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Overexpression of Sonic Hedgehog suppresses embryonic hair follicle morphogenesis

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Abstract

The Sonic Hedgehog (Shh) signalling pathway plays a central role in the development of the skin and hair follicle and is a major determinant of skin tumorigenesis, most notably of basal cell carcinoma (BCC). Various mouse models involving either ablation or overexpression of key members of the Shh signalling pathway display a range of skin tumours. To further examine the role of Shh in skin development, we have overexpressed Shh in a subset of interfollicular basal cells from 12.5 dpc under the control of the human keratin 1 (HK1) promoter. The HK1-*Shh* transgenic mice display a range of skin anomalies, including highly pigmented inguinal lesions and regions of alopecia. The most striking hair follicle phenotype is a suppression in embryonic follicle development between 14.0 and 19.0 dpc, resulting in a complete absence of guard, awl, and auchene hair fibres. These data indicate that alternative signals are responsible for the development of different hair follicles and point to a major role of Shh signalling in the morphogenesis of guard, awl, and auchene hair fibres. Through a comparison with other mouse models, the characteristics of the HK1-*Shh* transgenic mice suggest that the precise timing and site of Shh expression are key in dictating the resultant skin and tumour phenotype.

Keywords: Shh; Sonic hedgehog; Transgenic; Skin; Epidermis; Human keratin 1

Introduction

Hair follicle development is characterised by several inductive interactions between epithelial and mesenchymal cells. The first event in hair follicle development is marked by an epidermal thickening and mesenchymal condensation followed by subsequent invagination of the epithelium into the dermis. This first dermal message is "to make appendage here" and the epidermis responds by initiating thickenings (placodes). The second signal originates from the epithelium and induces mesenchymal aggregation. The third signal, once again relayed by the dermis, induces adjacent epithelial matrix cells to grow and differentiate into the mature hair follicle (Hardy, 1992). In the postnatal skin, the hair follicle cycles through phases of growth (anagen), regression (catagen), and resting (telogen) (Hardy, 1992). Both embryonic and adult hair follicle development utilise many of the same signalling pathways. Skin disorders and neoplasia may result due to perturbation of these signalling events in the adult skin.

The Sonic hedgehog (Shh) signalling cascade is one of the key pathways involved in skin and hair follicle development. Current models of hedgehog signalling propose that the transmembrane protein Patched (Ptc) acts as a receptor for Shh and their interaction triggers a signalling cascade modulated by Smoothened (Smo). It has been suggested that Smo activation leads to the dissociation of a cytosolic complex and the subsequent translocation of the

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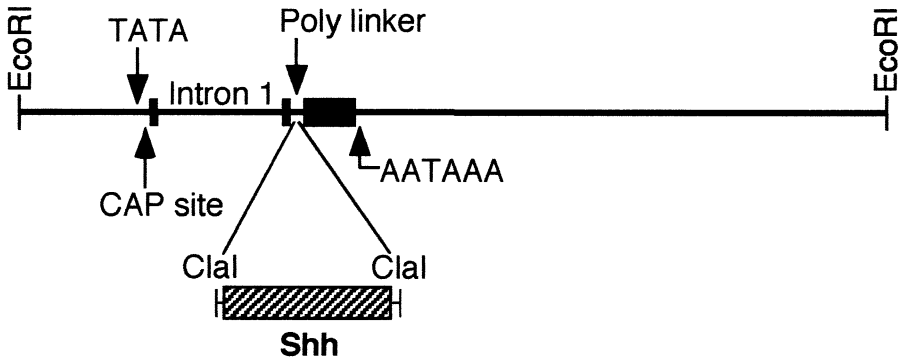

Fig. 1. Schematic representation of the HK1-*Shh* transgene construct. The HK1 transgene construct is based on a 12-kb *Eco*RI fragment of the human Keratin 1 gene as previously described (Greenhalgh et al., 1993a,b). Briefly, the transgene construct contains the first intron and all 5′ and 3′ flanking sequences and a polylinker 3′ to the first intron. At the *Cla*I site, we inserted the PCR product of the full-length rat Shh sequence (stipled box).

transcription factor Gli to the nucleus. In mammals, Shh signalling is mediated by three Gli proteins (Gli1, Gli2, and Gli3) in a complex regulatory network, the precise details of which remain to be elucidated (Wicking et al., 1999). This in turn results in the upregulation of a number of downstream target genes, including *Patched* and *Gli* as well as members of the *Wnt* and *TGFβ* gene families (Ingham, 1998; Ingram et al., 2002).

Shh is strongly expressed in the developing facial vibrissae and in later stages in the developing skin (Bitgood and McMahon, 1995; Goodrich et al., 1996). Examination of the skin from both *Shh*-null and *Shh* transgenic mice has demonstrated that Shh is not required for the first epidermal signal but is required for subsequent signalling from the epidermis to both epithelial and mesenchymal cells regulating proliferation and further downgrowth of follicular epithelium and dermal papilla development. (Chiang et al., 1999; St-Jacques et al., 1998). Furthermore, it is likely that the second dermal signal regulating proliferation and downgrowth is activated by Shh. It does appear that the formation of follicles may be Shh concentration-dependent, since defects in the differentiation of these structures are noted both in mice lacking *Shh* and in those overexpressing it (Chiang et al., 1999; Oro et al., 1997; St-Jacques et al., 1998). In addition to its role in embryonic hair follicle development, treatment of skin with Shh blocking antibodies results in reversible alopecia in which the adult follicles were arrested in the telogen phase of the hair cycle (Wang et al., 2000), thus indicating that Shh is also required for cycling in the postnatal skin.

The Shh signalling pathway has also been implicated in the processes of tumorigenesis. Of particular interest is the central role which activation of this pathway plays in the development of skin tumours both in humans and mice. Inactivating mutations in the gene encoding the hedgehog receptor Patched cause both human familial and sporadic basal cell carcinomas (BCCs) (Hahn et al., 1996; Johnson et al., 1996; Sato et al., 1999), as well as a number of other human skin cancers (Ping et al., 2001). Tumours of the skin reminiscent of BCCs are apparent in several mouse models in which the Shh signalling pathway is perturbed (Aszterbaum et al., 1999; Dahmane et al., 1997; Nilsson et al., 2000; Oro et al., 1997; Sheng et al., 2002; Xie et al., 1998).

Perinatal lethality in existing mouse models of *Shh* overexpression has hampered the study of postnatal effects on the epidermis and follicle. Overexpression of *Shh* from a keratin 14 (K14) promoter has been described by Oro and coworkers. K14-*Shh* mice die perinatally and at 18.5 dpc exhibit multiple BCC-like epidermal proliferations with the invaginating hair follicles (Oro et al., 1997). The expression of transgenes from early stages of development has clouded the issue of whether resultant phenotypes were a consequence of disrupted epidermal development rather than disrupted maintenance of the skin. In order to address these issues, we have generated transgenic mice expressing *Shh* in interfollicular basal cells under the control of the human keratin 1 (HK1) promoter. This promoter has several qualities that make it beneficial in studying epidermal develop-

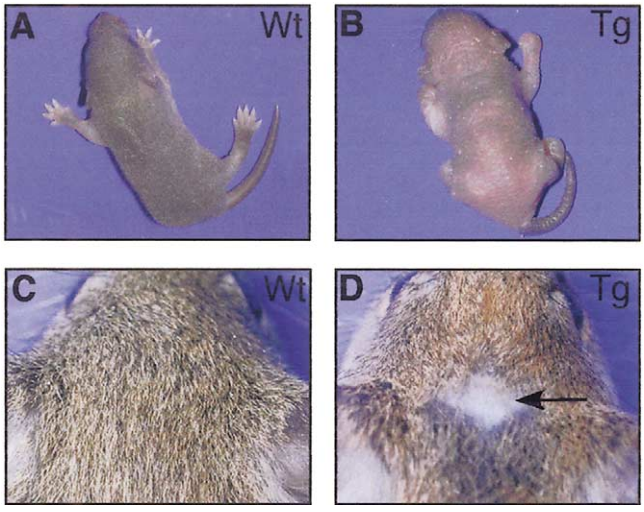

Fig. 2. Hair follicle phenotypes of the HK1-*Shh* transgenic mice. Five-day old (A) wild- type and (B) HK1-*Shh* littermates indicating lack of hair follicle eruption in the transgenic animal. One-month-old (C) wild-type and (D) HK1-*Shh* transgenic littermates showing the region of alopecia (arrow).

