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Modulation of Hair Growth with Small Molecule Agonists of the Hedgehog Signaling Pathway

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The hedgehog (Hh) family of intercellular signaling proteins is intricately linked to the development and patterning of almost every major vertebrate organ system. In the skin, sonic hedgehog (Shh) is required for hair follicle morphogenesis during embryogenesis and for regulating follicular growth and cycling in the adult. We recently described the identification and characterization of synthetic, non-peptidyl small molecule agonists of the Hh pathway. In this study, we examined the ability of a topically applied Hh-agonist to modulate follicular cycling in adult mouse skin. We report that the Hh-agonist can stimulate the transition from the resting (telogen) to the growth (anagen) stage of the hair cycle in adult mouse skin. Hh-agonist-induced hair growth caused no detectable differences in epidermal proliferation, differentiation, or in the endogenous Hh-signaling pathway as measured by *Gli1*, *Shh*, *Ptc1*, and *Gli2* gene expression when compared with a normal hair cycle. In addition, we demonstrate that Hh-agonist is active in human scalp *in vitro* as measured by *Gli1* gene expression. These results suggest that the topical application of Hh-agonist could be effective in treating conditions of decreased proliferation and aberrant follicular cycling in the scalp including androgenetic alopecia (pattern hair loss).

Key words: sonic hedgehog/hair follicle/androgenetic alopecia
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Hedgehog (Hh) was originally identified in Drosophila as a regulator of cell-fate determination during embryogenesis (Nüsslein-Volhard and Wieschaus, 1980). The three vertebrate Hh homologs, Sonic, Desert, and Indian (Shh, Dhh, and Ihh), perform essential roles in many of the fundamental processes that occur during the development and patterning of essentially every major organ (McMahon *et al*, 2003). In addition to its role during embryogenesis, Shh has been shown to be involved in the maintenance of stem cells in the adult (Beachy *et al*, 2004). Signaling activity in the Hh pathway begins with the binding of the Hh ligand to its receptor, Patched (Ptc), a 12-transmembrane domain protein (for a comprehensive review of the pathway, see Lum and Beachy, 2004). In the absence of the Hh ligand, Ptc suppresses the activity of the seven-transmembrane protein Smoothened (Smo); however, when Hh is present and binds to Ptc, the repression of Smo is suspended, resulting in the activation of the Gli family of transcription factors (Gli1, Gli2, Gli3). Upon pathway stimulation, these transcription factors translocate to the nucleus and effect target gene transcription. Members of the pathway, including Gli1 and Ptc1, are themselves transcriptional targets, and their own induction serves as a mechanism of pathway self-regulation.

During skin development, Shh plays a vital role in the morphogenesis of hair follicles. Mice lacking Shh activity exhibit follicles arrested at the hair germ stage of development (St-Jacques *et al*, 1998; Chiang *et al*, 1999). Although hair placodes are normal in spacing and number, dermal papillae fail to form correctly and proliferation in the follicular epithelium is impaired. Subsequently in the adult, Shh serves as a key regulator of follicular growth and cycling as it is able to induce the transition from the resting (telogen) to the growth stage (anagen) of the hair follicle cycle (Sato *et al*, 1999; Stenn and Paus, 2001). Conversely, antibodies that block the activity of Shh are able to prevent hair growth in adult mice (Wang *et al*, 2000). Although no hair is produced, the hair follicles do undergo limited proliferation that results in the formation of small, bulb-like structures that express markers of follicular differentiation. Similar results were observed when Shh $-/-$ skin was grafted onto recipient mice and analyzed (St-Jacques *et al*, 1998; Chiang *et al*, 1999). Collectively, these experiments indicate that Shh is not required to initiate anagen but is absolutely required for correct follicular morphogenesis and hair production. The Hh pathway has also recently been implicated in the regulation of sebaceous gland development and differentiation in the skin (Allen *et al*, 2003; Niemann *et al*, 2003).

The analysis of the Hh-signaling pathway in adult mouse skin has been complicated and hindered by the embryonic lethality of the majority of mouse models involving alterations in the pathway. *Shh*, *Gli2*, *Ptc1*, and *Smo* knockout mice all fail to develop to term (Chiang *et al*, 1996; Goodrich *et al*, 1997; Mo *et al*, 1997; Zhang *et al*, 2001). In addition, the analysis of transgenic mouse models in which various components of the Hh pathway have been expressed

Abbreviations: BrdU, bromodeoxyuridine; C57BL/6, C57BL/6NcrlBR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; Hh, hedgehog; Ptc1, patched-1; Shh, sonic hedgehog

in different contexts (Oro *et al*, 1997; Xie *et al*, 1998; Grachtchouk *et al*, 2000; Nilsson *et al*, 2000; Sheng *et al*, 2002; Grachtchouk *et al*, 2003; Mill *et al*, 2003; Hutchin *et al*, 2005), has primarily focused on the role of the Hh pathway in follicular morphogenesis and basal cell carcinoma (BCC) development and not on postnatal hair cycling.

We have previously described the identification and characterization of synthetic, non-peptidyl small molecules that bind to Smo and function as Hh pathway agonists (Frank-Kamenetsky *et al*, 2002). These molecules feature many properties that make them attractive as potential therapeutic agents including their low-nanomolar potencies and favorable pharmacokinetic profiles in targeted tissues. Importantly, the Hh-pathway agonists (Hh-agonist) can activate Hh-signaling in a wide variety of *in vitro* and *in vivo* assays (Frank-Kamenetsky *et al*, 2002; Wichterle *et al*, 2002; Gabay *et al*, 2003; Machold *et al*, 2003; Harper *et al*, 2004, unpublished data).

In this study, we have investigated the ability of topically applied Hh-agonist to modulate the Hh pathway in adult mammalian skin *in vivo*. Here we report that a single topical dose of Hh-agonist can stimulate the telogen to anagen transition in adult mouse hair follicles. When compared with a normal hair cycle, hair growth occurs without causing detectable differences in epidermal proliferation, differentiation, or in the expression of Hh-signaling pathway components. Furthermore, topical Hh-agonist can induce *Gli1* gene expression in human scalp *in vitro*. These results suggest that the topical application of Hh-agonist can be used to activate the pathway *in vivo* and may be a novel and effective method to treat conditions of decreased proliferation and aberrant follicular cycling in skin such as androgenetic alopecia (pattern hair loss).

Results

In order to determine if Hh-agonist could cause hair growth, 7-wk-old C57BL/6NCrlBR (C57BL/6) mice were topically treated with Hh-agonist. Hair growth in this strain has been extensively studied and the timing of the stages of depilation-induced anagen have been rigorously characterized (for a comprehensive review, see Müller-Röver *et al*, 2001). From weeks 7 to 12 of postnatal life, C57BL/6 mice are in the telogen, or resting stage, of the hair cycle, which provides a large window of time to assay for anagen induction in the absence of endogenous hair growth.

