Sonic hedgehog signaling is essential for hair development

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Background: The skin is responsible for forming a variety of epidermal structures that differ amongst vertebrates. In each case the specific structure (for example scale, feather or hair) arises from an epidermal placode as a result of epithelial–mesenchymal interactions with the underlying dermal mesenchyme. Expression of members of the Wnt, Hedgehog and bone morphogenetic protein families (*Wnt10b*, *Sonic hedgehog* (*Shh*) and *Bmp2*/*Bmp4,* respectively) in the epidermis correlates with the initiation of hair follicle formation. Further, their expression continues into either the epidermally derived hair matrix which forms the hair itself, or the dermal papilla which is responsible for induction of the hair matrix. To address the role of *Shh* in the hair follicle, we have examined *Shh* null mutant mice.

Results: We found that follicle development in the *Shh* mutant embryo arrested after the initial epidermal–dermal interactions that lead to the formation of a dermal papilla anlage and ingrowth of the epidermis. *Wnt10b*, *Bmp2* and *Bmp4* continued to be expressed at this time, however. When grafted to *nude* mice (which lack T cells), *Shh* mutant skin gave rise to large abnormal follicles containing a small dermal papilla. Although these follicles showed high rates of proliferation and some differentiation of hair matrix cells into hair-shaft-like material, no hair was formed.

Conclusions: Shh signaling is not required for initiating hair follicle development. Shh signaling is essential, however, for controlling ingrowth and morphogenesis of the hair follicle.

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Background

The skin is responsible for forming a variety of epidermal structures that differ among vertebrates. These include the scales present in many vertebrates and more elaborate appendages such as feathers in birds and hairs in mammals. In each case the specific structure arises from an epidermal placode as a result of epithelial–mesenchymal interactions with the underlying dermal mesenchyme. Defining the molecular and cellular nature of these interactions will provide important insights into developmental mechanisms and their evolutionary relationships.

In mammalian skin, grafting studies between hair-forming and non-hair-forming regions of the epidermis indicate that the dermal mesenchyme is most probably responsible for specifying hair development (reviewed in [1,2]). In response to mesenchymal signaling, the epidermal epithelium thickens and grows down into the dermis. Concurrent with these events, the epidermis is thought to induce the local condensation of dermal mesenchyme cells immediately below the hair placode. These cells are surrounded by the ingrowing epithelium and give rise to the dermal papilla of the resulting hair follicle. Transplantation of the dermal papilla indicates that it is responsible for induction of the hair itself [3]. Mesenchyme cells also contribute to the dermal sheath which surrounds the hair follicle.

The dermal papilla stimulates proliferation in overlying epithelial-derived cells of the hair matrix. These cells rise up the hair shaft where they differentiate into radially distinct populations of cells. The innermost cells give rise to the hair (medulla, cortex and cuticle) while the outermost cells form the inner root sheath (Henle's layer, Huxley's layer and cuticle). Finally, the outer root sheath, which is continuous with the basal keratinocytes of the skin, surrounds the hair follicle [2]. Stem cells, which are thought to participate in additional waves of folliculogenesis, reside in this population [4,5].

Several signaling factors are known to be expressed in the presumptive hair follicle before morphological signs of hair placode formation, consistent with a role for them in initiating hair follicle development. These include members of the Hedgehog (Sonic hedgehog, Shh) [6], bone morphogenetic protein (Bmp2, Bmp4) [6–8] and Wnt (Wnt10b; [9] and H.R.D. and A.P.M., unpublished observations) families whose members have been shown to participate in

regulatory networks controlling growth and pattern in *Drosophila* imaginal discs (reviewed in [10]). Expression of the genes for several of these proteins continues in either the hair matrix or dermal papilla consistent with later roles in forming the hair itself. The exact roles of these factors in hair development have not been determined but misexpression studies indicate that deregulated Bmp4 [11], Wnt [12] and Shh [13] signaling within the skin and hair follicle leads to highly abnormal development. Further, inappropriate activation of the Hedgehog pathway in skin appears to have a major role in the generation of basal cell carcinoma [13–16]. To further address the role of Shh in hair development, we have examined the hair follicles of Shh null mutant mice. We found that follicle development initiates correctly, but that it does not proceed normally. Although some elements of mature hair are present, the follicular structures are grossly abnormal and no hair is formed.

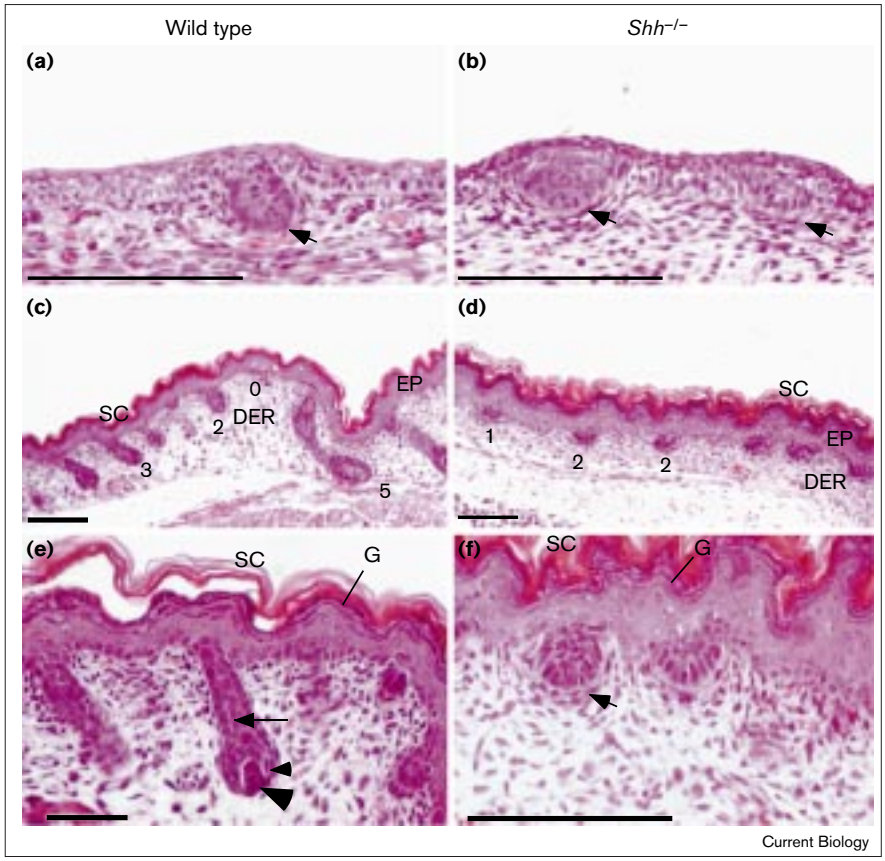
Results

Hair follicle development in *Shh* mutants

Skin development was examined in *Shh* mutants lacking exon 2, a region encoding over half of the highly conserved amino acid residues in the 19 kDa Shh signaling peptide. Embryos homozygous for this presumed null mutation were phenotypically similar to an earlier mutation also generated by gene targeting using embryonic stem cells [17]. Approximately half the expected number of mutant embryos survived to term but none lived beyond this time.

