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Expression in the Epidermis

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Sonic hedgehog (Shh) is expressed in the ectoderm of the forming hair follicle and feather bud during normal development. However, inappropriate activation of the Shh signal transduction cascade in human epidermis can cause basal cell carcinoma. Here we show that during normal development of avian skin, Shh is first expressed only after the responsiveness to this protein has been suppressed in most of the surrounding ectodermal cells. Forced expression of Shh in avian skin prior to this time causes a disorganized ectodermal proliferation. However, as skin begins to differentiate, the forced expression of Shh causes feather bud formation. Subsequently, expression of Shh in interfollicular epidermis has little or no morphological effect. Restricted responsiveness to Shh in developing skin has functional consequences for morphogenesis and may have important implications for cutaneous pathologies as well.

Key Words: **feather bud; pattern formation; Sonic hedgehog; basal cell carcinoma.**

INTRODUCTION

Sonic hedgehog (Shh) is an intercellular signaling molecule which plays an important role in organizing the formation of many structures in the vertebrate embryo including the neural tube, somite, limb, lung, and gut (reviewed in Johnson and Tabin, 1996; Apelqvist *et al.*, 1997; Hammerschmidt *et al.*, 1997). In at least some of these structures, Shh is thought to act as an instructive morphogen, eliciting different cell fates based on the level of Shh signal received (Roelink *et al.*, 1995; Marti *et al.*, 1995; Ericson *et al.*, 1996; Yang *et al.*, 1997). Changing responses of progenitor populations to the Shh signal may also contribute to the complexity of cell fates that may be elicited by Shh (Ericson *et al.*, 1996; Nelson *et al.*, 1996).

The mechanism by which Hh induces responses in receiving cells has been partially dissected in *Drosophila*. Hh signal is transduced by a complex of the transmembrane proteins patched and smoothened (Alcedo *et al.*, 1996; van den Heuvel and Ingham, 1996). In genetic terms, ptc represses smoothened. Shh binds to and represses ptc thereby de-repressing smoothened and leading to responses within the cell (Marigo *et al.*, 1996; Stone *et al.*, 1996). This results in the activation of the zinc finger transcription factor cubitus interuptus (Ci) and increased transcription of hedgehog-responsive genes (reviewed in Kalderon, 1997). The vertebrate homologues of ptc, smoothened, and hedgehog have been cloned and share these names, while the Gli genes have been identified as the vertebrate homologues of Ci (Orenic *et al.*, 1990; Hui *et al.*, 1994). Although there are at least three Gli genes in vertebrates, the Gli-1 protein appears to mediate transcriptional responses to Shh, while Gli-3 may act to oppose Gli-1 (Ruppert *et al.*, 1988; Hui *et al.*, 1994; Lee *et al.*, 1997; Hynes *et al.*, 1997; reviewed in Ruiz i Altaba, 1997).

Shh is expressed in the hair follicles of mammals and the feather buds of birds and is thought to play a role in the organization and growth of these structures. Forced expression of Shh in the skin can lead to alterations in feather bud development which have been interpreted as evidence that Shh promotes dermal condensation and organizes the oriented outgrowth of the bud (Ting-Berreth and Chuong, 1996).

However, aberrant activity of the Shh signaling pathway in skin can have deleterious consequences. The identifica-

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tion of inactivating mutations in the ptc gene as the cause of basal cell Nevus (Gorlin) syndrome demonstrated that altered activity of this pathway results in a predisposition to basal cell carcinoma (BCC) (Hahn *et al.,* 1996; Johnson *et al.,* 1996). Inactivation of the patched gene is also observed in the majority of sporadic BCCs (Johnson *et al.,* 1996; Galiani *et al.,* 1996; Wolter *et al.,* 1997). The antagonistic relationships between Shh and ptc, and ptc and smoothened predict that inactivating mutations of patched and gain of function mutations of Shh, smoothened, or Gli-1 will have similar consequences, although the Shh mutation would not be cell autonomous. Consistent with this model, activating mutations in smoothened have been found in sporadic BCCs (Xie *et al.,* 1998), and elevated Gli-1 expression was observed in a majority of BCCs as well (Dahmane *et al.,* 1997). While these mutations directly affect the responding cell, a potential gain of function point mutation in the Shh gene was found in a human BCC, although its role in tumor formation has not yet been confirmed (Oro *et al.,* 1996).

To study the role of Shh in skin development during embryogenesis, we have focused on the skin of the avian embryo which is more readily accessible to study than that of mammals. Heterospecific recombinations between ectoderm and dermis of avian and mammalian skin have demonstrated that the signals mediating the inductive interactions between these tissue layers to initiate the formation of a feather bud or hair follicle are conserved across classes (Garber and Moscone, 1964, 1968; Dhouailly 1973, 1975). Gene expression during feather bud and hair follicle formation is also generally similar. We have examined the expression of Shh during normal development of the skin and show that Shh is expressed in the ectodermal placode of the feather bud only after initial differentiation of this structure has restricted responsiveness to Shh in the ectoderm.

The accessibility of the avian embryo facilitates targeted misexpression of genes using retroviral vectors. We have employed this approach to express Shh in the ectoderm in restricted foci at successive developmental stages starting prior to its normal onset of expression in the skin. We find that forced expression of Shh at a level similar to that observed during normal development has profoundly different effects at different times in embryogenesis. At early stages while the skin is still a simple epithelium without an underlying dense dermis, forced expression of Shh can lead to formation of large disorganized ectodermal growths. Precocious expression of Shh in the ectoderm at slightly later stages can induce ectopic feather buds but not these disorganized growths. Subsequently, expression of Shh in the interfollicular ectoderm has little if any discernible effect on the differentiation of the skin in most instances. This is despite the fact that activation of patched transcription indicates the Shh signal is received and transduced in interfollicular ectoderm. These observations demonstrate that the response to Shh in the skin is closely regulated as the skin develops and that this restriction has important functional consequences for morphogenesis. They further suggest that a restricted subset of epidermal cells may be the targets of altered Shh signaling in cutaneous pathologies.

