The magnitude of hedgehog signaling activity defines skin tumor phenotype

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Gain-of-function mutations in *SMO* have been implicated in constitutive activation of the hedgehog signaling pathway in human basal cell carcinomas (BCCs). We used a truncated *keratin 5* (Δ*K5*) promoter to assess the potential role of the human M2SMO mutant in BCC development in adult transgenic mice. Δ*K5-M2SMO* mouse epidermis is hyperproliferative, expresses BCC protein markers and gives rise to numerous epithelial downgrowths invading the underlying dermis. Lesions strikingly similar to human basaloid follicular hamartomas develop, but BCCs do not arise even in elderly mice. Hedgehog target gene transcripts were only modestly upregulated in mouse and human follicular hamartomas, in contrast to the high levels detected in BCCs. Cyclins D1 and D2 were selectively upregulated in mouse BCCs. Our data suggest that the levels of hedgehog pathway activation and G_1 cyclins are major determinants of tumor phenotype in skin, and strongly implicate deregulated hedgehog signaling in the genesis of human basaloid follicular hamartomas. Expression of an activated SMO mutant in keratinocytes appears to be insufficient for the development and/or maintenance of full-blown BCCs.
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Introduction

Basal cell carcinomas (BCCs) are the most common cancers in light-skinned individuals, with >1 000 000 new cases diagnosed every year in the USA. The identification of *PTCH* mutations in human BCCs (Hahn *et al*., 1996; Johnson *et al*., 1996) provided the first clue to the molecular pathogenesis of these tumors. *Ptch* encodes a cell surface receptor for Sonic hedgehog (Shh), a secreted signaling molecule that regulates embryonic growth and patterning of various structures, including hair follicles (reviewed in Chuang and Kornberg, 2000; Ingham and McMahon, 2001). In addition to its many roles during development, transient activation of Shh signaling in postnatal skin has been implicated in the regulation of hair follicle growth (Sato *et al*., 1999; Wang *et al*., 2000).

Ptch normally maintains the hedgehog pathway in an inactive state by inhibiting the signaling effector Smo (reviewed in Ingham and McMahon, 2001). Shh activates signaling in target cells by inhibiting Ptch, resulting in derepression of Smo and activation of hedgehog target genes such as *Ptch* and *Gli1*. Whereas this pathway normally is regulated by the spatially and temporally restricted expression of Shh, loss-of-function *PTCH* mutations are associated with constitutive hedgehog signaling in human cancers, particularly BCCs (reviewed in Ruiz i Altaba *et al*., 2002). In addition, activating mutations in *SMO*, rendering SMO protein largely resistant to inhibition by PTCH (Murone *et al*., 1999; Taipale *et al*., 2000), have been identified in tumors that do not contain detectable alterations in *PTCH* (Reifenberger *et al*., 1998; Xie *et al*., 1998; Lam *et al*., 1999). One of these mutant *SMO* alleles, designated *M2SMO*, was isolated from a human BCC and can induce 'basal cell-like' proliferations in newborn mouse skin when driven by a *keratin 5* (*K5*) promoter (Xie *et al*., 1998). This phenotype is similar to that seen in *K14-SHH* transgenic mice, in which hedgehog signaling was constitutively activated using a *keratin 14* promoter to drive an *SHH* cDNA (Oro *et al*., 1997). However, the lethal phenotype of both of these transgenic strains has complicated analysis beyond the perinatal period. In particular, it is not known whether *M2SMO* expression in keratinocytes is sufficient for the development and maintenance of full-blown BCCs, or other skin tumors, in adult animals.

In an effort to study the consequences of deregulated Shh signaling in adult mouse skin, we generated transgenic mice expressing the human *M2SMO* mutant (Xie *et al*., 1998) using a truncated, 1.3 kb *K5* promoter (Casatorres *et al*., 1994; Brown *et al*., 1998) that is expressed in a subset of cells expressing the full-length *K5* promoter (Ramirez *et al*., 1994). This promoter has been used previously to drive the human H-*RAS* gene in skin, successfully circumventing the lethal phenotype seen with the full-length *K5* promoter (Bailleul *et al*., 1990) to yield viable, tumor-bearing mice (Brown *et al*., 1998). The epidermis of adult Δ*K5-M2SMO* mice exhibits several features in common with BCC keratinocytes; however, BCCs do not develop even in mice surviving for >1 year. Instead, there is an accumulation of primitive, hair follicle-like structures that appear remarkably similar to slow-growing, benign human skin tumors called basaloid follicular hamartomas. Comparison of BCCs arising in *K5-Gli2* mice (Grachtchouk *et al*., 2000) with follicular hamartomas revealed marked upregulation of Shh target genes only in BCCs. In addition, only BCCs exhibited upregulation of the G_1 cyclins D1 and D2, which recently have been implicated as direct targets of hedgehog
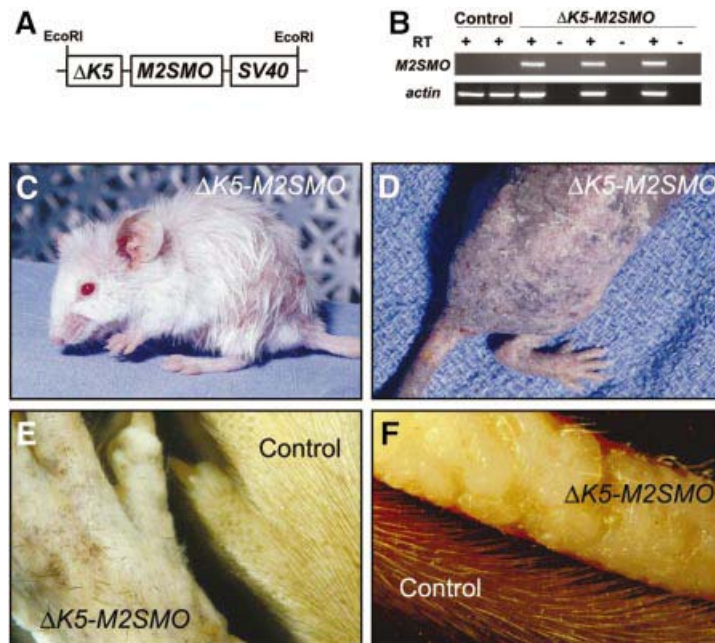
Fig. 1. Transgene design, characterization and gross phenotype of ΔK5-M2SMO mice. (**A**) *M2SMO* was subcloned into a transgenic cassette containing 1.3 kb of bovine *K5* promoter and *SV40 small t poly(A)* sequence with intron. (**B**) Expression of *M2SMO* mRNA in transgenic mouse skin detected by RT–PCR using transgene-specific primers, with β-actin used as an internal control. RT, reverse transcriptase. (**C**) Three-month-old ΔK5-M2SMO founder with patchy hair loss. (**D**) An F_2 ΔK5-M2SMO^{601} mouse exhibiting severe phenotype at ~5 months of age, with generalized alopecia, patchy pigmentation and dry, scaly skin. (**E**) Close-up of dorsal paw from a ΔK5-M2SMO founder, revealing the haphazard arrangement of the few remaining hair shafts at ~1 year of age. (**F**) Extensive alopecia and minute elevations on the surface of the tail from a ΔK5-M2SMO founder at 4.5 months of age, compared with an age-matched control tail.

signaling with central roles in the control of cell growth and proliferation (Kenney and Rowitch, 2000; Long *et al*., 2001; Duman-Scheel *et al*., 2002; Yoon *et al*., 2002). Our data point to a role for deregulated Shh signaling in the genesis of basaloid follicular hamartomas, and strongly support the concept that different levels of Shh signaling activity can result in formation of distinct tumor types in skin. Although the mutant *M2SMO* allele has been detected in a sizeable fraction of human BCCs, expression of this putative oncogene in cutaneous epithelium is not sufficient to drive BCC development in this mouse model.

