Conditional Disruption of Hedgehog Signaling Pathway Defines its Critical Role in Hair Development and Regeneration

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Members of the vertebrate hedgehog family (Sonic, Indian, and Desert) have been shown to be essential for the development of various organ systems, including neural, somite, limb, skeletal, and for male gonad morphogenesis. Sonic hedgehog and its cognate receptor Patched are expressed in the epithelial and/or mesenchymal cell components of the hair follicle. Recent studies have demonstrated an essential role for this pathway in hair development in the skin of Sonic hedgehog null embryos. We have further explored the role of the hedgehog pathway using anti-hedgehog blocking monoclonal antibodies to treat pregnant mice at different stages of gestation and have generated viable offspring that lack body coat hair. Histologic analysis revealed the presence of ectodermal placode and primodium of dermal papilla in these mice, yet the subsequent hair shaft formation was inhibited. In contrast, the vibrissae (whisker) development appears to be unaffected upon anti-hedgehog blocking monoclonal antibody treatment. Strikingly, inhibition of body coat hair morphogenesis also was observed in mice treated postnatally with anti-hedgehog monoclonal antibody during the growing (anagen) phase of the hair cycle. The hairless phenotype was reversible upon suspension of monoclonal antibody treatment. Taken together, our results underscore a direct role of the Sonic hedgehog signaling pathway in embryonic hair follicle development as well as in subsequent hair cycles in young and adult mice. Our system of generating an inducible and reversible hairless phenotype by anti-hedgehog monoclonal antibody treatment will be valuable for studying the regulation and mechanism of hair regeneration. ***Key words: anti-hedgehog antibody/hair follicle/hair morphogenesis/maternal transfer. J Invest Dermatol 114:901–908, 2000***

Members of the hedgehog (hh) gene family encode secreted signaling proteins that have been implicated in various developmental processes from fly to vertebrate. Significantly, mutations in the human hh gene and genes encoding its downstream intracellular signaling pathway result in diseases including holoprosencephaly, tumors, and postaxial polydactyly (Ming *et al*, 1998). Signaling by the hh was first described and most well studied in *Drosophila* (Nusslein-Volhard *et al*, 1980; Basler and Struhl, 1994; Diaz-Benjumea *et al*, 1994; Zecca *et al*, 1995; Blair and Ralston, 1997) where various molecules which participate in the signaling pathway have been identified: these include the cell surface coreceptors Patched (Ptc), a 12-pass transmembrane protein (Ingham *et al*, 1991; Marigo *et al*, 1996; Stone *et al*, 1996) and Smoothened (Smo), a seven-pass transmembrane, receptor-type protein (Alcedo *et al*, 1996; van den Heuvel and Ingham, 1996). It has been suggested that binding of hh to Ptc releases an inhibitory effect on Smo, which in turn leads to dissociation of a cytosolic complex composed of Fused (Fu), a serine/threonine kinase, Costal2 (Cos2), a kinesin-related protein, and Cubitus interruptus (Ci), a zinc finger-containing transcription factor. Following dissociation of the complex, Ci, is activated, moves to the nucleus and triggers expression of various target genes including Ptc, Decapentaplegic (Dpp), and Wingless (Wg) (Hammerschmidt *et al*, 1997; Ingham, 1998).

Three members of the hh family have been identified in vertebrates, they are Sonic, Indian, and Desert hh. Each of these proteins plays a unique part in the development of the central nervous system (Echelard *et al*, 1993; Roelink *et al*, 1994), chondrogenesis (Vortkamp *et al*, 1996), and spermatogenesis (Bitgood *et al*, 1996), respectively. The vertebrate homologs of Ptc (Ptc-1 and Ptc-2) (Carpenter *et al*, 1998; Motoyama *et al*, 1998a), Smo (Smo) (Akiyama *et al*, 1997), and Ci (Gli-1, Gli-2, and Gli-3) (Hui *et al*, 1994) also have been identified and were shown to be involved in the early development of neural tube, lung, gut, tooth, and skeletogenesis by knock-out and transgenic studies (Goodrich *et al*, 1997; Mo *et al*, 1997; Yang *et al*, 1997; Ding *et al*, 1998; Hardcastle *et al*, 1998; Litingtung *et al*, 1998; Motoyama *et al*, 1998b). Shh is the only hh member that was found to be expressed in the hair follicles (Iseki *et al*, 1996) and recently, two studies have demonstrated an essential role for Shh in hair follicle development (St-Jacques *et al*, 1998; Chiang *et al*, 1999). The early death of these genetically modified mice during embryogenesis or at birth, however, precludes analysis of the role of the Shh signaling

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Abbreviation: hh, hedgehog.

pathway in organ development in postnatal and adult mice. We have taken an independent approach employing maternal transfer of anti-hh monoclonal antibody (MoAb) to developing embryos and were able to generate viable offspring which lack body coat hair, supporting the absolute requirement of the Shh signaling pathway specifically in vertebrate body coat hair development. Furthermore, we have conditionally disrupted the hh pathway by postnatal administration of anti-hh MoAb, thereby establishing the Shh requirement for hair regeneration during different hair growth cycles in juvenile and adult animals.

MATERIALS AND METHODS

Antibodies The generation of anti-hh MoAb 5E1 was described previously (Ericson *et al*, 1996) and the hybridoma cell line was obtained from Ontogeny (Cambridge, MA) and Dr. Tom Jessell (Columbia University, NY). Antibodies AP.G6 and AC.D1 were generated at Biogen (Cambridge, MA). Briefly, the human N-terminal portion of the Shh (amino acids 23–197) was fused to the human IgG1 Fc domain and the resulting fusion protein (Shh-Ig) was stably expressed in Chinese hamster ovary cells. Mice were immunized three times with the subsequently purified Shh-Ig (50 μg per i.p. injection) coupled to protein A. Spleen cells were fused to FL653 myeloma cells and selected as previously described (Lerner, 1981). Monoclonal supernatants were screened for binding to human recombinant Sonic, Indian, or Desert hh N-terminal protein in enzyme-linked immunosorbent assay. Briefly, plates were coated overnight with relevant hh N-terminal protein (1 μg per ml), after which various anti-hh MoAb (500 ng per ml) were added and incubated for 90 min at room temperature, following incubation with peroxidase-conjugated goat antimouse antibody, the plates were developed with tetramethylbenzidine (TMB) (Sigma, St Louis, MO) and the absorbance were read at 450 nm. Levels of absorbance that were between 0 and 0.05 were assigned as "–", > 0.05–0.1 as "+/–", > 0.1–0.5 as "+", > 0.5–1.0 as "++", and > 1.0–1.5 as "+++" (**Table I**). The anti-hh MoAb were assessed for their ability to inhibit the hh-induced differentiation of C3H10T1/2 cells to bone lineage as described previously (Kinto *et al*, 1997; Pepinsky *et al*, 1998). All three antibodies also cross-react with mouse hh proteins (data not shown). 1E6, a mouse anti-human LFA-3 IgG1 antibody was used as an isotype matched control in this study (Majeau *et al*, 1994).