MATERIALS AND METHODS

Infection Protocol

The RCAS vector RCASBP(A) encoding the chick Sonic hedgehog protein (Riddle *et al.,* 1993) was used to generate stocks of viral inoculum of 1–3 $\times$ 10^{-8} infectious units/ml and prepared for microinjection as described (Morgan and Fekete, 1996). In embryos where the amnion had not yet closed, the needle was aligned parallel to the anterior posterior axis of the embryo and inserted through the opening in the amnion taking care to avoid damage to the ectoderm. In embryos where the amnion was closed, the injection needle was inserted through a small hole in the amnion generated above the right flank with a sharpened tungsten wire. Approximately 50 to 100 nl of viral suspension was delivered between the amniotic membrane and the surface of chick embryos, *in ovo*, at H + H stages 19–25 (Hamburger and Hamilton, 1951). Eggs were resealed with tape and incubated at 99.75°F until harvest.

Viral transcripts can first be detected in ectodermal cells 24 to 36 h after inoculation by *in situ* hybridization. Subsequent clonal expansion and infection of adjacent cells leads to expansion of these foci during the ensuing days of incubation. Secondary foci of infection also arise as virus shed into the amniotic fluid is carried to other sites. As a result, embryos infected prior to stage 22 will show productive patches of infection starting prior to feather tract formation (6.5–7 days incubation) but will also include secondary sites of infection from later stages. Embryos infected at stage 23/24 or later show infection during and after tract formation.

To precisely analyze the effects of stage of infection, embryos of assorted stages were inoculated in a single experiment. Of 14 embryos inoculated prior to stage 22, 8 harvested after day 8 showed "ectodermal growths," while 6 harvested earlier and sectioned showed early ectodermal growths. Of 12 embryos inoculated at stage 22/23,11 showed precocious feather bud formation and none showed ectodermal growths. Of 32 embryos inoculated after stage 23, none showed either growths or ectopic feather buds. Additional experiments performed separately with embryos inoculated at single embryonic stages gave similar results. This phenotypic analysis is based on gross morphology; numbers of sectioned specimens are supplied in the text.

Whole Mount in Situ Hybridizations

Protocols were modified from those previously described by reduction of proteinase K treatment to 2 min at room temperature (Burke *et al.,* 1995). For sequential *in situ* analysis, embryos were hybridized simultaneously with one digoxigenin- and one FITC-labeled riboprobe prepared as described. After detection with BCIP/NBT, samples were washed in three 30-min changes of NTMT at room temperature, then neutralized by incubation in PBST, pH 5.5, and transferred to TBST. Specimens were photographed, incubated with alkaline phosphatase conjugated anti-fluorescein and detected with BCIP/NBT. Photographs of the first and second detections were compared to reveal additional signal from the second hybridization. Control embryos hybridized only with the digoxigenin-labeled probe but taken through both detection protocols revealed some increase in signal intensity following the second detection, but no change in the pattern of signal was observed.

After whole mount *in situ* detection of RNA, some samples were cryosectioned to further characterize expression patterns. After the final dehydration cycle, samples were rehydrated into PBS and then TBS. Samples were infiltrated with 25% sucrose in 10 mM Tris, pH 7.5, followed by overnight infiltration at room temperature in OCT embedding compound. Samples were embedded in fresh OCT, frozen at −20°C, and cryosectioned at 5–10 μm.

Probe templates were as described in Noramly *et al.* (1996).

Serial section *in situ* hybridizations were performed on fixed frozen tissue according to the protocol devised by Dr. T. Ikeda (personal communication). Detailed protocols are available on the Morgan lab Web page at http://cbrc-a12.mgh.harvard.edu/CBRCpi.html.

RESULTS

Normal Expression of Shh in the Skin

The feather buds arise in discrete tracts (pterylae) at defined positions in the embryo starting at day 6.5 of incubation. Adjacent feather tracts converge but remain separated by featherless (apteric) regions. Within each tract a single row of feathers forms first and is referred to as the primary row. Additional rows of feather rudiments form sequentially such that the feather tract propagates laterally and the forming tract consists of buds at successive stages of development arrayed in adjacent rows (see Fig. 1). The first morphological indication of a nascent feather occurs when the presumptive feather bud ectoderm undergoes a transition from a simple cuboidal epithelium to a columnar and then stratified epithelium to form the ectodermal placode (Wessels, 1965). Shortly thereafter, the underlying mesenchyme forms the dermal condensation in response to signals from the placode. Reciprocal signaling between the ectoderm and mesenchyme leads to the outgrowth and patterning of the bud.

It has been proposed that Shh plays an important role in the initiation of bud formation based on its early activation in bud regeneration assays (Chuong *et al.*, 1996). However, during normal development Shh is not detected in the skin until after both the ectodermal placode and the dermal condensation have formed. The formation of an ectodermal placode precedes the induction of the dermal condensation and can be readily observed by the expression of BMP-2 in the ectoderm (Nohno *et al.*, 1995; Chuong *et al.*, 1996; Noramly and Morgan, submitted). BMP-4 is not expressed in the placode but is induced in the dermis during the early steps of condensation and serves as a marker for this structure (Wiedlitz *et al.*, 1997; Noramly and Morgan, 1998). Shh expression is not observed in the skin until after both of these markers have been activated in the ectoderm and dermis, respectively. When the left and right femoral tracts of an embryo are examined for BMP-2 and Shh expression, respectively, two rows of feather placodes expressing BMP-2 can be observed while only a single row of placodes express Shh (Figs. 1B and 1C). Sections through these samples show that two rows of morphologically distinct placodes have formed, but only the more mature placodes express Shh (not shown). Figure 1D shows a

