

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 Smoothed (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/6Ncr1BR; 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/6NCrIBR (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 μ M 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 μ g per μ L) was tested for its ability to induce hair growth. 25 μ L 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 μ g per μ L) 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 1A). In general, complete hair growth occurred in areas treated with Hh-agonist (Fig 1A) and was restricted to the area of topical application (Fig 1A). 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 1C). In contrast, hair follicles from vehicle-treated skin were all in telogen (Fig 1B).

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 2B and J to Fig 2A 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 2C, 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% \pm 1.3% compared with 36.9% \pm 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% \pm 1.6% in Hh-agonist-treated skin

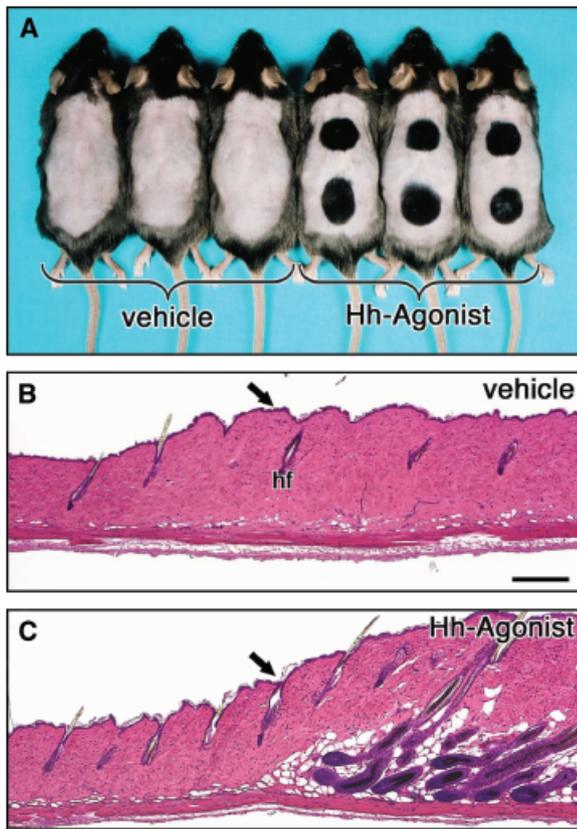


Figure 1
Anagen induction and hair growth in C57BL/6NcrIBR (C57BL/6) mice treated with a single topical dose of hedgehog (Hh)-agonist. (A) Seven-wk-old male mice were shaved and given a single 25 μ L topical application of vehicle (95% acetone/5% DMSO) or Hh-agonist (0.06 μ g per μ L in vehicle, 120 μ M) on their upper and lower dorsal regions. Hair growth in the mice pictured is 13 d after topical application. (B, C) Five- μ m 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 μ m.

compared with $33.1\% \pm 1.4\%$ 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 2A–D, I–L) when compared with depilated skin (Fig 2E–H, M–P) at any equivalent time point analyzed.

By day 18–19, both Hh-agonist and depilated skin had entered into catagen (Fig 2Q, R) and by day 23 had completed the hair cycle and the follicles were once again in telogen (Fig 2S, 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 (auchen, 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 3A, 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 3A, 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 (~ 100 -fold) was strikingly higher compared with that of the other genes analyzed (~ 20 – 25 -fold for *Gli1* and *Ptc1* (Fig 3C), and ~ 6 – 7 -fold for *Gli2* (Fig 3D)). 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 3C, 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 4A) both depilated (Fig 4F) and Hh-agonist-treated hair follicles (Fig 4K) 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