Mice and injection Time pregnant C57BL/6 or BALB/c mice were either purchased (Jackson Lab, Bar Harbor, Maine) or matings were set up in the mouse facility at Biogen Inc. The pregnant mice were injected with antibodies once every 2 d (6 mg per kg) by i.v injection. The subsequently born offspring continued to receive injection (3 mg per kg) once every 2 d intraperitoneally (i.p.) until the time of killing, or alternatively, the injections in some littermates were suspended after birth or at later stages as indicated in *Results*. The data shown in this study were obtained from BALB/C mice; however, identical phenotypes were observed in C57BL/6 mice.

For injections of young and adult mice, 3 wk old and 9 wk old mice were chosen (at the telogen phase of the hair cycle) (see *Results*). The mice were anesthetized with Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) and their back hair was shaved. These mice received MoAb injections i.p. (3 mg per kg for 3 wk old and 6 mg per kg for 9 wk old) immediately after shaving and injections were continued every 2 d for 21 d. In a separate experiment, 3 wk old (postnatal day 21 or P21) white mice (OF1, IffaCredo, France) received MoAb injection, depilated at P22 and injected MoAb at P23, P25, and P27. The mice were killed at P29. The skin samples were collected and were used for immunohistochemistry and *in situ* analysis.

Immunohistochemistry and *in situ* analysis Dorsal skin samples were harvested and cryosection (8 μm thickness) were prepared. For detection of injected MoAb in the hair follicle, sections were fixed in acetone for 10 min, then 5% H_2O_2 in Tris-buffered saline for 5 min. The sections were incubated for 30 min with rabbit anti-mouse immunoglobulin (DAKO, Carpinteria, CA), washed with Tris-buffered saline, and incubated for 30 min with mouse monoclonal PAP antibody (DAKO); the sections were then washed, developed with diaminobenzidine, and counterstained with hematoxylin. For staining of trichohyalin, samples were incubated with anti-trichohyalin antibody, AE15 (O'Guin *et al*, 1992), for 1 h at room temperature, washed in phosphate-buffered saline, then was incubated for 30 min at room temperature with fluorescein isothiocyanate conjugated rabbit anti-mouse IgG antibody (DAKO) and viewed under microscope equipped with epifluorescence (Zeiss Axioskop, Alto Instrument, MD). The *in situ* analysis were performed as described (Le Novere *et al*, 1996), frozen sections were hybridized with ^{33}P-labeled oligonucleotides of genes of interests:

MHKA1a:5′-AAGTCGTCTGCAGCCAGCTTGGACTTATCTATC-TGCACCACCA-AC-3′
MHKA1b:5′-TTTTTGTGGAACCAGAAGCAGGTCAGCCATCTG-GAGTGCTAAG-CC-3′
Hacl-1 : 5′-GCAAGCTCCCTCTTTCAGAGACACAAAAAGCAGAG-GCCAGGCAAT-3′
Hairless:5′-CGTGCACCAGGATACTGATTAGGTCAGACACCTCC-ACGCATAGAT-3′
Whn:5′-CCTCTGAGTGTAAGGCACATGATGAGCAGGTGTGTA-GAGCTTGAG-3′
Lef-1 : 5′-TCGCTTCGGTTTTCCTTCTGGAGGAGCATTTAATCT-GCTGGAGGG-3′

Histology Mice were killed, the skin removed and fixed in formalin. Paraffin-embedded sections of 5 μm thickness were prepared and hematoxylin and eosin staining was performed.

RESULTS

Disruption of hedgehog signaling pathway affect hair morphogenesis Maternal antibody treatment during embryogenesis has been employed to study the role of cell surface and secreted molecules during ontogeny (Rennert *et al*, 1996; Zoller *et al*, 1997). We took advantage of this approach to investigate the role of Shh proteins in hair follicle initiation and subsequent hair formation. Body coat hair development in mice has been shown to initiate at embryonic day 13.5 (E13.5) (Hardy, 1968; Godwin and Capecchi, 1998), hence, E12.5 pregnant mice were injected with anti-hh and control MoAb. Both the 5E1 and AP.G6 MoAb bind specifically to hh family members (**Table I**) and block their activity *in vitro* (**Fig 1**) (Ericson *et al*, 1996; Pepinsky *et al*, 1998). An irrelevant isotype-matched MoAb 1E6 and the MoAb

Table I. Anti-hedgehog monoclonal antibodies

mAb	Binding activity[a]		
	Shh	Ihh	Dhh
5E1	+++	+++	+
AP.G6	+++	+++	+/–
AC.D1	+++	++	+
1E6	–	–	–

[a]See *Materials and Methods*.

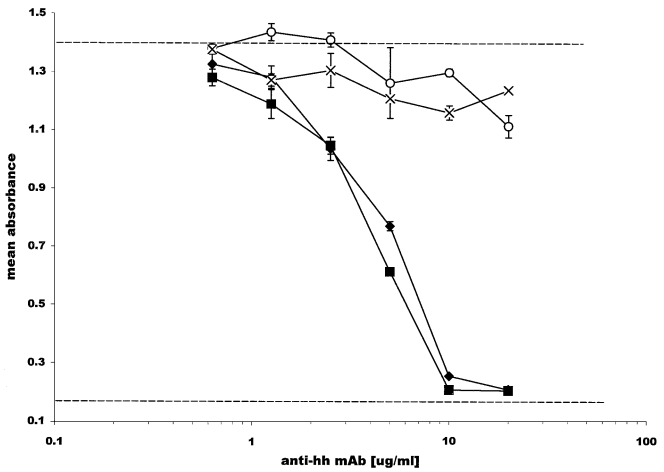

Figure 1. Anti-hh MoAb block Shh-induced differentiation of C3H10T1/2 cells. Serial 2-fold dilutions of the anti-hh MoAb 5E1 (n), AP.G6 (⧫), AC.D1 (X), and control MoAb 1E6 (¡) were incubated with the C3H10T1/2 cells and the Shh proteins (2 μg per ml) for 5 d and the resulting levels of alkaline phosphatase activity measured at 405 nm using the AP chromogenic substrate *p*-nitrophenyl phosphate. Mean absorbance of adding Shh protein alone (upper dotted line) or without any added proteins (lower dotted line) in the cells was also indicated.