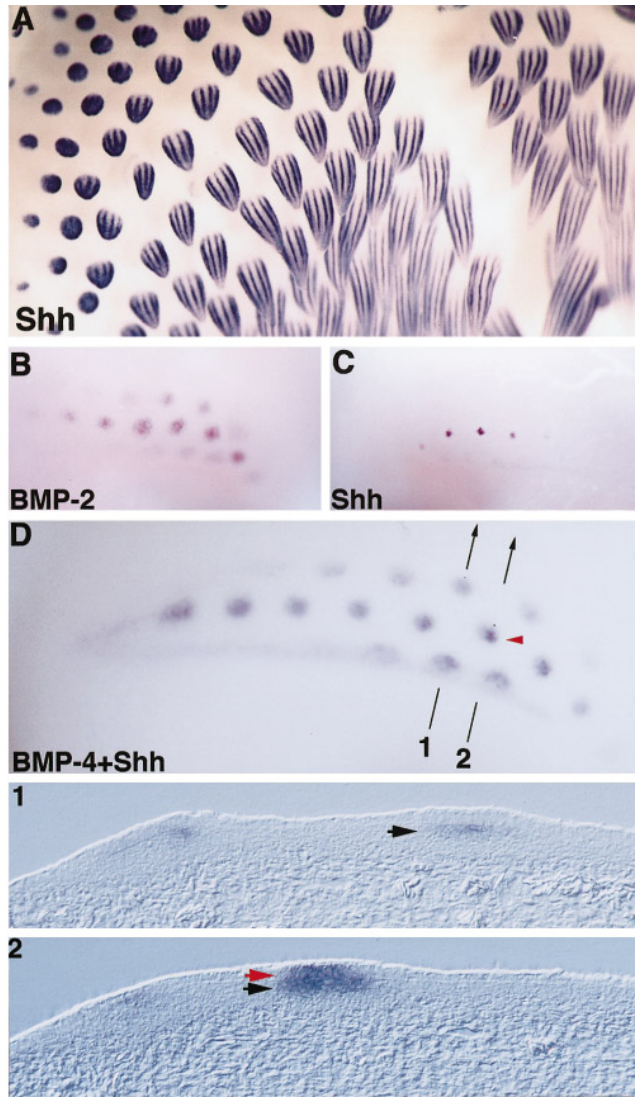


FIG. 1. (A) Whole mount *in situ* detection of Shh in a femoral feather tract shows the expression of Shh from early bud stage (upper left) through more mature filament stages (lower right). (B) Detection of BMP2 in the left leg of an embryo at day 7 shows two rows of buds expressing this gene. (C) In the right leg from the same embryo, only a single row of buds expresses Shh. (D) When genes are expressed exclusively in the ectoderm or dermis, their expression can be compared by hybridizing to both probes and sectioning the whole mount. This leg has been hybridized with Shh (ectoderm) and BMP4 (dermis). Viewed in whole mount, the faint spots are BMP4 expression, while the dark spot in the buds at the right of the first full row (e.g., red arrowhead) demarcate the ectodermal Shh transcripts. Note that this expression overlies the posterior half of the BMP4 expression domain and that Shh expression is asymmetric from its inception. The arrows mark the planes of section (1, 2) shown below with the arrowhead at the right of the photo. A section in plane 1 shows the transcripts of BMP4 in the dermis with no expression of Shh in the ectoderm of a developmentally younger bud (black arrow). A section through plane 2 shows both BMP4 (black arrow), and Shh (red arrow) in the more advanced bud.

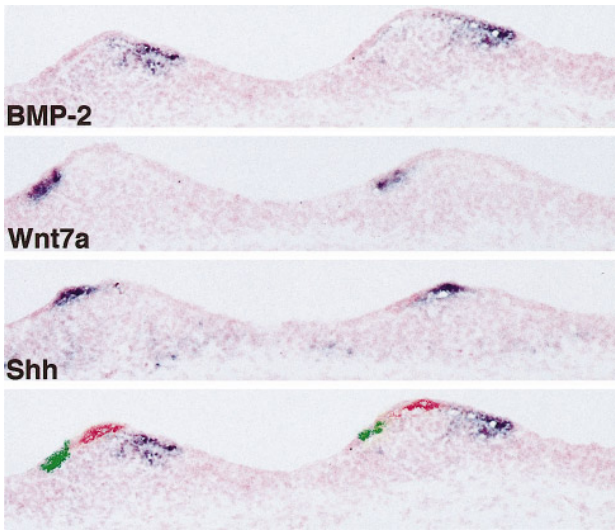


FIG. 2. Adjacent sections through the A/P axis (right to left) of feather buds detected for the expression of BMP-2, Wnt7a, and Shh. A false color overlay of these data (below) shows that Shh (red) is expressed in a domain bounded on the anterior side by cells expressing BMP-2 (purple) and on the posterior side by cells expressing Wnt7a (green). These sections were through the femoral tract of an embryo harvested at day 8 of incubation.

femoral tract hybridized with both Shh and BMP4. In whole mount view, the dermal expression of BMP-4 demonstrates that two rows of buds have progressed to the dermal condensation stage in this tract. Shh is expressed exclusively in the ectoderm and can be seen as the sharp dots of expression in the buds of the first row. Cross sections of this specimen show the expression of BMP-4 in the dermis of buds in both rows (Fig. 1D, 1 and 2), while Shh expression in the ectoderm is observed only in the primary row of buds (Fig. 1D, 2). This temporal lag suggests that BMP-4 or some other signal expressed in the dermal condensation may induce Shh in the overlying ectoderm. Note, however, that the expression of Shh is restricted to a small group of cells near the posterior of the placode, whereas BMP-4 expression is observed throughout the dermal condensation. The cells bordering this medial/posterior domain of expression on the anterior and posterior side can be distinguished from each other by the expression of Wnt7a and BMP-2. As shown in Fig. 2, Wnt7a is expressed in the ectoderm posterior to the domain of Shh expression while BMP-2 is expressed in ectoderm anterior to this region. Although at this stage neither BMP-2 nor Wnt7a is expressed in the Shh domain (Fig. 2), the initial expression of Shh is in cells expressing BMP2 but not Wnt7a (not shown). These results demonstrate that substantial patterning of the epidermis occurs prior to the normal onset of Shh expression in the skin.

This patterning includes local modifications in the cells of the ectodermal placode which limit their responsiveness to Shh. Patched protein inhibits transcription of the ptc gene and reception of the Shh signal relieves this repression. As a result of this relationship, elevated ptc transcript levels in the cell serve as an indication of Shh signaling (Goodrich *et al.*, 1996; Marigo *et al.*, 1996). In embryonic skin, one to two rows of buds expressing Shh have formed before elevated ptc transcripts are first observed in the feather tract which indicates a lag of approximately 6 h between initial detection of Shh transcripts and an observable transcriptional response to the encoded protein (Fig. 3A). At this stage ptc transcription is observed predominantly in the dermis, extending several cell layers beneath the ectodermal source of Shh (Fig. 3B). Patched is also induced in the ectoderm, but only very locally in the ectoderm posterior to the Shh-expressing cells (Fig. 3C). Neither the Shh-expressing cells nor ectoderm anterior to them show elevated levels of ptc transcripts. As development progresses, ptc transcripts are expressed in additional ectodermal cells posterior to the Shh-expressing region, but elevated ptc transcript levels are not observed in anterior or lateral ectoderm of the bud (data not shown). The lack of detectable expression of Shh until after the formation of the ectodermal placode and dermal condensation implies that Shh is not required for the specification of these structures. Patterning of the epidermis during the specification of these structures restricts responsiveness to Shh in the ectoderm.