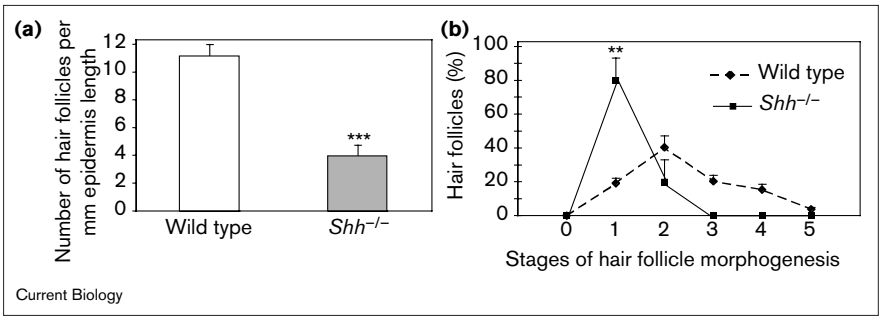
Those surviving to late gestation exhibited a completely penetrant hair follicle phenotype with no apparent variability in the sample analyzed. At 15.5 days post coitum (dpc), hair follicle development had begun on the back of the embryo. Epidermal cells formed a hair plug (stages 1 and 2) with a recognizable thickening of mesenchyme cells, the precursors of the dermal papilla, beneath this structure (Figure 1a). In *Shh* mutants the hair plug was smaller, but epidermal expansion into the dermis and dermal condensation of mesenchyme at the base of the hair plug were observed, indicating that the initial signaling events in folliculogenesis are independent of *Shh* (Figure 1b). However, by 18.5 dpc a dramatic difference was observed in hair development between *Shh* mutants and their wild-type siblings. Not only was the number of hair follicle anlagen in the skin reduced (Figure 2a), but their development was significantly retarded. In normal skin many hair follicles had initiated development of the inner root sheath from the hair matrix (stages 3–5 of follicular development; Figure 1c,1e,2b), but all hair follicles in *Shh* mutants had failed to progress beyond stage 2

Figure 1



Histological analysis of *Shh* mutant ($Shh^{-/-}$) hair follicles at 15.5 and 18.5 dpc. (a,b) Sections through 15.5 dpc hair follicles of (a) a wild-type embryo and of (b) a *Shh* mutant embryo. In the wild type, a stage 2 hair follicle is present whereas in the *Shh* mutant development is slightly delayed; only stage 1 and stage 0 hair follicles are visible. Dermal condensation (arrows) is present around both the wild-type and the mutant hair plugs. (c,d) Sections through 18.5 dpc skin of (c) wild-type and (d) *Shh* mutant embryos. Follicles have developed to stage 5 in wild-type skin, whereas only stages 1 and 2 are present in the mutant; numbers denote the stage of follicles above them according to Hardy [1]. Stratum corneum (SC), epidermis (EP) and dermis (DER) are present in both the wild type and the mutant. (e,f) High-power view of (e) wild-type and (f) mutant hair follicles and skin at 18.5 dpc. In the wild type, a stage 4/5 hair follicle with an inner root sheath (arrow), dermal papilla (large arrowhead) and hair matrix cells (small arrowhead) was apparent. In the mutant, a stage 2 hair follicle with epithelial invagination and dermal condensation was observed (arrow). Normal stratification of the skin was visible. Both wild type and mutant have a stratum corneum (SC), granulosa layer (G), and basal layer. Scale bars represent 0.1 mm.

Figure 2



Hair follicle morphogenesis is retarded in *Shh* mutant mice (18.5 dpc). (a) The number of hair follicles per mm of epidermal length in 8 μm thick cryosections of back skin from *Shh* null and wild-type mice was evaluated at 18.5 dpc. A dramatic decline in the number of induced hair follicles was found in *Shh* null mice, compared with wild-type animals (mean ± SEM; *** = $p < 0.001$). (b) The percentage of hair follicles at defined stages of morphogenesis was evaluated in hematoxylin and eosin stained paraffin-embedded skin sections, or in alkaline phosphatase stained cryostat sections (mean ± SEM; ** = $p < 0.01$).

(Figure 1d,1f,2b). Thus, loss of Shh signaling results in an apparent arrest of the embryonic hair follicle shortly after its induction.

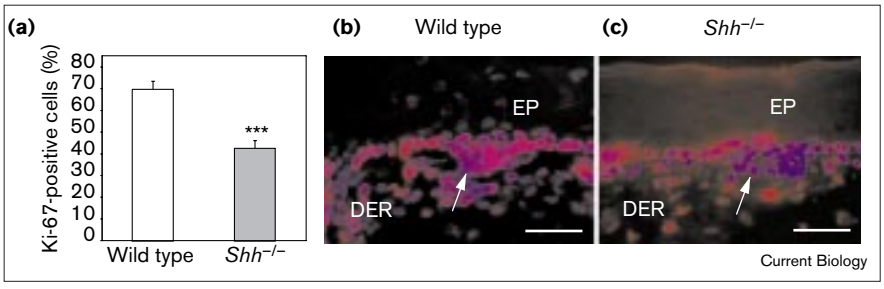
When the levels of matrix-associated proliferative antigen (Ki-67) were quantitated at 18.5 dpc as a measure of the proliferative index in follicles [18], Ki-67-positive cells were reduced by approximately 40% in *Shh* mutants (Figure 3). In contrast, no difference was observed in apoptosis as scored by quantitative terminal deoxynucleotidyl transferase mediated dUTP–biotin nick end labeling (TUNEL; [19] and data not shown). Together these results indicate that reduced proliferation in the ectoderm of the hair follicle may explain, at least in part, the arrest in follicle maturation. No differences in the thickness of the epidermis, or in the proportion of Ki-67-positive cells, were observed between the interfollicular epidermis of wild-type and *Shh* mutant siblings (data not shown). Thus, as might be expected from the follicle-restricted expression of *Shh*, the requirement for *Shh* in regulating proliferation is restricted to the hair follicle.

Cell interactions in hair follicles of *Shh* mutants

To determine how Shh signaling might act in regulating hair follicle development, we investigated the expression of a number of genes that serve as early markers of hair morphogenesis and of several others that are more directly implicated in Shh signaling. Formation of the epithelial hair bud is associated with the downregulation of keratin-14 expression in the hair placode [20] (Figure 4a). We observed a similar downregulation in the absence of *Shh* indicating that the initial modification of intermediate filament expression in the hair bud was independent of *Shh* activity (Figure 4b, arrow).