Prior to assaying for hair growth, Hh-agonist was prepared in a vehicle designed for effective delivery (95% acetone/5% dimethylsulfoxide (DMSO)) and the penetration and pharmacokinetic properties of Hh-agonist were investigated. Hh-agonist was topically applied to the shaved dorsal surface of 7-wk-old C57BL/6 male mice and the concentration in skin and plasma was determined at various times after application. Hh-agonist penetrated the skin quickly as quantitative absorption occurred 15 min after application and reached a maximum concentration (C_{max}) of 13.3 μM 4 h after application with a half-life in skin of 84 h ($T_{1/2}$). In addition, after either a single dose or multiple topical doses (eight), no Hh-agonist was detectable in the plasma at any time point analyzed (up to 8 d). Therefore, the topical application of Hh-agonist resulted in the quick, quantitative, and restricted delivery to the skin without systemic exposure.

Based on the pharmacokinetic data and previous studies (Frank-Kamenetsky *et al*, 2002), Hh-agonist at various concentrations (0.003–0.3 μg per μL) was tested for its ability to induce hair growth. 25 μL of Hh-agonist was topically applied once a day for 8 d to the shaved dorsal surface of 7-wk-old C57BL/6 mice. Beginning 7–8 d after application, the area of Hh-agonist application began to darken indicating that follicular melanogenesis was occurring (Slominski and Paus, 1993). Subsequently, hair began to emerge after 10–11 d in the Hh-agonist treated, but not in vehicle-treated skin (data not shown).

To determine the minimal number of doses required to initiate anagen, 7-wk-old C57BL/6 male mice were treated once a day with either vehicle or Hh-agonist (0.06 μg per μL) at a concentration that induced hair growth with similar kinetics to depilation (Müller-Röver *et al*, 2001) for 1–8 d on two areas of their shaved dorsal surface. Interestingly, one topical dose of Hh-agonist was sufficient to induce anagen and hair growth, whereas vehicle-treated skin remained in telogen (Fig 1*A*). In general, complete hair growth occurred in areas treated with Hh-agonist (Fig 1*A*) and was restricted to the area of topical application (Fig 1*A*). Light microscopy analysis of skin sections from Hh-agonist-treated mice revealed a clear demarcation between telogen and anagen follicles at the boundary of Hh-agonist application (Fig 1*C*). In contrast, hair follicles from vehicle-treated skin were all in telogen (Fig 1*B*).

In order to more accurately characterize Hh-agonist-induced hair growth, 7-wk-old shaved C57Bl/6 mice were treated with one topical dose of Hh-agonist and skin samples obtained at various time points over the course of the induced hair cycle were analyzed by light microscopy and immunohistochemistry (Fig 2). Skin samples from depilated mice were used as a reference since the timing of the stages of a depilation-induced hair cycle are well characterized and follow the same kinetics as a spontaneous, or naturally occurring, hair cycle (Müller-Röver *et al*, 2001).

Proliferation and growth of the hair follicles, as detected by bromodeoxyuridine (BrdU) immunohistochemistry, in response to Hh-agonist treatment was first observed beginning 2–3 d after topical application (data not shown). By day 4 there was a dramatic increase in the number of BrdU positive cells in the growing hair follicles of Hh-agonist-treated skin compared with either Hh-agonist-treated skin at day 1 (compare Fig 2*B* and *J* to Fig 2*A* and *I*) or vehicle-treated skin (data not shown) indicating that anagen had been initiated. By 8 d after topical application, Hh-agonist-treated skin exhibited correctly oriented hair follicles in anagen VI. The hair follicles extended deep into the dermis and BrdU positive cells were abundant in the follicular matrix and outer root sheath cells (Fig 2*C*, *D*, *K*, *L*). The percentage of proliferative matrix cells in Hh-agonist-treated skin based on BrdU labeling 8 d after treatment was 33.4% ± 1.3% compared with 36.9% ± 1.1% in depilated skin. When the same analysis was performed 12 d after treatment the percentage of proliferative matrix cells was still quite similar regardless of the method of anagen induction (35.7% ± 1.6% in Hh-agonist-treated skin

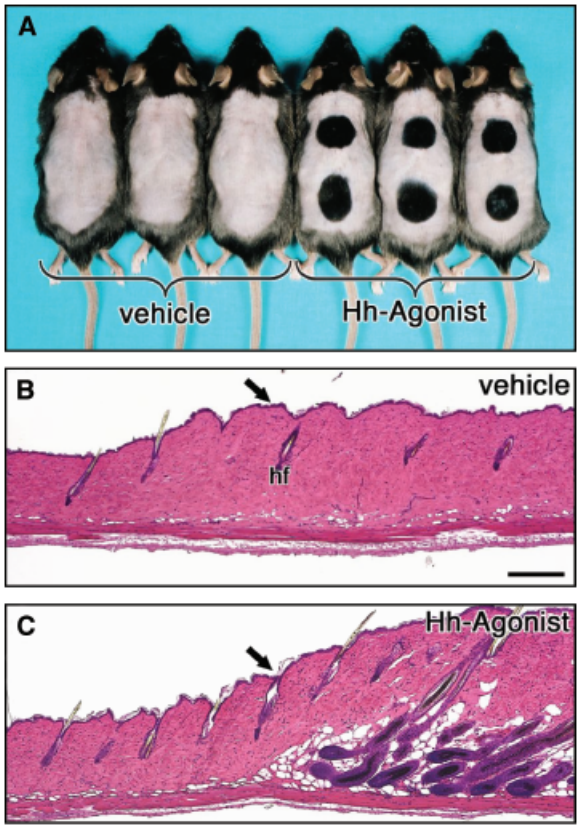

Figure 1
Anagen induction and hair growth in C57BL/6NcrlBR (C57BL/6) mice treated with a single topical dose of hedgehog (Hh)-agonist. (*A*) Seven-wk-old male mice were shaved and given a single 25 μL topical application of vehicle (95% acetone/5% DMSO) or Hh-agonist (0.06 μg per μL in vehicle, 120 μM) on their upper and lower dorsal regions. Hair growth in the mice pictured is 13 d after topical application. (*B*, *C*) Five-μm parasagittal paraffin sections were stained with hematoxylin and eosin and analyzed by light microscopy. Vehicle (*B*) or Hh-agonist (*C*) was applied to the right of the arrow. Skin was processed for analysis 14 d after treatment. Hair growth was restricted to the area of topical application. hf, hair follicle. *Scale bar* (*B*, *C*) = 200 μm.

compared with 33.1% ± 1.4% in depilated skin). In general, there was no evidence of abnormal proliferation or histology in the hair follicles, epidermis, or sebaceous glands of Hh-agonist-treated skin (Fig 2*A–D*, *I–L*) when compared with depilated skin (Fig 2*E–H*, *M–P*) at any equivalent time point analyzed.

By day 18–19, both Hh-agonist and depilated skin had entered into catagen (Fig 2*Q*, *R*) and by day 23 had completed the hair cycle and the follicles were once again in telogen (Fig 2*S*, *T*). In addition, when compared by light microscopy, hairs produced from Hh-agonist-treated and depilated mice exhibited no obvious differences in length, thickness, production and ratio of the four hair types (auchene, awl, guard, and zig-zag), and pigmentation patterns (data not shown). Thus, by all accounts, the hair cycle and hair produced by Hh-agonist treatment are similar to those of a normal hair cycle.