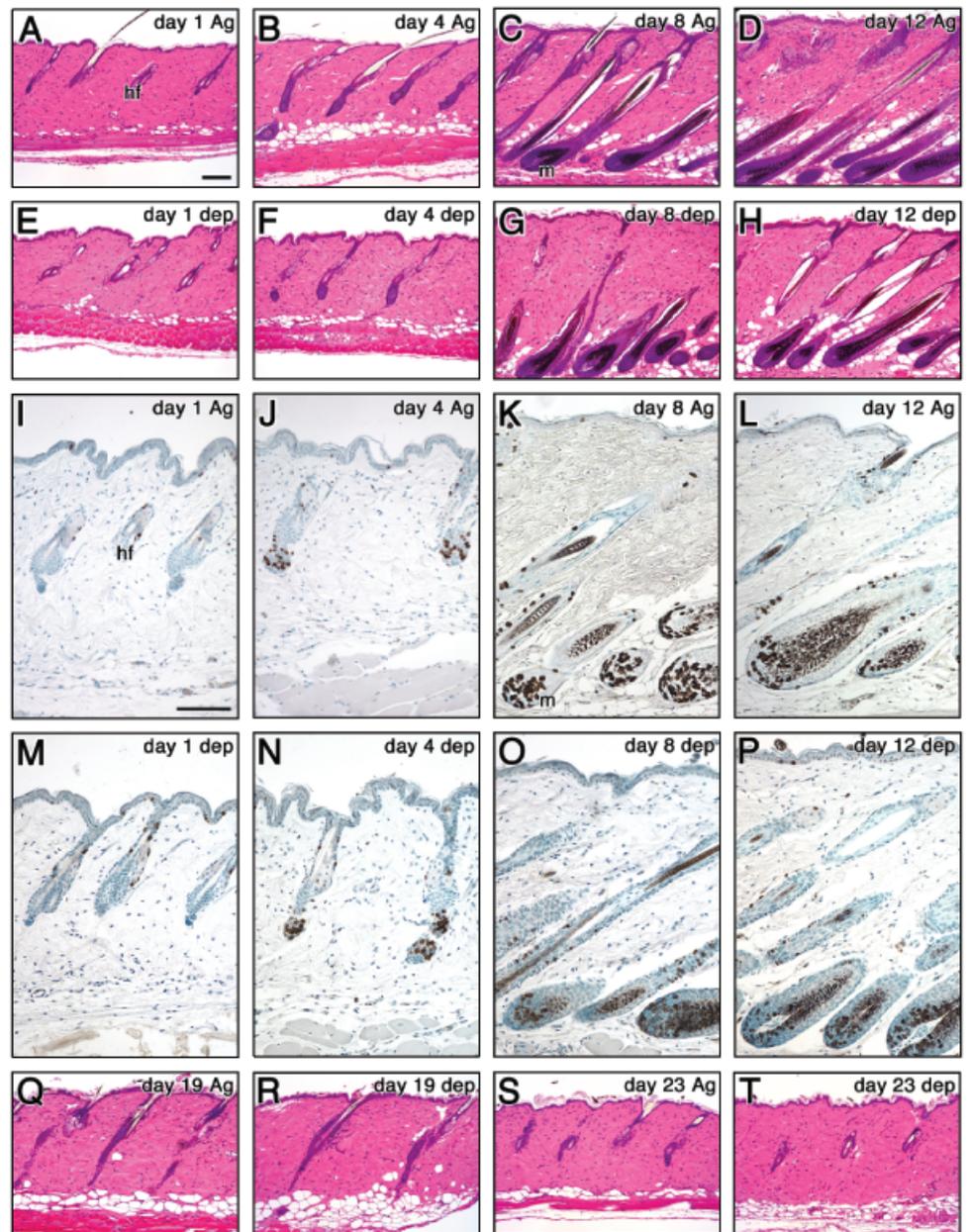


Figure 2
Light microscopy analysis of the hedgehog (Hh)-agonist-induced hair cycle. Five- μm parasagittal paraffin sections were stained with hematoxylin and eosin (A–H, Q–T) or immunostained with an antibody to BrdU (I–P) and analyzed by light microscopy. Seven-wk-old C57BL/6Ncr1BR (C57BL/6) male mice 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 4B, 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 4H), Hh-agonist-treated (Fig 4M) and vehicle-treated skin (Fig 4C). In addition, loricrin expression, in all three cases (Fig 4D, 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, Ker-

atin 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 5A)- and Hh-agonist (Fig 5B)-treated skin were in telogen. Proliferation in both vehicle- (Fig 5C) and Hh-agonist-treated (Fig 5D) skin, as analyzed by BrdU immunohistochemistry, was minimal. A few BrdU

