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An in vivo comparative study of sonic, desert and Indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis

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Summary

Despite the well-characterised role of sonic hedgehog (Shh) in promoting interfollicular basal cell proliferation and hair follicle downgrowth, the role of hedgehog signalling during epidermal stem cell fate remains largely uncharacterised. In order to determine whether the three vertebrate hedgehog molecules play a role in regulating epidermal renewal we overexpressed sonic (*Shh*), desert (*Dhh*) and Indian (*Ihh*) hedgehog in the basal cells of mouse skin under the control of the human keratin 14 promoter. We observed no overt epidermal morphogenesis phenotype in response to Ihh overexpression, however Dhh overexpression resulted in a range of embryonic and adult skin manifestations indistinguishable from Shh overexpression. Two distinct novel phenotypes were observed amongst *Shh* and *Dhh* transgenics, one exhibiting epidermal progenitor cell hyperplasia with the other displaying a complete loss of epidermal tissue renewal indicating deregulation of stem cell activity. These data suggest that correct temporal regulation of hedgehog activity is a key factor in ensuring epidermal stem cell maintenance. In addition, we observed *Shh* and *Dhh* transgenic skin from both phenotypes developed lesions reminiscent of human basal cell carcinoma (BCC), indicating that BCCs can be generated despite the loss of much of the proliferative (basal) compartment. These data suggest the intriguing possibility that BCC can arise outside the stem cell population. Thus the elucidation of *Shh* (and *Dhh*) target gene activation in the skin will likely identify those genes responsible for increasing the proliferative potential of epidermal basal cells and the mechanisms involved in regulating epidermal stem cell fate.

Key words: Shh, Epidermis, Stem cells, BCC, Proliferation, Dhh, Ihh

Introduction

The mechanisms involved in maintaining the mammalian epidermal stem cell population remain largely uncharacterised due in part to the lack of epidermal stem cell specific markers. Epidermal stem cells remain relatively quiescent in vivo, but are capable of generating new epidermal tissue via their daughter cells, otherwise known as transit amplifying (TA) cells, which have a low self-renewing capacity but cycle rapidly (Janes et al., 2002). Epidermal stem cell populations of mammalian skin reside in at least two locations: a specialised region of the hair follicle (HF) known as the hair bulge, and in a non-random distribution within the basal cells of the interfollicular epithelium (the keratinocyte stem cell (KSC) population). The KSC and TA cell populations comprise 70-80% of the basal cell population, express high levels of keratin 5 (K5) and keratin 14 (K14), β_1 integrin and $\alpha_6\beta_4$ integrin (Jones and Watt, 1993; Li et al., 1998), and represent the interfollicular epidermal progenitor compartment. The remaining 20-30% of basal cells comprise postmitotic-differentiating (PMD) cells, which have ceased cell cycle activity and begun the process of terminal differentiation, and non-epithelial cells such as leucocytes, and melanocytes. A well-accepted definition of quiescent stem cells in vivo is the ability to retain ^{3}H-Tdr label for 8 weeks or more (Albert et al., 2001; Bickenbach and Chism, 1998; Tani et al., 2000). These label-retaining cells (LRCs) can be distinguished from β_1 integrin^{bright} TA cells by their characteristic low levels of CD71 transferrin receptor expression in adult (but not embryonic) skin (Li et al., 1998; Tani et al., 2000). Melanoma chondroitin sulphate proteoglycan (MCSP) is also expressed in the human HF stem cell compartment, although its expression does not enrich a β_1 integrin^{bright} population for stem cells, indicating it is not a useful marker for interfollicular or murine epidermal stem cell populations (Legg et al., 2003). Although it has been suggested that the p53 homologue, p63, acts as a potential marker of epidermal KSCs (Pellegrini et al., 2001), its widespread expression throughout the basal layer is not consistent with the low incidence of LRCs. However, recent evidence suggests that p63 expression is required to maintain basal cell proliferative activity (Koster and Roop, 2004; McKeon, 2004), and is therefore useful as a marker of KSC

and TA cell populations. Although Shh has not yet been shown to directly influence the stem cell population of the skin, it is well characterised that mammalian Shh, normally expressed in the basal cells of the skin, promotes epidermal proliferation. Shh is required to induce the proliferation and downgrowth of follicular epithelium (Chiang et al., 1999; Karlsson et al., 1999; St-Jacques et al., 1998), as observed by a failure of HFs to grow down into the dermis of *Shh* null mice (St-Jacques et al., 1998). Overexpression of Shh in the skin results in epidermal hyperplasia by antagonising p21 cell cycle arrest (Fan and Khavari, 1999) and results in the proliferation of HF-like structures into basal cell carcinoma (BCC)-like growths (Fan et al., 1997; Oro et al., 1997). Recent evidence also suggests that Indian hedgehog (*Ihh*), another mammalian hedgehog homologue, has epidermal tumorigenic potential. Mice overexpressing ΔN-Lef1 in the basal cells of mouse skin, which blocks β-catenin signalling, develop sebaceous tumours that express Ihh (Niemann et al., 2003), leading to the hypothesis that Ihh may play a role in stimulating the proliferation of undifferentiated sebocytes. Thus it appears that both Shh and Ihh signalling pathways may play a role in the proliferation of different epidermal stem cell fate lineages.