Results

Progressive alopecia in ΔK5-M2SMO transgenic mice

Injection of the ΔK5-M2SMO transgene (Figure 1A) into oocytes yielded 14 transgene-positive mice from 58 potential founders, and RT–PCR confirmed the presence of *M2SMO* mRNA in skin from multiple founders (Figure 1B). Five of the 14 founders developed a grossly visible phenotype affecting their skin and/or hair. In the two most severely affected founders, several regions of skin never produced normal hairs, resulting in the appearance of hairless patches within 10 days of birth. Over time, the hair loss progressed (Figure 1C), and in the most severely affected mice resulted in generalized alopecia by 5 months of age (Figure 1D). Additional changes in the skin included a dry and scaly appearance, excoriations and focally increased pigmentation (Figure 1D). Other mice exhibited a similar but less dramatic phenotype resulting in varying degrees of hair loss. In older mice exhibiting a strong phenotype, the few

hairs remaining were disoriented and arranged in a haphazard manner, in striking contrast to the regular patterning and uniform orientation of hair shafts in control skin (Figure 1E). The phenotype in tail skin (Figure 1F) was typically more severe and appeared earlier than in other regions of the body. Interestingly, there was a conspicuous absence of tumors resembling BCCs (Grachtchouk *et al*., 2000; Nilsson *et al*., 2000) in ΔK5-M2SMO mice.

Although the truncated *K5* promoter enabled prolonged survival of multiple ΔK5-M2SMO transgenic mice into adulthood, founders gradually developed a wasted appearance, and three died of unknown causes by 6 months of age. Of three founders that produced transgene-positive offspring, only one transgenic line (ΔK5-M2SMO^{601}) was established successfully. Mice from this line have a phenotype similar to that seen in multiple founders as described above, and also never produced macroscopic tumors grossly resembling BCCs. Interestingly, the survival of ΔK5-M2SMO^{601} mice was influenced by genetic background. Offspring from initial crosses with C57BL/6 breeders (litters F_1–F_2) were smaller than control littermates and exhibited impaired viability. However, backcrossing onto an FVB background beginning with the F_2 generation gradually produced healthier offspring with a less severe skin phenotype, and these mice currently are robust breeders in the F_9 generation.

Impaired terminal differentiation and hyperplasia in adult ΔK5-M2SMO mouse epidermis

Proliferating cells in the basal layer of the epidermis give rise to several post-mitotic, differentiated cell types in the overlying spinous, granular and cornified layers

(Figure 2G, inset) (Fuchs and Raghavan, 2002; Niemann and Watt, 2002). Alterations in terminal differentiation are seen characteristically in a variety of cancers (Hanahan and Weinberg, 2000), including BCCs (reviewed in Miller, 1995). Examination of histological sections from several $\Delta K5$-$M2SMO$ founders revealed regions of thickened epidermis containing undifferentiated, basaloid-appearing cells with a high nuclear to cytoplasmic ratio (Figure 2A and B), reminiscent of BCC keratinocytes. A similar but more widespread phenotype was seen in descendents of founder 601, which were characterized in greater detail. The epidermis of $\Delta K5$-$M2SMO^{601}$ mice was appreciably thicker than in control littermates as early as 8 days after birth, and by 3 weeks of age was strikingly hyperplastic (Figure 2E and F). Interestingly, in some regions of transgenic epidermis, the nuclei of cells abutting the basement membrane were vertically elongated and crowded together in a pattern known as palisading, a characteristic feature of cells seen at the periphery of human BCC nodules (Figure 2C and D).

We further characterized the epidermal phenotype of $\Delta K5$-$M2SMO$ mice by examining the expression pattern of several protein markers. K5 normally is expressed almost exclusively in the basal layer of the epidermis and outermost layers of the hair follicle, the outer root sheath. The basal layer-specific expression of K5 is best appreciated in newborn mouse epidermis (Figure 2G, inset), which is several times thicker than adult epidermis (Figure 2G) and therefore has more conspicuous cellular compartments. In $\Delta K5$-$M2SMO^{601}$ mice, K5 expression was expanded from the basal layer to all living layers of the epidermis (Figure 2H). The expression pattern of K10 in epidermis is reciprocal to that of K5: K10 is detected in all epidermal spinous cells, beginning immediately above the basal layer (Figure 2I). However, K10 was not detected in the lower spinous cell layers of hyperplastic $\Delta K5$-$M2SMO^{601}$ epidermis, although it was present in the upper spinous cell compartment (Figure 2I and J). The persistent expression of K5 and delayed appearance of K10 suggest that M2SMO impairs the ability of epidermal keratinocytes to terminally differentiate. Expression of K17 and K6 normally is restricted to the outer root sheath (see Figure 3Q) of hair follicles, with K6 limited to the differentiated cell compartment (Rothnagel and Roop, 1995; McGowan and Coulombe, 1998); in addition, K17 is detected consistently in BCCs. K17 was highly expressed throughout the thickened epidermis of $\Delta K5$-$M2SMO^{601}$ mice, but K6 expression was undetectable in both control and transgenic epidermis (Figure 2K–N). The keratin expression profile in the lower layers of the hyperplastic $\Delta K5$-$M2SMO^{601}$ epidermis is thus strikingly similar to that reported in human and mouse BCCs, which express K5 and K17 but little or no K10 and K6 (Yoshikawa et al., 1998; Grachtchouk et al., 2000).

To assess the consequences of *M2SMO* expression on epidermal proliferation, we identified cells undergoing DNA synthesis by immunostaining for bromodeoxyuridine (BrdU) following a 1 h exposure. As expected, cell proliferation in control epidermis was restricted to the basal layer but, in the skin of $\Delta K5$-$M2SMO^{601}$ mice, BrdU-labeled cells were detected well into the spinous cell layers (Figure 2O and P). In addition, mitotic cells were seen occasionally in suprabasal epidermal layers of $\Delta K5$-

$M2SMO^{601}$ mice (Figure 2P, inset). Thus, the impaired capacity for terminal differentiation in the epidermis of $\Delta K5$-$M2SMO^{601}$ mice is coupled to inappropriate cell division in suprabasal cell layers.

$\Delta K5$-$M2SMO$ mice develop widespread hair follicle hamartomas, but not BCCs

Since allegedly oncogenic *SMO* mutations have been reported in up to 21% of human BCCs (Lam et al., 1999), we were surprised by the lack of grossly evident tumors in multiple $\Delta K5$-$M2SMO$ founders observed for at least 8 months. Even microscopic BCCs were not detected, based on hematoxylin and eosin (H&E) analysis of sections from multiple mice ranging in age from newborn to 14 months. These findings are in striking contrast to those obtained by *K5*-driven expression of the hedgehog transcriptional effectors GLI1 (Nilsson et al., 2000) or Gli2 (Grachtchouk et al., 2000): BCCs arose spontaneously in both of these transgenic models by several months of age.