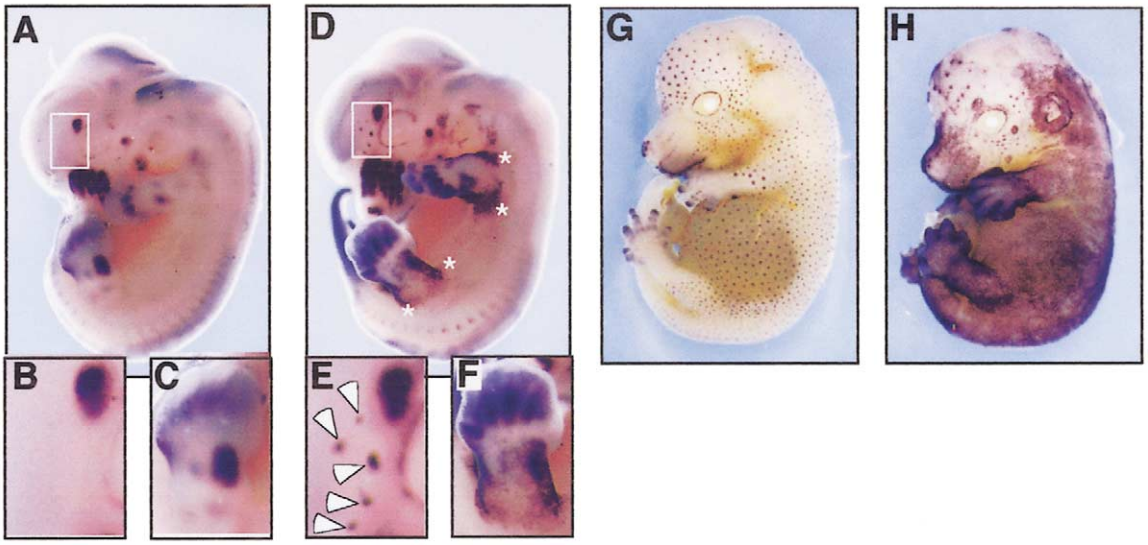

Fig. 3. In situ analysis of Ptc expression. (A, G) Wild-type embryos and (D, H) HK1- *Shh* transgenic littermates. (A–F) 12.5 dpc; (G, H) 15.5 dpc. Ectopic Ptc expression in the sinus follicles of (D, E) HK1-*Shh* compared with (A, B) wild type 12.5 dpc littermates. Boxes in (A, B) highlight region of higher magnification shown in (B) and (E), respectively. Arrowheads in (E) indicate ectopic Ptc expression. * in (D) indicates ectopic expression in the developing limbs. The hindlimb region is shown at a higher magnification in (C) wild type and (F) HK1-*Shh* 12.5-dpc littermates.

ment and function. In wild type skin, keratin 1 is expressed as keratinocytes differentiate in the suprabasal epidermal cell layers. As a transgenic promoter in mice, however, it is also expressed in 30–40% of proliferating basal cells, an obvious candidate cell type for overexpression of a potential oncogene (Rosenthal et al., 1991). Unlike the keratin 14 promoter construct previously used by Oro et al. (1997), the HK1 promoter is active only in the skin, thus avoiding in utero death due to transgene expression prior to epithelial differentiation (Imakado et al., 1995; Rothnagel et al., 1993). This specificity also means that the resultant transgenic phenotype is unlikely to be due to the secondary effects of altered embryonic development in other organs.

Here, we demonstrate that *Shh* under the control of the HK1 promoter results in the inhibition of embryonic hair follicle morphogenesis, but postnatal hair follicle development precedes normally, thus revealing a novel function of Shh in skin development. Additionally, although the skin of HK1-*Shh* mice exhibits increased proliferation, epithelial homeostasis is maintained to prevent a hyperproliferative skin phenotype. Interestingly, BCCs do not form in the skin of the transgenic mice under the control of the HK1 promoter, indicating that skin tumorigenesis requires Shh pathway activation in the correct temporal–spatial manner.

Materials and methods

Construction of HK1-Shh transgene

*Cla*I linkered primers (Shhfmk2 5′-ATC GAT ACC ACC ATG CTG CTG CTG CTG GC-3′, Shh1r 5′-ATC GAT TAG CTG GAC TGG ACT GCC-3′) were used to amplify the rat *Shh* complete open reading frame from nucleotides 315-1626 (GenBank Accession no. L27340). This product was subcloned into pGEM-T and sequenced completely with M13 forward and reverse primers and primers within *Shh* (Shh#3 5′-GGC CGA TAT GAA GGG AAG AT-3′, Shh#4 5′-ATC AGC CAC AGT GCA CCT G-3′, Shh#5 5′-TGT ACG TGG TGG CTG AAC G-3′, Shh#6 5′-TCT CGT AGC GTT ACG CTG TG-3′, Shh#7 5′-AGG AAG GTG AGG AAG TCG CTG-3′). This product was then subcloned into the *Cla*I site of the HK1 transgene (Rosenthal et al., 1991). The HK1 transgene construct is based on a 12-kb *Eco*RI fragment of the human Keratin 1 gene as previously described (Greenhalgh et al., 1993a,b). Transgenic mice were generated by pronuclear injection of embryos from a CBA/C57B16 F1 cross as described (Hogan et al., 1994). Transgenic mice were bred onto FVB or C57B16 backgrounds. All mice in this study were heterozygous for the transgene. DNA was screened for the Shh transgene by using primers which span intron 1 (Shh1F 5′-GCG ATT TAA GGA ACT CAC CCC C-3′, Shh2R 5′-TGC TTT CAC CGA GCA GTG G-3′).

Histology and immunofluorescence

Tissues were frozen directly in OCT tissue mounting media (Tissue-Tek) or fixed in 4% paraformaldehyde/PBS and paraffin-embedded. Blocks were sectioned between 5 and 12 μm and collected on Superfrost Plus-coated microscope slides (Menzel-Glaser). Haematoxylin and eosin staining was performed by using standard histological techniques. Immunofluorescence was performed by using stan-

dard protocols. Briefly, antigen unmasking (Vector labs) was performed on paraffin-embedded sections following manufacture's instructions. Primary antibodies were incubated overnight at 4°C followed by 1-h incubation with the appropriate Alexa-conjugated secondary antibody (Molecular Probes). Anti-Ptc antibodies were raised against synthetic peptides (Chiron Mimitopes) corresponding to residues 1440–1450 (CEERPRGSSSSN) in chicken (GenBank Accession No. U46155). The following primary antibodies were used: rabbit anti-mouse K10 (Babco), rabbit anti-mouse loricrin (Babco, clone AF62), rabbit anti-mouse filaggrin (Babco, clone AF111), and monoclonal anti-β-catenin (Sigma, clone 15B8).

Bromodexoyuridine (BrdU) immunohistochemistry

Pregnant females or pups were injected with 0.1 ml/g (vol/body weight) of BrdU labelling reagent (Zymed) 2 h prior to sacrificing. The tissues were processed for paraffin embedding as described. The sections were incubated for 1 h in 2 N HCl followed by a 1-h treatment at room temperature in 0.5× Trypsin (Sigma). Sections were incubated overnight at 4°C with 1:100 dilution of monoclonal mouse anti-BrdU (Zymed) and detected by using the Vectastain ABC Kit (Vector Labs) followed by DAB staining (Pierce).

In situ hybridisation

Whole-mount and section in situ hybridisations were performed as described previously (Christiansen et al., 1995; Fowles et al., 2003; Mahony et al., 2000), with minor modifications. Digoxigenin-labelled RNA antisense probes were synthesised from nucleotides 699-1414 of mouse *Ptc* (GenBank Accession No. U46155), nucleotides 36–678 of *Shh* (GenBank Accession No. L7340), and nucleotides 652–1476 of mouse keratin 14 (GenBank Accession No. NM 016958).

Results

HK1-Shh mice have hair follicle abnormalities

The HK1 promoter construct was derived from a 12-kb genomic fragment that includes 5′ and 3′ flanking sequence, the first intron including the splice site, but lacks the ATG codon and coding sequences (Rothnagel et al., 1993). The full-length rat homologue of Shh was cloned 3′ to the first intron–exon boundary (Fig. 1). Two lines were established in which transgenic pups were born at Mendelian frequency and were fertile. Of the two transgenic lines analysed, both presented with similar skin phenotypic features. In addition to the skin phenotype, the HK1-*Shh* transgenic mice also display fore- and hindlimb abnormalities that will be discussed elsewhere (unpublished observations).

At birth, the skin of transgenic animals was indistinguishable from wild type littermates; although by postnatal day 5, a lack of hair follicle eruption on the dorsal coat of transgenic animals could be detected (Fig. 2A and B). This delay in hair follicle morphogenesis was most apparent during the first weeks of life, and by the time of weaning, the lack of pelage hair was less obvious. In order to determine whether this defect was strain-specific, the transgene was bred onto both the white FVB and the agouti C57B16 backgrounds. On close examination of the dorsal coat of animals after weaning, the coat appeared sparser on both backgrounds. On the agouti background, transgenic coat colour was lighter than wild type littermates (data not shown). Hair from the dorsum of these animals was plucked and hair types were analysed. In wild type littermates, all four hair types were observed: guard, awls, auchenes, and zigzag. However, in HK1-*Shh* transgenic mice, the pelage hair consisted almost entirely of zigzag fibres (~90%) with only a few fibres that were unclassified (<10%), indicating that the long guard hairs, awls, and auchenes were absent. In addition, adult transgenic mice exhibited regions of alopecia on the top of the head (Fig. 2C and D) and dorsal midline (data not shown) irrespective of gender and background strain. Similar regions of alopecia were never observed on adult wild type littermates. The appearance of alopecia is not due to grooming effects but rather correlates with the lack of hair follicles observed (See Fig. 4K and L). The development of the whiskers was not obviously affected.