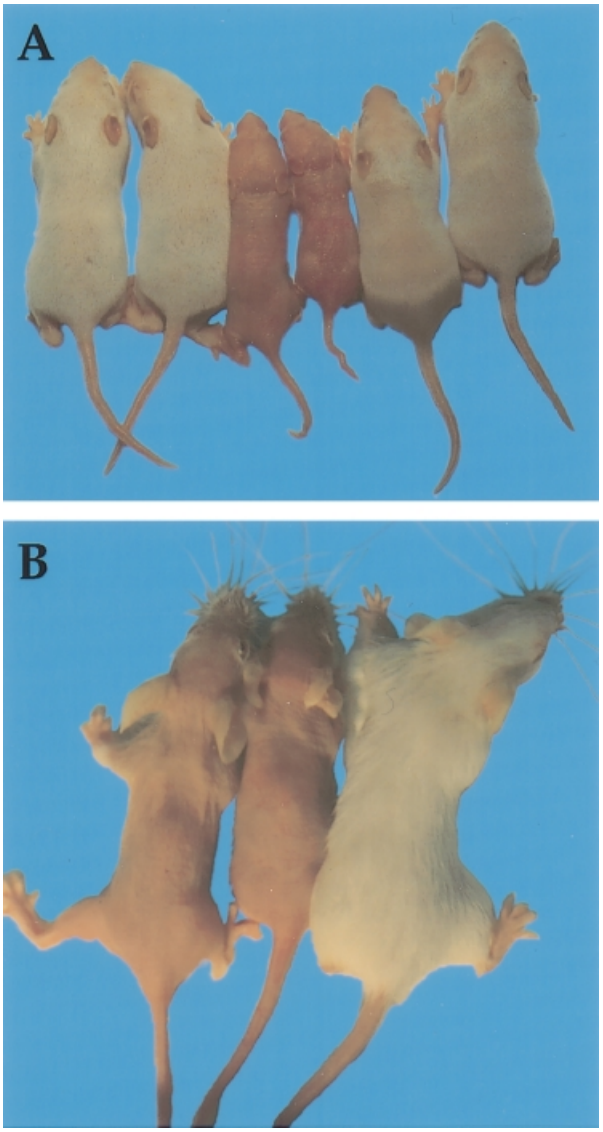

Figure 2. Lack of body coat hair formation in anti-hh MoAb-treated mice. (*A*) Representative offspring from pregnant mice (n = 3 for each MoAb) injected starting E12.5 of gestation with control MoAb, 1E6 (two mice on the left), nonblocking anti-hh MoAb, AC.D1 (two mice on the right) or blocking anti-hh MoAb AP.G6 (two mice at the center). Each pregnant mice gave rise to five to eight offspring with identical phenotype within the same MoAb injection. Mice are shown here at P12. Whiskers are present in these mice but do not show clearly in this figure. (*B*) Maintenance of the hairless body coat phenotype in mice which received continuous injection of AP.G6 (two mice on the left) at 5 wk old. Mice continuously treated with control MoAb 1E6 are shown on the right. Note the presence of whiskers in AP.G6-treated mice (see *Discussion*).

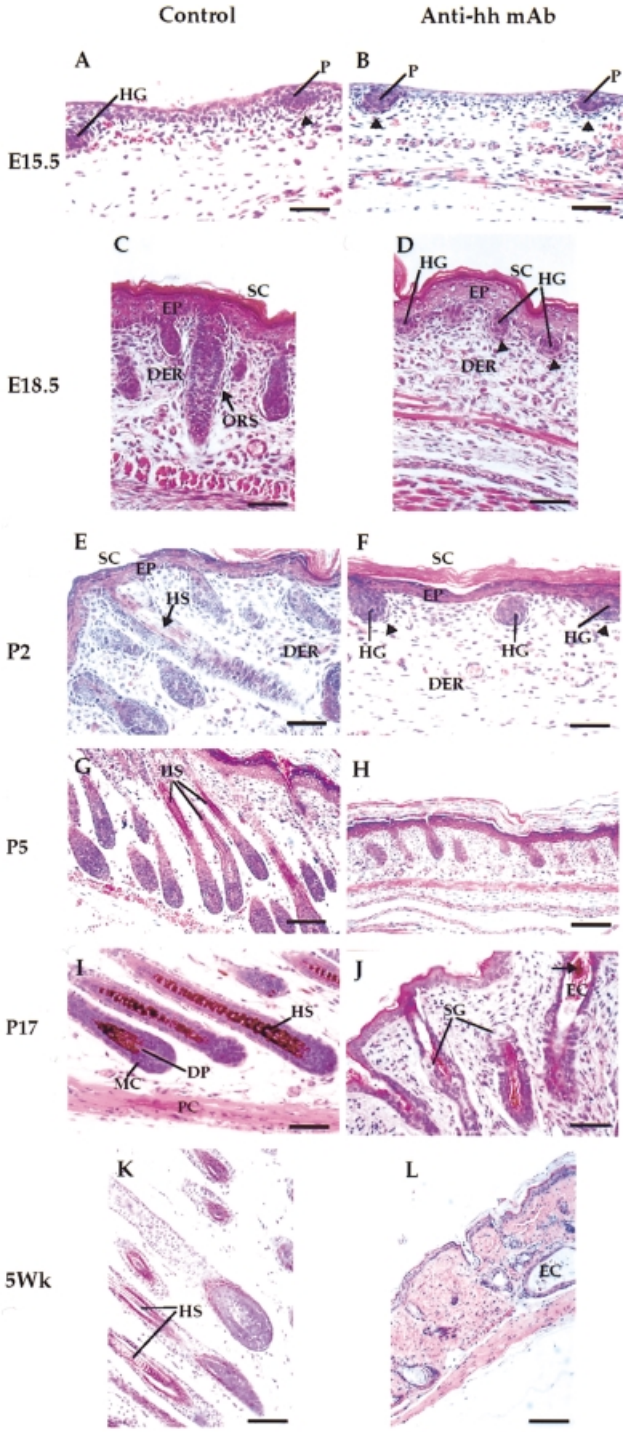

Figure 3. Inhibition of hair follicle morphogenesis but not initiation in blocking anti-hh MoAb-treated mice. Progression of hair follicle development in control 1E6 (*A*, *C*, *E*, *G*, *I*, *K*) and blocking anti-hh MoAb-treated mice (*B*, *D*, *F*, *H*, *J*, *L*) from E15.5 to postnatal (P) and 5 wk (Wk) old. At least three mice were examined at each developmental stage and similar results were observed. *Arrowheads* in (*A*), (*B*), (*D*), and (*F*) indicate dermal condensate. P, (ectodermal) placode; HG, hair germ; SC, stratum corneum; EP, epidermis; DER, dermis; ORS, outer root sheath; HS, hair shaft; DP, dermal papilla; MC, matrix cells; PC, panniculus carnosus; SG, sebaceous gland; EC, epidermal-like cyst. *Arrows* in *J* indicate hair shaft-like material. *Scale bar*: 50 μm.

