Sonic Hedgehog functions by localizing the region of proliferation in early developing feather buds

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Abstract

Feathers are formed following a series of reciprocal signals between the epithelium and the mesenchyme. Initially, the formation of a dense dermis leads to the induction of a placode in the overlying ectoderm. The ectoderm subsequently signals back to the dermis to promote cell division. Sonic Hedgehog (Shh) is a secreted protein expressed in the ectoderm that has previously been implicated in mitogenic and morphogenetic processes throughout feather bud development. We therefore interfered with Shh signaling during early feather bud development and observed a dramatic change in feather form and prominence. Surprisingly, outgrowth did occur and was manifest as irregular, fused, and ectopic feather domains at both molecular and morphological levels. Experiments with Di-I and BrdU indicated that this effect was at least in part caused by the dispersal of previously aggregated proliferating dermal cells. We propose that Shh maintains bud development by localizing the dermal feather progenitors.

Keywords: Feather bud; Shh; Cyclopamine; BMP; Proliferation

Introduction

Feathers, like hair, teeth, scales, taste buds, and mammary glands, are a product of ectodermal organogenesis. In keeping with their homologous origin, these diverse structures all share a common mechanism of development, in that they all rely upon a series of inductive and reciprocal signals between the epithelium (epidermis) and the mesenchyme (dermis). Feathers form in tracts or rows known as pteric regions (Sengel and Mauger, 1976) and are initiated to develop by signals originating from the dermis, which result in a morphologically distinct ectodermal thickening termed the epidermal placode. The placode then signals to the dermis to induce a dermal condensation, which proliferates to form a short, radially symmetrical bud. Reciprocal signaling between epidermis and dermis leads to coordinated proliferation and feather bud outgrowth (Chuong, 1993). Utilizing a feather reconstitution assay, Jiang et al. (1999) observed that rows of evenly spaced dermal condensations began to form simultaneously and develop into feather buds, indicating that dermal cells have an intrinsic ability to reform or self-organize into periodic patterns. Furthermore, it was demonstrated that this ability was dependent upon cell density, that once a critical mass of cells was achieved, dermal condensations formed and feather development progressed.

Sonic Hedgehog (Shh), a member of the highly conserved Hedgehog (Hh) family of secreted proteins, has been implicated in all stages of feather bud development from bud induction to the topological shaping of the feather filament and thus determination of feather type (Harris et al., 2002; Yu et al., 2002). Many of the components of Shh signaling have been previously identified. Shh binding to the Patched (Ptc) receptor, which was demonstrated by Taipale et al. (2002) to catalytically inhibit the seven pass transmembrane protein Smoothened (Smo). Binding of Shh to Ptc alleviates this inhibition resulting in the initiation of an intracellular signaling cascade leading to gene transcription. Shh signaling is therefore mediated via Smo, one particular target of which is Ptc; Ptc is upregulated in the presence of Shh signaling and is thought to sequester Shh and thus limit...
its diffusion and range of activity. Inappropriate activation of Shh signaling, either by overexpression of Shh or mutations that either constitutively activate Smo or inactivate Ptc, is associated with some tumor types (Hahn et al., 1996; Johnson et al., 1996; Oro et al., 1997; Xie et al., 1998).

Shh has been described as an activator of feather bud development, in that forced expression either by bead-mediated protein delivery or RCAS-mediated overexpression can result in both ectopic and enlarged feather buds (Jung et al., 1998; Ting-Berreth and Choung, 1996a,b). However, Morgan et al. (1998) demonstrated that at stages before dense dermis formation, forced Shh expression results in disorganized ectodermal growths, whereas forced expression at slightly later stages induces distinct ectopic bud growth, indicating that there are precise stage-specific effects of Shh signaling. Significantly, Shh expression is not natively expressed until after initial differentiation of the epidermal placode, indicating that the role of Shh in feather development must be post-placode formation. Indeed, several authors have proposed that the presence of Shh in the epidermis together with the detection of Ptc in the dermis, after the formation of the dermal condensation, suggests that Shh promotes proliferation in this region to control bud outgrowth (Jung et al., 1998; Morgan et al., 1998).