The Shh pathway is activated in HK1-Shh transgenic skin

Roop et al. have previously reported that the HK1 transgene is only detected in the epidermis from 15.0 dpc onwards by RT-PCR (Imakado et al., 1995; Rothnagel et al., 1993). Since numerous studies have indicated that *Ptc* is a robust downstream target of Shh (Murone et al., 1999; Ping et al., 2001), we analysed *Ptc* expression to determine pathway activation as a biological indicator of transgene expression. By whole-mount in situ analysis of *Ptc* in transgenic embryos (Fig. 3), it is apparent that the HK1-*Shh* transgene is activating Shh signalling as early as 12.5 dpc as observed by the upregulation of *Ptc* expression. Between 12.5 and 15.5 dpc, Ptc is expressed in wild type embryos in distinct sinus hair follicles on the face, pelage, the vibrissae (whisker), and areas of future cartilage condensation in the limbs (Fig. 3A and G). In the 12.5-dpc HK1-*Shh* transgenic embryo, *Ptc* expression appears to be upregulated in all domains (Fig. 3D) and ectopic expression is evident in sinus follicles (Fig. 3D and E) and in the overlying epidermis on the proximal limb (Fig. 3D and F). In the 15.5-dpc HK1-*Shh* embryos, *Ptc* expression is observed in some sinus hair follicles, but distinct pelage follicle expression was not detected and the *Ptc* expression domain in the transgenic skin is expanded, with expression dispersed throughout the overlying epidermis across the entire embryo (Fig. 3H). A similar pattern of expression was apparent at 13.5 dpc (data

not shown). Therefore, our results indicate that the HK1 transgene is expressed at least 2.5 days earlier in embryonic development than previously reported, and this leads to ectopic Shh signalling in the skin.

Ectopic Shh expression blocks all hair follicles derived during embryogenesis

To determine the effect of overexpression of *Shh* in suprabasal and mitotically active basal cells on the formation of pelage follicles, we examined hair follicles at various developmental stages (Fig. 4). Development of pelage follicles in the mouse initiates around 14.5 dpc, which is 1-2 days later than the whisker follicles (Hardy, 1992). Hair follicle development is divided into eight stages of morphogenesis (Hardy, 1992; Paus et al., 1999). Initial hair follicle development is characterised by the formation of the placode as an epidermal thickening with a localised increase of dermal fibroblasts. This placode forms into an epithelial bud and migrates into the dermis forming first hair germs concurrent with mesenchymal condensation. This condensing mesenchyme forms a dermal papilla that becomes surrounded by the follicle epithelium of the hair peg during stages 3–4. Further hair follicle morphogenesis results in the formation of the inner root sheath (IRS), hair shaft, and elongation of the follicle. By stage 8, the hair follicle has acquired its maximal length and a hair shaft emerges through the epidermis (Paus et al., 1999). In mice, fur consists of a number of different types of hair follicles characterised by both morphological differences and timing of morphogenesis. Pelage follicles begin to form prenatally at the crown of the head and extend in a wave-like manner over the body surface. In animals with a dense coat, this first wave is followed by further waves of smaller follicles producing smaller hairs (Hardy, 1992). Each adult hair fibre is associated with one of the successive time groups. The sinus hairs of the upper lip and face are the first to develop between 12 and 14 dpc. The coat of mice consists of four kinds of hair follicles: the overhairs, which consist of guard, awl, and auchene hairs, and the underhairs, which are known as zigzag hairs. The primary guard hair follicles of the mouse pelage, which constitute approximately 5–10% of the pelage, develop beginning at 14 dpc. In addition to guard hairs, awls and auchenes develop between 17 and 19 dpc. The final fibre to commence development after birth is the zigzag fibre of the undercoat (Botchkarev et al., 2002; Falconer et al.,).

In 14.5-dpc wild type skin, the earliest stages of hair development are observed as plugs invaginating from the epidermis with mesenchymal condensation (Fig. 4A). At 16.5 dpc, follicles are elongating. By 18.5 dpc, wild type hair follicles are observed in various stages of morphogenesis (Fig. 4C and E) with the majority in the earlier stages of development (stages 3–5), but a few follicles are seen that have almost acquired their maximal length. By contrast, hair follicles were never observed in any sections of the 14.5-(Fig. 4B) or 16.5-dpc (Fig. 4D) transgenic HK1 skin examined from multiple embryos. The 18.5-dpc transgenic skin showed relatively few areas of epidermal thickening (Fig. 4F) that appeared to migrate into the dermis as is indicative of the initial stage of hair follicle development (stage 1). Morphogenesis of the vibrissae follicles appeared relatively unaffected in the 14.5-dpc transgenic animals compared with wild type littermates (Fig. 4G and H), although the transgenic vibrissae appeared less organised. Histological analysis of newborn HK1-*Shh* skin revealed that postnatal day 2 (P2) skin had hair follicles that appeared to be in the later stages of morphogenesis, although the number of hair follicles was reduced in the transgenic skin (Fig. 4J) compared with the wild type littermates (Fig. 4I). Guard hair follicles, which are longer than other follicles, are visible in backskin of P2 wild type littermates (Fig. 4I) but absent in HK1-*Shh* skin (Fig. 4J). In addition, the epidermis of P2 transgenic skin showed hyperkeratosis (increased stratum corneum thickness) (Fig. 4J). Taken together, these data suggest that overexpression of Shh under the HK1 promoter leads to an approximate 4-day block in pelage hair follicle development, resulting in a complete deficiency of guard, awls, and auchene fibres which continues throughout adult life. The absence of guard hair follicles has been previously observed in mice with defects in Tumor necrosis factor (TNF) signalling mediated by Eda and Edar (Falconer et al.,; Headon and Overbeek, 1999; Kojima et al., 2000; Laurikkala et al., 2002; Naito et al., 2001; Nishioka et al., 2002; Yan et al., 2002).

Closer examination of skin sections revealed that the basal cells of the 16.5-dpc transgenic backskin had a high nuclear-cytoplasmic ratio, and the nuclei of the basal cells in contact with the basement membrane appeared vertically elongated and compacted (Fig. 4D). The vertical elongation of the cells is known as palisading and is a characteristic of the cells at the periphery of human BCCs (Oro et al., 1997). This palisading characteristic was also observed in the 18.5-dpc transgenic skin (Fig. 4F). Regions of alopecia on the head of adult HK1-*Shh* mice were also examined (Fig. 4L). The basal cell layer of this region appeared to be noticeably thickened, and an obvious lack of hair follicles was observed. The few follicular structures resemble sebaceous glands, although a few were noted to contain hair shafts. This indicates that postnatal hair cycling also has a requirement for correct Shh signalling as has been previously observed (Wang et al., 2000). By contrast, transgenic HK1-*Shh* skin adjacent to the region of alopecia appeared wild type (Fig. 4K).

Hair follicle development on the tail is also perturbed in the HK1-*Shh* mice. The tail skin is hyperproliferative and the number of hairs on the tail was reduced when compared with wild type littermates (Fig. 5A and B). In addition, the transverse skin folds, which give the characteristic appearance of tail rings, are absent from transgenic animals, most likely resulting from the reduction in the number of hairs.