AC.D1, which binds hh proteins but does not block the activity of hh *in vitro* were used as controls (**Table I**). The concentration of the injected MoAb was measured in the serum of the newborn mice and was in the range of 30 μg per ml for 5E1 and APG-6 and at 80 μg per ml for 1E6 treated offspring.

In contrast to the Shh knockout mice, which die just before or at birth (Chiang *et al*, 1996), offspring of the 5E1 and AP.G6 MoAb-treated mice were viable at least until the first week after birth. 5E1 MoAb-treated neonates died within the first week after birth with continued 5E1 treatment. On the contrary, the AP.G6 MoAb-treated mice were born normal in size during the first to third weeks but gradually became runted upon continued hh blocking antibody treatment (**Fig 2*A***). The reason for the longer lifespan of the AP.G6 MoAb-treated mice is unclear; this and the cause of the lethality and runting in these mice are being investigated.

Interestingly, an obvious and striking feature of these mice is that they lack body coat hair (**Fig 2*A***) and maintain this hairless phenotype with continued anti-hh MoAb injection (**Fig 2*B***). Histologic analysis of the 5E1-treated mice at different stages of

development reveal the appearance of the epithelial cell-derived ectodermal placode and dermal condensation of mesenchymal cells (primodium of dermal papilla) at the base of the placode at E15.5 comparable with that of the control mice (**Fig 3*A*, *B***). These data indicate that hh is dispensable for the initiation of folliculogenesis. At E18.5, the epithelial cells further grew downward into dermis and underwent hair morphogenesis to form the inner and outer root sheath in control mice (**Fig 3*C*, *E***) (Arnold *et al*, 1990). In contrast, the placodes of the 5E1 MoAb-treated mice only developed further to the hair germ stage (**Fig 3*D***) and remained at this stage at P2 (**Fig 3*F***). The 5E1-treated mice were severely runted after day 2; therefore, we examined the subsequent progression of hair morphogenesis in AP.G6 MoAb-treated mice. As shown in **Fig 3**(***H***), at P5, the ectodermal placode from AP.G6 MoAb-treated mice grew further downward into dermis as compared with 5E1 MoAb-treated mice at day 2 (**Fig 3*F***); however, the progression of the downward growth is significantly inhibited as compared with the control MoAb-treated mice at day 5 (**Fig 3*G***). The development of the interfollicular epidermis appears to be unaffected. By P17 and by 5 wk of age, the hair follicles from AP.G6 MoAb-treated mice began to differentiate, but instead of forming hair, numerous epidermal-like cyst structure enclosed with hair-shaft-like material were observed (**Fig 3*J*, *L***). The data indicate that Shh is not required for the initiation of folliculogenesis but is indispensable for the subsequent process of hair morphogenesis to form actual hair.

hh signaling pathway is also required for hair growth in young and adult mice The hairless phenotype seen in the prenatal anti-hh MoAb-treated mice indicate that Shh is involved in the earliest hair-growth (anagen) phase during embryonic development. Mouse hair cycles are more or less synchronized and each hair follicle goes through three distinct phases: growing phase (anagen); transitional phase (catagen); and resting phase (telogen) (Arnold, 1990). The first postnatal hair cycle is approximately 3 wk long whereas the second hair cycle is longer than 6 wk due to extended telogen phase (Miller *et al*, 1993). To assess whether the hh pathway is involved in the initiation of the anagen phase of subsequent hair cycles in older mice, anti-hh MoAb were administered postnatally to mice that were at the end stage of the first (3 wk old) and second (9 wk old) telogen phase of the hair growth cycle. Histologic analysis confirmed the regression of hair growth into foreshortened hair follicles, indicative of telogen phase of hair cycle in both 3 and 9 wk old mice (**Fig 4*B*** and data not shown) (Arnold, 1990). To highlight the effect on new hair growth, the back hair of these mice was shaved just prior to anti-hh MoAb treatment (day 0). The results show that the hair of the shaved area in control MoAb-treated mice grew back completely after 21 d post-treatment (**Fig 4*A***, upper row). In contrast, there is no hair in the corresponding shaved area of anti-hh MoAb 5E1-treated mice in both the 3 wk old and 9 wk old treatment groups at day 21 post-treatment (**Fig 4*A***, bottom row and data not shown). Consistent with the gross phenotype, skin sections of control mice revealed new hair follicle morphogenesis with hair shaft formation at day 21 post-treatment (**Fig 4*C***), whereas 5E1 MoAb-treated mice displayed foreshortened hair follicles and failed to complete the next new hair growth (anagen) cycle upon disruption of the hh signaling pathway (**Fig 4*D***). We did notice, however, the appearance of a hair bulb-like structure at the base of each foreshortened follicle in 5E1 MoAb-treated mice at day 21 post-treatment (**Fig 4*D***) that was not observed at day 0 of treatment (**Fig 4*B***). The formation of this structure most likely resulted from the interaction between the presumptive epithelial stem cells and the underlying mesenchymal cells, i.e., the first inductive event of a new hair growth cycle (Cotsarelis *et al*, 1990; Kobayashi *et al*, 1993; Rochat *et al*, 1994). Similar observations were also found in depilated mice treated with 5E1, in which hair morphogenesis was inhibited despite formation of hair bulb-like structures (**Fig 5*A*, *B***). Importantly, the defect in hair morphogenesis appears to be a direct result of 5E1 inhibition of the Shh/Ptc signaling pathway as