Although macroscopic tumors did not develop in $\Delta K5$-$M2SMO$ mice, there was a gradual loss of normal-appearing hair follicles and their replacement by immature follicle-like structures consisting of strands, cords and/or sheets of squamous epithelial cells filling the dermis (Figure 3A–F). Other histological findings included cysts, enlarged sebaceous glands and masses of undifferentiated, basophilic epithelial cells with scant cytoplasm, resembling hair matrix cells, located at the tips of epithelial downgrowths. Hair shafts or other morphological signs of advanced hair follicle differentiation were rarely seen in advanced lesions. In some of the deepest portions of these lesions, tumor epithelium was separated from surrounding mesenchyme by a cleft (Figure 3D, inset): similar retraction spaces are seen frequently surrounding human BCC nodules (Kirkham, 1997). Histological abnormalities were detected in tail skin earlier than other sites, and clearly revealed numerous epithelial invaginations originating directly from the epidermis (Figure 3E and F). Thus, in addition to activating expression of certain hair follicle markers in epidermis (Figure 2), M2SMO appears capable of triggering widespread morphological changes reminiscent of early stages in hair follicle development. Interestingly, the gross appearance and histological features of skin lesions arising in $\Delta K5$-$M2SMO$ mice are strikingly similar to those reported for human basaloid follicular hamartomas (Figure 3G) (Brownstein, 1992; Nelson et al., 1993; Walsh and Ackerman, 1993). Little is known presently about the molecular pathogenesis of these benign, slow-growing tumors.

Immunohistochemical analysis revealed expression of the follicle outer root sheath marker K17 in nearly all epithelial cells in the follicular hamartomas, with the exception of cells in the deepest portions that resemble hair matrix cells which are also negative for K17 in control hair follicles (Figure 3H, I and Q). K5 was also detected throughout the follicular hamartomas, but the distribution was not as widespread as for K17 (Figure 3J and K). The differentiation-specific keratin K10, expressed in inner root sheath cells of hair follicles, was detected in a subset of cells within the hamartomas (Figure 3L and M). This result suggests that follicular hamartomas contain cells with a greater potential for terminal differentiation than
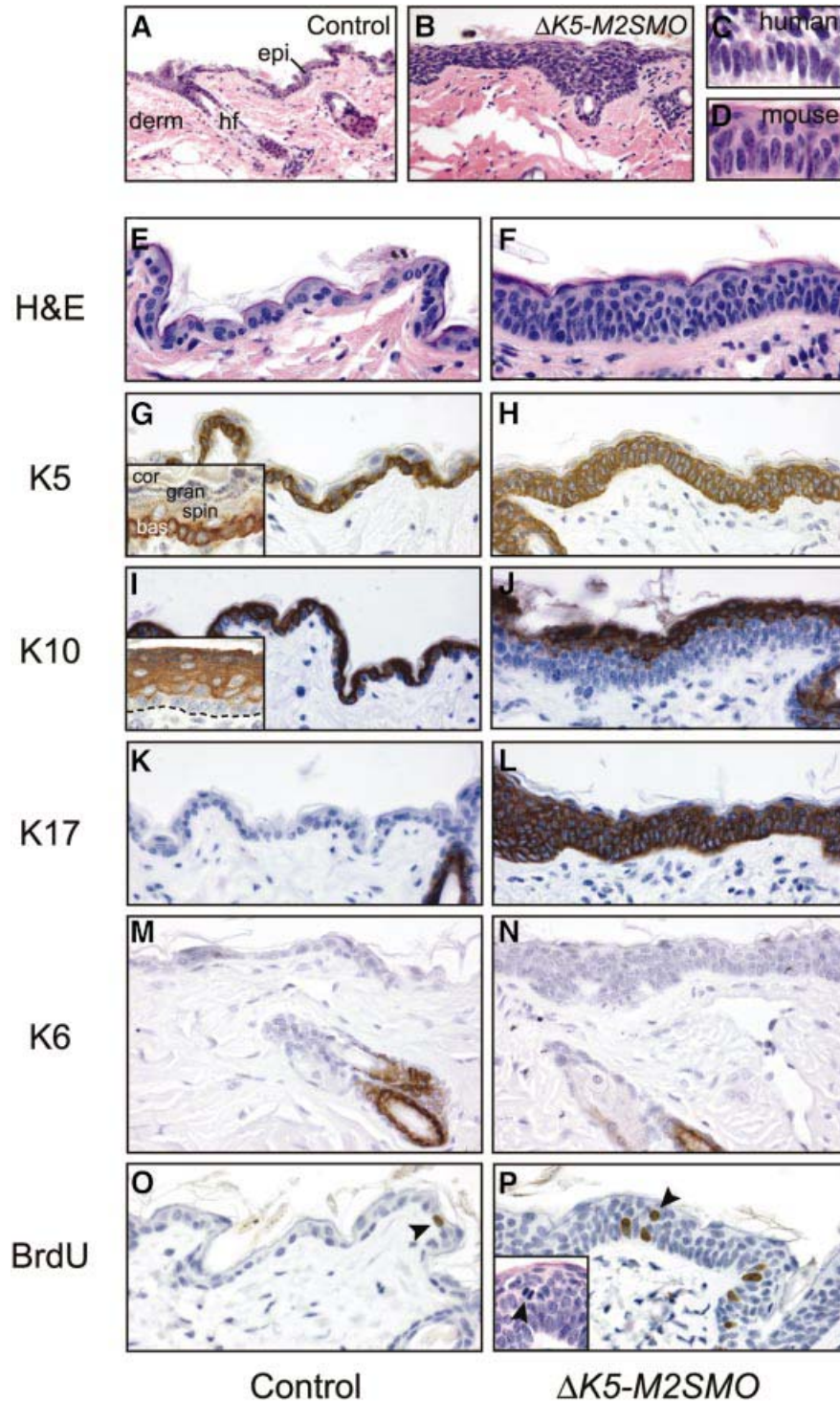
Fig. 2. Altered epidermal growth and differentiation in Δ*K5-M2SMO* transgenic mice. Epidermal hyperplasia (**B**) was seen in several Δ*K5-M2SMO* founders, when compared with age-matched controls (**A**). epi, epidermis; derm, dermis; hf, hair follicle. Epidermal basal cells in some regions of Δ*K5-M2SMO* mouse skin were crowded together and elongated (**D**) in a pattern resembling the peripheral palisading characteristically seen at the edge of human BCC tumor nodules (**C**). Altered growth and differentiation in skin from Δ*K5-M2SMO* mice (F, H, J, L, N and P) compared with littermate controls (E, G, I, K, M and O). Note the thickened epidermis and expansion of basal layer-like cells into upper strata (**E** and **F**). Expression of K5 is limited to the basal layer in control epidermis (**G**), but is expressed in multiple suprabasal cell layers in epidermis of Δ*K5-M2SMO* mice (**H**). The inset in (G) illustrates normal K5 expression largely limited to the basal layer compartment, even in hyperplastic newborn mouse epidermis. bas, basal cell layer; spin, spinous layers; gran, granular cell layers; cor, cornified cells. The appearance of the suprabasal cell marker K10 is delayed in Δ*K5-M2SMO* mice (**J**) compared with controls (**I**). The inset in (I) shows a normal K10 expression pattern in all suprabasal cell layers of newborn mouse epidermis, but not in the basal cell layer. The dashed line indicates basement membrane separating the epidermis from the dermis. K17 is ectopically expressed in epidermis of Δ*K5-M2SMO* mice (**L**) but not controls (**K**), which exhibit expression limited to the hair follicle outer root sheath. K6 is expressed in the same subset of hair follicle keratinocytes in control (**M**) and transgenic (**N**) skin sections. Increased proliferation in Δ*K5-M2SMO* mouse epidermis (O and P). Note the increased number of BrdU-labeled nuclei (arrowheads) in transgenic (**P**) versus control (**O**) mouse skin. BrdU-labeled nuclei and mitotic figures (inset) are seen in suprabasal layers of Δ*K5-M2SMO* epidermis (P).