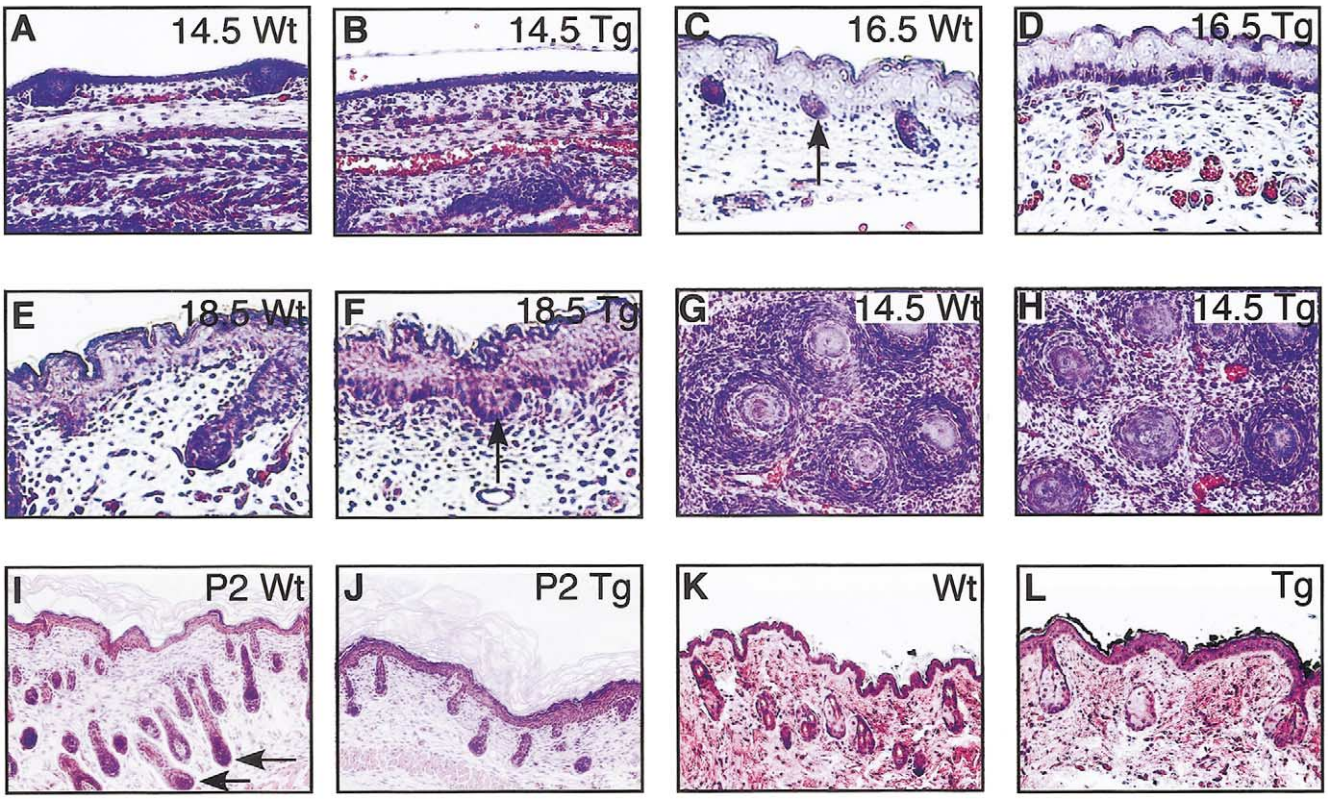

Fig. 4. Hair follicle development in wild-type and HK1-*Shh* transgenic littermates. Representative haematoxylin and eosin-stained sections of backskin from 14.5 dpc (A, B), 16.5 dpc (C, D), 18.5 dpc (E, F), and 2-day-old (I, J) wild-type (A, C, E, I) and HK1-*Shh* transgenic animals (B, D, F, J). Guard hair follicles observed in P2 wild type backskin (I, arrows). Haematoxylin and eosin-stained sections of vibrissae from 14.5- dpc wild-type (G) and HK1-*Shh* transgenic animals (H). Haematoxylin and eosin- stained sections from a HK1-*Shh* transgenic animal demonstrating the region of alopecia from the crown of the head (L) and immediately adjacent section (K). Arrow in (F) indicated a stage 1 hair follicle. The black staining in (L) was dye used to mark the region of alopecia.

The basal cell layer of the tail skin was thick and folded, unlike the relatively smooth layer seen in wild type mice. Many pigmented cells were noted in the tail skin dermis and throughout the basal and suprabasal cells (Fig. 5D and E). Ptc protein expression was detected by immunofluorescence in the basal cell layer and the hair follicles, although it was impossible to determine whether this expression was elevated in comparison to normal tail skin, which was also highly immunoreactive (data not shown). In addition, elevated levels of expression of exogenous rat *Shh* were detected in transgenic (Fig. 5F) but absent in normal tail skin (data not shown).

Absence of embryonic hair follicles in HK1-Shh mice is not due to a reduction in β-catenin

In view of the hair follicle block observed in HK1-*Shh* transgenic mice, we examined levels of β-catenin, which is a molecule known to be necessary in hair placode formation. A decrease in β-catenin is a possibility that would explain the lack of embryonic hair follicle morphogenesis in the HK1-*Shh* skin. In the absence of β- catenin, keratinocytic stem cells fail to differentiate into follicular cells but instead adopt epidermal cell fates, therefore resulting in a lack of hair follicles (Huelsken et al., 2001). In the absence of signalling, β-catenin is associated with cell-membrane adhesion complexes, and upon signalling, β-catenin translocates to the nucleus with subsequent activation of target genes (Huelsken and Birchmeier, 2001; Moon et al., 2002). In 16.5-dpc wild type skin, β-catenin is expressed strongly in hair placodes and germs and at lower levels in the surrounding basal cell layer. β-Catenin was localised to cell–cell borders and was not detected as nuclear (data not shown). This is in agreement with observations of Ridanpaa et al., (2001), who observe elevated β-catenin expression in the hair germs when compared with the epithelium and lack of nuclear β-catenin localisation in embryonic follicles. At 18.5 dpc, β-catenin was downregulated in the basal cell layer and hair follicle of wild type skin (Fig. 6A and B). In contrast to wild type, in 16.5- and 18.5-dpc HK1-*Shh* skin, β- catenin was elevated in the basal cell layer as compared with wild type littermates (Fig. 6C and D, and data not shown). In all cases, β-catenin was localised to cell–cell borders. Therefore, the block of embryonic hair follicle morphogenesis is not as a result of a decrease expression of β-catenin. However, whether the β-catenin present in the

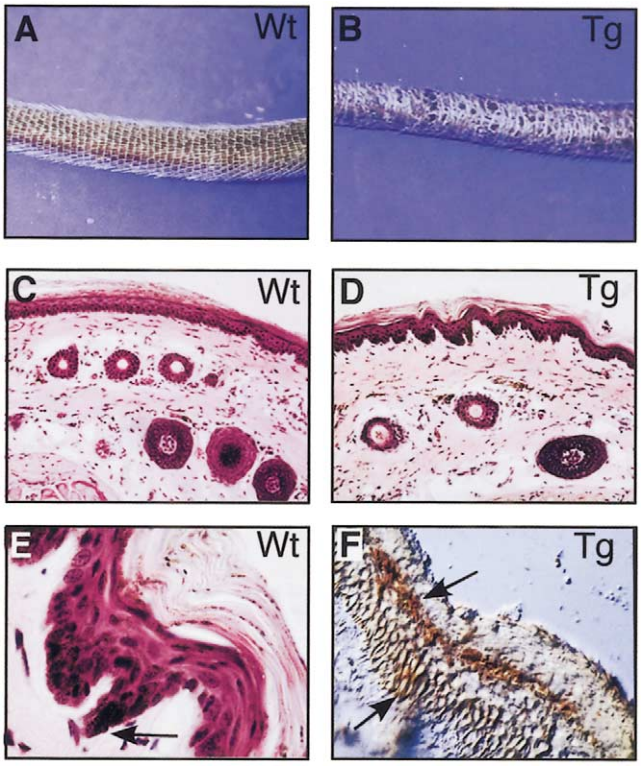

Fig. 5. Phenotypic analysis of the tail skin of HK1-*Shh* transgenic animals. (A) One- month-old tail of a wild-type mouse showing tail rings and hairs. (B) In comparison, a HK1-*Shh* transgenic littermate has a reduction in the number of hairs and an absence of tail rings. Haematoxylin and eosin-stained sections of tail skin of a (C) wild-type and (D) HK1-*Shh* transgenic littermate indicated a thickening of the basal cell layer. (E) Higher magnification of HK1-*Shh* transgenic tail skin reveal epidermal pigmentation (arrow) not observed in wild-type littermates. (F) In situ analysis identified ectopic Shh expression in basal and suprabasal cell layers (arrows) in the tail skin of a HK1-*Shh* transgenic animal

cell– cell borders of the basal cells of HK1-*Shh* mice is also able to signal remains to be elucidated.