In order to characterize, at a molecular level, the magnitude and temporal expression of the Hh-agonist-induced Hh-signaling pathway response in skin, the relative mRNA induction of *Gli1*, *Shh*, *Ptc1*, and *Gli2* were determined during the early events of anagen (days 0–10 after induction). Mice were treated with either one topical application of Hh-agonist, vehicle, or a depilatory agent to induce anagen and RNA from skin at each time point was obtained and analyzed by quantitative RT-PCR.

Induction of *Gli1*, an early and consistent indicator of Hh pathway activation (McMahon *et al*, 2003), and *Shh* was first observed 3 d after Hh-agonist treatment and 4 d after depilation (Fig 3*A, B*). By 2-way ANOVA analysis, a statistically significant induction of both *Gli1* and *Shh*, however, was observed for both treatments beginning 5 d after anagen initiation (Fig 3*A*, *B*, treatment, $p < 0.01$, time, $p < 0.01$). Relative mRNA levels remained elevated and appeared to level off at day 9–10 corresponding to the anagen VI stage of the hair cycle. The relative magnitude of *Shh* mRNA induction ($\sim$ 100-fold) was strikingly higher compared with that of the other genes analyzed ($\sim$ 20–25-fold for *Gli1* and *Ptc1* (Fig 3*C*), and $\sim$ 6–7-fold for *Gli2* (Fig 3 *D*)). The overall temporal pattern and relative level of *Gli1* and *Shh* induction over the time course was similar between the two methods of anagen initiation with the exception that induction of the two genes after Hh-agonist treatment occurred 1 d earlier.

Ptc1, the Hh protein receptor that is transcriptionally regulated in response to Hh pathway activation (McMahon *et al*, 2003), and *Gli2*, the functional mediator of Hh signal transduction in the hair follicle (Mill *et al*, 2003), were both induced at day 5 by Hh-agonist and depilation (Fig 3*C*, *D*). A statistically significant induction was observed beginning at day 6 for *Ptc1* and day 7 for *Gli2* (treatment, $p < 0.01$, time $p < 0.01$). In contrast to *Gli1* and *Shh*, the relative mRNA levels of *Ptc1* and *Gli2* appeared to still be increasing at day 10. The levels of all four mRNAs remained elevated until the onset of catagen at day 17–19, at which point they began to decrease. By 21 d after anagen initiation, the levels of all four mRNAs had returned to pre-treatment (telogen) levels (data not shown). Most importantly, the temporal pattern and magnitude of expression of *Gli1*, *Shh*, *Ptc1*, and *Gli2* in response to Hh-agonist treatment was similar to that observed with depilation-induced anagen.

Since it is well established that aberrant Hh pathway activity in skin can lead to abnormal differentiation and various follicular hyperplasias including BCC (Hahn *et al*, 1996; Johnson *et al*, 1996; Dahmane *et al*, 1997; Oro *et al*, 1997; Xie *et al*, 1998; Aszterbaum *et al*, 1999; Grachtchouk *et al*, 2000; Nilsson *et al*, 2000; Sheng *et al*, 2002), the effect of Hh-agonist on differentiation in the skin was analyzed. Seven-wk-old C57BL/6 male mice were either treated with one topical dose of vehicle, Hh-agonist, or depilated to induce anagen. Although hair follicles from vehicle-treated skin were still in telogen 10 d after treatment (Fig 4*A*) both depilated (Fig 4*F*) and Hh-agonist-treated hair follicles (Fig 4*K*) were in anagen VI of the hair cycle. At this substage of anagen, hair follicle size, proliferation, and hair production are at their maximum.

To determine if Hh-agonist treatment resulted in epidermal hyperproliferation, Keratin 6 (K6) expression was examined. K6 is normally expressed in the companion layer of the hair follicle (K6hf) but not in the inter-follicular epidermis (Winter *et al*, 1998). Under certain conditions including wounding and diseases that alter proliferation and differentiation, however, expression of K6 is induced in the suprabasal layers of the interfollicular epidermis (McGowan and

