary, longitudinal expression of *Shh* and *Bmp2* in the developing feather germ prepatterns barb ridge morphogenesis, exhibiting variations that mark a variety of branched barb morphologies in plumulaceous down. Coordinated utilization of two of these prepattern mechanisms, i.e., dorsal serial *Shh-Bmp2* stripe cessation coordinated with rachis formation and barb ridge fusion, and repeated *Shh-Bmp2* stripe bifurcation ventrally resulting in new barb ridge formation, produces helical growth of barb ridges, the rachis, serial barb fusion, the ventral new barb locus, indeterminate barb number, and the planar vane that characterize pennaceous feathers. Thus, control of *Shh-Bmp2* expression during feather morphogenesis is a fundamental component of the mechanisms determining feather form (e.g., plumulaceous and pennaceous structure).

Shh and Bmp2 signaling integration and function

The expression patterns of *Shh* and *Bmp2* shown above suggest that the functions of these two genes are related during feather and scale morphogenesis. Previous studies in the develop-

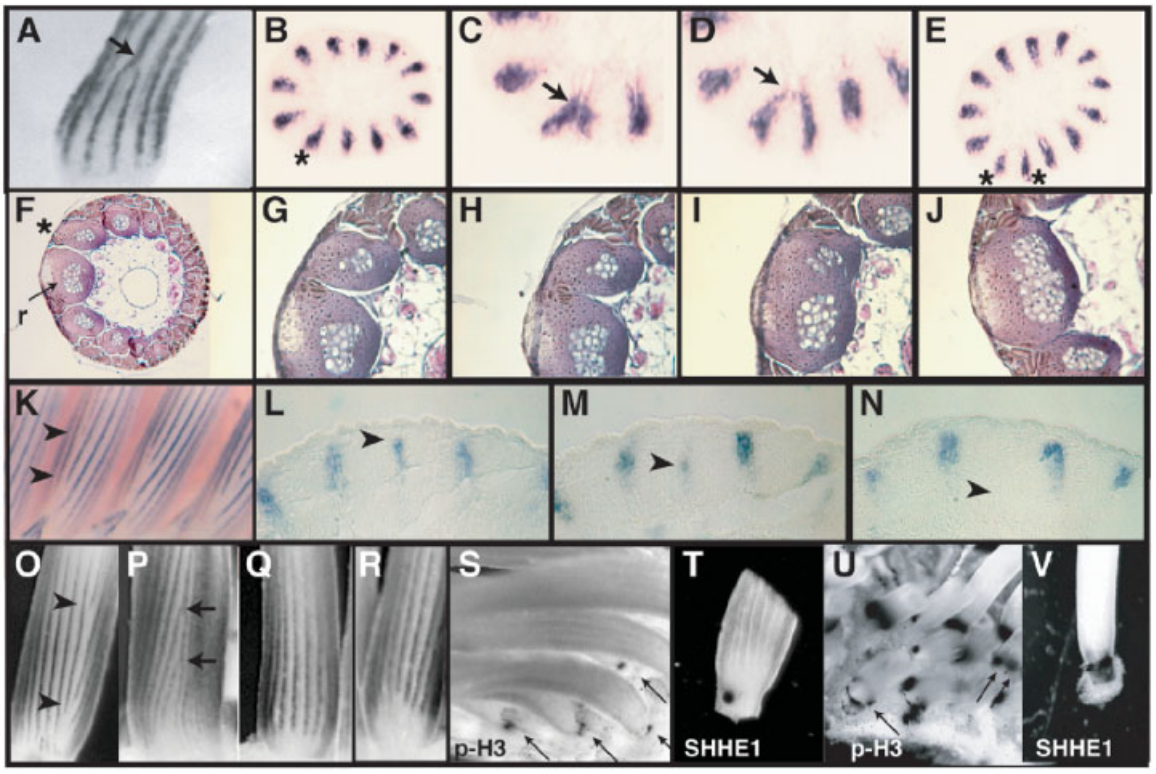

Fig. 4. *Shh* signaling and the formation of feather branched structure. (A), Close up of new barb ridge addition as indicated by division of longitudinal expression domain of *Shh*. (B–E), 10μm serial histological sections through a single feather hybridized for *Shh* showing the formation of a new barb ridge from distal (developmentally oldest) (B) to proximal (developmentally youngest) (E) region. Formation of a new barb ridge progresses from peripheral to central. (F–J) Serial 10μm histological sections of an E20 duck rectrix (tail feather) showing the formation of the rachis by fusion of neighboring barb ridges to the presumptive rachis ridge on the dorsal side of the feather germ (at the left). *r*, presumptive rachis; *, fusing barb ridge. Barb ridge formation shows a peripheral to central bias in morphogenesis as well. (K), *Shh* expression in E14 duck rectrices showing prepattern of rachis formation by barb fusion to the rachis through cessation of *Shh* expression (arrowheads). (L–N), Serial 10μm histological sections of a E14 duck rectrix showing