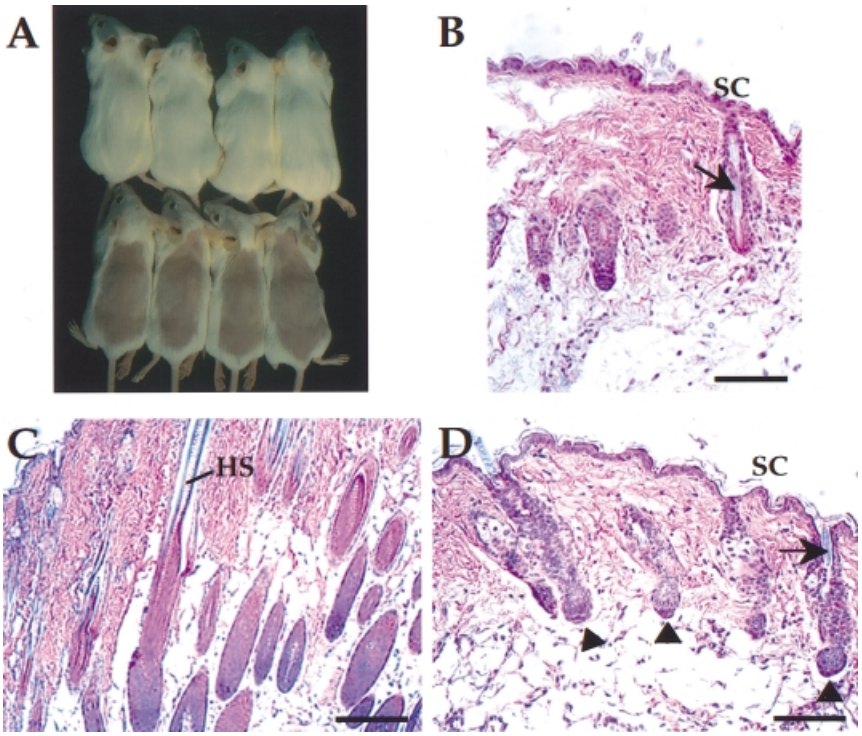

Figure 4. hh signaling pathway is required for hair growth in young and adult mice. (*A*) Three week old mice which were at the telogen phase of the hair cycle (see *Materials and Methods*) were treated with blocking anti-hh MoAb, 5E1 (n = 5), or control, 1E6 (n = 5). Note the failure to regenerate new hair-growth in 5E1 MoAb-treated (*bottom row*) but not control 1E6 MoAb-treated mice (*upper row*). Identical results were observed in mice treated at 9 wk old (data not shown). (*B*) Histology of hair follicle in shaved 3 wk old mice prior to MoAb treatment (day 0) and (*C*) control MoAb 1E6-treated mice after 21 d of treatment and (*D*) blocking anti-hh MoAb 5E1-treated mice after 21 d of treatment. *Arrows* in *B* and *D* indicate club hair (40). *Arrowheads* in *D* highlight the newly formed hair bulb structures. *Scale bars*: (*B*) 100 μm; (*C*, *D*) 200 μm.

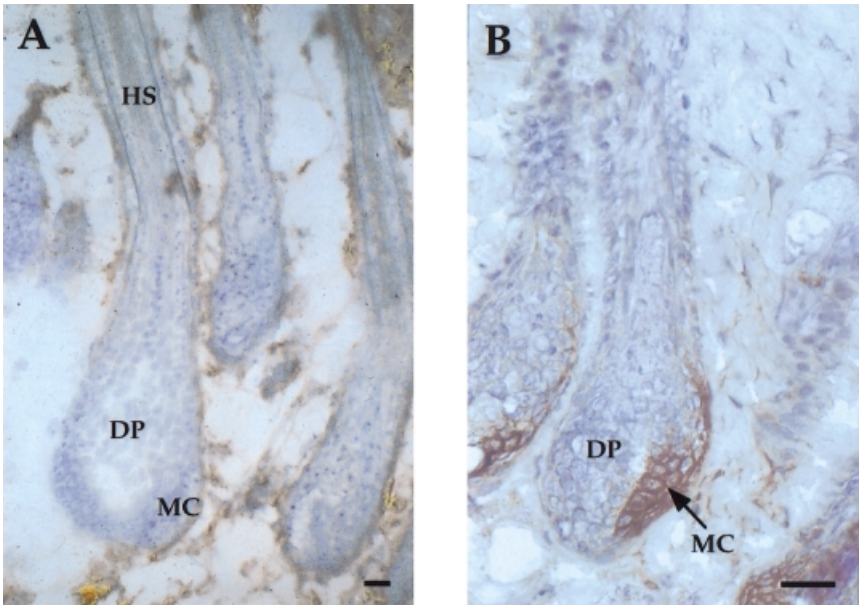

Figure 5. Specific bindings of anti-hh MoAb to hair follicles. Three week old mice were depilated and injected with (*A*) control MoAb 1E6, n = 6 or (*B*) anti-hh MoAb 5E1, n = 10, as described in *Materials and Methods*. Note the lack of elongated hair shaft (HS) in 5E1-treated hair follicle (*B*) as compared with (*A*). Also note that the presence of 5E1 bindings to matrix cells (MC and *arrow*) was only detected in 5E1 MoAb-treated hair follicles (*B*). DP: dermal papilla. *Scale bars*: (*A*, *B*) 25 μm.

evidenced by the binding of 5E1 MoAb in the region of epithelial cell-derived hair follicle matrix cells where Shh is known to be expressed (**Fig 5*A*, *B***) (Gat *et al*, 1998; Gambardella *et al*, 2000). These hair bulb-like structures, which are normally observed in early anagen phase of a normal hair cycle, were further characterized for expression of several cellular markers known to express in the hair follicles, for example, the transcription factors whn-1 (**Fig 6*A*, *B***), hairless (**Fig 6*C*, *D***), lef-1 (**Fig 6*E*, *F***), the hair keratins hacl-1 (**Fig 6*G*, *H***), MHKA-1 (**Fig 6*I*, *J***), and the keratin intermediate filament associated protein (trichohyalin) (**Fig 6*K*, *L***). The expression patterns of these cellular markers are as expected for normal hair follicles (**Fig 6**, *left panel*) and for regenerating hair-bulbs in early anagen phase (**Fig 6**, *right panel*) (St-Jacques *et al*, 1998; Gambardella *et al*, 2000) therefore results from these two independent analyses (**Fig 3*B***) indicate that the hh signaling pathway also is not required for the initiation of new hair follicles in subsequent hair growth cycles of older mice but is absolutely required for hair follicle morphogenesis to form new hair. Taken together, these data further demonstrate that in addition to the essential role of the hh pathway in neonatal hair formation, it also is required for hair growth (anagen) phases of the hair cycle in juvenile and adult mice.

The hairless phenotype is restored upon suspension of anti-hh MoAb treatment Our data indicate that hh proteins serve as an important factor in hair morphogenesis. To address whether the endogenous hh proteins can reverse the hairless phenotype, MoAb treatments were withdrawn in some pups which had received antibodies prenatally. Macroscopically, mice in which MoAb treatments were withdrawn immediately after birth exhibited intermediate hair growth at P12 as compared with littermates continuously treated with anti-hh MoAb and the control MoAb-treated mice (**Fig 7*A***). The delay in hair growth seen in the mice withdrawn from MoAb treatment is most likely due to the blockade of hair follicle development as a result of the prenatal antibody treatment. Indeed, by 5 wk of age, there is no discernible difference in the hair development between these mice and the control mice at both macroscopic and histologic levels (**Fig 7*B***). The reversibility of hair growth also is observed in mice which underwent continuous anti-hh treatment until 2 and 8 wk of age and were subsequently suspended of further treatment. (data not shown). These results indicate that the previously affected hair follicles were released from the blocked status and resume complete hair morphogenesis as soon as the functional endogenous hh signaling pathway was reactivated. Thus, our data underscore a direct involvement of hh signaling pathway in hair formation.