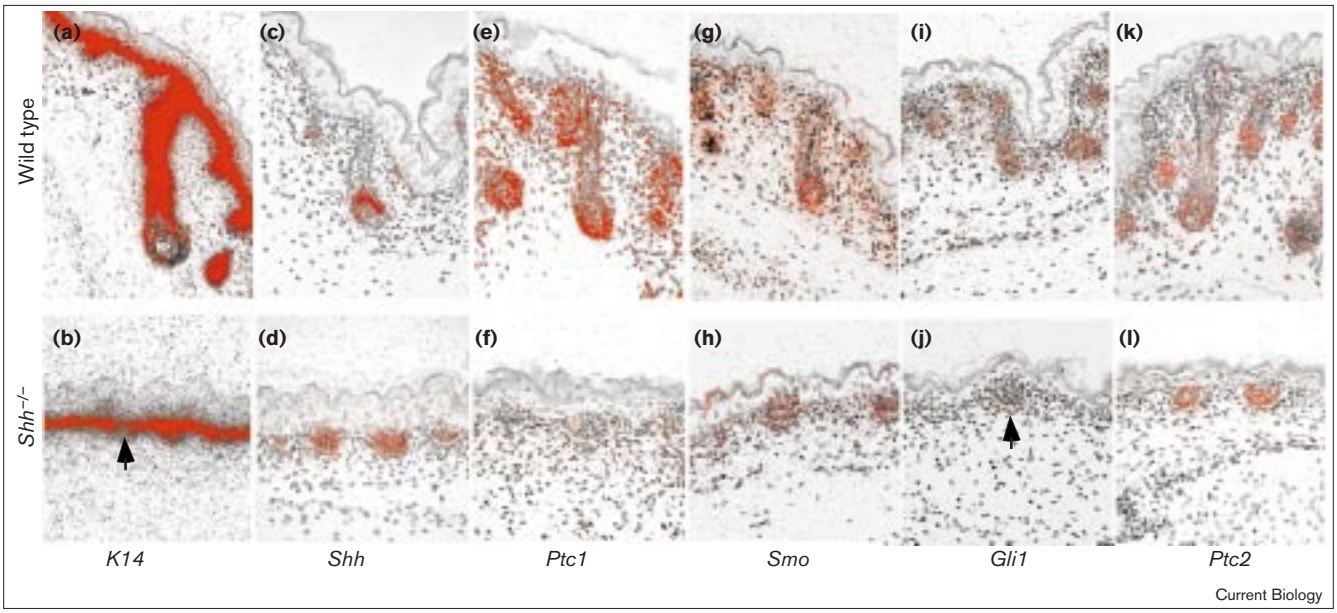
Hedgehog ligands are thought to interact with a receptor complex consisting of two multipass membrane proteins encoded by *Patched* (*Ptc1*) and *Smoothened* (*Smo*; for a review, see [21]). Hedgehog ligands are thought to bind Ptc1 and, in so doing, alleviate Ptc1-mediated repression of Smo, whose sequence bears homology to that of G-protein-coupled receptors. Recently, a second *Patched* gene (*Ptc2*) has been described in vertebrates but its properties have not been investigated [22,23]. Derepression of Smo results in the activation of transcriptional mediators of Hedgehog signaling, the Gli proteins, leading to transcription of Hedgehog target genes. Two general transcriptional targets of Hedgehog that are upregulated in a wide range of tissues are *Ptc1* and *Gli1* themselves [21]. Their activation is thought to modulate

Figure 3



The *Shh* null mutation selectively alters keratinocyte proliferation in developing hair follicle epithelium. (a) Percentage of proliferating cells in the hair follicle epithelium at stages 1–2 of hair follicle morphogenesis was evaluated in cryosections of wild-type and *Shh* mutant mice at 18.5 dpc, stained with antiserum against mouse Ki-67 and counterstained with Hoechst 33342 (mean ± SEM; *** = $p < 0.05$). (b) Ki-67 immunostaining in stage 1–2 hair follicles of wild-type skin; numerous proliferating cells are shown (arrow). (c) Ki-67 immunostaining in stage 1–2 hair follicles of *Shh* mutant skin. In contrast to wild-type skin, these developing hair follicles at stage 1–2 show a substantial decline of Ki-67-positive cells. DER, dermis; EP, epidermis. Scale bars represent 50 μm.

Figure 4



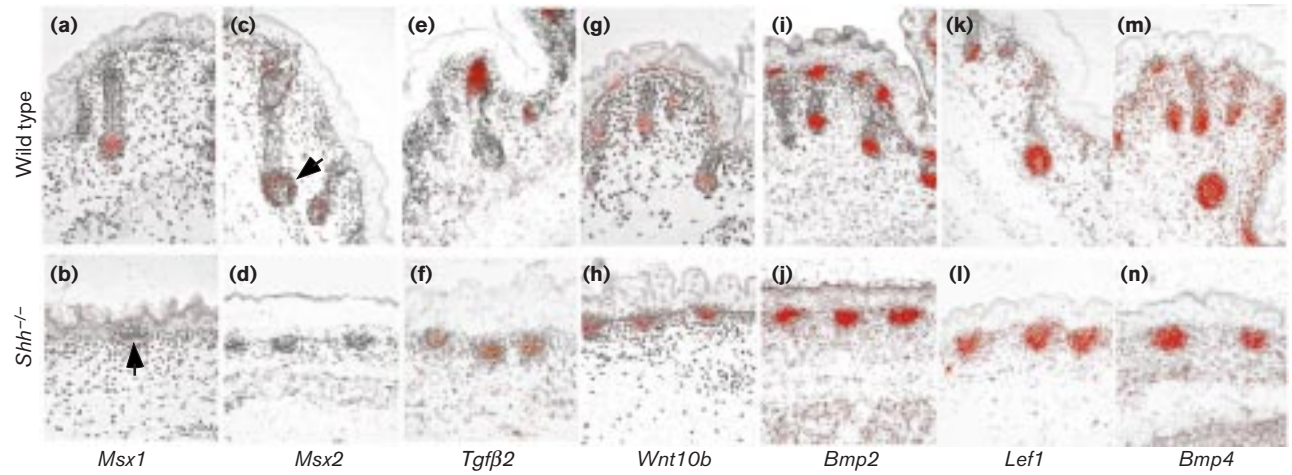
Analysis of gene expression in the skin of wild-type and *Shh* mutant embryos by *in situ* hybridization. Skin sections were analyzed at 18.5 dpc for expression of the genes indicated on the figure (where K14 corresponds to keratin-14). Regions that stain red correspond to regions that express the particular gene. Downregulation of keratin-14, which is associated with the formation of epithelial hair buds, is visible in the *Shh* mutant as shown by the arrow in (b). Weak *Gli1* expression is present in the dermal mesenchyme underlying the *Shh* mutant hair buds as shown by the arrow in (j).

signaling and gives an indication of which cells are directly responding to Hedgehog ligands.

In wild-type skin at 15.5 dpc we observed expression of *Ptc1*, *Smo*, *Gli1* and *Gli2* in both the epithelial and dermal components of the early hair follicle, although higher levels of *Ptc1* and *Gli1* were observed in dermal mesenchyme of the hair follicle (data not shown). In contrast, *Ptc2* expression was restricted to epithelial cells of the hair bud where it was coexpressed with *Shh* (data not shown, [23]). Thus, Shh may signal to either, or both, the epithelial or dermal component early in hair follicle development (stages 0–2). *Ptc1*, *Smo*, *Gli1* and *Gli2* were also detected at lower levels in keratinocytes and in the dermis of interfollicular regions consistent with the observation that these areas respond when *Shh* is ectopically expressed [13]. By 18.5 dpc, differentiation of the epidermally derived hair matrix cells is underway with the formation of the inner root sheath (stages 4–5). At these stages the major change was that *Ptc1* and *Gli1* expression became restricted to the dermal papilla and, to a lesser extent, the dermal sheath and hair matrix (Figure 4e,i). *Ptc2* continued to be coexpressed with *Shh* in hair matrix cells (Figure 4c,k). Lower levels were also present in the epithelium of the outer root sheath but no expression was observed in the dermal papilla (Figure 4k).