Forced Expression of Shh

In the avian embryo, retroviruses may be used to alter gene expression at successive stages of development. A replication competent virus encoding Shh was employed to express this protein in the ectoderm at abnormal times or positions in small patches which resemble the local expression observed during normal development. By injecting virus between the amnion and the ectoderm, we achieved scattered ectodermal infection (Fig. 4). Although the infection foci vary in size, even broad patches of infection are restricted to the ectoderm through the period of study (Fig. 5 and data not shown). Forced expression of Shh in the ectoderm prior to the development of dense dermis and initial differentiation of the skin can cause pronounced epidermal displasias. When embryos infected prior to stage 22 were examined 48 h after injection, elevated ptc transcripts can be observed in the ectoderm at the site of infection (Figs. 5A and 5C). Shortly thereafter, the simple cuboidal epithelium is converted to a disorganized ectodermal growth in the infected area while adjacent ectoderm continues to develop normally (Fig. 5C). These growths may invaginate into the forming dermis or protrude from the surface of the embryo (Fig. 5D). During these initial stages, the effect of Shh is restricted to the ectoderm. Elevated ptc transcripts are observed in this layer but ptc expression in the dermis remains undetectable (Figs. 5A and 5C). BMP2 is also induced in the infected ectoderm but not the underlying dermis (Fig. 5B). Other markers expressed in the dermal component of a forming feather bud also fail to

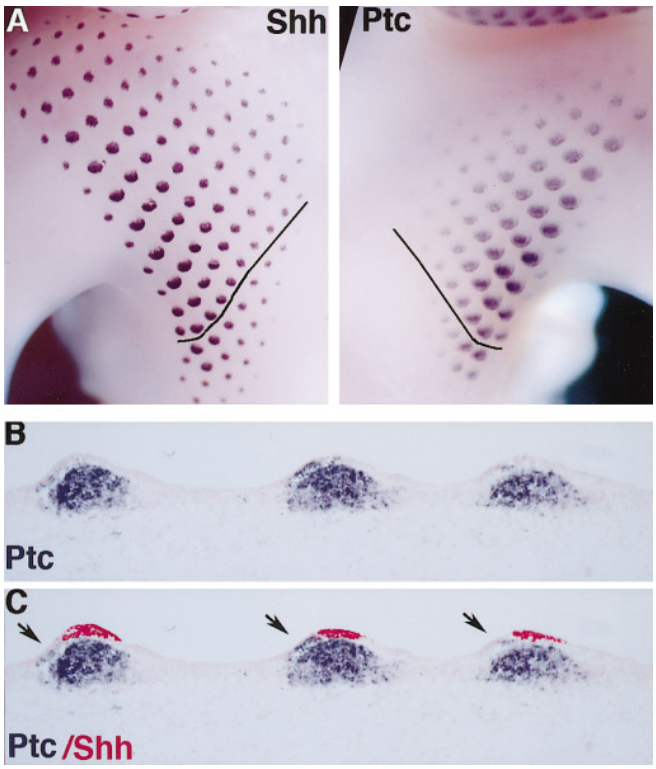


FIG. 3. Shh and ptc expression in the femoral feather tract at day 8.5 of incubation. (A) The expression of Shh (left) and ptc (right) detected in the left and right thighs of the same embryo. The black line indicates the corresponding series of buds in both legs. Shh expression in the ectoderm precedes elevated ptc expression. Although 10 buds expressing Shh can be seen in this line, only 8 buds expressing ptc are seen in the corresponding row. (B) ptc transcripts (purple precipitate) detected in a section through the femoral tract of an embryo at day 8.5 of incubation. This section is in the plane of the anterior/posterior axis of the buds through their midline (anterior to right). Although there is robust expression throughout the dermal condensation, expression of ptc in the ectoderm is restricted to a narrow strip on the posterior side of the bud. This strip of ectodermal expression is broader on more mature buds (left) than their developmentally younger counterparts (right). (C) The expression of Shh from an adjacent section (red) has been superimposed on this image. The Shh-expressing cells are anterior to the ptc-expressing cells in the ectoderm. Arrows indicate ptc expression in the posterior ectoderm. Ectoderm expressing Shh (C) does not exhibit elevated ptc expression (B). Sections are counterstained light pink to reveal nonexpressing tissue.

be expressed in the dermis underlying these infection foci. By day 9 of incubation, these growths can become quite large and may include a dermal component as well (Figs. 5 D–5F). The majority are composed of disorganized ectoderm (Figs. 5D and 5F), although in some cases (2 of 7 sectioned), lateral regions of the growth may form repeated folds after day 8 of incubation whose spacing and size resemble barb ridges of a feather bud while other regions of the growth remain disorganized.

Dramatically different results are observed when Shh is expressed at successively later stages of development. Inoculation at stage 22/23 leads to productive infection at the time of feather tract development. The most striking phenotype associated with the forced expression of Shh in the ectoderm at this stage is the formation of feather buds at abnormal positions or times (Fig. 6). This is most clearly observed in normally apteric regions. Expression of Shh in the ectoderm of the ventral midline can lead to the formation of buds in this normally featherless region (Figs. 6A and 6B). These abnormal feathers form the repeated barb ridges characteristic of a feather bud (data not shown). Unlike a wild-type bud where 12–13 evenly spaced ridges are formed, these oversized buds can form as many as 50 barb ridges. Shh expression in the ectoderm can also lead to the precocious formation of feather buds within the developing feather tracts, but they are more normal in structure and spacing than those generated in normally apteric regions (Figs. 6C and 6D). These induced buds include a dermal component which expresses BMP-2, -4, -7, and cek-1 (data not shown). All of these genes are expressed in the dermal condensation of a normal feather bud (Chuong *et al.*, 1996; Song *et al.*, 1996; Crowe *et al.*, 1998; Noramly and Morgan, 1998).