This interpretation of the role of Shh in feather development would appear to be consistent with the majority of reports regarding the role of Shh in other ectodermal organs, in particular, hair follicle development. Shh mutant mice appear to develop normally spaced hair placodes with associated dermal condensations, but then fail to go through subsequent stages of morphogenesis, with a marked reduction of proliferating cells in the hair follicle (Chiang et al., 1999; St.-Jacques et al., 1998). This observation was reiterated by Nanba et al. (2003), who inhibited Shh signaling in cultures of mouse skin and blocked follicular growth, again suggesting that Shh is required for cellular proliferation and the growth of both epidermis and dermis. Interestingly, Mill et al. (2003) demonstrated that the early defects seen in hair follicles of the Gli2 mutant mouse (Gli2 is a downstream effector of Shh signal transduction) were largely rescued by the restoration of Gli2, and hence Shh signaling, in the epidermis, suggesting that effects of Shh upon the dermis are indirect. Additionally, in vitro cell culture experiments have demonstrated that Shh can induce DNA replication and oppose cell cycle arrest (Fan and Khavari, 1999; Duman-Scheel et al., 2002).

However, certain lines of evidence suggest alternative functional significance of Shh signaling. Taste papillae are another ectodermal organ known to express Shh (Jung et al., 1999), and recently, Mistretta et al. (2003) demonstrated that the inhibition of Shh signaling in embryonic rat tongue cultures resulted in increased numbers of fungiform papillae. The authors state that there is the impression of proliferation and increased papillae size in the absence of Shh signaling. According to previous data, such phenotypes would be predicted only as a response to increased levels of Shh and are thus directly in contrast to the predicted effect of inhibiting Shh signaling. This could imply that Shh is a negative regulator of differentiation and growth of ectodermal organs, or could point simply to an organ specific difference in Shh function. Interestingly, St.-Jacques et al. (1998) report hyperplasia in the interfollicular epidermis of Shh mutant mice, a phenotype usually associated with tumor formation and inappropriate Shh pathway activation.

In light of the apparently contradictory reports on the function of Shh in ectodermal organogenesis, combined with the data regarding the potentially stage-specific nature of Shh signaling, we endeavored to assess the role of Shh in early feather bud development immediately post-placode formation. In this study, we provide evidence that Shh signaling is required for maintaining the distinct form of feather buds at both a molecular and morphological level. Shh may in part achieve this by constraining the domain in which proliferating dermal cells can reside.

Materials and methods

Explant culture of chick skin

Explant cultures were performed in a manner similar to that described by Jung et al. (1998). Briefly, dorsal skin from stage HH28-33 White Leghorn chicken embryos (staged according to Hamburger and Hamilton, 1951) was dissected in PBS and pinned dorsal side up onto nitrocellulose membranes (Millipore). Membranes were then placed on a solid 1% agarose in Dulbecco’s modified Eagle’s medium (DMEM-Gibco/BRL) base, which was supplemented with growth media-DMEM containing 10% fetal calf serum and 2% chick serum (Gibco/BRL). Explants were grown at the air–media interface at 37°C in an incubator containing 100% humidity and an atmosphere of 95% air/5% CO₂.

Application of cyclopamine to chick skin explant cultures was achieved by supplementation of the growth media. Cyclopamine (Toronto Research Chemicals) was dissolved in DMSO and added to a final concentration of 12.5 μM. This concentration was optimized in preliminary studies within the laboratory and is in line with that used by Mistretta et al. (2003) to disrupt papillae pattern in organ cultures of rat tongue. Control cultures were supplemented with an equivalent volume of DMSO. BrdU (Sigma) was added to the growth media to a final concentration of 150 μg/ml for 30 min before fixation.

Immunohistochemical detection of proliferation

BrdU-treated samples were fixed overnight in 4% paraformaldehyde in PBS. After washing in PBS, the samples were bleached in 1% H₂O₂ in PBS for 30 min and subsequently treated with 2 N HCl for 30 min. Samples
were then neutralized in 0.1 M Na$_2$B$_4$O$_7$ for 15 min, washed in PBS, and blocked for 2 h in 10% goat serum (Gibco/ BRL) in PBS. Monoclonal anti-BrdU antibody (Roche) was added at 1:100 overnight at 4°C. The following day after washing in PBS, the samples were incubated in a 2°C goat-anti-mouse-biotin antibody (Dako) in 10% goat serum in PBS for 1 h. After washing in PBS, samples were developed using an ABC-HRP kit according to manufacturers protocols (Vector Laboratories).