In contrast to wild-type follicles, those of *Shh* mutants showed only basal levels of *Ptc1* or *Gli1*, consistent with the general model that these genes are transcriptional targets of Hedgehog signaling (Figure 4f,j). *Shh*, *Smo* and *Ptc2* continued to be expressed at high levels in the hair plugs of mutants, however, indicating that their elevated expression in the epidermal component of the hair follicle was not dependent on *Shh* (Figure 4d,g,h,l). Weak expression of *Gli1* was observed in dermal mesenchyme underlying the hair plug even though no clear dermal papilla was formed, suggesting that some epithelial–mesenchymal interactions have taken place (Figure 4j, arrow). In agreement with this conclusion, *Msx1*, which is normally expressed exclusively in the dermal papilla at these stages (Figure 5a), was weakly expressed in the dermal mesenchyme of some hair follicles in *Shh* mutants (Figure 5b, arrow). In contrast, *Msx2*, which is normally expressed from stage 4 in conjunction with differentiation of the inner root sheath from hair matrix cells (Figure 5c, arrow), was not expressed in follicles of *Shh* mutants (Figure 5d). Thus, although there was some evidence of a weak dermal response in the hair follicle, this was not sufficient to trigger proximal epidermal cell differentiation by 18.5 dpc. In contrast, the gene encoding transforming growth factor β2 (*Tgfβ2*), which is normally activated in the distal epithelium of the follicle at this stage (Figure 5e), was expressed in *Shh* mutants (Figure 5f). Expression of *Tgfβ2* was observed throughout the hair bud of the mutant suggesting that *Shh* is required for the formation of proximal but not distal cell fates.

Figure 5



Analysis of gene expression in the skin of wild-type and *Shh* mutant embryos by *in situ* hybridization. Skin sections were analyzed at 18.5 dpc for expression of the genes indicated on the figure. Regions that stain red correspond to regions that express the particular gene. *Msx1* is weakly expressed in the dermal mesenchyme of the *Shh* mutant hair buds as shown by the arrow in (b). *Msx2* is expressed in the hair matrix cells of the wild-type hair bud as shown by the arrow in (c).

To determine how other signals, implicated from their expression in the regulation of folliculogenesis, may contribute to the phenotype, we examined the expression of Wnt and BMP family members in *Shh* mutants. Studies in *Drosophila* have demonstrated that the *Wnt1* orthologue, *Wingless*, and the *Bmp2/Bmp4* counterpart, *Decapentaplegic*, are transcriptional targets and, in the imaginal discs, long-range mediators of Hedgehog signaling [10]. In the wild-type hair follicle, *Wnt10b* and *Bmp2* showed a very similar pattern of expression to *Shh*. Their expression was initiated in the follicle epithelium at stage 0 and became localized to the hair matrix after epithelial ingrowth (Figure 5g,i). *Lef1*, a transcriptional effector of Wnt signaling that is required for later stages of hair morphogenesis [24] was upregulated in the early follicle epithelium and underlying mesenchyme and, after stage 3b, in their derivatives — the hair matrix and dermal papilla (Figure 5k, [24]). Finally, *Bmp4* was first activated in the dermal mesenchyme at stage 0. *Bmp4* expression continued in the dermal papilla, but was also observed in the hair matrix by stage 4 to 5 (Figure 5m).

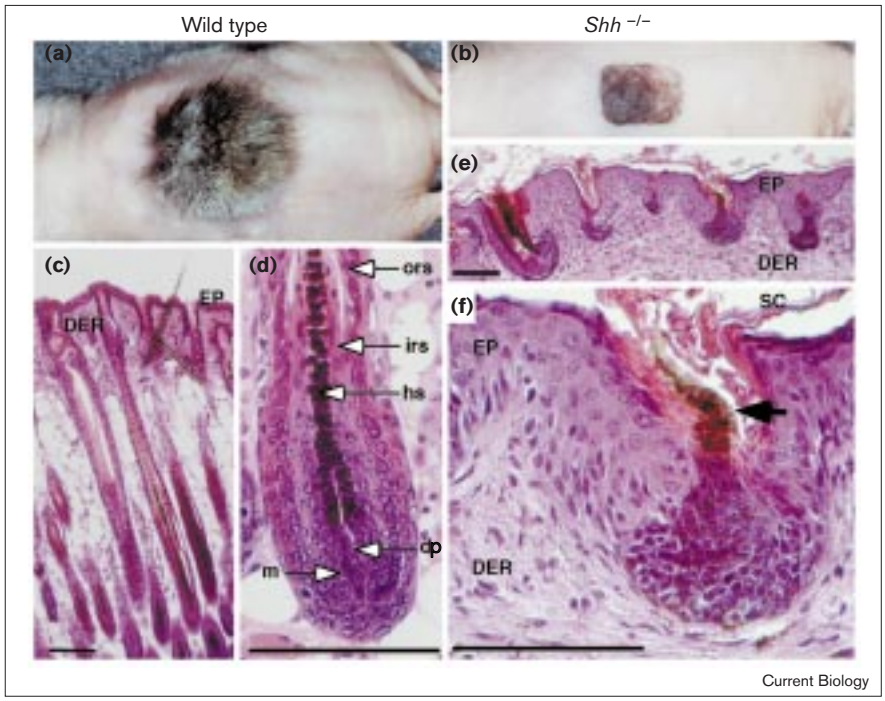
Interestingly, in *Shh* mutants, *Wnt10b*, *Bmp2*, *Lef1*, and *Bmp4* all showed correct spatial and temporal activation (data not shown). Moreover, their expression was maintained in the developmentally arrested hair follicles of mutants at 18.5 dpc (Figure 5h,j,l,n). Thus, Shh is not required for either the activation or the maintenance of these components of Wnt and BMP signaling in the hair follicle. From these results it seems unlikely that the observed phenotype results indirectly from the loss of these pathways. We did observe that *Bmp2* expression, which is normally restricted to and polarized within the tip of the ingrowing epidermis, was more broadly expressed throughout the epidermal component of the follicles of *Shh* mutants (Figure 5j). Thus, Hedgehog signaling could have a more subtle role in spatial refinement of signaling. Indeed, whereas wild-type follicles exhibit a typical polarized development along the anterior–posterior axis which prefigures the polarity of the hair in the adult coat, hair buds of *Shh* mutants did not seem to display an obvious polarity.

Hair follicle development in skin grafts

Although it is clear that hair follicle development is arrested at an early stage in *Shh* mutants, we cannot rule out that this arrest may not be permanent. Moreover, given the wide-ranging consequences of the loss of Shh signaling to development of many of the body's structures, there remained the possibility that some other systemic perturbation might contribute to the phenotype. To address these issues, we removed the skin (epidermis and dermis) from *Shh* mutant and wild-type siblings at 18.5 dpc and grafted it onto the back of *nude* mice (which lack T cells), and assayed development 2 and 3 weeks after grafting. All wild-type grafts (n = 11) and all but one from *Shh* mutants (n = 10) survived grafting. While all grafts of wild-type skin went on to form hair, all those from *Shh* mutants generated hairless, pigmented skin (Figure 6a,b).