Ectopic Shh expression results in increased epidermal proliferation

The pattern of hair follicle formation is a balance between proliferation, polarisation, differentiation, and apoptosis (Magerl et al., 2001). It has been proposed that Shh plays a role in both proliferation and survival (Fan and Khavari, 1999). Skin from both Shh−/− and Gli2−/− mice is arrested at the early hair plug stage. In both instances, a dramatic reduction in cell proliferation within the developing hair follicle had been implicated in the lack of further follicular epithelium downgrowth (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998). In order to determine whether the block of embryonic hair follicle development observed in the HK1-*Shh* transgenic mice was a result of a decrease in follicular epithelium proliferation, immunohistochemical analysis of bromodeoxyuridine (BrdU) incorporation into S- phase nuclei was undertaken. Equal numbers of proliferating cells were observed in both wild type and HK1-*Shh* undifferentiated epithelium at 14.5 dpc (Fig. 7A and B). In 16.5- and 18.5-dpc wild type skin, a limited number of proliferating cells are present in the undifferentiated basal layer of the epidermis (data not shown, and Fig. 7C). At these stages, the majority of proliferating cells in the wild type skin are contained within the developing hair follicle. This is in agreement with Paus and coworkers, who demonstrated that most proliferation occurring in the skin is confined to the distal and mid outer root sheath (ORS) and proximal hair matrix of the developing follicle (Magerl et al., 2001). In contrast to wild type littermates, a high percentage of basal cells are positive for BrdU incorporation in both the 16.5- and 18.5-dpc HK1- *Shh* transgenic skin (Fig. 7D, and data not shown). Therefore, our results indicate that the block of hair follicle development is not due to a lack of proliferation as observed in Shh−/− (Chiang et al., 1999; St-Jacques et al., 1998) and Gli2−/− (Mill et al., 2003) mice.

Epithelial homeostasis is maintained in the HK1-Shh transgenic skin

Although the transgenic epithelium exhibited increased proliferation as compared with wild type littermates, differentiation into the stratified epithelium occurred (Fig. 4). In addition, the HK1-*Shh* transgenic did not present a dramatic hyperproliferative phenotype nor an increase in apoptosis (data not shown) to compensate. To understand how interfollicular epidermal development preceded under these conditions, we analysed the expression patterns of a number of epidermal differentiation markers. In wild type skin, the interfollicular epidermis becomes stratified through the up-

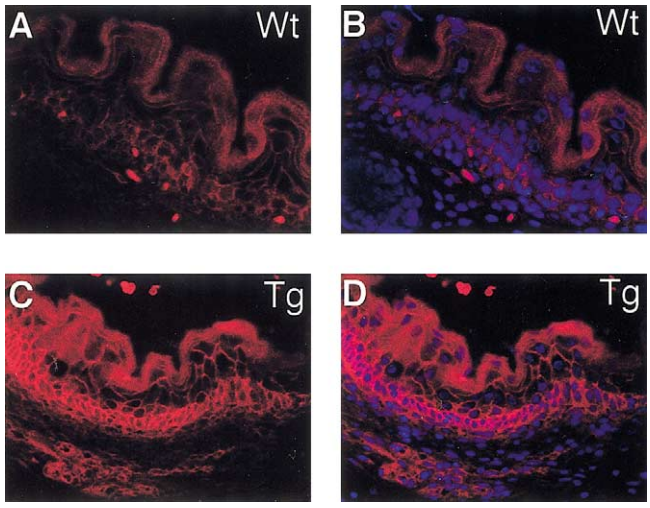

Fig. 6. Expression of β-catenin in 18.5-dpc backskin of (A, B) wild-type and (C, D) HK1-*Shh* transgenic littermate. β-Catenin antibodies (red in A–D) detected the protein at cell–cell borders in the hair follicles and basal cell layer of wild-type and transgenic animals. (B, D) Nuclei were visualized with DAPI (blue).

ward migration and differentiation of basal cells into the suprabasal layers. These cells differentiate, progressing through a stage-specific keratin expression program to form a keratinized layer that eventually sloughs off the surface (Byrne et al., 1994; Fuchs and Raghavan, 2002). The differentiation-specific keratin 10 (K10) protein was present in the suprabasal nucleated cells of the wild type and HK1-*Shh* transgenic 18.5-dpc epidermis (Fig. 8A and B). The thickness of the spinous layer in transgenic animals was not as consistent and organised as in the skin of wild type littermates. In addition, more nucleated cells were present in the transgenic skin (Fig. 8B). The expression of the late-stage differentiation markers filaggrin and loricrin was detected throughout the granular layer of transgenic and wild type littermate controls. However, the thickness of the granular layer and the number of granules appeared reduced in the HK1-*Shh* skin (Fig. 8C–F). In transgenic skin, keratin-14 expression is confined to the basal cell layer, which is similar to wild type expression (Fig. 8G and H). Taken together, these data suggest that the homeostasis of the hyperproliferative HK1-*Shh* epidermis is maintained by a reduction in the thickness of the individual layers of the differentiated stratified epidermis, and although there is an increase in the number of epidermal cells, these cells are more compacted.

HK1-Shh transgenic mice develop inguinal lesions but not BCCs

Unlike other models of Shh pathway activation, no evidence of tumours reminiscent of BCCs was observed on adult HK1-*Shh* mice up to 6 months of age. However, the hair on the inguinal regions of transgenic mice appeared sparse and 14/25 of the transgenic mice on the agouti background developed skin lesions in this region. The lesions appear as highly pigmented, discrete, and slightly raised forms which in the hind limb region often manifest as streaks which extend distally along the limb (Fig. 9A). Lesions were noted in mice as young as 3 weeks of age, and their visible numbers increased as the animals aged. The lesions did not form large masses nor adversely affect the health of the mice. Histological investigation of these lesions revealed that they are continuous with the basement membrane and are therefore not dermal in origin. The lesions are composed of a peripheral region of hyperplastic basaloid cells typically several cells thick, which taper towards the margins of the lesion. Aside from expansion of the basal cell layer, the overlying granular and spinous layers are apparently normal, although a degree of thickening of the stratum corneum is noted, consistent with a hyperproliferative basal cell population. The lesions also contain a preponderance of cells which resemble either sebaceous or follicular cells, although in the lesions examined to date, the former cell type is more commonly noted (Fig. 9B). In addition, masses of highly pigmented melanocytes were associated with the basaloid cells and rarely with the dermal pilosebaceous structures of the lesions. The lesions were found to express keratins 6 and 14 (Fig. 9C and D), but not the suprabasal marker loricrin nor the basement membrane protein BPAG, indicating that the lesions are epidermal in origin (data not shown).

Discussion

Shh regulates development of guard, awls, and auchene fibres

Increasing evidence suggests that the consequences of hedgehog expression in developing skin in transgenic mouse models leads to the formation of BCC-like tumours (Chiang et al., 1999; Oro et al., 1997; St-Jacques et al., 1998). In the chick, the epidermis is differentially responsive to *Shh* depending upon its developmental stage. Early epidermal *Shh* expression leads to disorganised epidermal growth. Similar expression as skin begins to differentiate promotes ectopic placode formation, whereas subsequent Shh expression in the interfollicular epidermis has no morphological effect (Morgan et al., 1998). This is not surprising as *Shh* expression is required after placode formation to direct proliferation of the epidermis to form the follicle (Chiang et al., 1999; St-Jacques et al., 1998).

In this paper, we demonstrate that the ectopic expression of Shh in mitotically active basal and suprabasal cells of the epidermis under the control of the human keratin 1 (HK1) promoter from 12.5 dpc leads to a block of pelage hair follicle development of approximately 4 days. This results in a lack of guard, awl, and auchene hair fibres at all stages from embryogenesis to adulthood. The hair follicle normally cycles in postnatal skin through growth, regression, and resting phases, resulting in the formation of a new hair fibre. Each follicle produces one type of fibre through successive cycles. HK1-*Shh* transgenic postnatal and adult skin continues to lack guard, awl, and auchene fibres due to follicle suppression during the embryonic stages of development. In wild type mice, agouti coat colour is influenced by the proportion of the differentially pigmented hairs. The overhairs, which account for approximately 20% of the pelage, are composed of the dark guard, awls, and auchenes, which are longer than the more abundant lighter coloured zigzag fibres and thus contribute more to coat colour. Thus, the light coat colour phenotype of the HK1-*Shh* transgenic mice is due to the pelage consisting entirely of zig-zag fibres.

It has been postulated that the Shh signalling pathway is involved in the formation of both guard and awl hairs (Yamago et al., 2001). Our results confirm and expand this idea to suggest that Shh signalling is important in the morphogenesis of the follicles, which undergo embryonic development between 14 and 19 dpc namely the guard, awl, and auchenes fibres. Abnormalities in the vibrissae and sinus follicles were not observed in HK1-*Shh* transgenic

mice as has been previously reported that vibrissae development is unaffected by Shh (Wang et al., 2000). The normal development of vibrissae and sinus follicles may be due to their morphogenesis being initiated prior to transgene expression. Expression of the transgene has been shown to be detectable in postnatal skin (Greenhalgh et al., 1993a,b), and ectopic Shh expression was observed in the tail skin of HK1-*Shh* adult mice (Fig. 5F). This expression postnatally does not explain why the normal morphogenesis of the postnatal zig-zag follicles is unaffected by Shh overexpression. This would suggest that the lack of guard, awl, and auchene follicles is not due to Shh overexpression during the initiation of hair follicle development from 14.0 to 19.5 dpc, but instead is due to the types of follicles being sensitive to Shh. This is further substantiated by the observation that guard hairs have a specific requirement for NFκB (Naito et al., 2001), mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Nishioka et al., 2002), and Eda/Edar signalling (Falconer et al.,; Headon and Overbeek, 1999; Kojima et al., 2000; Laurikkala et al., 2002; Naito et al., 2001; Nishioka et al., 2002; Yan et al., 2002). Taken together, these results indicate that initiation of the vibrissae, sinus, guard, awl, auchenes, and zig-zag fibres is controlled by different signalling events, with the formation of guard, awls, and auchenes influenced by Shh signalling and vibrissae, sinus, and zig-zag follicles resistant to Shh-overexpression.