Quantification of proliferation

After processing for immunohistochemical detection of BrdU, samples were embedded in paraffin, sectioned transversely and sagittally, and stained with hematoxylin according to standard histological procedures. Sections were viewed under a Leitz DMRB microscope, images captured on a Leica DC500 digital camera, and transferred into the QWIN Leica IM500 image manager. Beginning with control samples, bud and immediately adjacent interbud regions were defined and retained as a constant size throughout quantification. For cyclopamine-treated samples, these size parameters were maintained with the bud region defined as the predicted original site of a morphologically distinct bud, and the interbud region defined as that directly adjacent. The sites for bud location are easily predicted due to the regular manner in which each new feather bud appears morphologically relative to its neighbors. Furthermore, this method for analysis was used as although follicular patterns of gene expression were disrupted by cyclopamine treatment; there remained discrete regions with interfollicular morphology that also qualitatively appeared to have differing levels of BrdU-positive cells as compared to controls. Two independent observers counted the number of BrdU-positive nuclei within those defined regions. At least 30 different bud and interbud regions were scored for both control and cyclopamine-treated samples. Initial analysis revealed that no significant differences were observed in the epidermal compartments of control versus cyclopamine-treated samples (data not shown); therefore, although the numbers represent total BrdU-positive cells, any differences observed can be attributed to differences in the dermal compartment.

Data was entered into the SPSS for Windows statistical package, tested for equality of variance, and the means compared by independent samples t test.

Labeling of feather bud dermal cells with Di-I

Cultures were prepared as normal and individual buds injected via glass capillaries with 1–5 nl of 0.1% Di-I in 70% DMSO (Molecular Probes) using a PM1000 Cell Microinjector (MicroData Instruments Inc) and an Eclipse Micromanipulator. Stage HH30 and HH31 cultures were injected at time 0 and allowed to develop for a further 24 h before image capture. Stage HH33 cultures were allowed to develop for 48 h, injected with Di-I, and then allowed to develop for a further 24 h. Images were visualized using an Olympus BH-2 fluorescence microscope and captured on a Nikon 8600 digital camera.

Electron microscopy

Chick skin explants were rinsed in PBS and fixed overnight in 1% glutaraldehyde in PBS. Tissues were then rinsed in phosphate buffer and post-fixed in 1% OsO$_4$ for 1 h. Samples were then dehydrated through ascending alcohol series, with displacement of alcohol accomplished by three changes of acetone. Samples were then CO$_2$ critical point dried, mounted on specimen stubs, and gold sputter coated. Scanning electron micrographs were scanned as digital images and assembled in Adobe Photoshop.

Whole-mount in situ hybridization

Samples were washed in PBS and fixed overnight in 4% paraformaldehyde in PBS containing 0.1% Triton. Antisense RNA probes were labeled with digoxigenin and whole-mount in situ hybridization (ISH) was performed as described by Nieto et al. (1996). The following probes were used in this study: Shh full-length clone was a gift from Dr. Jane Dodd; Patched full-length clone was a gift from Dr. C Tabin; BMP-2, PCR-cloned fragment (nucleotides 1–797), and BMP-4, PCR-cloned fragment (nucleotides 1–953), were both gifts from Dr. A Graham. All samples, except those processed for electron microscopy, were subsequently photographed on a Zeiss SMZ1500 light microscope using a Nikon 8600 digital camera; plates were assembled in Adobe Photoshop.

Results

Ectodermal organogenesis results from sequential and reciprocal interactions between the epithelium and mesenchyme. Shh has previously been attributed some roles during feather development, in particular, during maturation of the buds and their subsequent topological transformation into distinct feather forms.

Inhibition of Shh signaling results in abnormal feather bud development

To analyze the potential significance of Shh signaling in the development of feather buds, we took advantage of a skin explant culture system whereby dorsal epidermis and dermis is grown on a nitrocellulose membrane, and follows exactly the same pattern of feather development as seen in ovo (Jung et al., 1998). Significantly, this system allows us to introduce compounds directly to the media supplementing the culture. We introduced 12.5 μM cyclopamine, a plant steroidal alkaloid that inhibits Shh signaling by antagonizing Smo signal transduction (Chen et al., 2002; Taipale...
et al., 2000) to skin cultures from stage HH28, 30, 31, and 33 with. We then examined these cultures by whole-mount in situ hybridization (ISH) for the presence of Ptc and Shh transcripts. As a transcriptional target of Shh signaling, Ptc expression was used to indicate the efficacy of cyclopamine treatment, as any reduction in Shh signaling must be accompanied by a concurrent reduction in Ptc expression. Shh expression was assayed as a marker of feather development as previous data have indicated that Shh does not regulate its own expression and remains present in ectodermal organs in the presence of cyclopamine (Mistretta et al., 2003).