Histological analysis demonstrated that, in contrast to wild-type skin grafts (Figure 6c,d), those from mutants were highly abnormal (Figure 6e,f). The most prominent

Figure 6



Skin grafts of wild-type and *Shh* mutant skin onto *nude* mice. (a,b) Photographs of grafted skin growing on the backs of *nude* mice. In a graft from a wild-type embryo (a), pigmented hair grows, whereas there is no hair growth in a graft from a mutant embryo (b). Pigmentation is present in both the wild-type and the mutant skin grafts. (c–f) Histological analysis of (c,d) wild-type and (e,f) mutant skin grafts. Normal hair growth and skin development proceed in wild-type skin grafts, whereas in the mutant grafts hair and skin development are both disrupted. A thickened epidermis with large disorganized ingrowths is present. There is formation of some keratinized pigmented hair-like material (arrow) distinct from the stratum corneum (SC). Abbreviations: dp, dermal papilla; DER, dermis; EP, epidermis; hs, hair shaft; irs, inner root sheath; m, matrix cells; ors, outer root sheath; SC, stratum corneum. Scale bars represent 0.1 mm.

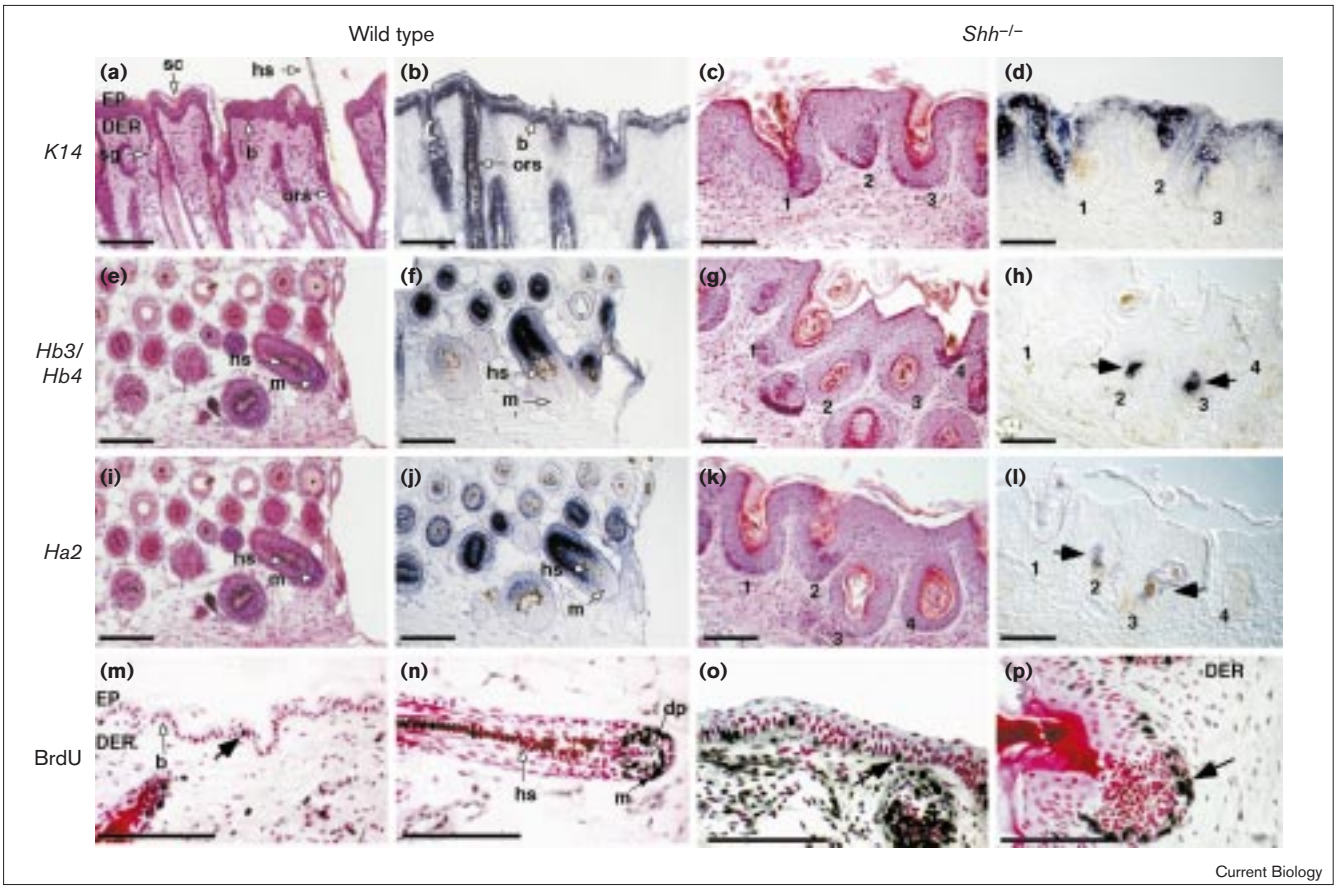
features were a thickened epidermis, a reduced dermal fat layer and large disorganized invaginations of the epidermis which lacked the normal epidermal lamination typical of the mature hair follicle (Figure 6e,f). The proximal region of these follicle-like structures displayed some evidence of hair matrix differentiation, including the formation of some keratinized material resembling hair matrix (Figure 6f, arrow), but no hair was formed.

At this stage *Shh* is normally expressed in the hair matrix (data not shown). In mutants, weak *Shh* transcription (the allele is transcribed but nonfunctional) indicated that some *Shh*-expressing cells were present in the follicles of *Shh* mutant skin grafts (data not shown). Expression of the gene for keratin-14, a marker of basal cells in the skin and outer root sheath in the follicle [20] (Figure 7a,b) extended into the abnormal follicles (Figure 7c,d). Surprisingly, the keratin-14 gene was not expressed in the most basal epidermal cells of the skin and follicle but in suprabasal cells instead (Figure 7d). The keratin-10 gene was also abnormally expressed in the granular layer and stratum corneum (data not shown). Thus, in the absence of Shh-mediated regulation, normal folliculogenesis and skin development was disrupted.

To examine epidermal differentiation within the hair follicle, we examined expression of a type II keratin (*Hb3* or *Hb4*) which is specific to the cortex of the hair (Figure 7e,f) [25] and *Ha2*, a type I keratin whose expression is normally restricted to the hair cuticle (Figure 7i,j) [26]. Both genes were expressed in *Shh* mutant follicles (Figure 7g,h,k,l, arrows). We also observed melanin-containing cells in the central part of the hair bud (Figure 8c), a characteristic of proximal hair follicle epithelium which is not normally observed in interfollicular or distal hair follicle epithelium [27]. Thus, some differentiation of hair matrix cells occurred in the absence of Shh, but this was not sufficient to generate an organized hair.