β-Catenin has been shown to be crucial for hair follicle development. In the absence of β-catenin, hair follicle morphogenesis is blocked at the early hair placode stage (Huelsken et al., 2001). Although the HK1-*Shh* embryonic skin lacks hair follicles, this defect is not due to a lack of β-catenin. Interestingly, overexpression of Shh under the control of the HK1 promoter resulted in increased expression of β-catenin in the cell–cell borders in late embryos. It is unknown whether this increased cell–cell border β-catenin staining masks nuclear β-catenin accumulation. In addition to the role of β-catenin in transcriptional activation, β-catenin is also a major structural component of adherens juctions, linking cadherins and α-catenin to the actin cytoskeleton (Conacci-Sorrell et al., 2002). The adhesion of cells determines cellular and tissue morphogenesis and can limit cell movement and proliferation. It is possible that the lack of hair follicles observed in Shh-overexpressing skin is the result of the decreased ability of cells to migrate downwards to form hair follicles due to the increased stability of the adherens junctions during embryonic development. Recently, it has been demonstrated that Notch1 inactivation in the epidermis results in an increase in the basal and suprabasal levels of the signalling competent form of β- catenin (Nicolas et al., 2003). Loss of epidermal Notch1 also resulted in the development of BCCs. Taken together with our results, this indicates that aberrant β-catenin signalling in the basal and suprabasal keratinocytes alone is not sufficient to induce BCCs.

HK1-*Shh* transgenic skin demonstrated an increase in basal cell proliferation. Fan and Khavari (1999) reported that Shh promotes epithelial cell proliferation by opposing p21-induced growth arrest in cell culture. In addition, Shh-expressing keratinocytes failed to exit S and G2/M phase in response to differentiation signals. Our data indicate that HK1-*Shh* transgenic skin does not lack embryonic hair follicles due to the lack of proliferation and subsequent failure of follicular downgrowths as observed for *Shh* and *Gli2* null mice (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998). In addition, K14-*Shh* skin, which overexpressed *Shh* earlier and in different cell types, demonstrated marked follicular epithelium proliferation (Oro et al., 1997). Taken together with our results, this indicates that differences in temporal, spatial, and/or concentration of Shh expression differentially affects basal cell proliferation. Perhaps the increased epithelial proliferation in HK1-*Shh* skin during a specific point in embryonic development influences the ability of the basal cell keratinocytes to embark on a program of differentiation and downward migration to form the guard, awl, and auchene follicles.

Studies on *Shh*-null mice have indicated that Shh is not required for the initiation of hair follicle development since hair was initiated but did not develop beyond early stages of morphogenesis, resulting in a complete lack of hair fibres (Chiang et al., 1999; St-Jacques et al., 1998). In contrast, our results demonstrate that overexpression of *Shh* in the suprabasal and a subset of mitotically active basal cells from 12.5 dpc results in inhibition of embryonic hair follicle morphogenesis. Keratinocytes of the basal cell layer have one of two fates (Taylor et al., 2000). These cells have the ability to terminally differentiate and migrate upwards into the stratified epithelium. The overexpression of Shh under the control of the HK1 promoter does not affect this process nor the number of stem cells as indicated by p63 staining (data not shown). Although the basal cell layer is actively proliferating in HK1-*Shh* transgenic skin, terminal differentiation to form normal stratified epithelium occurred. Conversely, the cells of the basal cell layer can also differentiate into hair follicles. Shh overexpression under the HK1 promoter blocks embryonic hair follicle development completely as indicated by the lack of hair follicle plugs until 18.5 dpc. Alternatively, overexpression of Shh may lead to the induction of genes that have a negative feedback on the formation of awl, auchene, and guard hair follicles. However, K14-Shh transgenic mice that overexpress Shh earlier and in more diverse cell types displayed plugs at 14.5 and 17.5 dpc (Oro et al., 1997). These results would refute that constitutive expression of Shh leads to a negative feedback loop on hair follicle development. In addition, 18.5-dpc K14-*Shh* grafted skin displayed hair follicles which may be reminiscent of our postnatal zig-zag follicles observed in the HK1-*Shh* skin. Once again, the exact timing, location, and concentration of Shh expression affects the resulting hair follicle phenotype. It appears that both models may lack embryonic hair follicles but have normal postnatal hair follicle development. It would be of interest to determine

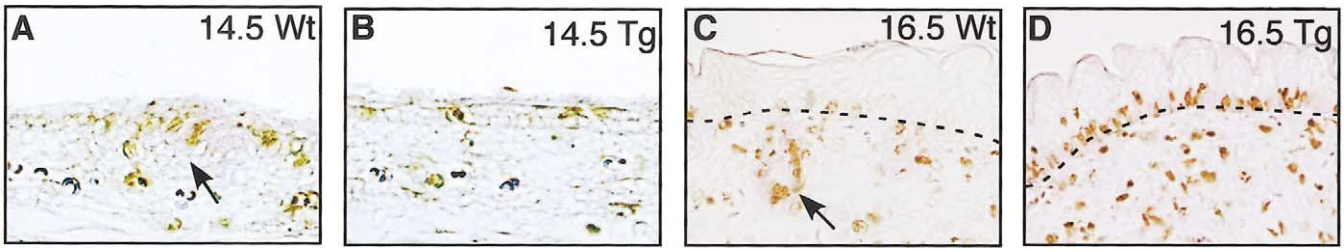

Fig. 7. Immunohistochemical analysis of bromodeoxyuridine (BrdU) incorporation in backskin from 14.5-(A, B) and 16.5 (C, D)-dpc wild-type (A, C) and HK1-*Shh* transgenic animals (B, D). Arrows in (A) and (C) indicate a developing hair follicle. Dashed line in (C) and (D) outlines the basal cell layer.

the hair follicle fibre types observed in the K14-*Shh* grafts as published by Oro and colleagues (Oro et al., 1997).

In addition, our data suggest that homeostatic mechanisms are present that compensate for the imbalance in proliferation induced by overexpression of *Shh* under the control of the HK1 promoter. The increased proliferation in the basal cell layer observed in embryonic HK1-*Shh* skin did not result in a drastic hyperproliferative phenotype as is observed in other mouse models with increased proliferation. Our results suggest that the skin of HK1-*Shh* transgenic mice compensated for the increased proliferation in three ways. First, there is an increase in the stratum corneum that is being sloughed off the skin. Secondly, there is reduction in the thickness of the individual layers of the stratified epidermis. Finally, although there appears to be an increase in the number of epidermal cells, these cells are more compact with the basal cells adopting an elongated, palisading morphology.

HK1-Shh transgenic mice do not develop BCC-like lesions

The HK1-*Shh* transgenic mice did not develop overt basal cell carcinoma-like tumours that have been previously observed in K14-*Shh* transgenic skin (Oro et al., 1997, C.A. et al., unpublished observations). However, the HK1-Shh transgenic animals did develop inguinal lesions which nonetheless express a similar profile of marker proteins as that of human BCCs (expression of K14 and K6 and reduced or no expression of loricrin and BPAG). It is also interesting to note that the basal cells of the HK1-*Shh* transgenic skin are arranged in a manner reminiscent of palisading cells characteristic of human BCCs. The appearance of skin lesions in these mice does indicate that dysregulation of the hedgehog signalling pathway later in embryonic development in a more limited cell type is capable of producing skin lesions. An unusual feature of transgenic mice previously generated using the HK1 promoter to overexpress oncogenes is the development of tumours on the limbs and inguinal and axillar areas, and in other areas associated with wounding and friction (Greenhalgh et al., 1993b; Rothnagel et al., 1993). This was thought to result from lack of epidermal stabilisation by hair follicles and subsequent induction of tumours by physical trauma. It is interesting to note the differentiation of complex, largely sebaceous structures observed within the lesions and regions of alopecia. Oro et al. (1997) had previously reported follicle and sebaceous gland differentiation in K14-*Shh* transgenic skin grown on nude