Treatment of skin cultures with 12.5 μM cyclopamine resulted in dramatic abnormalities of feather development at all stages assayed (Fig. 1). At stage HH30, the efficacy of the cyclopamine treatment was apparent within 8 h, whereby the presence of Ptc transcripts was greatly reduced as compared to controls (Fig. 1, compare A–B, n ≥ 30). This was further enhanced at 16 h (Figs. 1E–F, n ≥ 30), with expression almost entirely abolished at 24 (Figs. 1I–J, n ≥ 30) and 48 h (Figs. 1M–N, n = 12). It was noticeable that what little transcript could be detected appeared more diffuse than the control cultures. This was especially prevalent in the samples incubated with cyclopamine for 48 h where very faint levels of Ptc transcript could be detected in what appeared to be stripes of expression. Localization of Shh transcripts revealed the extent of feather bud abnormalities. At 8 h, little effect could be observed (Fig. 1, compare C–D, n ≥ 30); however, by 16 h, there appeared to be a generalized enlargement or spreading of the Shh expression domain in the cyclopamine-treated cultures as compared to the controls (Figs. 1G–H, n ≥ 30). At 24 and 48 h, this effect was dramatically enhanced with both a massive expansion of the Shh domain in individual buds and also apparent fusions between buds, resulting in stripes of expression (Figs. 1K–L, O–P, n ≥ 30).

We also assessed the effects of Shh signaling inhibition on older feather buds, at stage HH31 and HH33 (Fig. 2). At stage HH31, we observed similar effects to those seen a stage earlier. The presence of Ptc transcripts was

![Fig. 1. Cyclopamine causes severe abnormalities in feather development.](image-url)
abolished by 24 h in the cyclopamine-treated cultures as compared to the controls (Fig. 2, compare A–B, n = 22) and remained absent at 48 h (Figs. 2E–F, n = 12), whereas the expression of Shh again showed dramatic abnormalities. The expression domain of Shh in individual buds was expanded and irregular, and not restricted to the posterior domain but throughout the bud entirety, plus the presence of stripes of expression, presumably due to the fusion of several buds, was again detected (Figs. 2C–D and G–H, n = 22). At stage HH33, the pattern of abnormality was somewhat different compared to earlier stages. At this point, the feather buds are cylindrical and are in an advanced state of development as they grow in a posterior direction. The expression of Ptc was significantly reduced at both 24 (Fig. 2, compare I–J, n = 12) and 48 h (Figs. 2M–N, n = 12) in the cyclopamine cultures as compared to the controls; however, at 24 h, little effect could be observed between the conditions concerning the presence of Shh transcripts (Figs. 2K–L, n = 12). At 48 h, patches of Shh expression appeared in apteric regions of the skin cultures treated with cyclopamine (Figs. 2O–P, n = 12). These domains of Shh expression began to appear adjacent to the base of the feather buds and seemingly represented distinct ectopic sites of expression rather than fusions or expansions of existing domains.

Molecular markers of the feather program are maintained in the absence of Shh signaling