DISCUSSION

In summary, by injecting anti-hh MoAb into pregnant mice that subsequently crosses the placenta and yolk sac to developing embryos, we have generated a unique mouse model to study hh biology. We demonstrate in this study the absolute requirement of hh signaling pathway in hair growth in the embryonic, young, and adult mice. Our finding is consistent with the expression patterns of Shh and its receptor Ptc in the developing hair follicle (Iseki *et al*, 1996; St-Jacques *et al*, 1998) and the fact that we detected the presence of anti-hh MoAb in epithelial cells that make Shh (**Fig 5*B***) indicates that the defect observed is primary to the anti-hh MoAb blocking in hair follicles. Surprisingly, although both molecules are expressed in cell types which participate in the earliest stages of hair follicle initiation, the MoAb treatment did not inhibit the initiation of the hair follicle differentiation programs. Given the fact that a similar finding was reported recently in Shh knock-out mice (St-Jacques *et al*, 1998; Chiang *et al*, 1999), it is unlikely that the failure to inhibit hair follicle initiation observed in this system is due to incomplete blocking of the anti-hh MoAb. Thus, these data suggest that other signaling pathways are involved in the early process of the hair growth. Our data do however, indicate that hh signaling is required for further hair follicle expansion and maturation during embryonic development. We hypothesized that hh produced by epithelial (matrix) cells induces proliferation and differentiation of the mesenchymal dermal condensate into mature dermal papilla cells. In embryos exposed to the anti-hh blocking MoAb, hh binding to Ptc is interrupted and the expansion and maturation of the dermal condensate is arrested. These data are consistent with the inability of Shh knockout mice to develop mature hair follicles due to impaired expansion of dermal papilla cells (Chiang *et al*, 1999; St-Jacques *et al*, 1998). Because the cellular processes of the growth (anagen) phase of the hair cycle in postnatal mice resembles that of the embryonic skin, we also tested the effect of anti-hh MoAb in blocking hair regeneration in juvenile and adult mice. We showed that, as in the embryonic skin, hedgehog signaling pathway is not required for the initiation of hair growth but is absolutely required for the formation of actual hair and thus hair

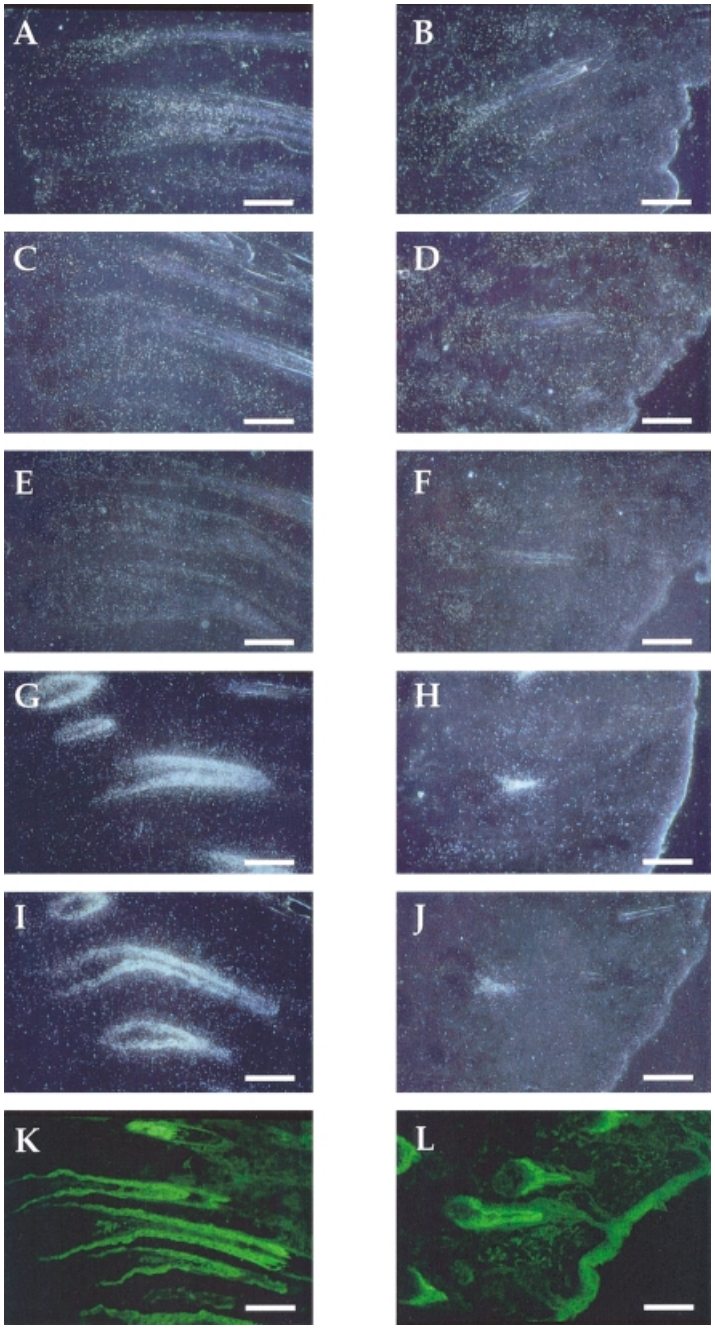

Figure 6. Expression of hair follicle markers in the hair bulbs of anti-hh MoAb-treated mice. *In situ* or immunohistochemistry analysis were performed on control 1E6 (*left panel*) and anti-hh MoAb 5E1 (*right panel*) treated skin sections. (*A*, *B*) Expression of whn-1 in the cuticle, the cortex, and the medulla of the hair. (*C*, *D*) Expression of hairless in the epithelial cells of the hair bulbs and the outer root sheath. (*E*, *F*) Expression of lef-1 in the upper portion of the dermal papilla, in the cuticle, the cortex, and the medulla of hair and in the cuticle of the inner root sheath. (*G*, *H*) Expression of hacl-1 and (*I*, *J*) MHKA-1 in the hair cortex and (*K*, *L*) expression of trichohyalin in the inner root sheath and the medulla of the hair. *Scale bars*: 100 μm.

regeneration. A complementary finding has been reported recently in which the transient expression of Shh in skin leads to hair growth in postnatal mice (Sato *et al*, 1999). In addition to blocking hair follicle maturation, our system allowed us to test the reversibility of inhibiting Shh signaling during hair follicle development. Removal of the blocking MoAb following birth releases the arrest of hair follicle development and follicles mature normally, suggesting that responsiveness to this pathway is preserved during the arrest period. Thus, the development of hair follicle is independent of other developmental events and pathways which occurred during the hh MoAb-mediated arrest of hair follicle development.