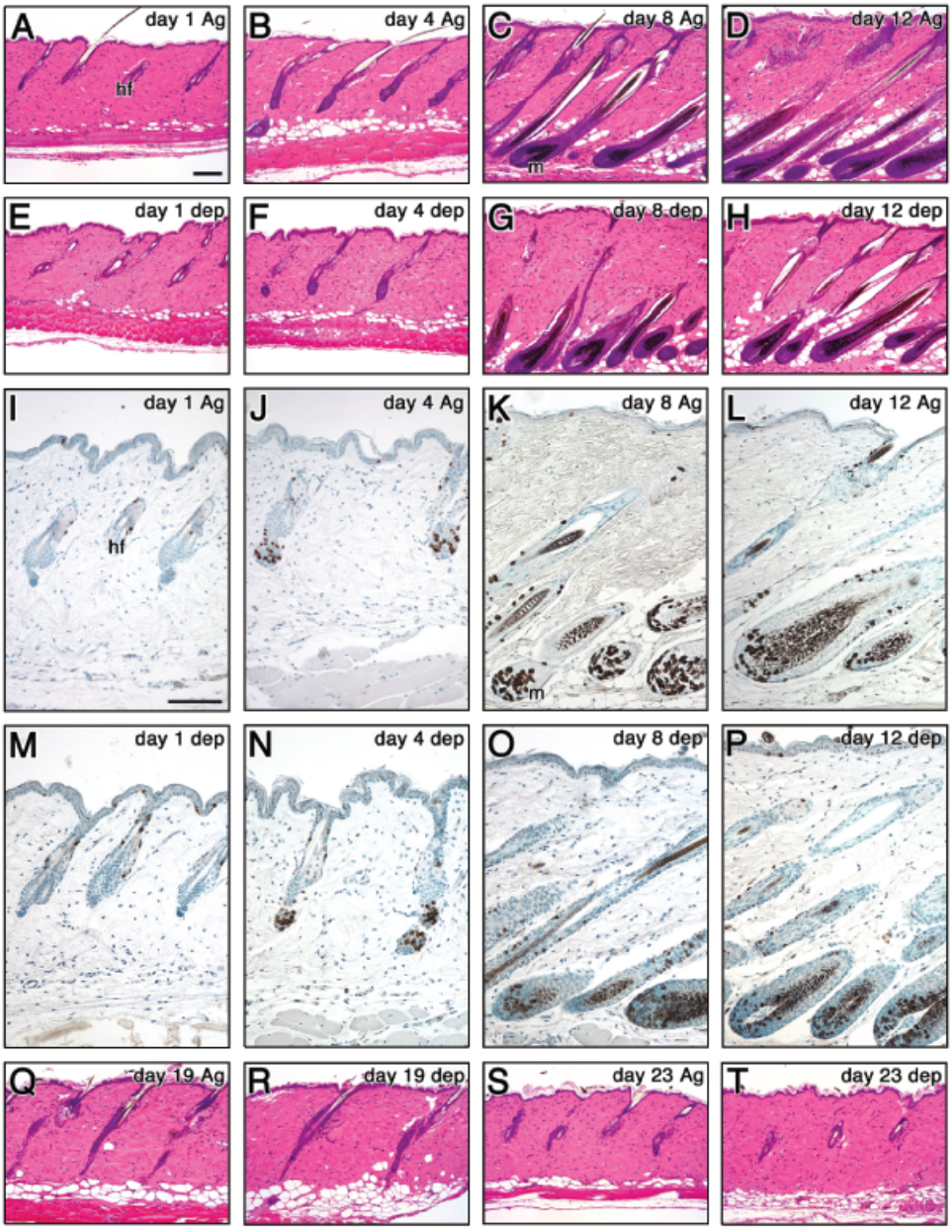

Figure 2
Light microscopy analysis of the hedgehog (Hh)-agonist-induced hair cycle. Five-μm parasagittal paraffin sections were stained with hematoxylin and eosin (*A–H*, *Q–T*) or immunostained with an antibody to BrdU (*I–P*) and analyzed by light microscopy. Seven-wk-old C57BL/6NcrlBR (C57BL/6) male mice were given a single 25 μL topical dose of Hh-agonist (0.06 μg per μL in vehicle) and skin was isolated at the indicated day over the course of the induced hair cycle. Depilated skin was used as a control for anagen induction. Hh-agonist (Ag)-treated skin: *A–D*, *I–L*, *Q*, *S*; depilated skin (dep): *E–H*, *M–P*, *R*, *T*. hf, hair follicle; m, matrix. *Scale bar* = 100 μm.

Coulombe, 1998). In all three groups analyzed (Fig 4*B*, *G*, and *L*), K6 expression was restricted to the hair follicle and there was no evidence of suprabasal expression indicating that Hh-agonist treatment did not cause epidermal hyperproliferation.

Markers of early and late terminal differentiation in the epidermis were also examined. Keratin 10 (K10) is expressed in the spinous layer of the epidermis as the process of terminal differentiation begins (Fuchs and Green, 1980) whereas loricrin, the major component of the cornified envelope, is expressed later in the granular layer (Mehrel *et al*, 1990). K10 expression was restricted to the suprabasal layers of the epidermis in both the depilated (Fig 4*H*), Hh-agonist-treated (Fig 4*M*) and vehicle-treated skin (Fig 4*C*). In addition, loricrin expression, in all three cases (Fig 4*D, I*, and *N*), was detected in the granular layer and stratum corneum. The correct expression of all three epidermal markers was observed not only at anagen VI but at all other stages of the induced hair cycle analyzed (data not shown). Finally, Keratin 14 (K14), a marker of the proliferative basal layer of the interfollicular epidermis and the outer root sheath of the hair follicle (Coulombe *et al*, 1989), exhibited proper expression in all skin samples analyzed (Fig 4 *E, J*, and *O*). The correct expression of epidermal differentiation markers coupled with the normal histology and proliferation observed (Fig 2) suggests that Hh-agonist treatment did not affect the normal epidermal proliferation and differentiation of the skin during anagen induction.

The long-term effects of Hh-agonist treatment on proliferation and differentiation in mouse skin were also examined. Seven-wk-old mice were treated with either one topical dose of vehicle or Hh-agonist to induce anagen. Approximately 1 y later, skin from both groups was analyzed by light microscopy and immunohistochemistry. Hair follicles from both vehicle (Fig 5*A*)- and Hh-agonist (Fig 5*B*)-treated skin were in telogen. Proliferation in both vehicle- (Fig 5*C*) and Hh-agonist-treated (Fig 5*D*) skin, as analyzed by BrdU immunohistochemistry, was minimal. A few BrdU

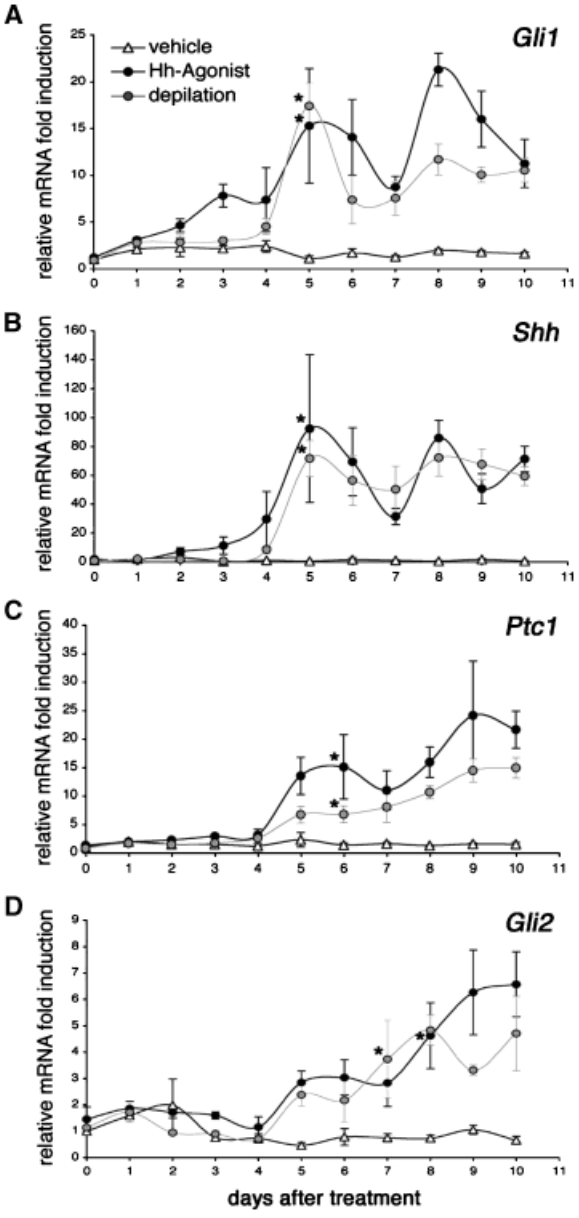

Figure 3
Time course of Sonic hedgehog (Shh) pathway gene expression in skin following anagen induction in hedgehog (Hh)-agonist-treated mice. The seven-wk-old C57BL/6NcrlBR (C57BL/6) male mice were treated once with either 25 μL of vehicle, Hh-agonist (0.06 μg per μL in vehicle), or a depilatory agent on their dorsal surface. Skin from each group ($n = 4$) was harvested at days 0–10 after treatment and RNA was isolated. Quantitative RT-PCR was used to analyze the expression of *Gli1* (*A*), *Shh* (*B*), *Ptc1* (patched-1) (*C*), and *Gli2* (*D*). Values are graphed as a relative fold induction compared with the vehicle-treated sample at time zero using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene. *GAPDH* levels did not vary significantly as a function of treatment or time. Statistical significance was determined by performing a two-way ANOVA analysis with Bonferroni post-tests ($p < 0.05$). Asterisk (*) indicates the time point at which statistically significant induction begins. There was no significant induction of any of the Hh pathway genes in the vehicle-treated samples. Error bars represent ± SEM.

positive cells were detected in both the telogen-staged hair follicles and the interfollicular epidermis (arrowheads in Fig 5*C* and *D*). In addition, the expression of the hyperproliferation-associated K6 was restricted to the hair follicle and was not detected in the suprabasal layers of the epidermis in either case (Fig 5*E* and *F*).