In light of the somewhat surprising observation that loss of Shh signaling appeared to lead to an expansion or fusion of the feather domains, we asked whether this was the case for other genes known to play a role in feather development (Fig. 3). BMP2 and BMP4 have been previously ascribed roles at both placode induction and topological transformation stages of feather development (Jung et al., 1998;
Noramly and Morgan, 1998). After 8 h in culture, little effect was observed on the expression pattern of either BMP2 (Fig. 3, compare A–B, n = 10) or BMP4 (Figs. 3C–D, n = 10) in the cyclopamine-treated samples as compared to the controls. However, at 24 h, dramatic effects were observed in the location of transcripts for both BMP2 (Figs. 3E–F, n = 10) and BMP4 (Figs. 3G–H, n = 10). As with the expression of Shh, in samples treated with cyclopamine, both BMP genes displayed irregular shaped and enlarged domains of expression in individual bud regions and also mimicked the expression pattern of Shh concerning the detection of transcripts in stripes of fused buds. Additionally, they were no longer restricted to the anterior domain but were expressed throughout the entirety of the abnormal buds. This molecular abnormality was also observed with stage HH33 skin explants cultured for 48 h in the presence of cyclopamine. BMP2 expression is detected in apteric regions lateral to the base of the cylindrical bud (Fig. 3I, n = 8), whereas BMP4 would appear to be expressed in an almost continuous stripe from medial to lateral (Fig. 3J, n = 8). In addition, we examined the expression of some other molecular markers of developing feathers at both HH31 and HH33 including Wnt3a, Wnt5a, Wnt7a, Msx1, and Follistatin (n = 6 in all cases), all of which continued to be expressed but showed abnormal patterns of expression, manifest as a loss of spatial restriction, expansion, and fusion of domains (I.M. and K.P., unpublished data).

Molecular changes observed in the absence of Shh signaling are reflected morphologically

We asked if equally dramatic morphological effects accompanied the expansion of molecular markers we observed. Examination of samples by electron microscopy revealed that the morphology of the feather buds was indeed affected by the presence of cyclopamine (Fig. 4). At stage HH30 the normal progression of development from 8 to 48 h consisted of small radially symmetrical buds becoming increasingly prominent and well defined, which at 48 h have a marked proximal–distal axis and are already showing growth biased toward the posterior (Figs. 4A–D, n = 4). In cyclopamine-treated cultures, little difference could be detected at 8 h (Fig. 4E); however, by 16 h, the buds already appeared less prominent and distinct and were displaying a less regular form (Fig. 4F, n = 4). By 24 h, this phenotype was significantly enhanced with very few distinct buds present; however, ridges of multiple fused
Buds were clearly evident (Fig. 4G, \( n = 4 \)). This effect was maintained at 48 h, at which point it was markedly clear that while buds could be enlarged in the anterior–posterior, medial–lateral axis, the prominence of both buds and ridges in the proximal–distal axis was significantly reduced as compared to control cultures (Fig. 4H, \( n = 4 \)). At stage HH31, cultures treated with cyclopamine morphologically mimicked the effect observed at stage HH30, with very few distinct buds detected as compared to controls (Fig. 4I–K, \( n = 4 \)). By 48 h, many ridges or stripes of fused buds were evident plus the difference in bud or ridge proximal–distal prominence between cyclopamine versus control cultures was greatly exaggerated (Figs. 4J–L, \( n = 4 \)). Stage HH33 cultures did not show any gross effects when examined at a molecular level after 24 h in culture; however, closer examination showed that while the cylindrical form and proximal–distal prominence of the cyclopamine cultures appeared normal, swellings were present around the base of each bud (Fig. 4O, \( n = 4 \)). After 48 h in culture in the presence of cyclopamine, a major morphological abnormal-
ity could be observed whereby the swellings appeared to develop into distinct, prominent ectopic buds situated in apertic regions (Fig. 4P, n = 4).

Molecular and morphological abnormalities observed in the absence of Shh signaling are reversible

We asked if once established, were these expanded or ectopic signaling domains autonomous and capable of fulfilling the feather program of development in the renewed presence of Shh signaling, or would they fail to sustain the feather program and instead regress? To address this question, we cultured dorsal skin explants in the presence of 12.5 μM cyclopamine for 24 (stages HH30, 31) or 48 h (stage HH33) before bisecting the explant along the dorsal midline. One-half was fixed for subsequent processing, and the other half was placed back into control culture conditions for a further 24 h (stages HH30, 31, 33) before fixation. Both halves of each culture were then processed either by ISH or electron microscopy. At each developmental stage examined, the withdrawal of cyclopamine resulted in a loss or regression of previously expanded, fused, or ectopic feather domains (Fig. 5). By comparing the expression pattern of Shh in two halves of one culture, there is a striking difference. The half fixed at 24 h showed the same effect as observed in Fig. 2D, whereby the expression domains are expanded, of irregular form, and fused (Fig. 5A, n = 12); however, the half that was placed back into culture for 18 h displayed a relatively normal pattern, with expression of Shh restricted to discrete domains (Fig. 5B, n = 12). Very faint levels of expression can be detected in expanded or fused locations, but it is clear that a near complete reversal of the cyclopamine effect has occurred. Accordingly, analysis by electron microscopy confirmed that the molecular observations were reflected morphologically. The most extreme morphological effect we had detected with cyclopamine was the presence of distinct, prominent, ectopic buds in stage HH33 cultures maintained for 48 h (Fig. 5D, n = 4). After being placed back into control culture conditions for only 24 h, these ectopic buds had significantly regressed (Fig. 5E, n = 4) and resembled more the swellings seen in Fig. 4O after HH33 explants had been cultured with cyclopamine for 24 h.