the pattern of *Shh* expression during the initial formation of the rachis by fusion of neighboring barb ridges from distal (L) to proximal (N). Cessation of *Shh* expression progresses from the periphery, inward (arrow heads). Formation of the rachis is accompanied by helical growth of barb ridges. (O –P), *Shh* prepattern in a pennaceous E16 duck feather germ demonstrates the ventrally localized area of new barb ridge addition, indicated by a series of bifurcations of *Shh* domains (P, arrows), juxtaposed to the dorsal barb ridge fusion, indicated by a series of *Shh* domain cessations (O, arrowheads). (Q–R), This pattern of helical barb ridge growth, ventral new barb ridge addition, and dorsal barb ridge fusion is not seen in E13 chick remiges which have a plumulaceous structure and lack a prominent rachis (Q–R, dorsal and ventral views respectively). In pennaceous duck feather germs, the foci of p-H3 (S) and SHH (T) are primarily seen on the ventral-proximal orientation (92%, n=36). In chick down (U–V), SHH protein and areas of cell division localize to discrete foci of E13 chicks in no particular orientation around the follicle.

ment of integumentary appendages of amniotes indicate that *Shh* maintains a consistent role in cell proliferation and patterning, whereas epidermal expression of *Bmps* are often associated with differentiation (Bitgood and McMahon, '95; St-Jaques et al., '98; Chiang et al., '99; Jung et al., '99; Park and Morasso, 2002). We suggest that *Shh* and *Bmp2* signaling functions as a conserved, integrated signaling system, or module, in the morphogenesis of epidermal appendages. To test this hypothesis, we analyzed the conservation of function of *Shh* and *Bmp2* during scale and feather morphogenesis.

We tested the effect of *Bmp2* on *Shh* function by placing beads loaded with recombinant human BMP2 (rBMP2) protein into follicles of duck E14 rectrices and assayed for *Shh* expression after they were cultured on the chorio-allantoic membrane (CAM) of chick hosts. Treatment of feather bud explants with ectopic rBMP2 protein caused localized, reduced *Shh* expression within the feather after 24 hours (Fig. 5A,B). Conversely,