Infection at this stage or later also leads to local expression of Shh in interfollicular ectoderm. However, after the

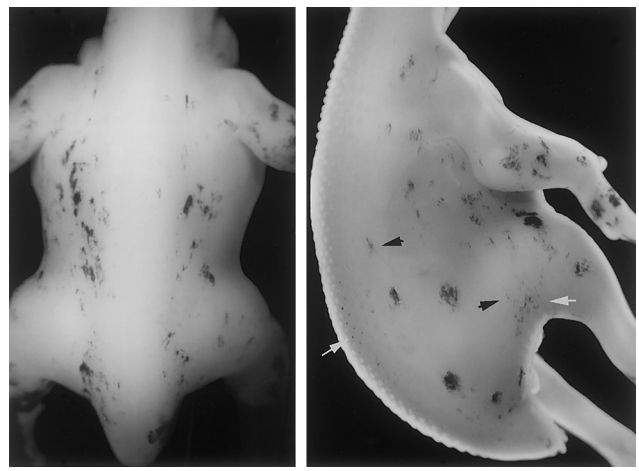
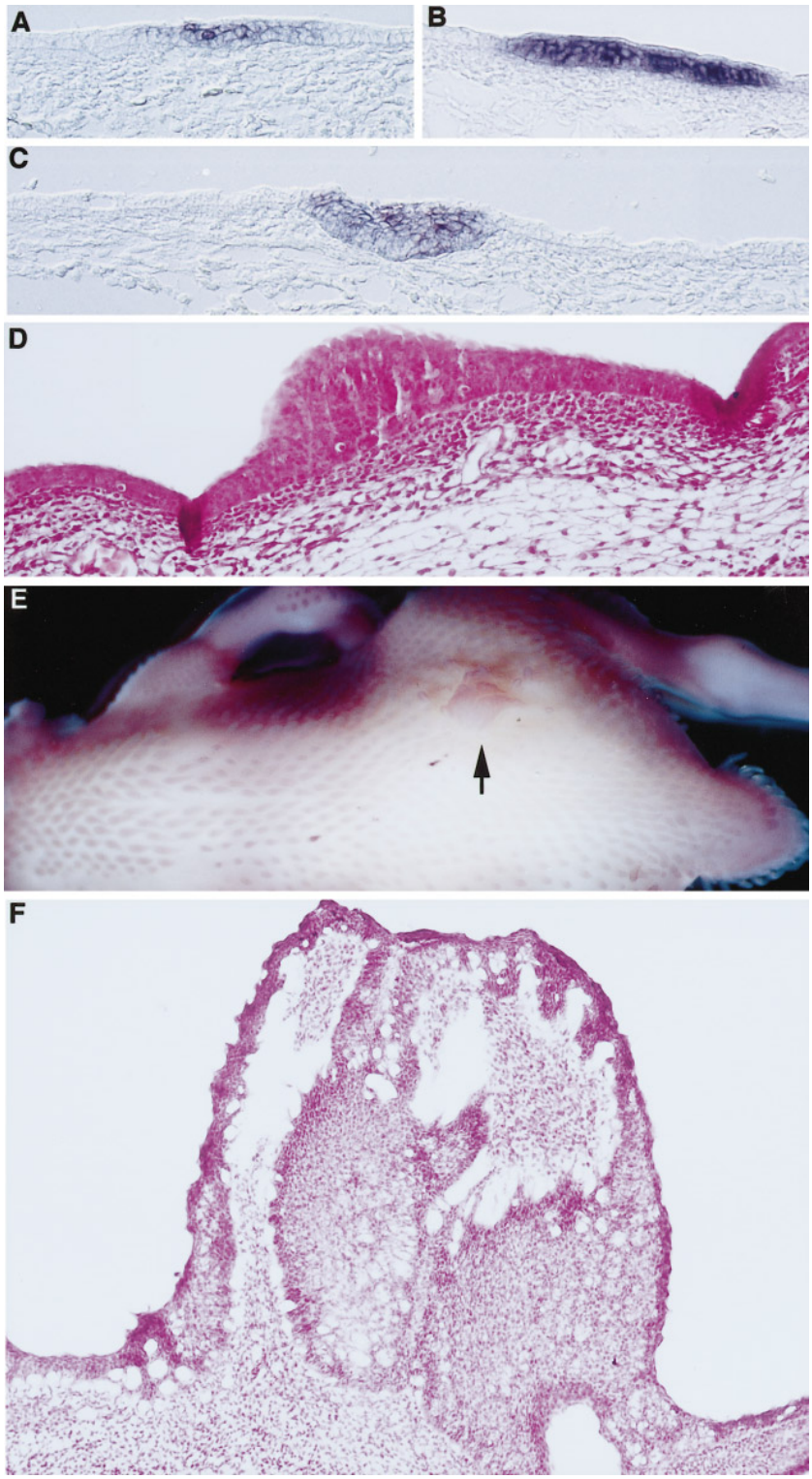


FIG. 4. Infections cause local expression of Shh in ectoderm. (A) Expression of Shh in an embryo inoculated at stage 21 and harvested at day 6.75 of incubation. Multiple foci of infection are scattered across the skin of the embryo, but individual foci of infection range from a few cells to slightly larger than the size of a feather placode. (B) Infection at later stages (23/24) results in local infection within forming feather tracts both in areas that have already begun bud formation (white arrows) and in areas that will soon begin this process (black arrows) as shown by detection of viral transcripts in an embryo harvested at day 8.25 of incubation.