In addition to Shh, several other signaling pathways also have been shown to be involved in the hair or feather development, these include fibroblast growth factors, bone morphogenetic proteins (BMP), Notch-1/Delta-1 and Wnt signaling pathways. The interrelationships of molecules and targets involved in these pathways and that of the Shh pathway have been investigated (Oro and Scott, 1998). For example, expression of members of the Wnt (Wnt 10b) and Bmp (Bmp2/Bmp4) family were not affected in Shh null mice (St-Jacques *et al*, 1998). In addition, mice overexpressing epidermal promoter controlled β-catenin which associates with lymphoid enhancer factor 1 (Lef-1/Tcf) and functions in the Wnt/*wingless* pathway exhibit *de novo* hair follicle morphogenesis and hair tumors (Gat *et al*, 1998). Interestingly, expression of Shh was aberrant in the skin of these mice correlating with misangling of the *de novo* hair follicles and formation of hair tumors. Collectively, these results suggest that Shh activation is most likely downstream of the Bmp or Wnt-Lef/Tcf-β-catenin pathway. In addition to the utility of the loss or gain-of-function systems in these studies, our mouse model of conditional disruption of the Shh pathway will add to the understanding of these interactions in hair development.

In addition to being expressed in body coat hair follicles, Shh is also expressed in developing vibrissae (whisker) follicles (Iseki *et al*, 1996). Shh knockout mice manifest severe craniofacial defects, thus precluding the analysis of the hh signaling in vibrissae development (St-Jacques *et al*, 1998). Recent *in vitro* analysis in E13.5 vibrissae

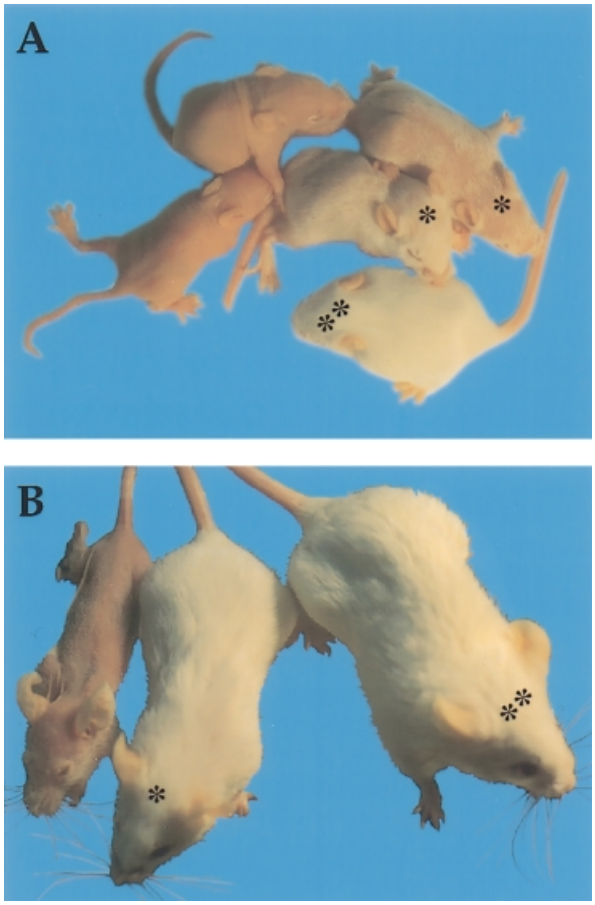

Figure 7. Suspension of anti-hh MoAb treatment restores hair growth. E12.5 pregnant mice were treated with either blocking anti-hh MoAb, AP.G6 (n = 2), or control MoAb, 1E6 (n = 2), by maternal transfer (see *Materials and Methods*). Starting at P0, half the pups (n = 6) from each litter were treated with the same MoAb received *in utero* and half (n = 6) were given no injection. Representative mice are shown at P12 (*A*) and 5 wk old (*B*). Mice treated *in utero* and subsequently with AP.G6 are unmarked. Mice treated only *in utero* with AP.G6 are indicated with a single asterisk. Note the restoration of body coat hair growth in these mice. Mice treated *in utero* and subsequently with control MoAb 1E6 are indicated with a double asterisk.

explant cultures demonstrated impairment of vibrissae follicle morphogenesis upon cyclopamine treatment to block shh signaling (Chiang *et al*, 1999). As anti-hh MoAb-treated mice have normal craniofacial development, we assessed vibrissae development in these mice. Surprisingly, the vibrissae development in the anti-hh MoAb-treated mice that were injected from E12.5 of gestation appear to be indistinguishable from control MoAb-treated mice both macroscopically (**Fig 2*B***) and histologically (data not shown) suggesting Shh signaling is not required for vibrissae development *in vivo*. Therefore, unlike Hoxc-13 (Godwin and Capecchi, 1998) and Lef-1/Tcf (van den Heuvel and Ingham, 1996; Zhou *et al*, 1995), which are required for the development of body coat hair as well as vibrissae, the hh signaling pathway appears to be specifically required for body coat hair morphogenesis.

Taken together, the generation of the inducible and reversible hairless phenotype observed in these mice is especially valuable for studying the regulation and mechanism of the mouse hair growth cycle mediated by the hh signaling pathway. Our application of the maternal administration method using anti-hh MoAb into developing embryos provides rapid assessment of the role of hh pathway in various organ development. This system also allows for conditional disruption of the hh signaling pathway at various stages of development, and therefore allows for the assessment of this pathway's role in the maintenance of cell proliferation, differentiation, survival, and/or function of many organs in embryonic, young, and adult mice.