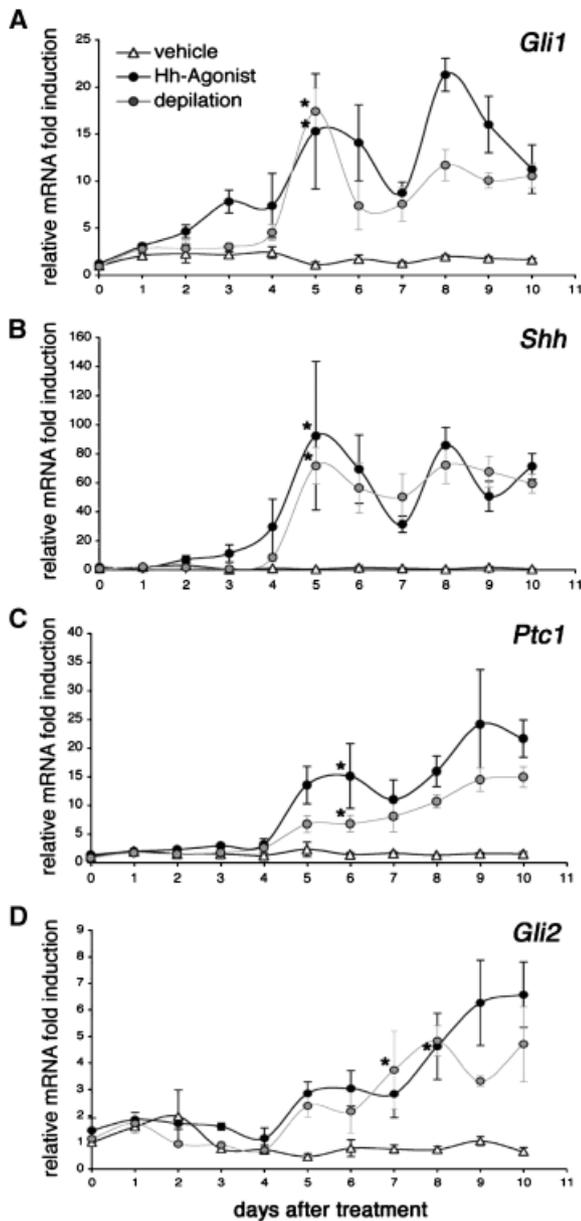


Figure 3
Time course of Sonic hedgehog (Shh) pathway gene expression in skin following anagen induction in hedgehog (Hh)-agonist-treated mice. The seven-wk-old C57BL/6Ncr1BR (C57BL/6) male mice were treated once with either 25 μ L of vehicle, Hh-agonist (0.06 μ g per μ L in vehicle), or a depilatory 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 \pm SEM.

positive cells were detected in both the telogen-staged hair follicles and the interfollicular epidermis (arrowheads in Fig 5C 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 5E 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 5G and H), whereas loricrin was detected in the granular layer and stratum corneum (Fig 5I 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 5K) and Hh-agonist-treated skin (Fig 5L). 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*, *Ptc1*, *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