BCC cells, which rarely express K10. Immunostaining for BrdU to identify proliferating cells revealed significant heterogeneity with follicular hamartomas. While some regions contained numerous cells with labeled nuclei, other regions contained very few labeled cells (Figure 3N and O).

In addition to alterations involving epidermis and hair follicle epithelium, other cell types were affected in skin of ΔK5-M2SMO transgenic mice including the mesenchymal dermal papilla required for proper hair follicle growth

(reviewed in Jahoda and Reynolds, 1996), and neural crest-derived melanocytes that provide pigment to growing hairs. Hair follicles in adult mice are usually in the resting phase of the hair cycle (reviewed in Stenn and Paus, 2001) called telogen (e.g. Figure 3A and C): in these quiescent follicles, the dermal papilla is a small cluster of cells at the tip of the follicle epithelium (Figure 3A). In actively growing anagen hair follicles, dermal papilla cells are embedded within abundant extracellular material and are surrounded by proliferating matrix cells of the hair
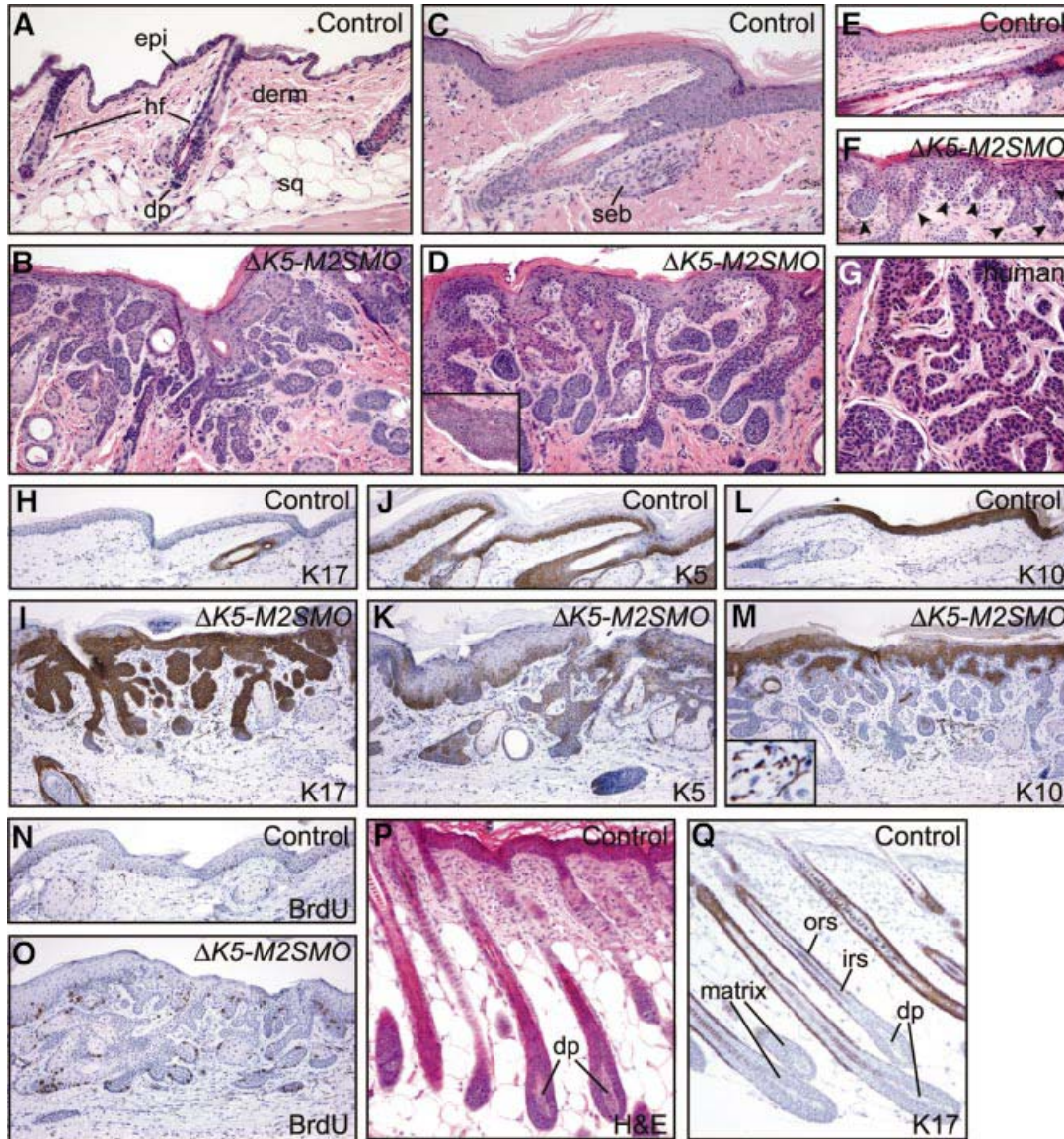
Fig. 3. Histology, marker expression and increased proliferation rate of basaloid follicular hamartomas in ΔK5-M2SMO mice. H&E-stained skin sections from control (**A**, **C** and **E**) and ΔK5-M2SMO (**B**, **D** and **F**) mice at ~14 weeks (A–D) and 23 days (E and F) of age. Basaloid follicular hamartomas were detected throughout the integument, including dorsal (A and B) and tail (C–F) skin. In some areas, a cleft separated abnormal epithelium from the surrounding mesenchyme (inset in D), mimicking changes seen in human BCCs. Hair follicles in age-matched control skin (A and C) are in the resting phase of the hair cycle, telogen, with compact clusters of dermal papilla cells (dp) at the tips of the follicles. epi, epidermis; derm, dermis; hf, hair follicle; sq, subcutaneous fat; dp, dermal papilla; seb, sebaceous gland. Analysis of tail skin from young mice (23 days) revealed multiple epithelial downgrowths originating directly from ΔK5-M2SMO epidermis (arrowheads in F, compared with control in E exhibiting a single hair follicle). Note the similar morphology of mouse lesions and human basaloid follicular hamartoma in (**G**). Marker analysis and BrdU labeling in control (**H**, **J**, **L** and **N**) and ΔK5-M2SMO transgenic (**I**, **K**, **M** and **O**) tail skin. Note the widespread expression of K17 in hamartomas (I); expansion of K5 expression (K), which is more evident in immunofluorescence studies (not shown); and focal expression of K10 (M). Scattered pigmented cells frequently are present in the dermis of ΔK5-M2SMO mice (inset in M). Immunostaining for BrdU revealed focally increased proliferation in transgenic mouse skin (O) compared with control (N). (P and Q) Normal growing (anagen) hair follicles. H&E-stained section (**P**) reveals dermal papilla cells with abundant extracellular material enveloped by hair matrix cells. K17 is expressed in the outer root sheath (**Q**), but not matrix cells or inner root sheath. ors, outer root sheath; irs, inner root sheath.
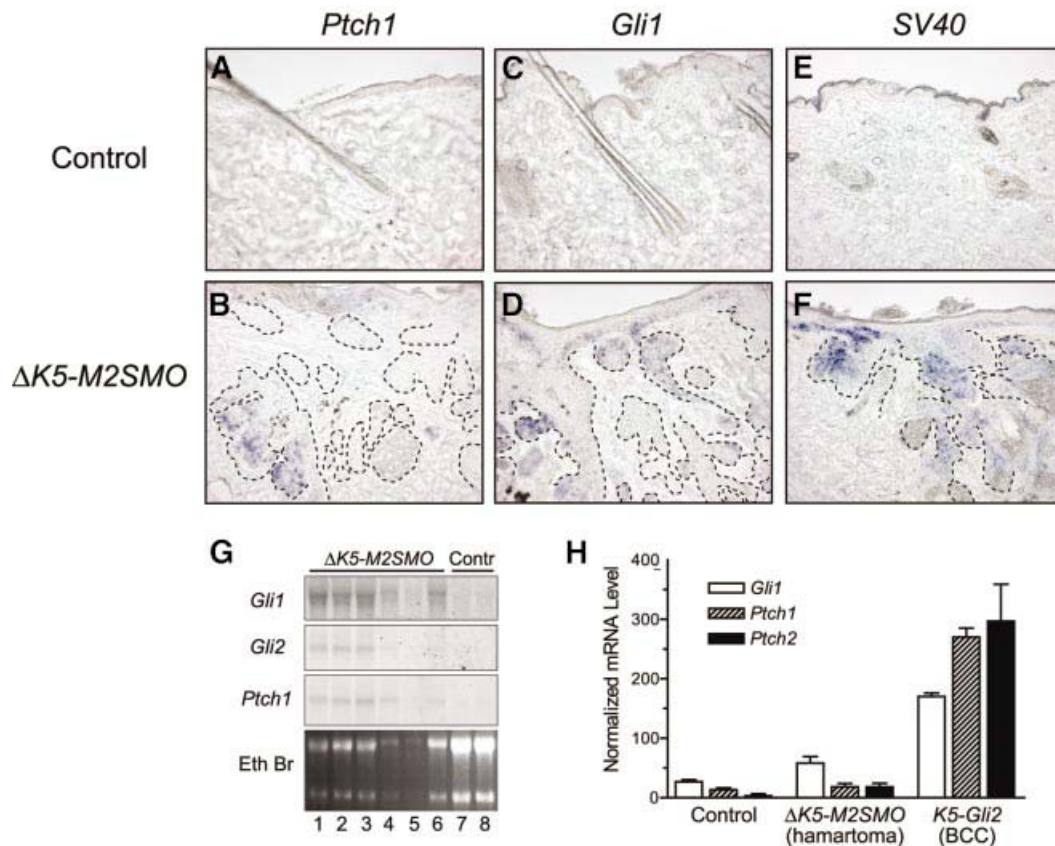
Fig. 4. Expression of transgene and Shh target genes in follicular hamartomas arising in Δ*K5-M2SMO* transgenic mice. *In situ* hybridization of control (A, C and E) and Δ*K5-M2SMO* skin sections (B, D and F), with epithelial cell islands outlined using dotted lines. Antisense riboprobes hybridizing to the Shh target genes *Ptch1* (**A** and **B**) and *Gli1* (**C** and **D**), as well as the transgene-encoded mRNA which contains SV40-derived *poly(A)* sequence (**E** and **F**), revealed patchy expression (blue-purple staining) in a fraction of epithelial cells in follicular hamartomas. Control skin containing resting (telogen) hair follicles did not contain detectable levels of any of these mRNAs. (**G**) Northern blot analysis revealed elevated expression of *Gli1*, *Gli2* and *Ptch1* in RNA samples from Δ*K5-M2SMO* mouse skin (lanes 1–6), compared with control skin (lanes 7 and 8). (**H**) Expression of Shh target genes *Gli1*, *Ptch1* and *Ptch2*, measured by quantitative PCR (TaqMan). RNA was isolated from control skin (*n* = 3), follicular hamartomas arising in Δ*K5-M2SMO* mice (*n* = 6) and BCCs from *K5-Gli2* mice (*n* = 3). Note the dramatic increase in Shh target gene expression in BCCs, with only modest elevations in follicular hamartomas. Standard errors are indicated by bars: the increase in mRNA levels is statistically significant for BCCs ($P < 0.05$), but not follicular hamartomas.