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REFERENCES

Akiyama H, Shigeno C, Hiraki YH, *et al*: Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5. *Biochem Biophys Res Commun* 235:142–147, 1997

Alcedo J, Von Ayzenzon M, Ohlen T, Noll M, Hooper JE: The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* 86:221–232, 1996

Arnold HL, Odom RB, James WD: Disease of the skin, clinical dermatology. In: *The Skin: Basic Structure and Function*. Philadephia: WB Saunders, 1990, pp 1–13

Basler K, Struhl G: Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein [see comments]. *Nature* 368:208–214, 1994

Bitgood MJ, Shen L, McMahon AP: Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr Biol* 6:298–304, 1996

Blair SS, Ralston A: Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of *Drosophila*. *Development* 124:4053–4063, 1997

Carpenter D, Stone DM, Brush J, *et al*: Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc Natl Acad Sci USA* 95:13630–13634, 1998

Chiang C, Litingtung Y, Lee E, *et al*: Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383:407–413, 1996

Chiang C, Swan RZ, Grachtchouk M, *et al*: Essential role for sonic hedgehog during hair follicle morphogenesis. *Dev Biol* 205:1–9, 1999

Cotsarelis G, Sun TT, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61:1329–1337, 1990

Diaz-Benjumea FJ, Cohen B, Cohen SM: Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs [see comments]. *Nature* 372:175–179, 1994

Ding Q, Motoyama J, Gasca S, *et al*: Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* 125:2533–2543, 1998

Echelard Y, Epstein DJ, St-Jacques B, *et al*: Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417–1430, 1993

Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM: Two critical periods of Sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* 87:661–673, 1996

Gambardella L, Schneider-Maunoury S, Voiculescu O, Charnay P, Barrandon Y: Dynamic pattern of expression of the transcription factor Krox-20 in mouse hair follicle: implications for hair growth. *Mech Dev* 2000, in press

Gat U, DasGupta R, Degenstein L, Fuchs E: De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell* 95:605–614, 1998

Godwin AR, Capecchi MR: Hoxc13 mutant mice lack external hair. *Genes Dev* 12:11–20, 1998

Goodrich LV, Milenkovic L, Higgins KM, Scott MP: Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 277:1109–1113, 1997

Hammerschmidt M, Brook A, McMahon AP: The world according to hedgehog. *Trends Genet* 13:14–21, 1997

Hardcastle Z, Mo R, Hui CC, Sharpe PT: The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. *Development* 125:2803–2811, 1998

Hardy MH: Glandular metaplasia of hair follicles and other responses to vitamin A excess in cultures of rodent skin. *J Embryol Exp Morphol* 19:157–180, 1968

van den Heuvel M, Ingham PW: smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* 382:547–551, 1996

Hui CC, Slusarski D, Platt KA, Holmgren R, Joyner AL: Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev Biol* 162:402–413, 1994

Ingham PW: Transducing Hedgehog: the story so far. *EMBO J* 17:3505–3511, 1998

Ingham PW, Taylor AM, Nakano Y: Role of the *Drosophila* patched gene in positional signalling. *Nature* 353:184–187, 1991

Iseki S, Araga A, Ohuchi H, *et al*: Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth. *Biochem Biophys Res Commun* 218:688–693, 1996

Kinto N, Iwamoto M, Enomoto-Iwamoto M, *et al*: Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. *FEBS Lett* 404:319–323, 1997

Kobayashi K, Rochat A, Barrandon Y: Segregation of keratinocyte colony-forming cells in the bulge of the rat vibrissa. *Proc Natl Acad Sci USA* 90:7391–7395, 1993

Le Novere N, Zoli M, Changeux JP: Neuronal nicotinic receptor α6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *Eur J Neurosci* 8:2428–2439, 1996

Lerner EA: How to make a hybridoma. *Yale J Biol Med* 54:387–402, 1981

Litingtung Y, Lei L, Westphal H, Chiang C: Sonic hedgehog is essential to foregut development. *Nat Genet* 20:58–61, 1998

Majeau GR, Meier W, Jimmo B, Kioussis D, Hochman PS: Mechanism of lymphocyte function-associated molecule 3-Ig fusion proteins inhibition of T cell responses. Structure/function analysis in vitro and in human CD2 transgenic mice. *J Immunol* 152:2753–2767, 1994

Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ: Biochemical evidence that patched is the Hedgehog receptor. *Nature* 384:176–179, 1996

Miller SJ, Sun T-T, Lavker RM: Hair follicles, stem cells, and skin cancer. *J Invest Dermatol* 100:288S–294S, 1993

Ming JE, Roessler E, Muenke M: Human developmental disorders and the Sonic hedgehog pathway. *Mol Med Today* 4:343–349, 1998

Mo R, Freer AM, Zinyk DL, *et al*: Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* 124:113–123, 1997

Motoyama J, Takabatake T, Takeshima K, Hui C: Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. *Nat Genet* 18:104–106, 1998a

Motoyama J, Liu J, Mo R, *et al*: Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. *Nat Genet* 20:54–57, 1998b

Nusslein-Volhard C, Lohs-Schardin M, Sander K, Cremer C: A dorso-ventral shift of embryonic primordia in a new maternal-effect mutant of Drosophila. *Nature* 283:474–476, 1980

O'Guin WM, Sun TT, Manabe M: Interaction of trichohyalin with intermediate filaments: three immunologically defined stages of trichohyalin maturation. *J Invest Dermatol* 98:24–32, 1992

Oro AE, Scott MP: Splitting hairs: dissecting roles of signaling systems in epidermal development. *Cell* 95:575–578, 1998

Pepinsky RB, Zeng C, Wen D, *et al*: Identification of a palmitic acid-modified form of human Sonic hedgehog. *J Biol Chem* 273:14037–14045, 1998

Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS: Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. *J Exp Med* 184:1999–2006, 1996

Rochat A, Kobayashi K, Barrandon Y: Location of stem cells of human hair follicles by clonal analysis. *Cell* 76:1063–1073, 1994

Roelink H, Augsburger A, Heemskerk J, *et al*: Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* 76:761–775, 1994

Sato N, Leopold PL, Crystal RG: Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. *J Clin Invest* 104:855–864, 1999

St-Jacques B, Dassule HR, Karavanova I, *et al*: Sonic hedgehog signaling is essential for hair development. *Curr Biol* 8:1058–1068, 1998

Stone DM, Hynes M, Armanini M, *et al*: The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 384:129–134, 1996

Vortkamp A, Lee K, Lanske B, *et al*: Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 273:613–622, 1996

Yang JT, Liu CZ, Villavicencio EH, *et al*: Expression of human GLI in mice results in failure to thrive, early death, and patchy Hirschsprung-like gastrointestinal dilatation. *Mol Med* 3:826–835, 1997

Zecca M, Basler K, Struhl G: Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* 121:2265–2278, 1995

Zhou P, Byrne C, Jacobs J, Fuchs E: Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes Dev* 9:700–713, 1995

Zoller M, Herrmann K, Buchner S, *et al*: Transient absence of CD44 expression and delay in development by anti- CD44 treatment during ontogeny: a surrogate of an inducible knockout? *Cell Growth Differ* 8:1211–1223, 1997