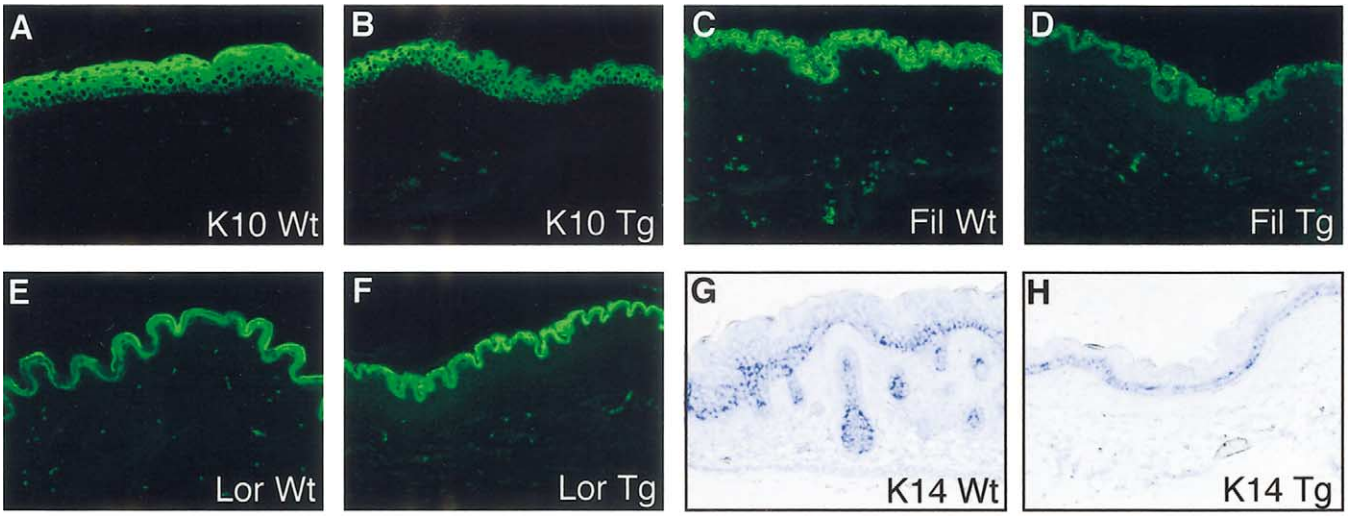

Fig. 8. Analysis of markers of hair and epidermal differentiation in backskin of 18.5- dpc wild-type (A, C, E, G) and HK1-*Shh* transgenic littermates (B, D, F, H). Immunofluorscence of (A, B) keratin 10, (C, D) filaggrin, (E, F) loricrin, and (G, H) in situ analysis of keratin 14 expression.

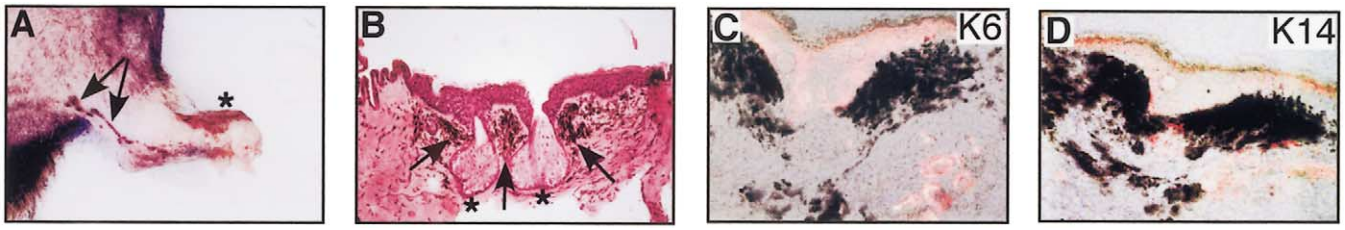

Fig. 9. Analysis of lesions in HK1-*Shh* transgenic animals. (A) Discrete pigmented lesions develop on the transgenic hindlimbs (arrows and unusual pigmentation of the foot skin is also often noted (*). (B) Haematoxylin and eosin-stained sections of an inguinal lesion. Notice the appearance of melanocytes surrounding the lesion (arrows) and the differentiation of gland-like structure (*). In situ analysis of (C) keratin 6 and (D) keratin 14 expression of inguinal lesions.

mice. It should be noted, however, that in human BCCs, differentiation into sebaceous or follicle-like structures is occasionally observed. Pilosebaceous differentiation in the skin of HK1-*Shh* mice does indicate that cells of the interfollicular epidermis are still competent to form these elements. While the unique histology of the lesions does not completely parallel a human dermatological condition, some aspects, particularly the sebaceous differentiation, are similar to human sebaceous nevi. Recent reports investigating loss of heterozygosity in these rare skin tumours have reported loss of chromosome 9q22.3, the interval which harbours the *PATCHED* gene (Xin et al., 1999). While mutations of *PTCH* have yet to be described in these tumours, the lesions apparent on this mouse model do suggest that dysregulation of the hedgehog pathway may lead to the formation of complex sebaceous epidermal structures. Further, they strengthen the potential role of *PTCH* mutation as a causative mechanism in the formation of sebaceous nevi, a tumour type with the potential to differentiate into classic basal cell carcinomas.

What difference in the mode of *Shh* overexpression between the K14 and HK1 transgenic models could account for the differences in neoplasia? One possibility lies in the fact that the HK1-*Shh* transgenic mice lack guard, awl, and auchene fibres. We postulate that zig-zag follicles do not correlate to the type of follicle from which BCCs develop in humans, and that one possibility as to why HK1-*Shh* transgenic mice do not develop BCCs is the lack of guard, awl, and auchene follicles. Further investigation into the molecular differences between the various mouse hair fibres, their correlation to human fibres, and which follicles develop BCC-like lesions in the mouse needs to be elucidated. One major difference between these two modes of *Shh* overexpression in the skin is the timing and site of *Shh* overexpression. In the HK1- *Shh* model, *Shh* is overexpressed in cells that have become committed to terminal differentiation. Namely, these are cells that have switched from expressing K14 to K1. In contrast, in the K14 transgenic model, Shh is overexpressed from 9.5 dpc in both the follicular and interfollicular epithelium (Oro et al., 1997). This suggests that perturbation of the Shh signalling pathway at a critical point in development of the hair follicle may be crucial for the development of BCC-like lesions. In addition, it is possible that Shh overexpression in the undifferentiated K14-positive basal cell is necessary for BCC induction. Although Shh is a secreted long-range signal (Gritli- Linde et al., 2001; Zeng et al., 2001), the concentration of Shh activity in the undifferentiated K14-positive basal cell may not be sufficient to induce BCC-like tumours in the HK1-*Shh* transgenic mice.

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References

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

Bitgood, M.J., McMahon, A.P., 1995. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. Dev. Biol. 172, 126–138.

Botchkarev, V.A., Botchkareva, N.V., Sharov, A.A., Funa, K., Huber, O., Gilchrest, B.A., 2002. Modulation of BMP signaling by noggin is required for induction of the secondary (nontylotrich) hair follicles. J. Invest. Dermatol. 118, 3–10.

Byrne, C., Tainsky, M., Fuchs, E., 1994. Programming gene expression in developing epidermis. Development 120, 2369–2383.

Chiang, C., Swan, R.Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E.K., Cooper, M.K., Gaffield, W., Westphal, H., Beachy, P.A., Dlugosz, A.A., 1999. Essential role for Sonic hedgehog during hair follicle morphogenesis. Dev. Biol. 205, 1–9.

Christiansen, J.H., Dennis, C.L., Wicking, C.A., Monkley, S.J., Wilkinson, D.G., Wainwright, B.J., 1995. Murine Wnt-11 and Wnt-12 have temporally and spatially restricted expression patterns during embryonic development. Mech. Dev. 51, 341–350.

Conacci-Sorrell, M., Zhurinsky, J., Ben-Ze'ev, A., 2002. The cadherin–catenin adhesion system in signaling and cancer. J. Clin. Invest. 109, 987–991.

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

Falconer, D.S., Fraser, A.S., King, J.W.B. The genetics and development of "Crinkled" a new mutant in the house mouse. J. Genet. 50, 324–346.

Fan, H., Khavari, P.A., 1999. Sonic hedgehog opposes epithelial cell cycle arrest. J. Cell Biol. 147, 71–76.

Fowles, L.F., Bennets, J.S., Berkman, J.L., Williams, E., Koopman, P., Teasdale, R.D., Wicking, C., 2003. Genomic screen for genes involved in mammalian craniofacial development. Genesis 35, 73–87.

Fuchs, E., Raghavan, S., 2002. Getting under the skin of epidermal morphogenesis. Nat. Rev. Genet. 3, 199–209.

Goodrich, L.V., Johnson, R.L., Milenkovic, L., McMahon, J.A., Scott, M.P., 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes Dev. 10, 301–312.

Greenhalgh, D.A., Rothnagel, J.A., Quintanilla, M.I., Orengo, C.C., Gagne, T.A., Bundman, D.S., Longley, M.A., Roop, D.R., 1993a. Induction of epidermal hyperplasia, hyperkeratosis, and papillomas in transgenic mice by a targeted v-Ha-ras oncogene. Mol. Carcinog. 7, 99–110.