follicle bulb (Figure 3P and Q). Established follicular hamartomas in Δ*K5-M2SMO* mice did not contain morphologically recognizable dermal papillae, suggesting a requirement for normal follicle architecture in maintenance of this critical cell population. In normal dorsal mouse skin, pigmented melanocytes are localized almost exclusively to the anagen hair bulb, where they transfer pigment to hair shaft progenitor cells. They regress together with the follicle epithelium during the catagen phase of the hair cycle, and are not detected in resting, telogen, hair follicles (Slominski *et al.*, 1994). In striking contrast, and consistent with the gross appearance of adult Δ*K5-M2SMO* mice (Figure 1D), the dermis surrounding follicular hamartomas frequently contained pigmented cells, and many of these exhibited a dendritic morphology reminiscent of melanocytes (Figure 3M, inset, and Figure 5I).

M2SMO upregulates Shh target genes in transgenic mouse skin in vivo
To assess whether the *M2SMO* transgene was functioning to activate Shh signaling in adult mouse skin, we examined expression of Shh target genes by *in situ* hybridization,

northern blotting and real-time PCR (Figure 4). *In situ* analysis revealed upregulation of *Ptch1* and *Gli1* in follicular hamartomas in a patchy distribution (Figure 4B and D). To investigate the potential basis for this patchy expression of Shh target genes, we generated a transgene-specific probe against the *SV40 small t poly(A)* sequence (Brown *et al.*, 1998). *In situ* analysis revealed that only some of the epithelial downgrowths expressed the *M2SMO* transgene (Figure 4F), which is in keeping with the expected expression pattern for the truncated *K5* promoter (Brown *et al.*, 1998). Essentially no signal was detected for *Ptch1*, *Gli1* or the *SV40 poly(A)* sequence in skin from control mice (Figure 4A, C and E). Northern blot analysis revealed elevated expression of *Gli1*, *Gli2* and *Ptch1* in skin from Δ*K5-M2SMO* mice when compared with controls (Figure 4G), confirming increased Shh signaling in transgenic skin. Since high-level expression of Shh target genes is a hallmark of BCCs, we also performed real-time PCR to compare Shh pathway activity in control skin, follicular hamartomas arising in Δ*K5-M2SMO* mice and BCCs from *K5-Gli2* mice (Grachtchouk *et al.*, 2000). Interestingly, while BCCs exhibited the expected upregulation of Shh target genes *Gli1*, *Ptch1* and *Ptch2*,

only a marginal increase was seen in $\Delta K5$-$M2SMO$ samples that was not statistically different from controls (Figure 4H). The discrepancy between northern blotting and real-time PCR results, which were performed using different sets of RNA samples, reflects the low level and variable expression of Shh target genes in $\Delta K5$-$M2SMO$ transgenic mice.