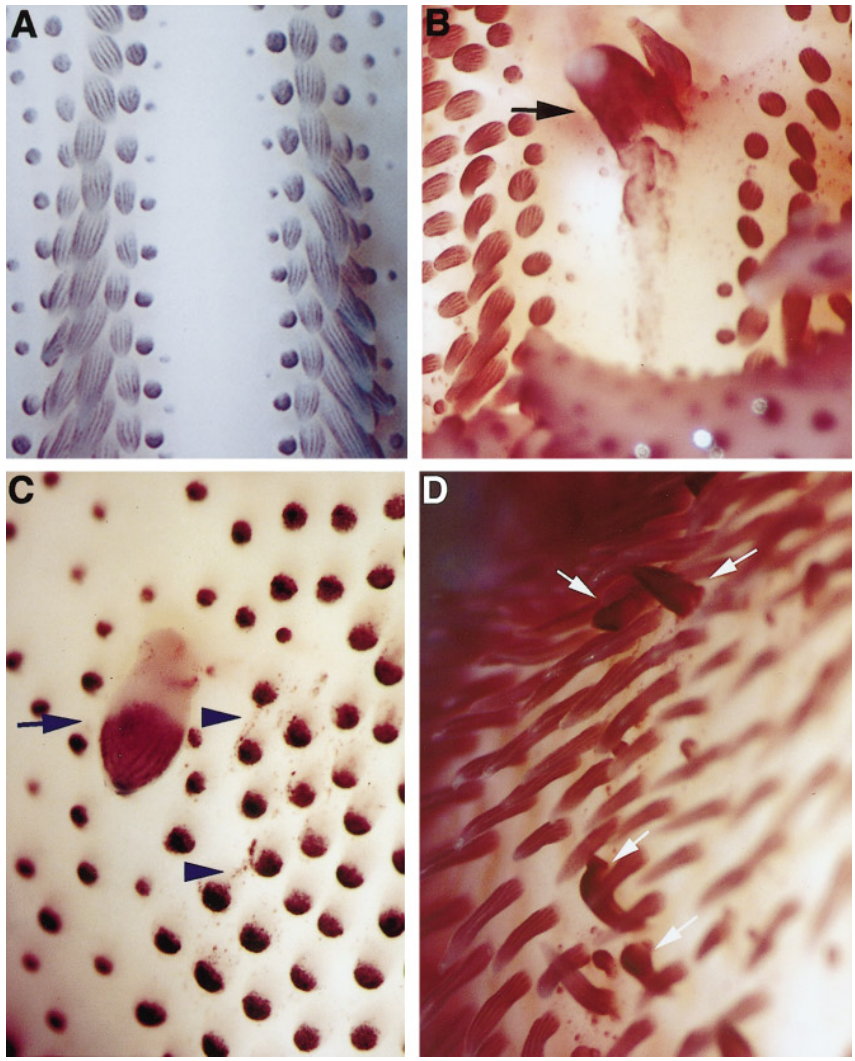


FIG. 6. Forced expression of Shh in the ectoderm has different effects at different stages. Expression of Shh in the ectoderm caused by inoculation at stage 23 can lead to the formation of feather buds in normally apteric areas. Whole mount *in situ* detection of Shh transcripts are shown to demarcate the feather buds. (A) The skin of the day 10 embryo has two tracts of feather buds expressing Shh separated by the midventral apterium. (B) Expression of Shh in the skin of the ventral midline leads to the formation of large, abnormal feather buds. Cross sections through this bud at day 10 of incubation showed 38 evenly spaced barb ridges instead of the normal 12–13 (not shown). (C) Buds induced precociously within a presumptive tract are more normal in appearance (C, D) . (C) A large bud induced at the edge of the femoral tract is more regular than its counterparts in the midventral apterium. Blue arrowheads indicate Shh expression in interfollicular ectoderm of this embryo at day 9.5 of incubation that will not result in a morphological response. (D) Precocious buds induced within a femoral shown at day 9.5 of incubation may be abnormally oriented or shaped (arrows) suggesting a role for Shh in later stages of bud morphogenesis.

FIG. 5. Forced expression of Shh leads to ectodermal proliferations. Embryos were inoculated at stage 21 and harvested at day 7 (A, B, C), day 9 (D), and day 9.5 (E, F) of incubation. (A) Outside the area of infection the ectoderm consists of periderm (small cells) overlying a simple cuboidal epithelium. In the infected region, the ectodermal cells express elevated levels of ptc RNA (purple) and have begun to form a stratified epithelium. The dermal cells underlying this area do not express elevated levels of ptc. (B) BMP2 expression is also induced in the ectoderm by infection with the Shh virus. No BMP2 expression is observed in the dermis after infection at this time. (C) A slightly more advanced response detected for both Shh and ptc shows that at this stage, nether the virus nor elevated ptc transcripts are observed in the underlying dermis. (D) Section through a growth at day 9 of incubation stained with hematoxylin and eosin shows that it is composed of disorganized ectoderm. (E) An embryo harvested at day 9.5 of incubation shows a pronounced ectoderm growth (arrow). (F) A section through another example shows that the majority of this growth consists of disorganized ectoderm.

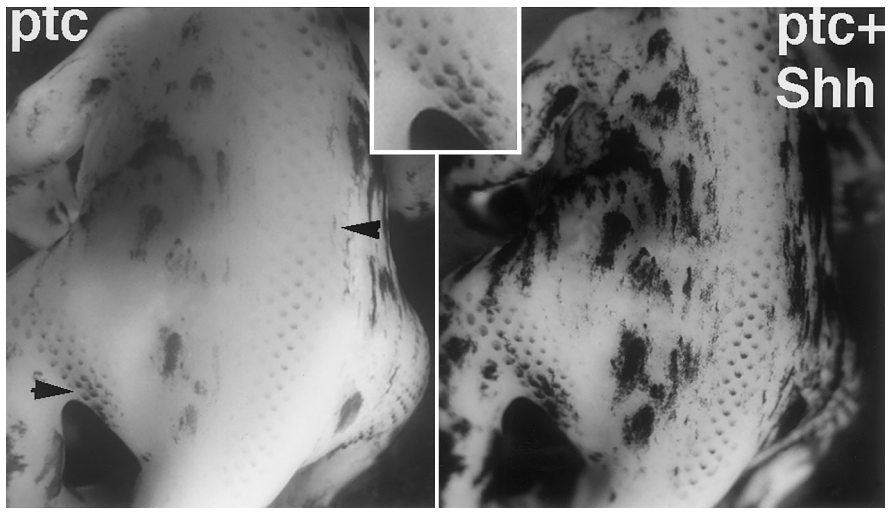


FIG. 7. Forced Shh expression at later stages induces ptc. An embryo inoculated with Shh virus at stage 23/24 and detected first for ptc (left) and subsequently for Shh (right) at day 8 of incubation. ptc transcripts are detected wherever Shh is expressed including apteric regions, presumptive feather tracts, and interfollicular epidermis. The inset shows a higher magnification view of the left femoral tract to demonstrate ptc transcripts in interfollicular ectoderm. The more extensive expression of Shh compared to ptc may reflect the lag between Shh expression and ptc induction in recently infected sites. Of 32 embryos inoculated after stage 23 and harvested at days 8.5 through 10 of incubation, none showed a morphological response to forced expression of Shh in the epidermis.