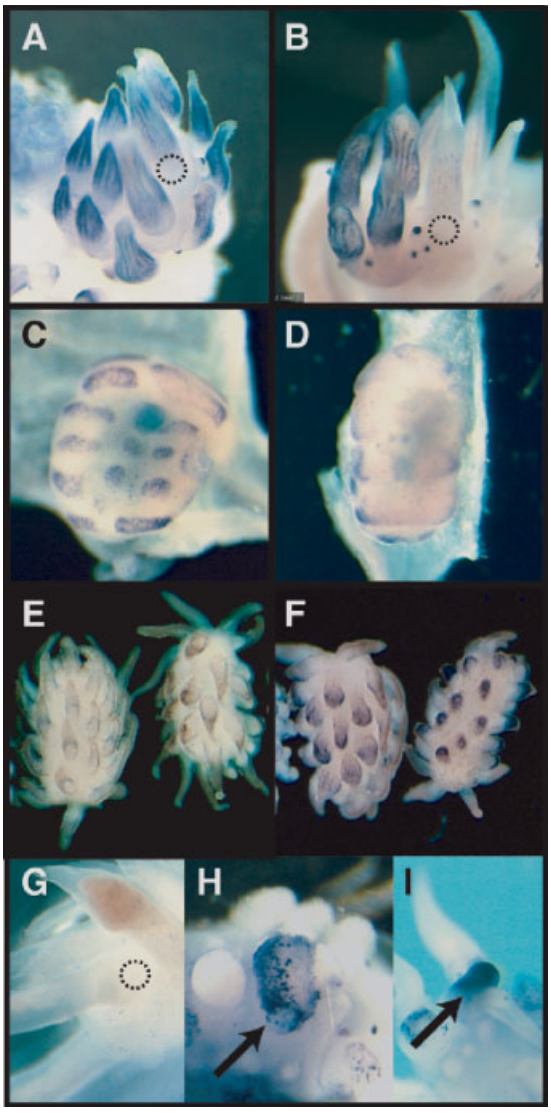

Fig. 5. *Shh* and *Bmp2* signal integration and effect on cell proliferation. (A–B), WMISH detection of *Shh* transcripts 24 hours after bead implantation (circles) show a specific reduction of *Shh* expression by (B) rBMP2 (85%; n=7) in embryonic feathers when compared to (A) PBS control beads (0%; n=6); circles indicate location of bead. In a similar experiment in embryonic scale rudiments, rBMP2 treatment leads to a reduction of (D) *Shh* transcripts (80%; n=5) when compared to (C) control (16%; n=6). (E–F), WMISH detection of *Shh* transcripts in chick feather explants treated with (E) PBS (0%; n=6) or (F) Noggin (75%; n=8) indicate that BMP signaling is necessary for regulation of the level of *Shh* transcripts in feather germs. (G–I), Increased *Shh* signaling in the feather by treatment with (H) N-SHH (100%, n=4) or (I) Noggin (100%, n=5) loaded on beads (arrows) leads to an increase in cell division as measured by detection of p-H3 when compared to (G) control beads (0%, n=4). A cicle outlines the location of the bead in (G).

treatment of feather bud explants in vitro with the BMP antagonist, Noggin, caused increased *Shh* expression only within forming feather germs and not in inter-follicular tissue (Fig. 5E,F). Thus, BMP2 is sufficient and necessary to negatively regulate *Shh* activity within the forming feather germ. Similar to our results in the feather germ, there is evidence that *Noggin* overexpression in the epidermis of the early placode of the feather can lead to the expansion of *Shh* expression domains leading to trilobed feathers (Noramly and Morgan, '98). Given the conservation of expression of *Shh* and *Bmp2* between feather and scale placodes, we tested the effect of rBMP2 protein on *Shh* expression in forming chick scales to see if regulation of *Shh* may be similar to that seen in the feather follicle and feather placode. Treatment of chick scutate scale CAM explants with rBMP2 protein loaded beads suppressed *Shh* expression in forming scale placodes demonstrating conservation of the signaling integration between *Shh* and *Bmp2* in both integumentary structures (Fig. 5C,D).

Several lines of evidence indicate that *Shh* plays an important role in regulating epidermal proliferation and patterning. Increased *Shh* signaling leads to epidermal overgrowth, or cancer (Dahmane et al., '97; Fan et al., '97; Oro et al., '97; Xie et al., '98), and altered specification of epidermal cell fates (Rowitch et al., '99). Additionally, *Shh*, and its *Drosophila* ortholog *hedgehog* are necessary for pattern formation and differentiation within epithelia of forming ectodermal appendages (Basler and Struhl, '94; St-Jaques et al., '98; Chiang et al., '99). Given the conservation of integrated *Shh* and *Bmp2* signaling documented above, we examined the role of *Shh* and *Bmp2* in regulating the cell cycle and differentiation of epidermis during feather morphogenesis.

Previous studies using ectopic expression of *Shh* in chick epidermis indicate that an increase in the levels of *Shh* signaling lead to an overgrowth of the feather germ (Ting-Berreth and Choung, '96; Morgan et al., '98). Similarly, we report that duck feathers treated with heparin beads loaded with N-SHH protein showed a distinct hypertrophy of the epidermis surrounding the bead (Fig. 6B). Analysis of explants 48 hours after bead placement showed an increase of cells staining for the mitotic epitope p-H3, indicating that the phenotype is due, in part, to increased cell division (Fig. 5H). Feather follicles treated with beads loaded with Noggin protein showed an increase in p-H3 labeling supporting the conclusion that BMP

signaling abrogates *Shh* activity (Fig. 5I). In contrast, beads loaded with rBMP2 protein induced a localized increase in epidermal differentiation of the feather follicle (Fig. 6C). This altered histology was demonstrated by a thickened stratum corneum around the bead. These data suggest that there is a negative feedback control mechanism between *Shh* and *Bmp2* signaling which functions in the homeostasis of epidermal growth and differentiation during appendage development. *Bmp2* regulates *Shh* expression in developing feather germs and, thus, cell division. In addition, BMP2 treatment leads to an increase in differentiation, which may be a consequence of suppressing cell proliferation, but *Bmp2* may also affect immediate downstream genes associated with differentiation [e.g., Dlx3 (Park and Morasso, 2002)]. A recent report (Kulessa et al., 2000) showed similar effects of *Noggin* misexpression in hair follicles; they demonstrate a release of inhibition on the expression of *Shh* as well as increased proliferation in hair follicles. Along with our data, these observations raise the possibility that the regulatory mechanism seen in feathers and scales may be common to the whole class of vertebrate epidermal appendages.