The magnitude of Shh signaling activity defines skin tumor phenotype

Given the relatively low expression level of Shh target genes in follicular hamartomas arising in $\Delta K5$-$M2SMO$ mice, additional studies were performed to explore more rigorously the potential relationship between Shh signaling activity and skin tumor phenotype. We previously reported that mice overexpressing the transcription factor Gli2 using a $K5$ promoter developed multiple BCCs by 3–4 months of age (Grachtchouk *et al.*, 2000). We have generated additional $K5$-$Gli2$ founders and established three transgenic mouse lines that spontaneously develop BCCs similar to those described in our initial report, but these mice are healthier and have therefore allowed a more complete characterization well into adulthood. As these mice age, some of them develop abnormal-appearing skin on their tails and ears even in the absence of grossly evident BCCs, and H&E analysis revealed follicular hamartomas similar to those arising in $\Delta K5$-$M2SMO$ mice. The appearance of follicular hamartomas in $K5$-$Gli2$ mice suggests that upregulation of endogenous Gli activity, albeit at a relatively low level (Figure 4G and H), is responsible for development of follicular hamartomas in $\Delta K5$-$M2SMO$ mice. The focal appearance of BCCs in $K5$-$Gli2$ mouse lines enabled analysis of both BCCs and follicular hamartomas in the same tissue section (Figure 5A and B). *In situ* hybridization revealed high levels of *Ptch1* and *Gli1* expression in the BCCs, with markedly weaker or undetectable signals in adjacent follicular hamartomas (Figure 5C and D). Upregulation of Shh target genes in BCCs is associated with high transgene expression levels, based on the abundance of *Gli2* mRNA in these tumors relative to the adjacent follicular hamartomas (Figure 5E).

Several reports have implicated G_1 cyclins in hedgehog-mediated growth control, in both *Drosophila* (Duman-Scheel *et al.*, 2002) and vertebrates (Kenney *et al.*, 2000; Long *et al.*, 2001; Yoon *et al.*, 2002). We therefore examined cyclin D1 and D2 levels to see whether differences in their expression could help explain the relatively slow growth of follicular hamartomas when compared with BCCs. Immunostaining of control skin or follicular hamartomas revealed cyclin D1 and D2 levels to be low or undetectable (Figure 5F–I); in contrast, BCC keratinocytes expressed both D-type cyclins (Figure 5J and K). These data suggest that enhanced expression of cyclin D1 and D2, driven by high levels of Shh signaling activity, may be required for the sustained, expansive tumor growth seen in BCCs.

Given the fact that basaloid follicular hamartomas arise in both $\Delta K5$-$M2SMO$ and $K5$-$Gli2$ mice, we were interested in assessing whether deregulation of SHH signaling may also play a role in the genesis of the corresponding human lesions. To explore this possibility, we examined the expression of *PTCH1* and *GLI1* mRNA in a series of
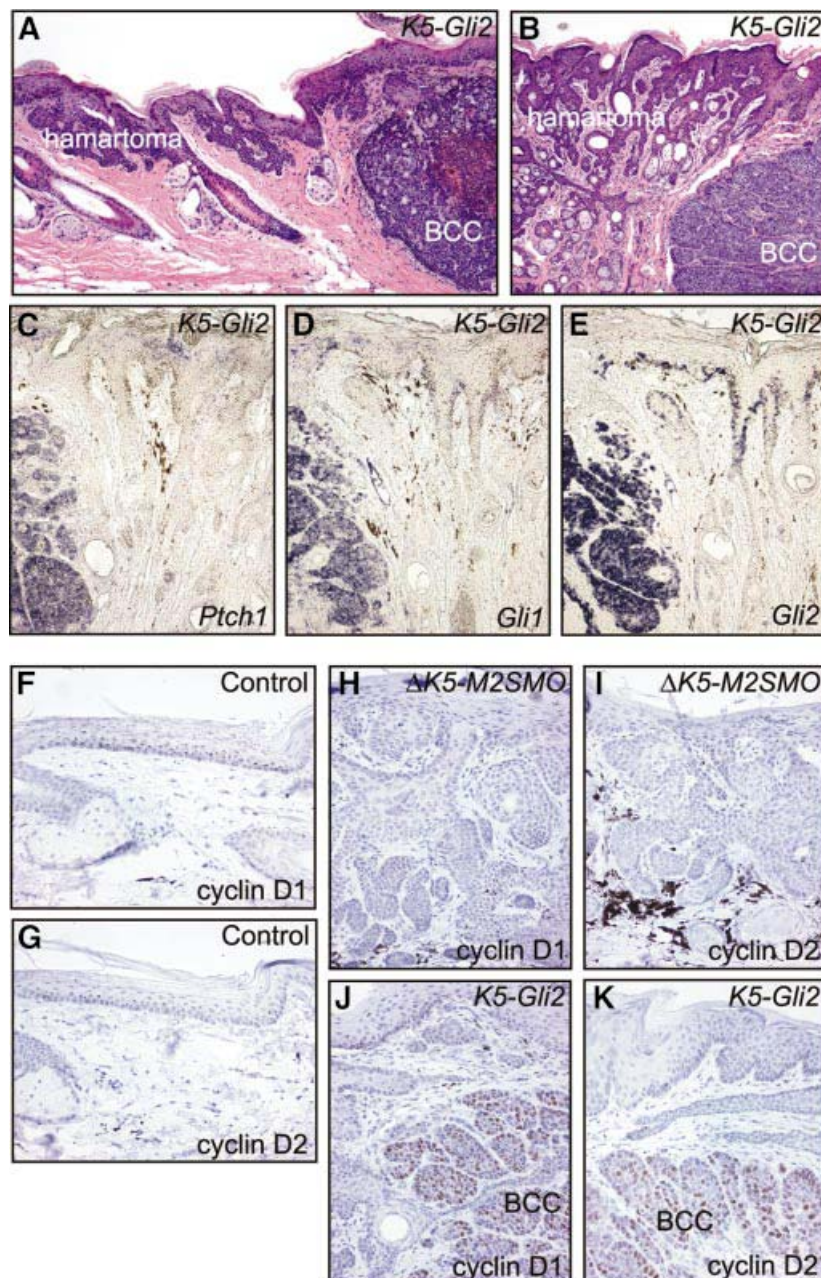
human BCCs ($n = 5$) and basaloid follicular hamartomas ($n = 5$). Expression of both SHH target genes was readily detected in essentially all BCC tumor cells (Figure 6A and C). In contrast, *PTCH1* was detected at low levels and in only a fraction of cells comprising the follicular hamartomas (Figure 6B). *GLI1* mRNA was also detected in follicular hamartomas at substantially lower levels than in BCCs (Figure 6D). Collectively, our findings strongly support the notion that modestly deregulated hedgehog signaling plays a role in the development of basaloid follicular hamartomas in both mice and humans.

Discussion

The consistently elevated expression of Shh target genes in BCCs suggests that uncontrolled Shh signaling plays a crucial role in the initiation and/or maintenance of these tumors. We have shown that different levels of Shh pathway activity are associated with different epithelial tumor types in the skin of mice and humans, and provide evidence implicating modestly deregulated Shh signaling in the genesis of basaloid follicular hamartomas. Despite the occurrence of activating *SMO* mutations in humans BCCs, our findings argue that expression of *M2SMO* in keratinocytes is not sufficient for full-blown BCC development.