Greenhalgh, D.A., Rothnagel, J.A., Wang, X.J., Quintanilla, M.I., Orengo, C.C., Gagne, T.A., Bundman, D.S., Longley, M.A., Fisher, C., Roop, D.R., 1993b. Hyperplasia, hyperkeratosis and benign tumor production in transgenic mice by a targeted v-fos oncogene suggest a role for fos in epidermal differentiation and neoplasia. Oncogene 8, 2145–2157.

Gritli-Linde, A., Lewis, P., McMahon, A.P., Linde, A., 2001. The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. Dev. Biol. 236, 364–386.

Hahn, H., Wicking, C., Zaphiropoulous, P.G., Gailani, M.R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A.B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D.J., Gerrard, B., Goldstein, A.M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., Bale, A.E., 1996. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell 85, 841–851.

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

Headon, D.J., Overbeek, P.A., 1999. Involvement of a novel Tnf receptor homologue in hair follicle induction. Nat. Genet. 22, 370–374.

Hogan, B., Beddington, R., Constantini, F., Lacy, E., 1994. Manipulating the Mouse Embryos. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Huelsken, J., Birchmeier, W., 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. 11, 547–553.

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., Birchmeier, W., 2001. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105, 533–545.

Imakado, S., Bickenbach, J.R., Bundman, D.S., Rothnagel, J.A., Attar, P.S., Wang, X.J., Walczak, V.R., Wisniewski, S., Pote, J., Gordon, J.S., et al., 1995. Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. Genes Dev. 9, 317–329.

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

Ingram, W.J., Wicking, C.A., Grimmond, S.M., Forrest, A.R., Wainwright, B.J., 2002. Novel genes regulated by Sonic Hedgehog in pluripotent mesenchymal cells. Oncogene 21, 8196–8205.

Johnson, R.L., Rothman, A.L., Xie, J., Goodrich, L.V., Bare, J.W., Bonifas, J.M., Quinn, A.G., Myers, R.M., Cox, D.R., Epstein Jr., E.H., Scott, M.P., 1996. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science 272, 1668–1671.

Kojima, T., Morikawa, Y., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Senba, E., Kitamura, T., 2000. TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. J. Biol. Chem. 275, 20742–20747.

Laurikkala, J., Pispa, J., Jung, H.S., Nieminen, P., Mikkola, M., Wang, X., Saarialho-Kere, U., Galceran, J., Grosschedl, R., Thesleff, I., 2002. Regulation of hair follicle development by the TNF signal ectodysplasin and its receptor Edar. Development 129, 2541–2553.

Magerl, M., Tobin, D.J., Muller-Rover, S., Hagen, E., Lindner, G., McKay, I.A., Paus, R., 2001. Patterns of proliferation and apoptosis during murine hair follicle morphogenesis. J. Invest. Dermatol. 116, 947–955.

Mahony, D., Karunaratne, S., Cam, G., Rothnagel, J.A., 2000. Analysis of mouse keratin 6a regulatory sequences in transgenic mice reveals constitutive, tissue-specific expression by a keratin 6a minigene. J. Invest. Dermatol. 115, 795–804.

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

Moon, R.T., Bowerman, B., Boutros, M., Perrimon, N., 2002. The promise and perils of Wnt signaling through beta-catenin. Science 296, 1644–1646.

Morgan, B.A., Orkin, R.W., Noramly, S., Perez, A., 1998. Stage-specific effects of sonic hedgehog expression in the epidermis. Dev. Biol. 201, 1–12.

Murone, M., Rosenthal, A., de Sauvage, F.J., 1999. Sonic hedgehog signaling by the patched-smoothened receptor complex. Curr. Biol. 9, 76–84.

Naito, A., Hisahiro, Y., Nishioka, E., Satoh, M., Azuma, S., Yamamoto, T., Nishikawa, S.-I., Inoue, J., 2001. TRAF6-deficient mice display hypohidrotic ectodermal dysplasia. Proc. Natl. Acad. Sci. USA 99, 8766–8771.

Nicolas, M., Wolfer, A., Raj, K., Kummer, J.A., Mill, P., van Noort, M., Hui, C., Clevers, H., Dotto, G.P., Radtke, F., 2003. Notch1 functions as a tumour suppressor in mouse skin. Nat. Genet. 33, 416–421.

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

Nishioka, E., Tanaka, T., Yoshida, H., Matsumura, K., Nishikawa, S., Naito, A., Inoue, J., Funasaka, Y., Ichihashi, M., Miyasaka, M., 2002. Mucosal addressin cell adhesion molecule 1 plays an unexpected role in the development of mouse guard hair. J. Invest. Dermatol. 119, 632–638.

Oro, A.E., Higgins, K.M., Hu, Z., Bonifas, J.M., Epstein Jr., E.H., Scott, M.P., 1997. Basal cell carcinomas in mice overexpressing sonic hedgehog. Science 276, 817–821.

Paus, R., Muller-Rover, S., Van Der Veen, C., Maurer, M., Eichmuller, S., Ling, G., Hofmann, U., Foitzik, K., Mecklenburg, L., Handjiski, B., 1999. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J. Invest. Dermatol. 113, 523–532.

Ping, X.L., Ratner, D., Zhang, H., Wu, X.L., Zhang, M.J., Chen, F.F., Silvers, D.N., Peacocke, M., Tsou, H.C., 2001. PTCH mutations in squamous cell carcinoma of the skin. J. Invest. Dermatol. 116, 614–616.

Ridanpaa, M., Fodde, R., Kielman, M., 2001. Dynamic expression and nuclear accumulation of beta-catenin during the development of hair follicle-derived structures. Mech. Dev. 109, 173–181.

Rosenthal, D.S., Steinert, P.M., Chung, S., Huff, C.A., Johnson, J., Yuspa, S.H., Roop, D.R., 1991. A human epidermal differentiation-specific keratin gene is regulated by calcium but not negative modulators of differentiation in transgenic mouse keratinocytes. Cell Growth Differ. 2, 107–113.

Rothnagel, J.A., Greenhalgh, D.A., Wang, X.J., Sellheyer, K., Bickenbach, J.R., Dominey, A.M., Roop, D.R., 1993. Transgenic models of skin diseases. Arch. Dermatol. 129, 1430–1436.

Sato, N., Leopold, P.L., Crystal, R.G., 1999. Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. J. Clin. Invest. 104, 855–864.

Sheng, H., Goich, S., Wang, A., Grachtchouk, M., Lowe, L., Mo, R., Lin, K., de Sauvage, F.J., Sasaki, H., Hui, C.C., Dlugosz, A.A., 2002. Dissecting

the oncogenic potential of Gli2: deletion of an NH(2)-terminal fragment alters skin tumor phenotype. Cancer Res. 62, 5308–5316.

St-Jacques, B., Dassule, H.R., Karavanova, I., Botchkarev, V.A., Li, J., Danielian, P.S., McMahon, J.A., Lewis, P.M., Paus, R., McMahon, A.P., 1998. Sonic hedgehog signaling is essential for hair development. Curr. Biol. 8, 1058–1068.

Taylor, G., Lehrer, M.S., Jensen, P.J., Sun, T.T., Lavker, R.M., 2000. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102, 451–461.

Wang, L.C., Liu, Z.Y., Gambardella, L., Delacour, A., Shapiro, R., Yang, J., Sizing, I., Rayhorn, P., Garber, E.A., Benjamin, C.D., Williams, K.P., Taylor, F.R., Barrandon, Y., Ling, L., Burkly, L.C., 2000. Conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. J. Invest. Dermatol. 114, 901–908.

Wicking, C., Smyth, I., Bale, A., 1999. The hedgehog signalling pathway in tumorigenesis and development. Oncogene 18, 7844–7851.

Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., Hynes, M., Goddard, A., Rosenthal, A., Epstein Jr., E.H., de Sauvage, F.J., 1998. Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 391, 90–92.

Xin, H., Matt, D., Qin, J.Z., Burg, G., Boni, R., 1999. The sebaceous nevus: a nevus with deletions of the PTCH gene. Cancer Res. 59, 1834–1836.

Yamago, G., Takata, Y., Furuta, I., Urase, K., Momoi, T., Huh, N., 2001. Suppression of hair follicle development inhibits induction of sonic hedgehog, patched, and patched-2 in hair germs in mice. Arch. Dermatol. Res. 293, 435–441.

Yan, M., Zhang, Z., Brady, J.R., Schilbach, S., Fairbrother, W.J., Dixit, V.M., 2002. Identification of a novel death domain-containing adaptor molecule for ectodysplasin-A receptor that is mutated in crinkled mice. Curr. Biol. 12, 409–413.

Zeng, X., Goetz, J.A., Suber, L.M., Scott Jr., W.J., Schreiner, C.M., Robbins, D.J., 2001. A freely diffusible form of Sonic hedgehog mediates long-range signalling. Nature 411, 716–720.