Our data indicate that *Shh* and *Bmp2* signaling are integrated to control proliferation and differentiation within forming feathers and that negative regulation of *Shh* expression, and hence function, by BMP signaling is conserved in the initial development of scale and feather placodes. To test the role of these integrated signaling systems on barb ridge morphogenesis, we used antagonists of *Shh* signaling on feather explants grown in culture or grafted to the CAM of the chick. The alkaloid cyclopamine is a potent inhibitor of *Shh* signaling (Incardona et al., '98). Cyclopamine treatment of feather CAM explants altered feather morphogenesis showing an inhibition of epidermal invagination (a morphological indicator of barb ridge formation) in histological sections (Fig. 6D–G). A comparable result was achieved by treating explants with the *Shh* blocking antibody 5E1 showing similar inhibition of epidermal invagination (data not shown). These data demonstrate that *Shh* signaling is necessary for barb ridge formation.

To further investigate the effects of *Shh* and *Bmp2* on barb ridge morphogenesis, we used viral constructs to locally increase the expression of negative mediators of both *Shh* and *Bmp2* signaling within forming feathers. Protein kinase A (PKA) is a cAMP dependent kinase that has been found to be a general inhibitor of *Shh* signaling in many developing systems, including feathers (Hammerschmidt et al., '96; Noveen et al., '96). We found that areas of forced overexpression of a constitutively active variant of PKA (RCAS-CaP-KA) in the epidermis of embryonic chick feathers led to aberrant feather growth demonstrated by sharp bends in the growing feather germ that localized to foci of viral expression (Fig. 6I). These deformed feathers showed a consistent alteration of cell differentiation within the epidermis that localized to areas of viral infection. In histological section, regional differentiation towards a ''barbule-like'' fate was seen in areas of viral infection in a non cell-autonomous manner (see eosinophilic cells, white arrowheads, Fig. 6L). Parallel experiments using virus containing a dominant negative *Bmpr1B* variant that abrogates BMP signaling (RCAS-dnBMPR1B; Zou and Nisewander, '96) resulted in feathers with bends in the follicle that were associated with an overgrowth of the follicular epidermis, similar to the overgrowths seen around N-SHH loaded beads (Fig. 6J). Analysis of histological sections revealed that, in areas of viral infection, a noncell autonomous effect on differentiation is evidenced by the presence of noneosinophilic ''centralized'' cells in the peripheral epithelium and/or heightened growth of the sheath ectoderm. In the dnBMPR1B infected feather, nuclei are seen throughout the epidermis suggesting an inhibition of cell differentiation in the cornefied cell layers (Fig. 6M). These data demonstrate that *Shh* and *Bmp* signaling is necessary for feather barb morphogenesis and suggest that integrated *Bmp2* and *Shh* signaling controls polarity and differentiation within the forming barb ridges (Fig. 6N). Interestingly, Haake et al. ('84) describe a peripheral to internal wave of differentiation in the forming feather with beta keratin expression in peripheral domains similar to expression of *Shh* responsive genes that we report in this study.

We conclude that *Shh* and *Bmp2* are an integrated signaling system that mediates cell growth and differentiation of the feather. Early polarity in the expression of these signaling systems may permit differential signaling within epithelia. *Bmp2* is expressed prior to *Shh* and may limit the expression of *Shh* to the posterior domain of forming placodes and to the central domains of barb ridge epidermis. Our results suggest that the effect of *Bmp2* on *Shh* expression, and hence *Shh* function, permits controlled morphogenesis of epidermal appendages.