Proliferation activity is not restricted to discrete bud regions in the absence of Shh signaling

The observation that inhibiting Shh signaling leads to expanded, fused, and ectopic bud domains led us to investigate whether this resulted from altered or ectopic patterns of proliferation or cell movement. To this end, we incubated stage HH31 dorsal skin explants in either control or 12.5 μM cyclopamine supplemented media for 24 h, and pulsed each sample with BrdU, which was used immunohistochemically as a marker of proliferating cells (Fig. 6). Control cultures contained BrdU-positive nuclei throughout the feather bud with an increased intensity in the posterior region; very few positive nuclei could be detected in interbud regions (Figs. 6A and C, n = 8). A clear disruption was evident in the pattern of proliferation observed in the presence of cyclopamine. Positive nuclei were no longer detected just in discrete feather bud

Fig. 5. Molecular and morphological effects caused by cyclopamine are reversible. Stage HH31: the half culture treated only with cyclopamine (A) showed the expected expansions (arrowhead) and fusions (arrows) of Shh expression. The half placed back into control media showed an expression pattern comparable to control cultures with expression restricted to discrete feather buds (B, arrowheads. Arrow indicates very faint residual ectopic expression of Shh). The reversal effect is most clearly seen in a side-by-side comparison of both halves (C). Stage HH33: the half culture treated with cyclopamine for 48 h shows distinct and prominent ectopic buds (D, arrows). The half placed back into control media for only 18 h showed a considerable regression in prominence of the ectopic buds (E, arrows) (scale bar = 100 μm).
domains but instead appeared to recapitulate the molecular and morphological patterns described earlier, in that the domains of proliferating cells were expanded and fused as compared to controls. In addition, many BrdU-positive nuclei could be detected throughout what natively would represent interbud regions but were now displaying morphological abnormalities, and in regions not displaying morphological abnormalities (Figs. 6B and D, \( n = 8 \)). To further dissect this phenomenon, we sectioned explants and quantified the number of BrdU-positive nuclei in both bud and interbud regions. Observation of tissue sections stained with hematoxylin post-BrdU processing revealed a generalized decreased cell density and proximal–distal prominence in bud regions of the cyclopamine samples as compared to controls. Conversely, the interbud region of the cyclopamine samples appeared thicker than that of the control samples (Figs. 6E and F, \( n \geq 30 \)). Statistical analysis revealed a complex pattern of effects upon proliferation caused by cyclopamine within the dermis of skin explants. Initially, it was observed that both the overall level of proliferation (Fig. 6G) and the level of proliferation within bud domains (Fig. 6H) were significantly
reduced in the presence of cyclopamine. However, the number of BrdU-positive nuclei in interbud regions was significantly increased in those samples treated with cyclopamine (Fig. 6I). Furthermore, although the level of proliferation in bud regions was significantly higher than interbud regions in both control (Fig. 6J) and cyclopamine (Fig. 6K) cultures, the difference between bud and interbud regions was also significantly lower in those cultures treated with cyclopamine.

To assess cell movement, we labeled the dermal cells of pairs of adjacent feather buds (stage HH30 and HH31), with the lipophilic dye Di-I, used in living tissue to mark and subsequently trace cells. Dermal cells in stage HH30 control cultures appeared to remain tightly confined within the feather buds 24 h after labeling (Figs. 7A and B, n = 12/12). However, dermal cells from stage HH30 cultures treated with cyclopamine appeared to show a generalized spreading away from their initial location giving the appearance of a loss of discrete form (Figs. 7C and D, n = 10/12). This effect was observed even more dramatically with stage HH31 cultures whereby labeled dermal cells from separate feather buds could be seen intermingling, representing the fusions of buds seen morphologically (Figs. 7E and F, n = 6/12). In HH31 cultures, cell migration was observed to occur in all directions, but most commonly toward the posterior–lateral aspect. Stage HH33 dorsal skin was cultured for 48 h in the presence of cyclopamine, at which point, ectopic and existing buds were labeled with Di-I before the cultures were placed back into control conditions for a further 24 h. In all cases, the ectopic bud regressed morphologically after a further 24 h culture post-labeling, and the cells of the ectopic buds did not remain tightly confined but could be observed to disperse in nonuniform directions, similar to that seen in the stage HH30 cultures. Interestingly, in 50% of cases, a significant proportion of cells were observed to be moving toward and fusing with the existing native bud location (Figs. 7G and H, n = 4/8).