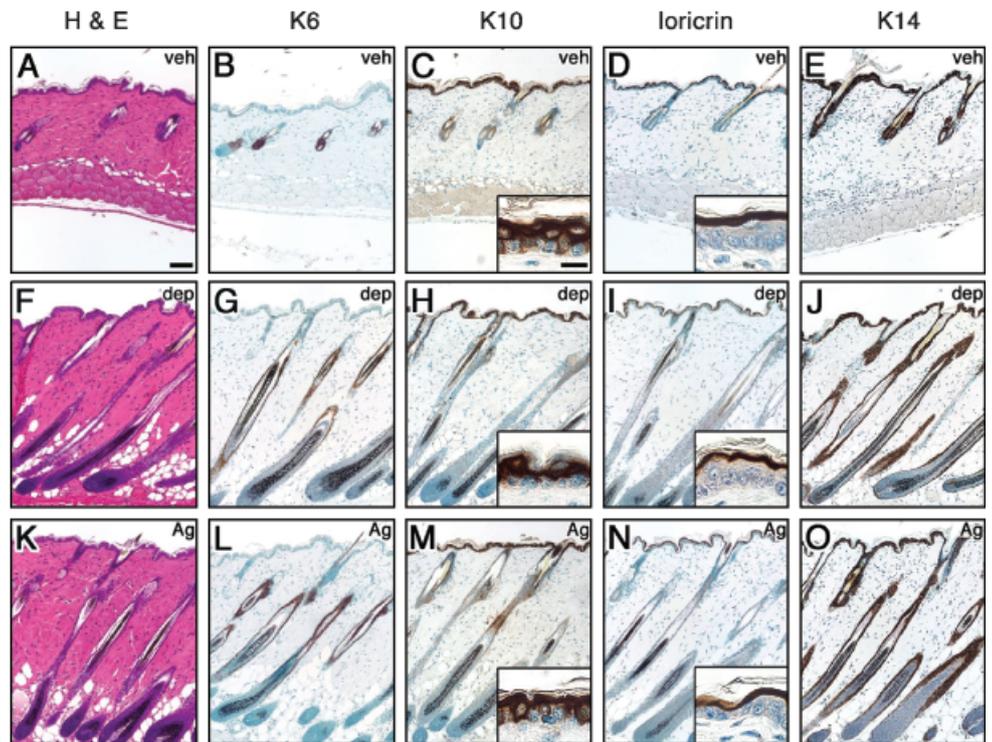
We have reproduced a defined biological role of Shh in the adult mouse, namely the ability to modulate hair growth and follicular cycling, by the topical application of a small molecule agonist of the Hh-signaling pathway. This work further validates the concept that Hh-signaling in the adult vertebrate 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- μ m 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/6Ncr1BR (C57BL/6) 6 male mice were given a single 25 μ L topical application of either vehicle or Hh-agonist (0.06 μ g per μ L 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 μ m. Scale bar in inset (C, D, H, I, M, and N) = 10 μ m.



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 1A, 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