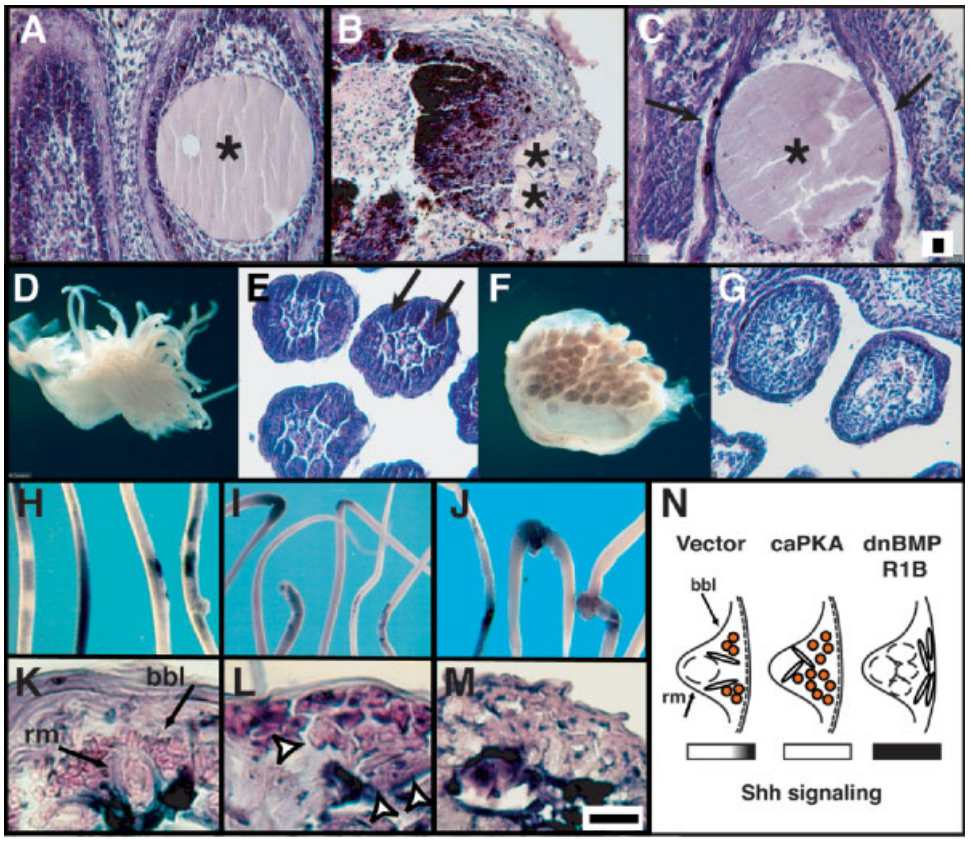

Fig. 6. Conserved *Shh* and *Bmp2* signaling module controls proliferation and differentiation during feather morphogenesis. (A–C), Hematoxylin and eosin stained histological sections of Khaki Campbell duck rectrix explants treated with beads containing (A) PBS (0%; n=3), (B) N-SHH (75%; n=4), or (C) rBMP2 (100%; n=2) and grafted to the chorio-allantoic membrane (CAM) of chick hosts. The beads are marked with an asterisk; bar equals 10 μm in all three histological sections. Arrows in (C) point to areas of increased differentiation of the stratum corneum. (D–G), Chick feather germ explants to the CAM treated with (F–G) cyclopamine show inhibition of feather morphogenesis in comparison to (D–E) diluent control. Analysis of histological sections shows a specific inhibition of barb ridge invagination by (G) cyclopamine (52%, n=21; X^2, $P<0.05$) when compared to (E) diluent group (9%; n=21). Arrows point to forming barb ridges in a feather of control explant. (H–M), Phenotypes of chick natal down expressing (H, K) RCAN control, (I, L) RCAS-caPKA, and (J, M) RCA-BMPR1B viruses. Foci of viral expression were detected by looking at viral GAG protein expression (stained purple). (I), RCAS-caPKA infection showed a reduction of the filament circumference and bending of the feather germ. (J), RCAS-dnBMPR1B infection foci exhibited proximal epidermal overgrowth as seen in (B) N-SHH bead studies. (K), Hematoxylin and eosin stained transverse histological sections of RCAN control samples showed no specific change in phenotype that correlated with epidermal viral infection shown by blue-purple NBT-BCIP precipitate (0%, n=4) (*rm*, barb ramus; *bbl*, barbules; bar equals 10 μm). (L, M), transverse histological sections of areas of bends associated with viral infection shows a specific alteration in ramus and barbule morphology in both (L) RCAScaPKA (75%; n=4) and (M) RCAS dnBMPR1B (100%, n=4) infected feathers. (N), Model of the effect of altering *Shh* signaling on barb ridge polarity. RCAN control (vector) infected barb ridges exhibit normal polarity containing a central ramus (ovals, *rm*) and barbules (red circles, *bbl*) positioned more peripherally. The bars represent the levels of *Shh* signaling along the central to peripheral axis. In RCAN treated feathers, the normal level and polarity of *Shh* signaling within the forming barb ridge is modeled as demonstrated in WMISH analyses (Fig 1). The data from histological analyses support a model in which a localized reduction of *Shh* signaling by CaPKA expression leads to a 'peripheralization' of the forming barb ridge as evidenced by the central presence of eosiniphilic (tissue staining red), ''barbule-like'' cells in the epidermis (red circles). This effect of CaPKA expression on *Shh* signaling in the forming barb ridge is modeled as a loss of polarity within the marginal plate epithelium (bar schematic). Conversely, a localized activation of *Shh* signaling in the barb ridge (modeled in bar schematic) by abrogation of BMP signaling (dnBMPR1B) leads to a 'centralization' in the forming barb ridge with an increase in noneosinophilic cells (clear circles and ovals) in the epidermis and a lack of proper epithelial differentiation.