Discussion

In this study, we describe the role of Shh during early development of feather buds. Inhibition of Shh signaling in early-middle stages of bud development resulted in a decrease in proliferation and the dispersal of the dermal condensation, with a concurrent expansion of placodal and dermal condensation gene expression. In later stage of buds, the effect is more modest but results in the initiation of ectopic buds in the interbud space.

Inhibition of Shh signaling and abnormal molecular feather bud development

We used the steroidal alkaloid cyclopamine as an inhibitor of Shh signaling (Chen et al., 2002; Taipale et al., 2002). The almost complete ablation of Ptc transcripts at all times and stages indicated to us that Shh signaling was severely or completely inhibited. The majority of previously described data implies a role for Shh of stimulating feather growth by proliferation in early feather development; in addition, Shh mutant mice develop hair placodes that maintain the correct spatiotemporal molecular profile, but which fail to grow (St.-Jacques et al., 1998). The prediction therefore would be that in the absence of Shh signaling, the feather buds would be small, would have a normal discrete bud form, and would maintain anterior–posterior molecular polarity.

It was somewhat surprising then (and potentially may indicate a fundamental difference in the biology of feather and hair development) that contrary to the observations in the Shh mutant mice, cultures treated with cyclopamine...
showed dramatic spatial abnormalities of gene expression. All genes examined at stages HH30-31 had expanded or fused expression domains often forming into long stripes of expression. Furthermore, the extreme nature of the molecular change was reflected by extreme morphological change. Significantly, in normal bud development, BMP2 and BMP4, along with Msx1, become restricted to the anterior region of the bud. Shh is expressed in the central domain of the epidermis of feather buds at the placode stage before becoming restricted to the posterior domain together with Wnt7a, Wnt5a, and Follistatin (Harris et al., 2002; Morgan et al., 1998; Noveen et al., 1995; Patel et al., 1999; Ting-Berreth and Chuong, 1996a,b; Widelitz et al., 1999; I.M. and K.P., unpublished data). In all cases, the spatial restriction of expression was abolished with transcripts for each gene detected throughout all domains of the feather buds, suggesting that anterior–posterior polarity of gene expression was lost. Older cultures of stage HH33 skin displayed distinct ectopic buds with discrete dermal condensations. Close examination of marker gene expression in these ectopic structures revealed that anterior–posterior polarity was also absent with transcripts detected throughout the entirety of the bud (BMP2, BMP4, Msx1, Follistatin—data not shown).

Taken together, these data suggest that Shh signaling is not required to maintain the gross molecular feather program, as all markers of feather development examined (with the obvious exception of Ptc) remain present in the absence of Shh signaling. Furthermore, the observation that the epidermal (Shh, BMP2) and dermal (BMP4) compartments were exhibiting spatial abnormalities concurrently suggests that feather development is also functioning correctly concerning reciprocal interactions and induction. In addition, these data concur with two previously described observations in mouse. Firstly, that Shh is not required for the initiation of feather buds, as both placodes and dermal condensations form and express feather-specific markers in the lateral regions of stage HH30 explants (at the start of the culture period, these explants had three rows of buds at the midline) cultured for 24–48 h in the presence of cyclopamine (as seen in Fig. 1), and in stage HH28 explants cultured in the presence of cyclopamine for 36 h (data not shown). Secondly, that Shh is not responsible for the maintenance of its own expression (Nanba et al., 2003; St.-Jacques et al., 1998). However, the loss of region-restricted transcripts at all stages examined would suggest that Shh does play some role in the induction or maintenance of polarity of gene expression.