Hair follicle development requires proliferation by hair matrix precursors to generate the post-mitotic epidermal populations of the inner root sheath and hair [1,2]. To determine whether an absence of proliferation might underlie the failure in hair formation in *Shh* mutants, we monitored bromodeoxyuridine (BrdU) incorporation into wild-type and mutant grafts. Interestingly, proximal epithelial cells in the follicles of *Shh* mutants showed high rates of proliferation (Figure 7p) comparable to those observed in the germinative epithelium surrounding the dermal papilla in wild-type skin grafts (Figure 7n). Thus, an absence of proliferation at this stage is unlikely to account for the failure in hair development. Surprisingly, we observed higher rates of BrdU incorporation amongst basal keratinocytes in the skin and follicles of *Shh* mutants (Figure 7o) than in wild-type grafts (Figure 7m) indicating an upregulation of proliferation in the skin. As proliferation and differentiation of the hair matrix is thought to be controlled by interactions with the dermal papilla, these

Figure 7



Examination of gene expression and proliferation in skin grafts. (a,b) In wild-type grafts keratin-14 is expressed in the basal layer of the skin and in the outer root sheath. (c,d) In *Shh* mutant skin grafts, keratin-14 is abnormally expressed in the suprabasal layer, as well as in follicular ingrowths. Expression is not observed in the basal epidermal layer or in the proximal epithelium of the follicles. (e–h) Expression of *Hb3* or *Hb4*, a type II keratin, in the cortex of the wild-type hair (e,f) and in the abnormal follicular structures of the mutant (g,h) as shown by the arrows in (h). (i–l) Expression of *Ha2*, a type I keratin, in the hair cuticle of the wild-type hair (i,j) and weak expression of *Ha2* in the abnormal follicular structures of the mutant (k,l) as shown by the arrows in (l). Hematoxylin and eosin stained sections are shown (a,c,e,g,i,k) with *in situ* hybridizations using probes for the indicated genes in (b,d,f,h,j,l). (m,n) BrdU incorporation into (m) basal keratinocytes (arrow) and into (n) the germinative hair matrix cells of the hair follicles in the wild type. (o) Increase in incorporation of BrdU amongst basal keratinocytes in the thickened *Shh* mutant skin (arrow). (p) Incorporation of BrdU in epithelial cells at the periphery of the base of the abnormal follicles (arrow). Abbreviations: b, basal keratinocytes; DER, dermis; dp, dermal papilla; EP, epidermis; hs, hair shaft; m, matrix cells, ors, outer root sheath; sg, sebaceous gland; sc, stratum corneum. Scale bars represent 0.1 mm.

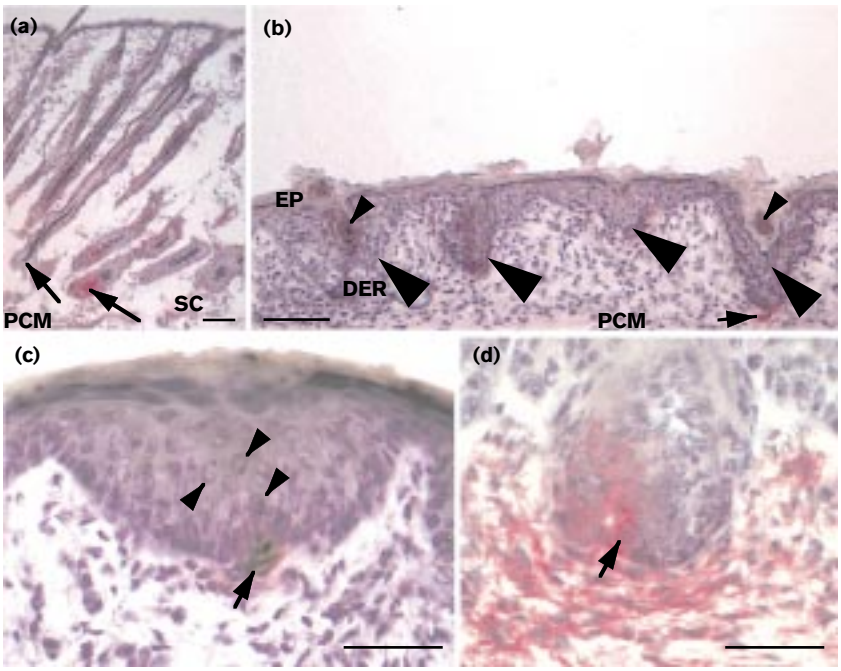
results suggest that dermal papilla cells were most likely present in the hair follicles of *Shh* mutant skin grafts. Indeed we detected small groups of dermal cells positive for alkaline phosphatase (Figure 8a–c), neural cell-adhesion molecule (NCAM; Figure 8d) and the p75 neurotrophin receptor (data not shown), all of which are markers of dermal papilla fibroblasts [28]. These data indicate that a rudimentary dermal papilla was present, consistent with the analysis of late embryonic skin.

Discussion

We have explored the role of *Shh* in mouse skin. We find that *Shh* is not required for hair placode formation or initial ingrowth of the epidermis but is essential for the formation of hair. Thus, Shh signaling is a critical component of the signaling pathways that govern hair follicle morphogenesis.

Examination of skin in *Shh* mutants just before birth indicated that hair follicle formation appeared to be arrested after the formation of the hair bud. *Shh* is normally expressed in the proximal tip of the ingrowing epithelium of the hair follicle where interactions with the underlying mesenchyme of the dermal papilla are thought to occur [6,13]. The decreased proliferative activity in the follicle at this early stage, the absence of proximal epithelial differentiation, as judged by the failure to detect *Msx2* expression, and the identification of only a rudimentary dermal papilla, all indicate that epithelial–mesenchymal

Figure 8



Grafts of *Shh* mutant skin transplanted onto *nude* mice show development of giant hair-bud-like structures and evidence of dermal papilla formation. (a) All hair follicles in wild-type grafts have completed their morphogenesis and displayed their first catagen development (arrows point to narrowing hair matrix as an indicator of early catagen development [19]). (b) Thickening of epidermis and formation of numerous giant hair-bud-like structures (large arrowheads), some with hair-shaft-like material (small arrowheads), were found in *Shh* mutant grafts. Note the alkaline phosphatase positive dermal-papilla-like cells associated with one of the hair buds (arrow); alkaline phosphatase positivity is a typical feature of dermal papilla fibroblasts as opposed to non-follicle-associated dermal fibroblasts [28]. (c) Cluster of alkaline phosphatase positive mesenchymal cells (arrow) becomes embedded into the proximal part of the epithelial hair bud in a *Shh* mutant skin graft. Note the melanin-containing cells (arrowheads) in the central part of the hair bud. (d) Numerous NCAM-positive cells are located in dermis around the hair bud, while some of NCAM-positive fibroblasts (arrow) are surrounded by hair bud keratinocytes. Abbreviations: DER, dermis; EP, epidermis; PCM, panniculus carnosus muscle; SC, subcutis. Scale bar represents 50 μm.

interactions are disrupted in *Shh* mutants. As two general Shh targets, *Ptc1* and *Gli1* are normally expressed at highest levels in the dermal papilla, it is reasonable to speculate that *Shh* may regulate early folliculogenesis through this cell population. Yet, as some dermal papilla cells appear to form and condense around the hair bud in the absence of *Shh*, it is unlikely that Shh has a role in inducing these cells. However, *Shh* could be required for their subsequent expansion. In this respect, Hedgehog signaling has been implicated in the regulation of proliferation in other aspects of embryonic development [10]. Further, in chick skin where *Shh* expression in the epidermis is implicated in an analogous process of feather morphogenesis, ectopic expression of Shh results in a dramatically expanded dermal mesenchymal population consistent with a model in which Shh may play a mitogenic role [29]. The continued expression of *Bmp2*, *Bmp4* and *Wnt10b* in the epidermis of hair buds in *Shh* mutants suggests that one or more of these factors may play a role in dermal papilla induction. The finding that ectopic expression of *Lef1*, a transcriptional mediator of Wnt signaling [30], can lead to ectopic hair formation [12] implicates Wnt signals, but hair induction is very limited in these experiments, suggesting the requirement for other factors. Whatever the role of these *Bmps* and *Wnt10b*, our data demonstrate that *Shh* is not required for activation or maintenance of their expression unlike many aspects of epithelial patterning regulated by their counterparts in the *Drosophila* embryo [10].