DISCUSSION

Previous studies have investigated the molecular mechanisms of early feather and scale development at the level of the placode and feather bud. Here we show that *Shh* and *Bmp2* expression and the negative regulation of *Shh* by *Bmp2* is conserved among feather and scale placode primordia. We demonstrate that the general temporal and spatial regulation of *Shh* and *Bmp2* expression is also conserved in the formation of alligator scales. Thus, the initial formation of the placode and the molecular determinants of placode formation are likely to be pleisomorphic within archosaurs. The extension of our study to encompass an analysis of the later and derived mechanisms of feather formation has shown that the complexity of feather branched morphology stems from the iterative expression and function of the pleisomorphic integrated signaling between *Shh* and *Bmp2* in novel developmental contexts. We propose that the reuse of this signaling module to control cell division and differentiation of epithelia led to the morphogenesis of novel integumentary structures.

Our observations suggest that changes in cell division and cell differentiation under control of an integrated *Shh* and *Bmp2* signaling module lead to discrete changes in feather morphology. *Shh* and *Bmp2* are expressed in a prepattern that is a prelude to the development of the barb ridges in a feather germ, and their signaling is necessary for barb morphogenesis. It is a critical observation that these expression domains exhibit variations within a feather germ that correlate specifically with the variation in branching patterns seen in plumulaceous chick natal down and pennaceous embryonic duck feathers. In addition, we provide the first evidence of the mechanism of helical growth and the differentiation of branched form between plumulaceous and pennaceous feathers. We show that differential spatial control of cell division in feather morphogenesis, which is associated with the formation of plumulaceous and pennaceous feather morphologies, corresponds with localized regulation of SHH protein expression. Variations in the rate of new barb ridge formation, barb ridge diameter, and the angle of helical growth are hypothesized to be among the determinants of the shape of the pennaceous feather vane (Prum and Williamson, 2001). We propose that regulation of the *Shh-Bmp2* signaling module and thus, proliferative capacity, is involved in, and essential for, determining the tremendous diversity of pennaceous feather sizes and shapes found in extant birds.

The polarized expression of *Shh* and *Bmp2* in placode epithelia is conserved across archosaur integumentary appendages and this polarity is observed in the marginal plate epithelia of forming feathers as well. Although several signaling systems are involved in specification of placode asymmetry upstream of *Shh* and *Bmp2*, β-catenin mediated signaling may be a critical mediator of this early expression, as overexpression of transcriptionally activated β-catenin is sufficient to induce expression of *Shh* and *Bmp2* in feather placode epidermis and to initiate the establishment of polarity in their expression (Noramly et al., '99). It is notable that the control of *Shh* and *Bmp2* expression in the marginal plate epithelium of forming barbs is unlikely to be controlled by this mechanism, since β-catenin is expressed in complementary domains to *Shh* and *Bmp2* within the barb ridge (Widelitz et al., 2000). Thus, variation in upstream regulation of the expression of the *Shh -Bmp2* module is likely to be critical for the evolution of morphological diversity in branched feather form.

Interestingly, as we show in feathers, *Shh* is thought to act as a prepattern for tooth cusp formation and its expression is differentially regulated in teeth of varied morphologies (Jernvall et al., 2000). *Shh* and *Bmp2* are both expressed within the dental epithelium during the formation of a tooth anlagen and within patterning of tooth cusps. *Shh* signaling is sufficient for epithelial invagination and is necessary for tooth initiation and patterning (Hardcastle et al., '98; Dassule et al., 2000; Cobourne et al., 2001). *Shh* is thought to regulate proliferation within the forming tooth and *Bmp2* is associated with regulating differentiation factors [e.g., MSX and p21 (Dassule and McMahon, '98; Jernvall et al., '98)]. It is possible that the antagonistic relationship of *Shh* and *Bmp2* signaling is conserved in the formation of mammalian teeth, however, existing data are not sufficient to make a clear assessment of integrative *Shh* and *Bmp2* signaling within the forming tooth anlagen.