subdivision of embryonic skin into follicular and interfollicular ectoderm, Shh expression in the interfollicular regions has no obvious effects on morphology. These results demonstrate that the response to Shh in the skin changes as development proceeds. One possible explanation for the lack of response after feather tract formation is that the ectoderm no longer expresses the Shh signal transduction apparatus. However, as shown in Fig. 7, elevated ptc transcripts are observed in interfollicular ectoderm after Shh infection at stages which do not cause morphological responses. Although the expression of Shh (Fig. 7, right) is slightly more widespread than ptc (Fig. 7, left), the lag between Shh expression and induced ptc transcription may explain this difference. Elevated ptc transcripts are observed in apteric regions, presumptive feather tracts, and within the interfollicular ectoderm. In this specimen and in sibling embryos allowed to develop further, no morphological defects were observed in these regions which had begun feather tract development. The majority of the cells in the epidermis, including the interfollicular ectoderm, remain capable of responding to Shh, although interfollicular ectoderm shows no morphological response.

DISCUSSION

Our analysis of the expression of Shh and ptc demonstrates that Shh is not active until after the initial specification and patterning of the feather bud. The ability of Shh to induce feather bud development as well as its early expression in bud regeneration assays (Chuong *et al.*, 1996) suggested a role for Shh in the normal initiation of feather bud formation. However, the spacing of buds within a field and their initial development occurs prior to the expression of Shh in the skin. The predominance of ptc expression in the dermal condensation suggests that a major role for Shh in normal bud development may involve signaling from the ectodermal placode to promote proliferation in the dermal condensation. Although the initial formation of the dermal condensation is the result of cell migration rather than proliferation (Desbiens *et al.*, 1991), the subsequent outgrowth of the bud entails rapid proliferation of the dermal core. Previous experiments in which widespread expression of Shh in the dermis caused feather defects were interpreted as showing a role for Shh in "dermal condensation" (Ting-Berreth and Chuong, 1996). Since the normal expression of Shh in the ectoderm and response of ptc in the dermis begins after this condensation phase, we conclude that it is more likely that Shh promotes proliferation of the dermal condensation. Shh is mitogenic for somitic mesoderm (Fan *et al.*, 1995) and our preliminary experiments expressing Shh in the forming dermis suggest that it is mitogenic for this tissue as well (not shown). ptc transcription is also observed in the posterior ectoderm of the bud. This region corresponds to the region of the ectodermal placode where preferential proliferation is occurring at this stage (Chan *et al.*, 1997). The local expression of Shh posterior to the midline of the bud may coordinate the posteriorly directed outgrowth of epidermis and dermis. This might explain why abnormally oriented buds were observed after forced

expression of Shh in the mesenchyme (Ting-Berreth and Chuong, 1996) or the ectoderm (e.g., Fig. 6D).

Jung *et al.* (1998) have recently published data which they interpret to mean that prior to both placode formation and BMP2 expression, Shh is expressed in the skin and ptc transcription is increased locally. They further conclude that BMPs are not expressed until after placode formation. We have examined expression of these genes from stages 27 through 33 by *in situ* hybridization to both whole mounts and sectioned tissue and find no evidence of expression of Shh or ptc until that described above. Extension of the chromogenic reaction beyond that required to detect Shh expression in the placode does not reveal Shh expression in the skin prior to placode formation. Although this failure could be ascribed to a lack of sensitivity in our experiments, comparison of the signal intensity in data presented here with similar data from Jung *et al.* suggests that our experiments have sufficient sensitivity to detect this reported expression. The detection of diffuse BMP2 expression prior to placode formation (Normaly and Morgan, 1998) also indicates superior sensitivity in our experiments. The sequence of events documented here, including the initiation of BMP-2 expression and formation of the placode and condensation prior to detectable Shh expression, are inconsistent with models in which Shh initiates bud formation during normal development.

Forced expression of Shh in the ectoderm leads to four distinct responses depending on the developmental state of the skin at the time of expression. Prior to day 6.5 of incubation, the dermis is not developed and the ectoderm is a simple epithelium. Expression at this stage causes large disorganized ectodermal hyperplasias. Qualitatively different responses are observed in the presumptive apteria and in the actively forming feather tracts as skin matures. Forced expression of Shh at the time the feather tracts are forming (days 6.5–7.5) leads to induction of feather buds in the normally apteric regions which are larger and more abnormally shaped than those induced precociously in the forming feather tracts. Tissue recombination experiments have shown that the ectoderm from either apteric regions or presumptive feather tracts has equivalent developmental potential and both can form feathers when recombined with dermis from a feather tract. The difference between pterylae and apteria is specified by the dermis (reviewed in Sengel, 1975) and differences in the responsiveness of epidermis to Shh presumably reflect the influence of underlying dermis.

This observation, coupled with the fact that during normal feather bud development responsiveness to Shh is spatially restricted within the ectoderm, leads us to interpret these differences as evidence that patterning of the epidermis includes inhibitory signals which restrict responsiveness to Shh and downstream effectors. In the apteria, these patterning mechanisms are not active until after the expression of Shh in the ectoderm initiates the feather bud formation pathway. As a part of that cascade, inhibitory signals are generated which restrict responsiveness to Shh and prevent the formation of a disorganized ectodermal growth. In contrast, in the pterylae, these inhibitory mechanisms are already active as part of the mechanism by which ectodermal placodes are generated in a regular array. Although at the early stages, Shh expression can override this inhibition and promote feather bud development, the prior activation of these inhibitory mechanisms restricts the response to generate a more normal feather bud.