The expression of epidermal differentiation markers in both vehicle- and Hh-agonist-treated skin was also similar. K10 was restricted to the suprabasal layers of the epidermis (Fig 5*G* and *H*), whereas loricrin was detected in the granular layer and stratum corneum (Fig 5*I* and *J*). Furthermore, K14 expression was detected in the basal layer of the epidermis and outer root sheath of the hair follicles in both vehicle (Fig 5*K*) and Hh-agonist-treated skin (Fig 5*L*). Thus, Hh-agonist treatment did not appear to result in any long-term effects on the ability of the epidermis and hair follicles to differentiate and proliferate normally.

In order to determine if Hh-agonist is active in human skin, 6 mm skin punches from fetal scalp (18–22 wk) were treated with Hh-agonist *in vitro*. At this stage of embryonic development the hair follicles are already producing hair (lanugo type). The agonist was topically applied to the exposed surface of the skin once daily and activity was determined by examining transcriptional targets of the Hh-pathway using quantitative RT-PCR. After 4 d in culture, treatment with vehicle did not induce *Gli1*, *Ptch1*, *Gli2*, or *Shh* expression relative to untreated skin (Fig 6). Treatment with Hh-agonist, however, resulted in the statistically significant induction of *Gli1*, *Gli2*, and *Ptc1* (Fig 6 (*Gli1*, *Ptc1* 3.5–4-fold; *Gli2* 2-fold)).

Discussion

We have reproduced a defined biological role of Shh in the adult mouse, namely the ability to modulate hair growth and follicular cycling, by the topical application of a small molecule agonist of the Hh-signaling pathway. This work further validates the concept that Hh-signaling in the adult vertebrate can be modulated in order to achieve a desired biological effect. One application of the Hh-agonist was able to induce anagen in adult C57BL/6 mice. The resultant hair cycle was indistinguishable from a normal hair cycle (depilation model) with no detectable differences in the cycle length, histology, expression of Hh-pathway genes, epidermal proliferation, differentiation, melanogenesis, or, ultimately, in the hair produced. In addition, Hh-agonist treatment did not appear to have any long-term effects on the skin. Furthermore, we have also demonstrated that the Hh-agonist is active in human scalp as measured by the induction of Hh-pathway gene expression.

In addition to its necessity during hair follicle morphogenesis (St-Jacques *et al*, 1998; Chiang *et al*, 1999), the Hh-signaling pathway is re-utilized during the postnatal cycling of hair follicles (Sato *et al*, 1999; Wang *et al*, 2000). During anagen, the Hh pathway components *Gli1*, *Ptc1*, *Gli2*, and *Shh* have been localized by *in situ* hybridization to the matrix, dermal papillae, and outer root sheath cells of the hair follicle (Gat *et al*, 1998; Sato *et al*, 1999; Botchkarev *et al*, 2001; Oro and Higgins, 2003; Ikram *et al*, 2004; Lo Celso *et al*, 2004). Subsequently, expression of the Hh-pathway genes is downregulated when the hair follicles are in telogen (Botchkarev *et al*, 2001; Oro and Higgins, 2003; this study).

We have determined the temporal Hh-pathway activation that occurs during the course of a post-natal hair cycle us-

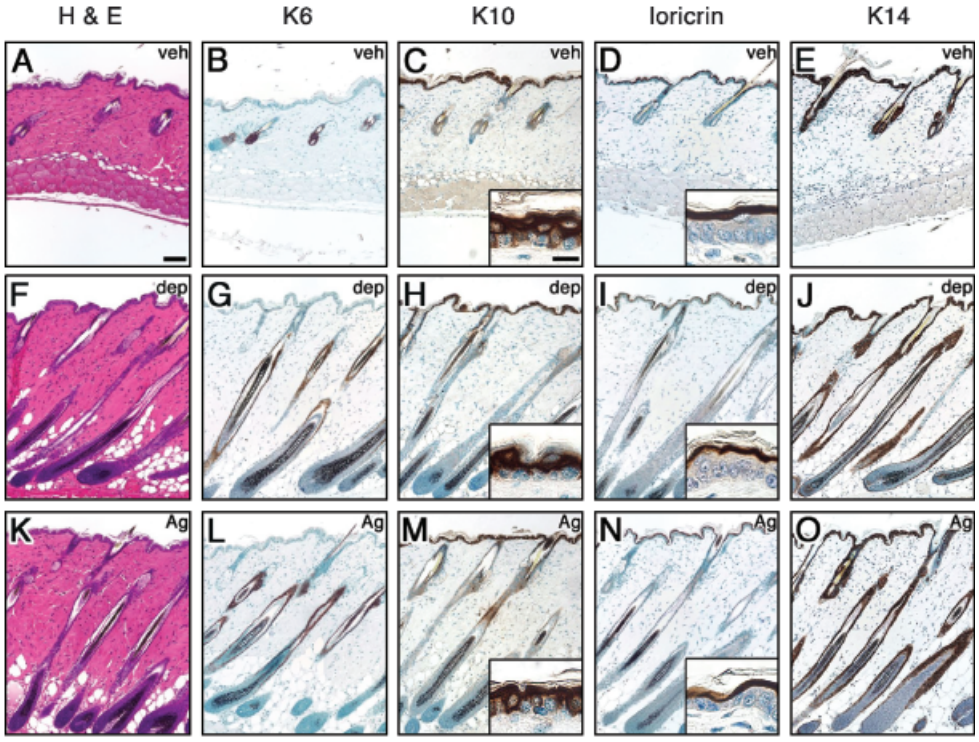

Figure 4
Epidermal differentiation in hedgehog (Hh)-agonist-treated mouse skin. Five-μm parasagittal paraffin sections from vehicle, depilated, or Hh-agonist-treated mouse skin were stained with hematoxylin and eosin (*A*, *F*, *K*) or immunostained with antibodies to K6 (*B*, *G*, *L*), K10 (*C*, *H*, *M*), loricrin (*D*, *I*, *N*), and K14 (*E*, *J*, *O*) and subsequently analyzed by light microscopy. Seven-wk-old C57BL/6NcrlBR (C57BL/6) 6 male mice were given a single 25 μL topical application of either vehicle or Hh-agonist (0.06 μg per μL in vehicle). Depilated skin was used as a control for anagen induction. Skin was processed for analysis 10 d after treatment during the anagen VI stage of the hair cycle. Vehicle (veh)-treated skin: *A–E*; depilated skin (dep): *F–J*; Hh-agonist (Ag)-treated skin: *K–O*. *Scale bar* (*A–O*) = 100 μm. *Scale bar* in inset (*C*, *D*, *H*, *I*, *M*, and *N*) = 10 μm.

ing two distinct methods to initiate hair growth. Hh-pathway gene expression was first detected 3–4 d after anagen initiation (Fig 3; see also Sato *et al*, 1999). The timing of Hh-pathway signaling activation coincides with the transition from the anagen II to anagen III stage of the hair cycle. At this stage, the follicular matrix cells begin to surround the dermal papillae cells and form the hair bulb. Proliferation in the bulb increases, formation of the inner root sheath and hair shaft begins, and the hair follicles extend deep into the subcutis (Müller-Röver *et al*, 2001). Previous work has shown that in the absence of Shh activity, both the epithelial and mesenchymal (dermal papillae) portions of the bulb structure do not form properly despite the expression of markers of mature follicular differentiation (St-Jacques *et al*, 1998; Chiang *et al*, 1999; Wang *et al*, 2000). Taken together, these data suggest that Shh is required for the proper formation of the hair follicle bulb and hair production during the postnatal hair cycle. Expression of *Gli1*, *Shh*, *Ptc1*, and *Gli2* continued over the course of anagen and their levels decreased as the hair follicles entered catagen and had returned to baseline when the follicles were once again in telogen (Fig 3; data not shown). Hh-signaling may therefore also be required for the sustained proliferation of matrix cells that occurs over the course of anagen and hair production.