Based on our results, we hypothesize that feathers originated and diversified from a primitive archosaurian scale through the repeated co-option, or reutilization, of a plesiomorphic *Shh-Bmp2* signaling module in new developmental contexts (Fig. 7). The elongate, tubular feather germ evolved by the derived, distal co-expression of *Shh* and *Bmp2* that mediates the elongation of

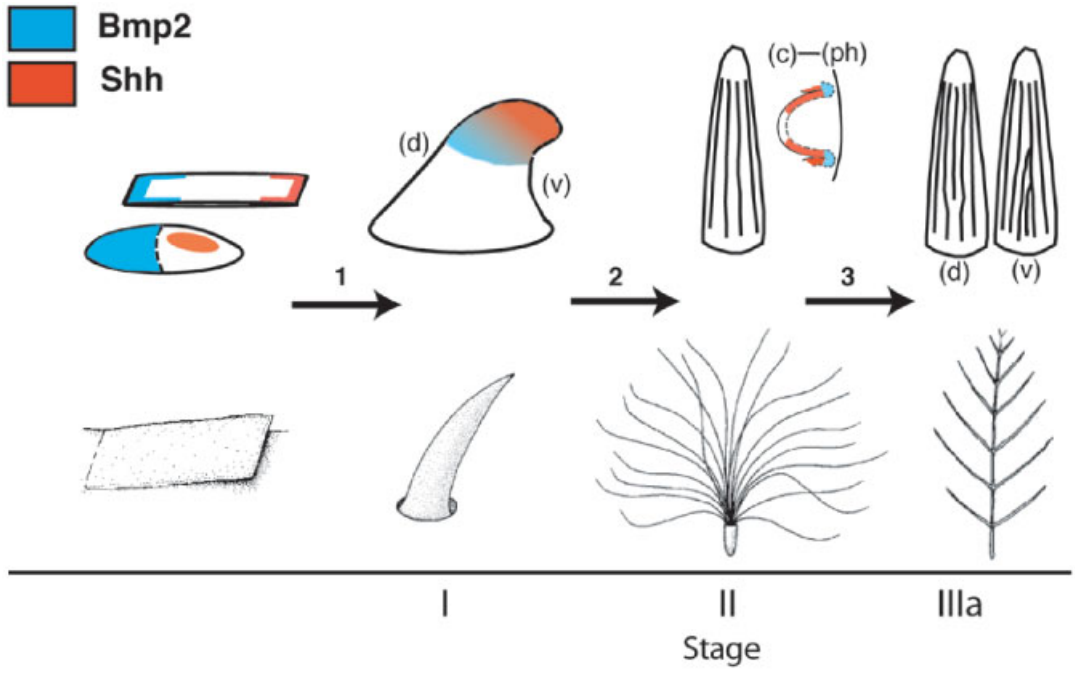

Fig. 7. Sequential redeployment of *Shh-Bmp2* module during the evolution of a feather. Congruence between patterns of expression of the *Shh-Bmp2* module in feathers and archosaurian scales (top), and the developmental theory of the origin and early evolution of feathers (bottom) (Prum, '99). The Stages I-IIIa of the developmental theory of feather evolution are proposed to have evolved by novel regulation of *Shh-Bmp2* module expression. Stage I– the first, elongate tubular feather evolved from a primitive archosaurian scale by the derived distal *Shh-Bmp2* co-expression (Event 1). Stage II- the first, branched plumulaceous feather evolved by the origin of derived longitudinal *Shh-Bmp2* expression domains (Event *2*) that created differentiated filaments from the tubular epithelium of the feather germ. The central-peripheral (*c-ph*) polarity of *Shh-Bmp2* expression in the marginal plate epithelium between the barb ridges is shown in the inset. Stage IIIa evolved by the controlled dorsal (*d*) cessation and ventral (*v*) division of the longitudinal *Shh-Bmp2* expression domains (Event *3*) producing helical growth of barb ridges, indeterminate barb number, a rachis, serial fusion of barbs to the rachis, and a planar vane. Subsequent events in the development and evolution of feathers, e.g., origins and differentiation of barbules, will require further investigation.

the placode. Subsequently, the evolution of a novel pattern of longitudinal stripes of polarized *Shh-Bmp2* expression resulted in the differentiation of the conical epithelium of the feather germ into barb ridges, yielding the primary branched structure of the feather. The differential expression of this signaling module in separate longitudinal domains creates several inherently different patterns of barb ridge morphogenesis (Fig. 3A–E). Two of these, new barb ridge formation and barb ridge fusion (Fig. 3A–B), produced potentially advantageous morphologies that subsequently combined to create the pennaceous feather with a planar vane composed of a rachis and barbs.