Prior to the formation of epidermal placodes there is no morphological or identified molecular distinction between presumptive follicular and interfollicular ectoderm and tissue recombination experiments support the assumption that epidermis is equipotent at this stage. There is no apparent spatial restriction in the response to Shh at this time; all cells will induce ptc and participate in disorganized ectodermal growths. As the tract begins to form, Shh can induce precocious bud formation in regions that have not yet initiated placode formation. Although presumptive follicular and interfollicular ectoderm cannot be rigorously identified at this stage, their positions can be inferred by extrapolating from the regular array of forming buds in other regions of the tract. This analysis suggests that there is no distinction between the response of presumptive follicular and interfollicular ectoderm just prior to normal bud formation. However, after interfollicular ectoderm is defined by the specification of surrounding buds, expression of Shh in this tissue does not result in a morphological response. This is despite the fact that interfollicular ectoderm retains the capacity to participate in follicle formation at least transiently after the initial patterning of the tract and can be forced to assume a follicular fate by repositioning the ectoderm relative to the dermis (Novel, 1973; Sengel, 1975). The failure of Shh to induce a response in interfollicular skin suggests a dominance of inhibitory signals over Shh signaling at this stage. The induction of ptc suggests that this inhibition in interfollicular ectoderm is mechanistically distinct from that which generates refractive areas within the ectodermal placode where no ptc induction is observed after activation of the endogenous Shh gene.

It should be noted that our ability to analyze the activity of Shh in interfollicular ectoderm is restricted by the inactivation of viral expression in differentiating interfollicular ectoderm. Subsequent to the differentiation of interfollicular epidermis, viral transcripts are detected with reduced frequency compared to that observed in sibling embryos harvested at earlier stages. It is possible that prolonged Shh signaling is required to induce an ectodermal growth and that differentiation of the skin inactivates Shh expression before this can occur. However, viral expression does persist for the 48 h which is sufficient to generate the morphological response after infection in a younger embryo (Fig. 5C). Furthermore, once induced, the epidermal growths can continue to express viral transcripts well after the surrounding skin has differentiated (data not shown). Although we cannot assess the effect of prolonged Shh signal-

ing, interfollicular skin is clearly more refractive to Shh than the ectoderm of the forming tracts or apteria.

Shh and BCC

The involvement of ptc in BCC stimulated investigation of the role of Shh in this disease. Shh was placed under the control of the keratin 14 gene promoter/enhancer to force its expression in basal cells of the mouse epidermis from the earliest stage of skin development (Oro *et al.*, 1997) . These mice exhibit an extensive epidermal proliferative disorder with many of the hallmarks of BCC. Forced expression of Shh in human keratinocytes also results in a BCC-like phenotype in skin reconstituted from these cells on a nude mouse host (Fan *et al.*, 1997). Overexpression of an activated mutant form of smoothened in the basal cells of transgenic mice under the control of the keratin 5 promoter leads to a similar BCC-like phenotype (Xie *et al.*, 1998). Overexpression of Gli-1 in the ectoderm of *Xenopus* embryos also results in epidermal tumors, although forced expression of Shh may not be sufficient to cause ectodermal tumors in frogs (Ruiz i Altaba, 1995; Dahmane *et al.*, 1997). Although all of these experiments demonstrate that activation of the Shh signaling pathway can cause a BCC-like phenotype in epidermis, they all activate this pathway in embryonic ectoderm prior to its differentiation into mature skin. In this regard, our experiments with avian skin suggest that this distinction may be important for the response observed.

The experiments reported here are most similar to the transgenic mouse experiments driving the expression of Shh in the ectoderm (Oro *et al.*, 1997). Our early infection protocol drives the expression of Shh in the ectoderm at a similar stage of skin development. However, the response to Shh may vary with the level of signal received (Roelink *et al.*, 1995; Marti *et al.*, 1995; Yang *et al.*, 1997) and one major distinction between these experiments may be the level of Shh expressed in the ectoderm. Comparison of the viral transcripts with endogenous Shh expression in the feather buds suggests that the level of forced Shh expression is similar to that present during normal development of the feather bud. This is likely to be lower than the level achieved using keratin regulatory sequences to drive expression in the transgenic mice. Nevertheless, the level of expression achieved with the virus is sufficient to cause a disorganized ectodermal growth which is morphologically similar to that observed in the mice. These mice also exhibit spina bifida and limb abnormalities and both defects may also be observed after widespread infection of the chick ectoderm with a Shh-expressing virus at this stage (data not shown). However, by limiting the expression of Shh in our experiments to discrete foci on the skin, BCC-like phenotypes are readily separable from these other defects. These observations demonstrate that the level of Shh expression achieved by the virus, although probably lower than that achieved in the transgenic mice, is sufficient to generate the types of phenotypes reported after expression of Shh in mouse epidermis at a similar stage of development. Furthermore, restricted expression of Shh in discrete patches is sufficient to induce the formation of ectodermal displasias at this early stage and most if not all of the foci of infection result in abnormal ectodermal development. Therefore, it appears that the majority of epidermal cells will differentiate abnormally in response to Shh signal at this stage. In contrast, expression of similar levels of Shh which leads to a corresponding induction of ptc transcription at later stages of development does not result in this phenotype.

If these results can be extrapolated to mammalian skin, they suggest that although the link between ptc inactivation and BCC is unequivocal, it is possible that deregulated expression of Shh in mature epidermis will be insufficient to cause BCC because of modulatory influences on Shh signaling. The fact that ptc transcripts may be induced in maturing avian epidermis without the formation of ectodermal proliferations suggests that this modulation occurs downstream of signal transduction across the cell membrane and affects a subset of responses to Shh. Complete inactivation of the ptc protein may suffice to overcome this modulation. Further analysis of the changing responsiveness to Shh in developing skin will help to identify these modulatory influences and their implications for BCC.

CONCLUSIONS

During normal development, Shh is not expressed in the skin until after patterning events have restricted responsiveness to this potent modulator of ectodermal development. The induction of ptc transcription suggests that the primary target of Shh signaling during skin development is the dermal condensation, but a small group of cells in the posterior ectoderm of the bud also induce ptc transcription and this response may be important in the outgrowth of the ectodermal component of the feather as well. Forced expression of Shh prior to this stage suggests that this restriction in responsiveness has important functional consequences as in its absence Shh causes a disorganized ectodermal growth. The nature of these restrictions in responsiveness both within the placode and in interfollicular skin will have important implications both for pattern formation during normal development and the role of this pathway in cutaneous pathologies.

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