Interestingly, the temporal pattern and magnitude of *Gli1*, *Shh*, *Ptc1*, and *Gli2* gene expression were similar whether anagen was initiated with Hh-agonist or by depilation. This suggests that the Hh-pathway transcriptional activation that occurs 3–4 d after Hh-agonist treatment is due to the endogenous response of the proliferating hair follicles as they enter anagen (this study; Sato *et al*, 1999) and not primarily as a direct result of Hh-agonist stimulation. How then does Hh-agonist initiate anagen? In telogen skin there must be a small population of a particular cell type(s) that is responsive to Hh-agonist early after topical application. In a fashion consistent with the paradigm of Hh-signaling (McMahon *et al*, 2003), direct stimulation of this cell type may then influence the same or adjacent cell type(s) to mediate the early events of anagen through a different signaling pathway. Further experiments will be necessary to determine the exact cell type(s) in the skin that are being stimulated by the Hh-agonist and how that stimulation mediates the early events of anagen induction.

The non-peptidyl, small molecule Hh-agonists contain multiple characteristics that make them attractive as potential therapeutic agents (Frank-Kamenetsky *et al*, 2002). When applied topically, the Hh-agonist is absorbed quickly and quantitatively. Importantly, topical application of the Hh-agonist results in restricted delivery and activity to the applied area while avoiding systemic exposure (Fig 1*A, C*). By regulating the amount of Hh-agonist delivered the biological effect can be regulated. In the case of anagen induction, a small amount is enough to trigger hair growth that occurs without causing any detectable differences when compared with a normal hair cycle. Collectively, these properties of the Hh-agonist class of compounds make them suitable candidates for potential therapeutic indications that require topical application.

Androgenetic alopecia (also called male pattern hair loss) is the most common type of genetic-based hair loss. Pattern hair loss is characterized by both a progressive shortening of anagen and an increased time period between exogen (hair shedding) and anagen induction that leads to a decrease in hair density. As affected hair follicles continue to cycle, they undergo a process of progressive miniaturization. Concurrently, the size of the dermal papillae, which is known to correlate with the size of the hair follicle and hair shaft produced, also decreases (Hardy, 1992; Paus and Cotsarelis, 1999; Messenger, 2003). The end result is the

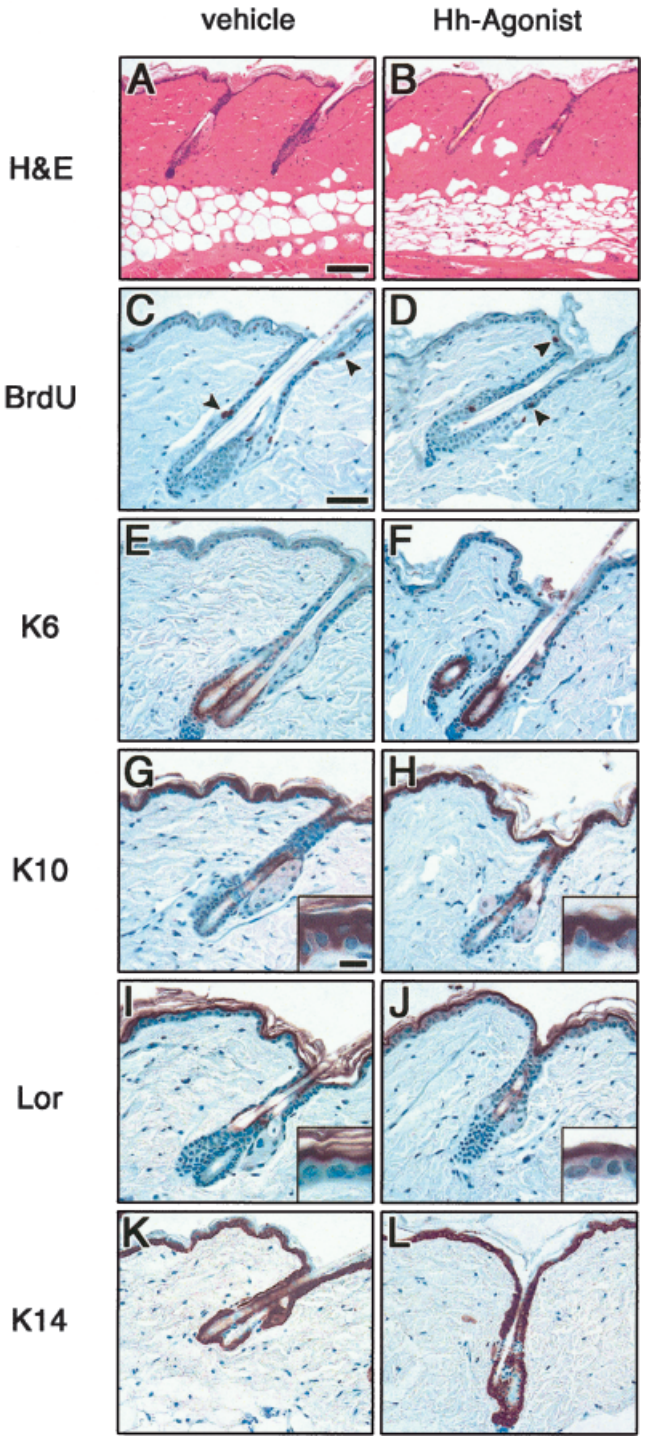

Figure 5
Long-term effects of hedgehog (Hh)-agonist treatment on epidermal differentiation in mouse skin. Five-μm parasagittal paraffin sections from vehicle-(*A*, *C*, *E*, *G*, *I*, *K*) or Hh-agonist-treated (*B*, *D*, *F*, *H*, *J*, *L*) mouse skin were stained with hematoxylin and eosin (*A*, *B*) or immunostained with antibodies to BrdU (*C*, *D*), K6 (*E*, *F*), K10 (*G*, *H*), loricrin (*I*, *J*), and K14 (*K*, *L*) and subsequently analyzed by light microscopy. Seven-wk-old C57BL/6NcrlBR (C57BL/6) male mice were given a single 25 μL topical application of either vehicle or Hh-agonist (0.06 μg per μL in vehicle). Skin from vehicle- and Hh-agonist-treated mice was obtained and processed for analysis ~ 1 y after treatment. *Scale bar* (A, B) = 100 μm. *Scale bar* (*C*–*L*) = 50 μm. *Scale bar* in inset (*G*–*J*) = 10 μm. Arrowheads indicate BrdU positive cells (*C* and *D*).