As expected when Shh signaling is absent, expression of *Ptc1* and *Gli1* returns to basal levels. In contrast, high levels of *Ptc2* are maintained in the follicle epithelium in the absence of Shh signaling. Although the exact role of this gene has not been determined, our results indicate that the simple model in which all *Ptc* genes are transcriptional targets of Hedgehog signaling is not likely to be correct.

Although examination of skin in *Shh* mutants at 18.5 dpc indicated that epidermal development of the hair follicle was arrested shortly after the ingrowth (stage 2), grafting studies demonstrated that the epithelium can undergo further development. When skin was grafted to *nude* mice and analyzed 3 weeks later, large disorganized superficial follicles were present throughout the skin graft. The superficial nature of the follicles is consistent with the failure of normal proximal–distal elongation of the hair follicle observed at embryonic stages. Thus, unlike wild-type skin where extensive ingrowth of the epithelial component of the follicle leads to deep placement of the hair bulb in the

dermis, in *Shh* mutants a hair-bulb-like population remains in close association with the epidermis of the skin.

Several lines of evidence indicate that *Shh* mutants form a hair-bulb-like structure despite the fact that its mor phology (convex epithelium in mutant, concave in wild type) and position is highly abnormal. Hair matrix cells in the hair bulb show very high proliferative rates, generating post-mitotic daughter cells which move distally up the hair shaft differentiating into specific components of the hair or inner root sheath [1,2]. In *Shh* mutants, epithelial cells at the proximal limit of the follicle-like ingrowths exhibit comparable rates of proliferative activity. Above these cells are histologically distinct, post-mitotic cells which show evidence of differentiation into specific components of the hair (cuticle and cortex), and in some cases hair-shaft-like material. Finally, we observe evidence of proximally located dermal papilla cells in association with the abnormal follicles. Thus, although *Shh* activity at this time is neither essential for proliferation, nor for some aspects of differentiation, the failure to form hair indicates an essential role for *Shh* in morphogenesis of the hair. Whether the absence of hair reflects a role for Shh signaling in the control of morphogenetic interactions that take place between the germinative cells of the hair bud and the dermal papilla after birth, or whether it reflects the disruption of the early stages of follicle morphogenesis, is unclear. Distinguishing between these alternative explanations will require the development of a more refined genetic analysis, for example the use of post-natal tissue-specific gene targeting.

Interestingly, abnormalities in skin development were not restricted to the hair follicles in grafts of *Shh* mutant skin; we observed hyperplasia and abnormal keratin expression in the interfollicular epidermis. Hyperplasia and tumor formations have been associated with inappropriate activation of the Shh pathway in the epidermis [13–16]. Consequently the hyperplasia observed in the absence of signaling is surprising. Proliferation is localized, as in wild-type skin, to keratinocytes in the basal layer which normally express keratin-14. Although proliferation is appropriately restricted to basal cells in *Shh* mutants, more cells appear to be undergoing DNA synthesis and keratin-14-expressing cells are observed in suprabasal positions in the abnormally thickened epidermis. Although the significance of the change in keratin expression is unclear, both observations indicate that interfollicular skin development is abnormal. It is doubtful that these results reflect a normal role for Shh, a protein that is tethered at the cell surface [31–33], in long-range interactions in the skin. Rather, the phenotype may relate to the abnormal juxtaposition and poor physical separation between hair follicle and skin following abnormal morphogenesis of the hair shaft in *Shh* mutants. Alternatively, we observed that *Smo*, which encodes the signal-transducing component of the Hedgehog receptor, remained upregulated in the epidermis of the *Shh* mutant follicles which lack Ptc1, a repressor of Smo activity [21]. If Ptc2 does not modulate Smo function, it is possible that deregulated Smo activity, which can lead to basal cell carcinoma [16], may contribute to the observed epidermal phenotype.

Conclusions

We provide evidence that Shh signaling is required for growth and morphogenesis of the hair follicle. Hair follicle induction is independent of *Shh*, but *Shh* is essential for ingrowth of the epidermis and consequently for morphogenesis of the hair shaft. The resulting superficially localized hair follicles are capable of localized growth and differentiation, which is reminiscent of hair matrix cells in the hair bulb, but they are unable to form a hair. This raises the possibility that localized Shh signaling in the hair bulb may control later aspects of hair morphogenesis. Our results provide compelling evidence that *Shh* has a pivotal role in the cell–cell interactions that govern hair development.

Materials and methods

Generation of a Shh *mutant*

Generation of a *Shh* null mutant will be described more fully elsewhere. Briefly, exon 2 was deleted by gene targeting in the R1 embryonic stem (ES) cell line. The targeted alleles from two independently isolated ES cell lines were transmitted through the germline. Embryos homozygous for these had identical phenotypes, similar to those previously documented for a similar likely null allele [17]. The mutant allele was maintained on a mixed (129/Sv; C57BL6/J; CBA/J) background.

Histology

Tissues were either fresh frozen or fixed overnight in 4% paraformaldehyde in PBS, 10% neutral-buffered formalin or Bouin's fixative depending on the application. Cryostat sections were prepared for immunohistochemistry. Wax sections were prepared for *in situ* hybridization and histology, and counterstained with either hematoxylin and eosin or toluidine blue.

In situ *hybridization*

In situ hybridization using ^{35}S-labeled riboprobes was essentially carried out as described [34]. Digoxygenin *in situ* hybridization to skin sections was performed according to published procedures with the probes indicated in the text [35].

Immunohistochemistry

Ki-67 immunoreactivity was studied in acetone-fixed or paraformaldehyde-fixed cryostat sections (8 μm) of fetal back skin of *Shh* mutant ($n = 4$) and wild-type mice ($n = 4$, 18.5 dpc), using rabbit antiserum against murine nuclear matrix-associated proliferation-related antigen (Ki-67; 1:100, Dianova) and TRITC-conjugated goat anti-rabbit or goat anti-rat IgG as secondary antibodies (1:200; Jackson ImmunoResearch), as described in [18]. Sections were counterstained by Hoechst 33342 (Sigma; 10 mg/ml in TBS, 30 min, room temperature) for identification of cell nuclei [19]. For quantitative histomorphometry, the percentage of Ki-67-positive cells in epidermis was determined by counting at least 2500–3000 cells each in wild-type and mutant skin in > 30 microscopic fields, derived from four animals per group. For analysis of the percentage of Ki-67-positive cells in the developing hair placodes, 40–50 hair follicles at stages 1 and 2 of hair follicle morphogenesis were analyzed in four *Shh* mutant and four wild-type mice at E18.5. NCAM and p75NTR immunoreactivity were assessed using rat anti-mouse monoclonal antibodies by the avidin–biotin method as described [19].