The diffuse epidermal phenotype seen in $\Delta K5$-$M2SMO$ mice (Figure 2) suggests that deregulated Shh signaling can dramatically alter the differentiation program of interfollicular keratinocytes, in keeping with previous studies both in transgenic mice and in human skin grafts (Fan *et al.*, 1997; Oro *et al.*, 1997; Xie *et al.*, 1998). Interestingly, the expression pattern of certain keratins in epidermis of $\Delta K5$-$M2SMO$ mice is distinctly different from that seen in epidermal hyperplasia triggered by other stimuli, such as wound-healing or tumor-promoting phorbol esters. While K17 and K6 are normally upregulated coordinately in these settings, K6 is conspicuously missing in epidermis of $\Delta K5$-$M2SMO$ mice. Together with the expanded distribution of K5 into suprabasal cell layers and delayed appearance of K10, this keratin profile mimics that which is seen in both BCCs and the proliferating compartment of follicle outer root sheath (Yoshikawa *et al.*, 1998; Grachtchouk *et al.*, 2000). Since Shh signaling is activated in both of these settings, albeit only transiently in the anagen outer root sheath, additional studies are warranted to investigate the potential role of Shh signaling in bringing about these characteristic changes in marker expression.

The absence of BCCs in $\Delta K5$-$M2SMO$ mice is particularly interesting in light of the well-documented resistance of mice to BCC development. While mice treated with chemical or physical carcinogens readily develop squamous tumors such as papillomas and carcinomas, BCCs are almost never seen; in contrast, BCCs are common in rats exposed to a variety of carcinogens (reviewed in Dlugosz *et al.*, 2002). The resistance of adult mouse skin to BCC formation has only been circumvented recently by genetically modulating the Shh pathway. $K5$ promoter-driven overexpression of the transcription factors GLI1 (Nilsson *et al.*, 2000) or Gli2 (Grachtchouk *et al.*, 2000; Sheng *et al.*, 2002) results in the spontaneous development of grossly visible tumors within several months. $K14$-SHH

Fig. 5. Distinct skin tumor phenotypes (basaloid follicular hamartoma versus BCC) are associated with different expression levels of Shh target genes and G₁ cyclins. (**A** and **B**) H&E staining showing basaloid follicular hamartomas, on the left side of each panel, and BCCs, on the right, in sections from *K5-Gli2* mice. (**C–E**) *In situ* analysis reveals abundant expression of Shh target genes *Ptch1* and *Gli1*, and the transgene *Gli2*, in BCCs on the left side of each panel, but not follicular hamartomas on the right. Negligible expression level of G₁ cyclins in control (**F** and **G**) and *ΔK5-M2SMO* (**H** and **I**) tail skin. In *K5-Gli2* mouse skin, only the BCCs expressed high levels of cyclin D1 (**J**) and cyclin D2 (**K**).

mice produce BCC-like tumors at birth (Oro *et al.*, 1997), but it is not yet known whether SHH is sufficient for BCC tumorigenesis in adult mice. While macroscopic BCCs arise in *Ptch+/–* mice, they do so after prolonged intervals and require exposure to either UV or ionizing radiation (Aszterbaum *et al.*, 1999). Thus, while nuclear mediators of Shh signaling readily produce BCCs when targeted to mouse skin, deregulation of this pathway at a proximal level may be less effective at generating skin tumors. These data are consistent with the intriguing notion that mice harbor a defect in responsiveness to pathological Shh signaling that lies downstream of Ptch and Smo.

The appearance of basaloid follicular hamartomas in skin of *K5-Gli2* mice strongly suggests that the development of these lesions in *ΔK5-M2SMO* mice is due to increased transcriptional activity of endogenous Gli proteins. In keeping with this idea, *Gli1* and *Gli2* mRNA levels are increased in skin from *ΔK5-M2SMO* mice (Figure 4G). However, upregulation of Shh target genes is far greater in BCCs than in follicular hamartomas both in mice (Figures 4H, and 5C and D) and in humans (Figure 6), suggesting that a critical threshold of signaling activity must be achieved for full-blown BCC development and growth. These results also suggest that reduction of Shh

PTCH1 GLI1

Basal Cell Carcinoma

Basaloid Follicular Hamartoma
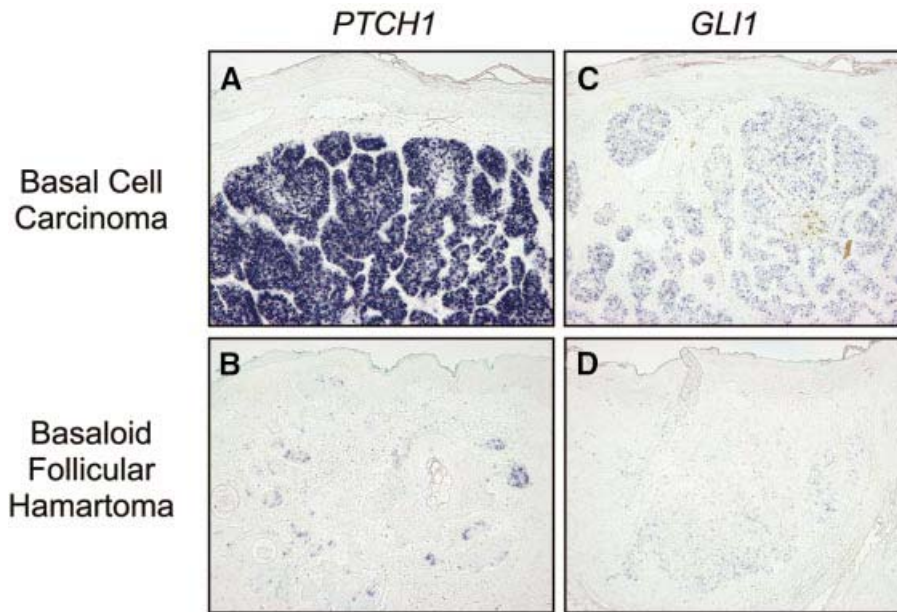
Fig. 6. Low level expression of SHH target genes in human basaloid follicular hamartomas relative to BCCs. (A and B) *In situ* analysis for *PTCH1* mRNA reveals high expression in essentially all tumor cells in human BCC (**A**), with low expression levels detected in a fraction of cells comprising basaloid follicular hamartoma (**B**). (C and D) Low expression of *GLI1* mRNA in human follicular hamartoma (**D**) compared with BCC (**C**). Note the absence of *PTCH1* or *GLI1* signal in overlying epidermis of all samples. *In situ* analysis of five BCCs and five basaloid follicular hamartomas revealed substantially lower *PTCH1* and *GLI1* expression in all follicular hamartomas.

signaling activity, rather than total suppression, may be sufficient to reverse the locally invasive behavior of BCC. Although the downstream effectors driving proliferation in cancers caused by deregulated hedgehog signaling are not yet known, recent studies suggest a role for the G_1 cyclins in physiological cell growth (Kenney *et al.*, 2000; Long *et al.*, 2001; Duman-Scheel *et al.*, 2002) and transformation (Yoon *et al.*, 2002) mediated by the hedgehog pathway. In addition, cell cycle withdrawal normally associated with terminal differentiation is blocked in human keratinocytes overexpressing SHH (Fan and Khavari, 1999). Upregulation of cyclins D1 and D2 in mouse BCCs, compared with follicular hamartomas and normal skin, suggests that modulation of G_1 cyclin expression contributes to tumor growth associated with hedgehog pathway activation.

Follicular hamartomas in humans may be solitary or multiple, and either congenital or acquired; generalized forms, inherited as an autosomal dominant trait, have also been described (reviewed in Requena *et al.*, 1999; Morohashi *et al.*, 2000). Lesions may be associated with extensive hair loss, reminiscent of changes seen in ΔK5-M2SMO mice. The slow growth of human follicular hamartomas and absence of local invasion are also mimicked by the murine lesions described in this report. In many cases, the epithelium of human follicular hamartomas is connected to epidermis rather than hair follicles, suggesting that these lesions arise in a manner similar to those in mice. Their appearance in both ΔK5-M2SMO and K5-Gli2 mice strongly implicates deregulated Shh signaling in the development of murine basaloid follicular hamartomas, and the presence of low-level *PTCH1* and *GLI1* mRNA in human follicular hamartomas points to a role for aberrant Shh signaling in the genesis of

these lesions as well. If this hypothesis is correct, future studies may yield insight into a specific molecular defect underlying the development of these interesting skin lesions.