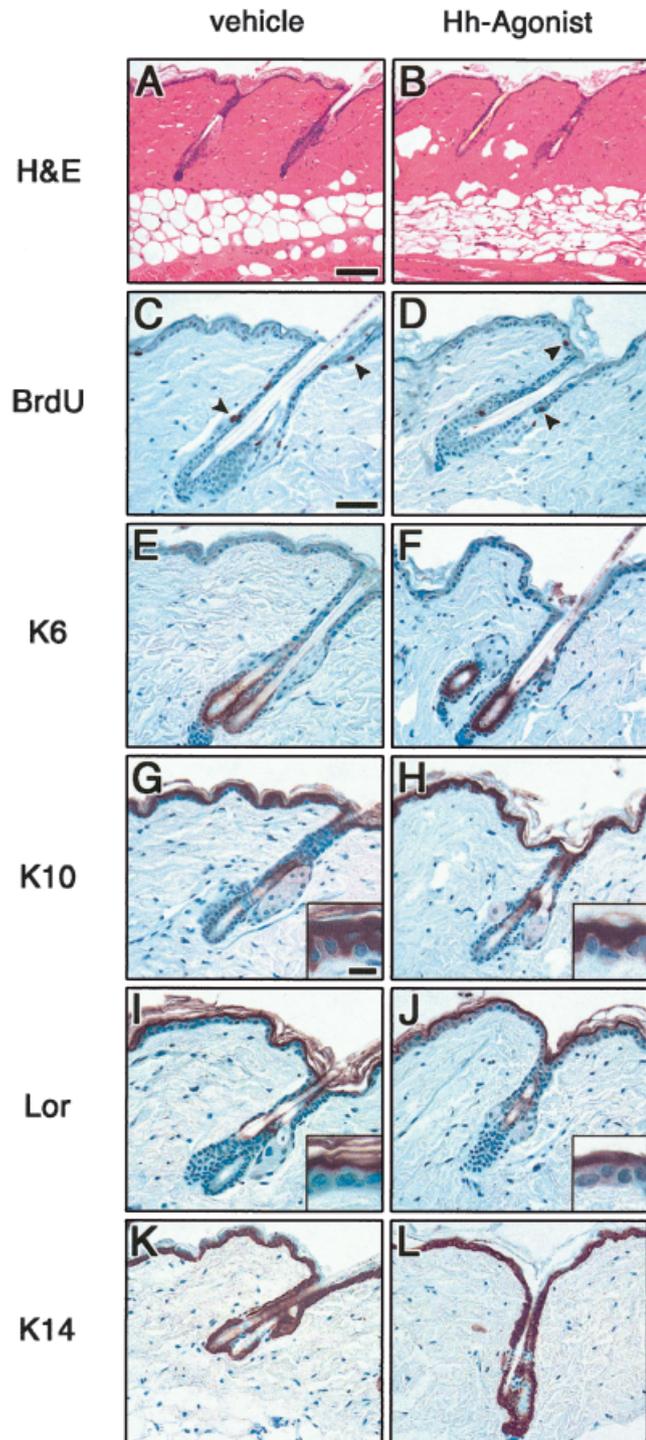


Figure 5
Long-term effects of hedgehog (Hh)-agonist treatment on epidermal differentiation in mouse skin. Five- μm parasagittal paraffin sections from vehicle-(A, C, E, G, I, K) or Hh-agonist-treated (B, D, F, H, J, L) mouse skin were stained with hematoxylin and eosin (A, B) or immunostained with antibodies 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/6Ncr1BR (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).

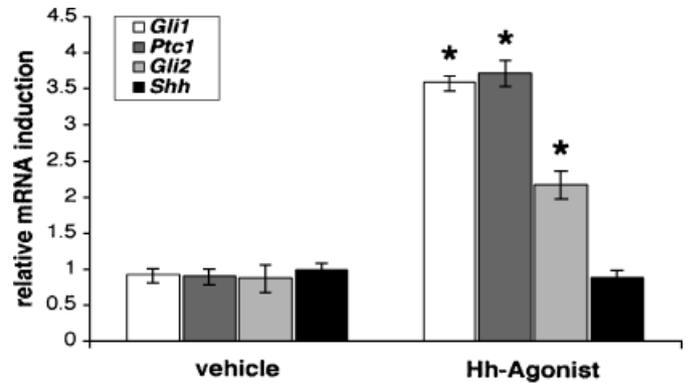


Figure 6
Hedgehog (Hh) pathway gene induction in hedgehog (Hh)-agonist-treated fetal scalp. Human fetal scalp skin was cultured and topically treated once daily with either 8 μL of vehicle or Hh-agonist (0.15 μg per μL in vehicle). RNA was isolated after 4 d in culture. The relative mRNA fold induction 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 \pm 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 μ L 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 H₂O. 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 μ L topical dose of Hh-agonist (0.06 μ g per μ L in vehicle, 120 μ M) 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 μ L of acetonitrile. To isolate the Hh-agonist from blood, 100 μ L of plasma was mixed with 200 μ L of acetonitrile, vortexed, and centrifuged for 10 min \times 8,160 g. The supernatant was isolated and subjected to LC-MS/MS. 10 μ L of an internal standard was added to each sample. Chromatographic separation was achieved using a 5 μ m, 2.1 \times 30 mm XTerre MS C₁₈ 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 μ m 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 μ g per μ L 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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