To evaluate apoptotic cells, we used an established, commercially available TUNEL kit (ApopTag) [19]. Negative controls for the TUNEL staining were made by omitting TdT, according to the manufacturer's protocol. Positive TUNEL controls were run, as described [36], by comparison with tissue sections from the thymus of young mice, which display a high degree of spontaneous thymocyte apoptosis [19]. After washing in TBS, all sections were mounted with Immunomount medium (Shandon). Sections were examined under a Zeiss Axioscope microscope, using the appropriate excitation–emission filter systems for detecting the fluorescence induced by Hoechst 33342, FITC or TRITC. Photodocumentation was done with the help of a digital image analysis system (ISIS Metasystems). BrdU incorporation into graft tissue was performed as described [37].

Quantitative histomorphometry

The number of hair follicles per unit length of epidermis was calculated in skin cryostat and paraffin sections of *Shh* mutant skin ($n = 5$) at 18.5 dpc, and was compared with that of age-matched wild-type controls ($n = 5$). To be sure that each assessed microscopic field contained new hair follicles, only every tenth section from each animal was analyzed. The percentage of hair follicles in different stages of morphogenesis was assessed and defined on the basis of accepted morphologic criteria [1,2]. In order to identify the defined substages of hair follicle morphogenesis as precisely as possible, in addition to hematoxylin–eosin staining, histochemical detection of endogenous alkaline phosphatase activity [27] was used in some sections, as this highlights the developing dermal papilla as a useful morphological marker for staging hair follicle development and cycling [2]. At least 60–80 longitudinal hair follicle sections in 50–60 microscopic fields derived from five *Shh* mutant animals were analyzed and compared to those of 200–250 hair follicles from five age-matched wild-type mice. Thickness of interfollicular epidermis was assessed in hematoxylin–eosin stained cryostat sections of five *Shh* mutant and five wild-type littermates at 18.5 dpc. At least 100 measurements from 2–3 sections per animal were performed and compared with wild-type skin using digital image analysis (ISIS Metasystems).

All sections were analyzed at 200× magnification; means and SEM were calculated from pooled data. Differences were judged as significant if the p value was < 0.05, as determined by the independent Student's t test for unpaired samples.

Skin grafts

Skin grafts were performed using a method modified from published procedures [36–39]. Full-thickness skin (epidermis and dermis) approximately 10×10 mm in size was removed from the back of 18.5 dpc *Shh* mutants and normal littermates and placed onto back skin excisions of a similar size (up to but not including the panniculus carnosus) in 8–10 week old female *nude* mice anesthetized with 0.35–0.4 ml 0.1 M tribromoethanol. Grafts were protected by covering with vaseline gauze and silicon transplantation chambers. The chambers were removed 4–5 days after grafting and grafts biopsied 2 weeks and harvested 3 weeks after grafting.

Supplementary material

A supplementary figure showing gene expression patterns in the wild-type and mutant hair follicles at 15.5 dpc is published with this paper on the internet.

Acknowledgements

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Supplementary material

Sonic hedgehog signaling is essential for hair development

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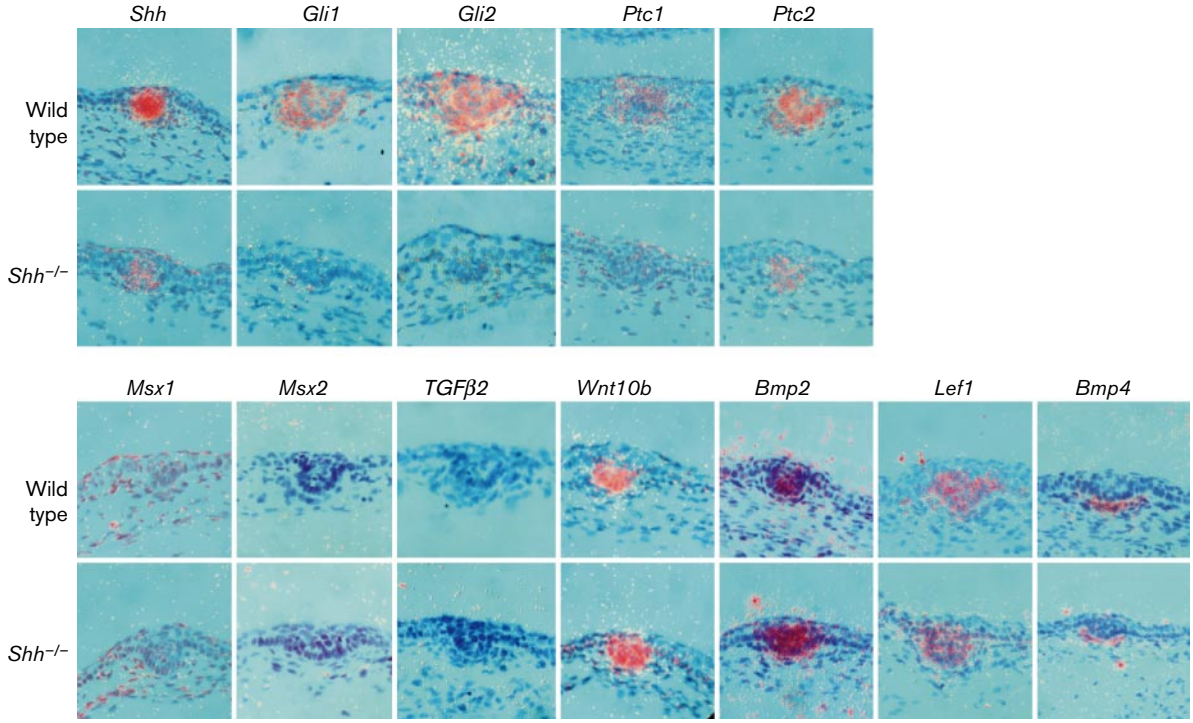
Figure S1



Expression patterns of *Shh*, *Gli1*, *Gli2*, *Ptc1*, *Ptc2*, *Msx1*, *Msx2*, *TGFβ2*, *Wnt10b*, *Bmp2*, *Lef1* and *Bmp4* in the wild-type and the mutant hair follicles at 15.5 dpc. *Shh, Ptc2, Wnt10b* and *Bmp2* are expressed in the epithelium of both wild-type and mutant hair follicles. *Lef1* is expressed in the epithelial and dermal components of the wild-type and mutant hair follicles. *Gli1, Gli2* and *Ptc1* are expressed in the epithelial and the dermal components of the wild-type hair follicle, but are downregulated to basal levels in the mutant follicles. *Bmp4* is expressed in the dermal component of the wild-type and mutant hair follicles. *Msx1, Msx2* and *TGFβ2* are not expressed in wild-type or mutant follicles.