The absence of BCCs in ΔK5-M2SMO mice was unanticipated given the proposed role for this mutant allele as an oncogene capable of driving human BCC development. The simplest interpretation is that *M2SMO* expression is not sufficient for BCC formation either in humans or in mice, but the inherent resistance of mice to BCC development remains an important caveat. Transgenic studies designed to express *M2SMO* in rats would get around this issue, and provide a direct way of testing for species-specific differences in susceptibility to BCC development using a genetic approach. Another potential explanation for the lack of BCCs is that sufficiently high transgene expression levels were not achieved in ΔK5-M2SMO mice, but this may not be a valid argument since only a single copy of mutant *M2SMO* is proposed to be sufficient for human tumorigenesis. Although the human and mouse Smo proteins exhibit 93% identity (Akiyama *et al.*, 1997), it is possible that the human protein is incapable of signaling at sufficiently high levels to drive tumorigenesis in mice. Future studies using a mouse mutant analogous to M2SMO, designated SmoA1 (Taipale *et al.*, 2000), should help resolve this issue. The lack of BCCs is probably not due to use of the ΔK5 promoter, since we have seen the same phenotype in adult mice that conditionally express *M2SMO* under the control of the full-length K5 promoter (M.Allen, M.Grachtchouk, V.Grachtchouk, E.DeLassus, A.Wang, L.Wei and A.A.Dlugosz, manuscript in preparation). It is conceivable that the absence of BCCs relates to differences in the manner in which *SMO* activation takes place

in humans versus mice. During human BCC development, *SMO* mutation is likely to occur in a single keratinocyte in adult skin, not in a large number of cells beginning during embryogenesis as occurs in many conventional transgenic mouse strains. The importance of timing with regard to Shh responsiveness was elegantly demonstrated in a previous study using chick skin, which showed that ectopic hedgehog signaling produces vastly different results based on when it is activated during development (Morgan *et al.*, 1998). To model human BCC development more accurately, an inducible transgenic model enabling activation of *M2SMO* in a limited number of adult skin cells will be needed.

Materials and methods

Production of transgenic mice and tissue harvests
The *M2SMO* cDNA (Xie *et al.*, 1998), encoding a constitutively active human SMO protein, was subcloned into the *Bgl*II site of the K5pola transgenic cassette containing the 1.3 kb bovine *K5* promoter and *SV40 small t poly(A)* signal with intron (Brown *et al.*, 1998). Subcloning was verified by sequencing. The insert was released using *Eco*RI, and DNA was microinjected into C57/BL6 × SJL F_2 embryos by members of the University of Michigan Transgenic Core. Founders were crossed onto either C57/BL6 or FVB/n breeders (Charles River), as discussed in the Results. Animals were housed and maintained according to institutional guidelines and sacrificed by CO_2 asphyxiation. Some mice were injected intraperitoneally with BrdU (100 µg/g) 1 h prior to sacrifice (Dlugosz *et al.*, 1995). Tissue was fixed overnight in either neutral-buffered formalin (NBF) or Carnoy's solution for H&E staining and routine immunohistochemistry. Mouse tissue harvested for *in situ* analysis was fixed in 4% paraformaldehyde at 4°C overnight, transferred to 30% sucrose at 4°C for 24–48 h, and frozen in OCT embedding medium (Miles). Additional tissue samples for RNA isolation were homogenized in TriZol solution (Invitrogen) according to the manufacturer's instructions.

Immunostaining
Immunostaining was performed with the following antibodies at the indicated concentrations: K5 and K10 (Covance), 1:2000; K6 (Covance), 1:1000; K17 (kindly provided by Pierre Coulombe), 1:2000; BrdU (Zymed), 1:1000; cyclin D1 (Neomarkers Ab-4), 1:100; cyclin D2 (Santa Cruz, sc-181), 1:100. All antibodies are rabbit polyclonals except anti-BrdU, which is a mouse monoclonal. Sections from Carnoy's-fixed tissues were used for keratin antibodies (K5, K6, K10 and K17). NBF-fixed tissue sections were used for cyclin D1, cyclin D2 and BrdU immunostaining, following antigen retrieval using boiling citrate buffer (Chiang *et al.*, 1999). Immunostaining was performed with Vectastain ABC kits (Vector) using 3,3′-diaminobenzidine (DAB) to detect horseradish peroxidase (HRP)-conjugated streptavidin bound to biotinylated secondary antibodies. Immunostained sections were counterstained with hematoxylin.

In situ hybridization
Detailed protocols for *in situ* hybridization using mouse and human tissue, obtained according to University of Michigan Institutional Guidelines, are included in the Supplementary data available at *The EMBO Journal* Online. Probes for mouse *Gli1* and *Ptch1* were as previously described (Hui *et al.*, 1994; Goodrich *et al.*, 1996), and were kindly provided by Dr C.-c.Hui. An ~0.5 kb transgene-specific riboprobe was designed to detect the *SV40 small t poly(A)*, using T3 or T7 promoters and primers just downstream of the small t intron (5′-attccaacctatggaact-gat-3′) and near the 3′ end (5′-gcattcattttatgtttc-3′). For *in situ* analysis of human tissue, T3 or T7 promoters were used to generate riboprobes against human *GLI1* (nucleotides 3285–3598) with the following primers: forward primer (5′-cagctctggacatacccc-3′), reverse primer (5′-gatg-cagttcctttattatc-3′). For the human *PTCH1* probe (nucleotides 216–805), a forward primer with flanking *Eco*RI site (5′-acggaattccggaaagcgcc-3′) and a reverse primer with an internal *Eco*RI site (5′-ttaactcttccaggaattc-caaagg-3′) were used for PCR amplification of reverse-transcribed human RNA. The resultant fragment was subcloned following *Eco*RI digestion into pBluescript II SK (Stratagene). Digoxigenin-UTP-labeled riboprobes were generated by *in vitro* transcription (Roche).

Northern blotting, semi-quantitative RT–PCR and real-time PCR analysis
Northern blot analysis was performed using total RNA isolated from TriZol lysates. A 20 µg aliquot of total RNA was loaded per lane on 1% agarose gels containing 2.2 M formaldehyde and a MOPS-based buffer system. RNA was transferred onto Zeta-probe (Bio-Rad) nylon membranes, and pre-hybridization performed for at least 3 h at 42°C in buffer containing 6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 µg/ml salmon sperm DNA and 50% formamide. Hybridization was performed overnight at 42°C using probes labeled with ^{32}P by random priming (Roche), according to the manufacturer's instructions. *Gli1* probe for northern blotting was a 500 bp *Nco*I 3′ cDNA fragment (Park *et al.*, 2000); *Gli2* probe was a 1 kb *Eco*RI fragment (Hui *et al.*, 1994); and the *Ptch1* probe was the same 0.8 kb fragment used for *in situ* hybridization studies. Filters were washed at a final stringency of 0.1× SSC with 0.1% SDS, at 68°C. Methods for semi-quantitative RT–PCR and real-time PCR analysis are described in the Supplementary data.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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