Our combination of comparative and experimental investigations strongly support these evolutionary conclusions about integumentary appendage evolution. The experimental data document that the *Shh-Bmp2* signaling module is necessary for appropriate feather germ, barb ridge, and rachis morphogenesis. Thus, the evolution of the novel expression patterns of this module was a necessary and critical component of the evolution of a tubular feather germ and feather branched structure. An evolutionary model of the origin of feathers must incorporate the origin of novel regulatory mechanisms of the expression of the *Shh-Bmp2* module and its effects on controlled cell proliferation and differentiation within the epithelia of the forming integumentary appendage.

Our data and model are consistent with the hypothesis that feathers evolved from scutate scales. The anterior-posterior polarized pattern of *Shh-Bmp2* expression that is shared by all archosaur integumentary appendage rudiments supports their homology (Fig. 2K). However, we emphasize that the feather and archosaurian scale are homologous only at the placode stage. Essentially all feather structures formed after the

placode stage are evolutionary novelties that have no homologs within archosaurian or reptilian scales. Nevertheless, while there is divergence in structure after the placode stage, the developmental module of integrated *Shh* and *Bmp2* signaling used in placode specification of the archosaurian scale has been re-utilized in the formation of derived feather structures. Thus, this signaling modularity provides an inherent homology of developmental process among archosaurian integumentary appendages (Gilbert et al., '96).

The developmental model of the origin of feathers hypothesizes that feathers originated and diversified through a series of evolutionary novelties in developmental mechanisms (Prum, '99). This theory predicts that the most primitive feathers were undifferentiated tubular cylinders (Stage I), which were followed by a downy, basal tuft of barbs (Stage II), and subsequently, by a pennaceous vane with a rachis and barbs (Stage IIIa) (Fig. 7). The predicted morphologies and hierarchical sequence of the stages of the developmental model are entirely congruent with the molecular developmental results presented here. Stages I, II, and IIIa are each associated with an evolutionarily novel pattern of expression of the *Shh-Bmp2* signaling module during development (Fig. 7). Furthermore, the contingency of longitudinal *Shh-Bmp2* expression patterns upon the prior formation of an elongate feather predicts that an undifferentiated tubular feather was evolutionarily primitive to a basally branched, or downy, structure. Likewise, helical growth, rachis formation, and indeterminate barb number are developmentally contingent upon the prior establishment of these longitudinal *Shh-Bmp2* expression patterns. Therefore, our data support the predictions of the developmental model (Prum, '99) that a less organized, tufted, downy feather morphology preceded the origin of the pennaceous feather with a rachis and a planar vane (Fig. 7).

Recent paleontological discoveries have documented that feathers evolved in coelurosaurian theropod dinosaurs before the origin of birds (Chen et al., '98; Ji et al., '98; Xu et al., '99; Ji et al., 2001; Xu et al., 2001; Norell et al., 2002). The morphologies of these primitive, nonavian feathers support predictions of the developmental theory of feather evolution (Prum, '99; Ji et al., 2001; Sues, 2001), and their phylogenetic distributions provide an emerging picture of the early history of feather evolution (Sereno, '99; Padian, 2001; Prum and Brush, 2002). Our data suggest that the evolutionarily novel patterns of *Shh-Bmp2* signaling hypothesized here were derived in the late Jurassic within specific lineages of basal and higher coelurosaurian theropod dinosaurs that were ancestral to birds.

The evidence of the repeated evolutionary utilization of integrated *Shh* and *Bmp2* signaling, reflects an inherent potential of this developmental signaling module to permit controlled morphogenesis within epidermal appendages of archosaurs. Thus, the re-utilization of this signaling module would facilitate the evolution of the phenotypic diversity of epidermal structures in amniotes. Our data support the conclusion that qualitatively distinct morphological evolutionary novelties can originate from the expression of plesiomorphic molecular signaling systems in a novel context. Further, we present a mechanistic scenario for how the redeployment of an integrated signaling module can foster the evolution of novel morphological structures.

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