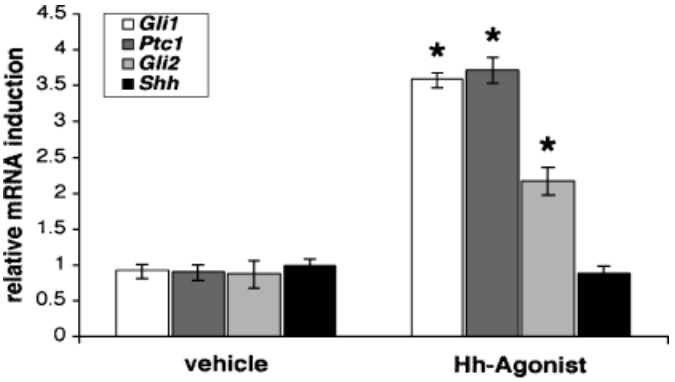

Figure 6
Hedgehog (Hh) pathway gene induction in hedgehog (Hh-)agonist-treated fetal scalp. Human fetal scalp skin was cultured and topically treated once daily with either 8 μL of vehicle or Hh-agonist (0.15 μg per μL in vehicle). RNA was isolated after 4 d in culture. The relative mRNA fold induction of *Gli1*, *Ptc1* (patched-1), and *Gli2* due to Hh-agonist treatment was statistically significant compared with vehicle ($^*p < 0.01$, $n = 4$) as analyzed by the student's t-test. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene and did not vary significantly due to treatment. The relative mRNA fold induction was determined by using no treatment as a reference. The results are the average of four different experiments. Each experiment was performed in duplicate. Error bars represent ± SEM.

production of smaller, unpigmented vellus hairs instead of larger, pigmented terminal hairs. Although the follicles do progressively get smaller, the overall scalp follicle density appears to be preserved until late in the course of hair loss when follicles are eventually destroyed (for a thorough description, see Olsen, 2003).

Since scalp affected with pattern hair loss has a higher percentage of hair follicles in telogen, the ability of Hh-agonist to promote anagen suggests that it may be useful as a potential therapeutic agent. The Hh-agonist may also enhance follicular proliferation and the size, proliferation, and/or organization of the dermal papillae in hair follicles affected by pattern hair loss. Ultimately, this potential combination of increasing the percentage of follicles in anagen and helping to restore a more normal follicular architecture may have a positive effect on hair growth. We have shown that the Hh-agonist is able to activate the Hh pathway in human scalp (Fig 6). Preliminary experiments suggest that adult scalp, and in particular, alopecic scalp, is also responsive to Hh-agonist as measured by *Gli1* induction (data not shown). Although these data are compelling, the physiological response to Hh-pathway activation in normal and human scalp affected with pattern hair loss needs to be determined in an *in vivo* context. In conclusion, we propose that the use of small molecule agonists of the Hh pathway may be a potential therapeutic agent in the treatment of male and female pattern hair loss.

Materials and Methods

Animal studies All animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at Curis, Inc. (Cambridge, Massachusetts). C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Massachusetts). Hh-Ag 1.8, a soluble derivative from the recently characterized, Biaryl class of Hh agonists (Frank-Kamenetsky *et al*,

2002) was used in all of these experiments. Details of the Hh-agonist class of small molecules are presented in the issued US Patent No. US 6,683,192 which can be examined at the following website: http://www.uspto.gov/patft/. Hh-Ag 1.8 is available for research use from Curis under a material transfer agreement. For all experiments, Hh-Ag 1.8 (subsequently referred to as Hh-agonist) was diluted in a vehicle of 95% acetone/5% DMSO (Sigma, St Louis, Missouri) from an original 10 mg per mL DMSO stock. A volume of 25 μL of Hh-agonist or vehicle was applied with a pipetman to the shaved dorsal surface of 7-wk-old male mice. As a control for anagen induction, 7-wk-old C57BL/6 male mice were depilated with Nair (Carter-Wallace, New York, New York). The mice were shaved on the dorsal surface and the depilating agent was applied for 2 min, removed, and the depilated area was rinsed with H_2O. During all procedures the mice were temporarily anesthetized using a mixture of isoflurane (Baxter, Deerfield, Illinois) and oxygen.

Hh-agonist concentration determination in skin and plasma Seven-wk-old C57BL/6 male mice were treated with a single 25 μL topical dose of Hh-agonist (0.06 μg per μL in vehicle, 120 μM) or vehicle. Skin and blood samples were collected at various time points after topical administration. Treated skin was cleaned three times with alcohol pads (Kendall, Mansfield, Massachusetts) prior to isolation in order to remove any Hh-agonist that may not have penetrated the skin. Approximately 20–30 mg of treated skin was homogenized in 1 mL of phosphate buffered saline (PBS). Three 1.5 mL ethyl acetate extractions of the homogenate were obtained and condensed by evaporation. The compound extracts were then reconstituted in 100 μL of acetonitrile. To isolate the Hh-agonist from blood, 100 μL of plasma was mixed with 200 μL of acetonitrile, vortexed, and centrifuged for 10 min × 8,160 g. The supernatant was isolated and subjected to LC-MS/MS. 10 μL of an internal standard was added to each sample. Chromatographic separation was achieved using a 5 μm, 2.1 × 30 mm XTerre MS C_{18} column (Waters, Milford, Massachusetts) with a gradient mobile phase of (A) acetonitrile:water:formic acid (5:95:0.1, vol/vol/vol) and (B) acetonitrile:water:formic acid (95:5:0.1, vol/vol/vol). The analytes were detected with a PE Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, California). Precursor and product ions of the Hh-agonist (m/z 570.1, m/z 212.0) and the internal standard (m/z 436.2, m/z 150.9) were monitored in the multiple reaction monitoring (MRM) model. Standards were prepared by adding known amounts of the Hh-agonist to known amounts of untreated skin and plasma samples. The Hh-agonist was detectable to levels of 1 ng per mL. Measurements of each time point and tissue were performed in triplicate.

Quantitative PCR analysis RNA from mouse and human skin was isolated using the RNeasy mini kit (Qiagen, Valencia, California) and cDNA was synthesized using established protocols. The cDNA was assayed by quantitative PCR using primers and probes specific for mouse and human *Gli1*, *Gli2*, *Ptc1*, *Shh*, and *GAPDH* (Qiagen and Applied Biosystems). All quantitative PCR assays were performed using an ABI prism 7700 sequence detector and the data were analyzed using ABI prism sequence detections systems version 1.7 (Taqman; Applied Biosystems). Statistical analysis of the data was performed using the GraphPad Prism program (version 4.0a, GraphPad Software, San Diego, California).

Histological analysis and immunohistochemistry Mouse skin was fixed overnight at 4°C with either 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) in PBS or Bouin's fixative (Sigma) and subsequently processed for paraffin embedding and sectioned. For BrdU analysis, mice were given an intraperitoneal injection of BrdU (Zymed, South San Francisco, California) 2–3 h prior to obtaining tissue. For general histological analysis, 5 μm parasagittal paraffin sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed according to standard procedures with antibodies against K6, K10, K14, and loricrin (Covance, Berkeley, California). BrdU immunohistochemistry was performed using a detection kit from Zymed. The percentage of proliferating epidermal cells in the hair follicle matrix was determined by dividing the number of BrdU positive matrix cells by the total number of matrix cells in a given follicle and multiplying by 100. Four random anagen VI staged-hair follicles from three separate mice in each treatment group (day 8 and 12, depilation and Hh-agonist) were chosen and the BrdU positive and total matrix cells were counted.