BMPs are believed to be responsible for the spacing of the feather pattern by mediating a lateral inhibition mechanism (Noramly and Morgan, 1998). It may seem surprising that a protein ascribed this function would be expressed so strongly in buds that have expanded and fused laterally into interbud regions. Alternatively, the fact that the BMP is detected only in expanded or fused buds, and not in the morphologically interbud regions, may suggest that BMP is still mediating a lateral inhibition signal, in a bud versus interbud mechanism, but that in the absence of Shh signaling, the mechanism is overridden, suppressed, or secondary to another mechanism caused by our manipulation. Our data do not directly address this issue and so can neither refute nor confirm any of these possibilities.

In the absence of Shh signaling, cell proliferation and migration are affected

Significantly, invariably, the phenotypic buds were smaller than those in the control cultures in the proximal–distal axis; thus, while feather bud domains may have been greatly expanded, they were less prominent. This raised a question regarding cell number, whether the same number of “feather-forming cells” were present but had simply spread further. In line with previous data (St.-Jacques et al., 1998), we also detected a significant reduction (40–50%) in the number of proliferating cells in bud regions of skin explants cultured with cyclopamine, in keeping with our observations that the expanded domains were less prominent. Furthermore, the significant increase (40–50%) of proliferating cells in interbud regions in those same samples would appear to explain the increased thickness of adjacent interbud regions. Interestingly, the total reduction of BrdU-positive cell number in the presence of cyclopamine, although significant, was only 20%. It is tempting to ascribe this increase in interbud proliferation to a failure of lateral inhibition; however, by labeling dermal cells with Di-I, we were able to ascertain that this altered level of bud–interbud proliferation and general pattern of cell proliferation may at least in part be due to the migration of dermal cells away from the original bud position. Intriguingly, while the direction of migration was never consistent or uniform, it appeared to occur most commonly in a lateral direction. Furthermore, in stage HH33 cultures, we observed a proportion of cells that once were resident in the distinct ectopic domains migrating back toward the original bud.

Feather bud development in the absence of Shh signaling

Taken together, these observations would appear to indicate that Shh certainly maintains molecular polarity of feather buds and induces or maintains a level of proliferation necessary for normal feather development. The migration of cells away from bud locations may suggest this is the result of a breakdown in the integrity of the dermal condensation. It is tempting to speculate therefore that Shh induces altered cell–cell adhesion in feather buds; however, analysis of such an effect is beyond the scope of this study. If over-expression of Shh results either in disorganized growth or ectopic buds (Jung et al., 1998; Morgan et al., 1998; Ting-Berreth and Chuong, 1996a,b), why does inhibition of Shh achieve the same effect, as it would appear that manipu-
loration of Shh signaling from contrary ends of the spectrum appears to result in the same phenotype?

While Shh certainly appears to localize and maintain the integrity of follicle development, as evidenced by the migration of dermal cells, we tentatively suggest these questions may be explained by a primary effect on cell proliferation rather than adhesion. Since Shh is locally expressed, its effects are normally restricted; hence, cells would aggregate due to secondary effects on cell adhesion caused by the polarizing and proliferative action of Shh. In the absence of Shh, polarization would be lost and more cell migration. This would result in areas of increased density leading to the formation of large, malformed dermal condensates. A further possibility is that dispersal of dermal condensations is the secondary effect of changes in the distribution of inductive or instructive signals in the epidermis as a result of changes in Shh signaling. Indeed, the data clearly show redistribution in the expression of growth factors capable of affecting proliferation, in both epidermis and dermis. In addition, as mentioned previously, the fact that restoration of Shh signal transduction components in the epidermis rescues hair follicle phenotype (Mill et al., 2003) would imply that the effects we observe in the dermis by inhibiting Shh activity are likely to be indirect. Finally, we cannot dismiss the possibility that the difference observed in the phenotypes between chick and mouse is due to fundamental differences in their biology, or to an undefined effect of cyclopamine that extends beyond its inhibition of Shh activity.

Conclusion

Previous data had implicated Shh as having numerous roles in feather development, from actively promoting proliferation post-placode formation, to the later morphological transformations involved in determining feather type. We examined the role of Shh post-placode formation by knocking out Smo signal transduction with cyclopamine. We have demonstrated that Shh is responsible for maintaining the growth of feather buds at least in part by localizing and containing the area in which the cells of the dermal condensation reside.

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References