Human scalp skin explants Fetal human scalp (18–22 wk of gestation) was procured by Advanced Bioscience Resources (Alameda, California). Six-mm skin punches were made using dermal biopsy punches (Miltex, Bethpage, New York) and cultured in Biocoat Collagen I coated 3.0 micron Transwell tissue culture plates (Becton Dickinson Labware, Bedford, Massachusetts). Skin punches were cultured in RPMI 1640 media (changed daily) supplemented with 10% fetal bovine serum, glutamax, penicillin, streptomycin, and gentamicin (Gibco-BRL, Gaithersburg, Maryland) for 4 d. Hh-agonist (0.15 μg per μL in vehicle) was applied topically to the air-exposed, epidermal surface of the skin punches once a day. Prior to RNA isolation, the skin punches were quick frozen in liquid nitrogen.

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References

Allen M, Grachtchouk M, Sheng H, *et al*: Hedgehog signaling regulates sebaceous gland development. Am J Pathol 163:2173–2178, 2003

Aszterbaum M, Epstein J, Oro A, Douglas V, LeBoit PE, Scott MP, Epstein EH Jr: Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in patched heterozygous knockout mice. Nat Med 5:1285–1291, 1999

Beachy PA, Karhadkar SS, Berman DM: Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324–331, 2004

Botchkarev V, Botchkareva NV, Nakamura M, *et al*: Noggin is required for induction of the hair follicle growth phase in postnatal skin. FASEB J 15:2205–2214, 2001

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA: 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

Coulombe PA, Kopan R, Fuchs E: Expression of keratin K14 in the epidermis and hair follicle: Insights into complex programs of differentiation. J Cell Biol 109:2295–2312, 1989

Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A: Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. Nature 389:876–881, 1997

Frank-Kamenetsky M, Zhang XM, Bottega S, *et al*: Small-molecule modulators of Hedgehog signaling: Identification and characterization of Smoothened agonists and antagonists. J Biol 1:10.1–10.19, 2002

Fuchs E, Green H: Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell 19:1033–1042, 1980

Gabay L, Lowell S, Rubin LL, Anderson DJ: Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells *in vitro*. Neuron 40:485–499, 2003

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

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

Grachtchouk M, Mo R, Yu S, Zhang X, Sasaki H, Hui CC, Dlugosz AA: Basal cell carcinomas in mice overexpressing Gli2 in skin. Nat Genet 24:216–217, 2000

Grachtchouk V, Grachtchouk M, Lowe L, *et al*: The magnitude of hedgehog signaling activity defines skin tumor phenotype. EMBO J 22:2741–2751, 2003

Hahn H, Wicking C, Zaphiropoulos PG, *et al*: Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell 85:841–851, 1996

Hardy M: The secret life of the hair follicle. Trends Genet 8:55–61, 1992

Harper JM, Krishnan C, Darman JS, *et al*: Axonal growth of embryonic stem cell-derived motoneurons *in vitro* and in motoneuron-injured adult rats. Proc Natl Acad Sci USA 101:7123–7128, 2004

Hutchin ME, Kariapper MST, Grachtchouk M, *et al*: Sustained hedgehog signaling is required for basal cell carcinoma proliferation and survival: Conditional skin tumorigenesis recapitulates the hair growth cycle. Genes Dev 19: 214–223, 2005

Ikram MS, Neill GW, Regl G, *et al*: GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. J Invest Dermatol 122:1503–1509, 2004

Johnson RL, Rothman AL, Xie J, *et al*: Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science 272:1668–1671, 1996

Lo Celso C, Prowse DM, Watt FM: Transient activation of β-catenin signaling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. Development 131:1787–1799, 2004

Lum L, Beachy PA: The Hedgehog response network: Sensors, switches, and routers. Science 304:1755–1759, 2004

Machold R, Hayashi S, Rutlin M, *et al*: Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937–950, 2003

McGowan KM, Coulombe PA: The wound repair-associated keratins 6, 16, and 17. Insights into the role of intermediate filaments in specifying keratinocyte cytoarchitecture. Subcell Biochem 31:173–204, 1998

McMahon AP, Ingham PW, Tabin CJ: Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 53:1–114, 2003

Mehrel T, Hohl D, Rothnagel JA, *et al*: Identification of a major keratinocyte cell envelope protein, loricrin. Cell 61:1103–1112, 1990

Messenger AG: The control of hair growth and pigmentation. In: Olsen EA (ed). Disorders of Hair Growth. Diagnosis and Treatment. New York: McGraw-Hill Company, 2003; p 49–74

Mill P, Mo R, Fu H, Grachtchouk M, Kim PCW, Dlugosz AA, Hui CC: Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. Genes Dev 17:282–294, 2003

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

Müller-Röver S, Handjiski B, van der Veen C, *et al*: A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol 117:3–15, 2001

Niemann C, Unden AB, Lyle S, Zouboulis ChC, Toftgård R, Watt FM: Indian hedgehog and β-catenin signaling: Role in the sebaceous lineage of normal and neoplastic mammalian epidermis. Proc Natl Acad Sci USA 100:11873–11880, 2003

Nilsson M, Unden AB, Krause D, Malmqwist U, Raza K, Zaphiropoulos PG, Toftgård R: Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. Proc Natl Acad Sci USA 97:3438–3443, 2000

Nüsslein-Volhard C, Wieschaus E: Mutations affecting segment number and polarity in Drosophila. Nature 287:795–801, 1980

Olsen EA: Pattern hair loss in men and women. In: Olsen EA (ed). Disorders of Hair Growth. Diagnosis and Treatment. New York: McGraw-Hill Company, 2003; p 321–362

Oro AE, Higgins K: Hair cycle regulation of Hedgehog signal reception. Dev Biol 255:238–248, 2003

Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH Jr, Scott MP: Basal cell carcinomas in mice overexpressing sonic hedgehog. Science 276: 817–821, 1997

Paus R, Cotsarelis G: The biology of hair follicles. N Engl J Med 341:491–497, 1999

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

Sheng H, Goich S, Wang A, *et al*: Dissecting the oncogenic potential of Gli2: Deletion of an NH_2-terminal fragment alters skin tumor phenotype. Cancer Res 62:5308–5316, 2002

Slominski A, Paus R: Melanogenesis is coupled to murine anagen: Toward new concepts for the role of melanocytes and the regulation of melanogenesis in hair growth. J Invest Dermatol 101:90S–97S, 1993

Stenn KS, Paus R: Controls of Hair Follicle Cycling. Physiol Rev 81:449–494, 2001

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

Wang LC, Liu ZY, Gambardella L, *et al*: Conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. J Invest Dermatol 114:901–908, 2000

Wichterle H, Lieberam I, Porter JA, Jessell TM: Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397, 2002

Winter H, Langbein L, Praetzel S, *et al*: A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. J Invest Dermatol 111:955–962, 1998

Xie J, Murone M, Luoh SM, *et al*: Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 391:90–92, 1998

Zhang XM, Ramalho-Santos M, McMahon AP: Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. Cell 106:781–792, 2001