In order to determine the tumorigenic potential of Shh, Dhh and Ihh, and to establish whether they play a role in regulating the epidermal stem cell population of the interfollicular, hair follicle or sebaceous lineages, we overexpressed each of the hedgehog genes in the basal layer of the skin via the keratin 14 promoter. A subset of *Shh* and *Dhh* transgenic embryos presented with a marked expansion of epidermal progenitor cells, an increase in basal cell proliferative activity and a delay in basal cell differentiation. In addition, we observed several *Shh* and *Dhh* transgenic embryos that lacked epidermal proliferative activity and cell/tissue renewal potential. Our results show for the first time that hedgehog signalling in the proliferative compartment of the epidermis plays an important role in regulating homeostatic cell renewal of the skin, and indicate that manipulation of the hedgehog pathway may provide a useful tool for the experimental investigation of epidermal tissue regeneration and for the ex-vivo expansion of epidermal progenitors for clinical applications.

Materials and methods

Production of transgenic mice

*Bgl*II linkered primers were used to PCR amplify the complete open reading frame of mouse *Shh* (*Shh5′*: GAA GAT CTT CAC CAT GCT GCT GCT GCT GGC CAG, *Shh3′*: GAA GAT CTT CTC AGC TGG ACT TGA CCG CCA), mouse *Dhh* (*Dhh5′*: CAG AGA TCT CCC ACC ATG CCC GAG CGG ACC, Dhh3′: CTA GAT CTT CAG CCC ATT AAC TCC TCG), and mouse *Ihh* (*Ihh5′*: CCA AGA TCT CCC AGC ATG GAG TCC CCA AGA G, *Ihh3′*: CGC AGA TCT GTT CCA GGT GGG CAG), and cloned into the *Bam*HI linearised human keratin 14 (K14) promoter and confirmed by direct sequencing. The hedgehog-transgene (*hK14-Hh*) inserts were released from the plasmid backbone by *Eco*RI/*Hin*dIII double digests and pronuclear injection of the DNA performed on E0.5 BCBF1 × Quackenbush embryos. Injected embryos were transferred into the uteri of pseudo pregnant female CD1 recipients. All transgenic progeny were identified via PCR analysis of tail tip DNA using human K14 specific primers: (*K14AF*: 5′-TCT CGC CTC TCT CTG GTC AT-3′ and K143′R: 5′-CCT GAT CAC AAA AAC ATC AGG A-3′). These primers generated a 328 bp fragment when cycled under the following conditions: 94°C 2 minutes, then 94°C 45 seconds, 50°C 45 seconds, 72°C 45 seconds for 30 cycles.

Histology and immunohistochemistry

Back skin from E17.5 or E18.5 embryos were fixed overnight in 4% paraformaldehyde in PBS, and embedded in paraffin. Histological analysis was performed on 4 μm sections stained with haematoxylin and eosin. Antibody markers were analysed on 4 μm tissue sections via standard immunofluorescence and immunohistochemistry techniques using the following antibodies: K14 (1/10,000), K6 (1/500), K10 (1/500) loricrin (1/500) (Babco), Ki67 (1/500) (Novo Castra), PCNA (1/100) (ZYMED laboratories), p63 (1/100) (gift from Dr F. McKeon), Ihh (1/30) (C-15; Santa Cruz).

In situ hybridisation

RNA in situ analyses were performed on 14 μm paraffin-embedded skin sections using Digoxigenin (DIG)-labelled RNA probes. Sections were rinsed in xylene then hydrated through a graded ethanol series. Sections were treated with 10 μg/ml proteinase K for 10 minutes at 37°C, rinsed in PBS, fixed in 4% PFA for 10 minutes, rinsed in PBS, acetylated in 0.1 M triethanolamine/0.25% acetic anhydride for 10 minutes. Sections were then dehydrated through a graded ethanol series and xylene, then allowed to air dry. Then 500 μg of RNA probe plus 1 mg/ml of salmon sperm were heated at 80°C for 5 minutes, and added to 500 μl of hybridisation solution solution (50% formamide, 10% dextran sulphate, 10 mM Tris-HCl, 10 mM EDTA, 0.6 M NaCl, 10 mM dithiothreitol (DTT), 1×Denhardt's solution, 0.25% sodium dodecyl-sulphate (SDS) and 200 μg/ml yeast total RNA). RNA probes were hybridised overnight at 64°C in a humidified chamber. Washing and blocking steps were performed using DIG wash and block buffer set (Roche) as per manufacturer's instructions. Colour detection was performed using Nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in the presence of 50 mM $MgCl_2$ and 10% polyvinyl alcohol. Samples were then fixed in 8% formamide for 60 minutes, dehydrated through a graded ethanol series and xylene, and mounted in xylene-based mounting media. Transgene RNA expression was detected using a 328 bp antisense RNA probe, made from PCR amplification of the K14 poly A region of the promoter (see primer sequences above). *Ptc2*, *Gli1*, *Gli2*, *Gli3* and *Shh* RNA probes were kindly provided by Dr C. C. Hui. The *Ptc1* probe has been previously described (Hahn et al., 1996).

Skin grafts

Skin samples for grafting were prepared and grafted onto 6- to 10-week-old female, SCID recipient mice as previously described (Mayumi et al., 1988). Recipient mice were shaved, and the graft dressed and bandaged. Dressings were removed seven days post-graft and skin phenotype noted weekly.

Keratinocyte cell culture

Primary keratinocytes were isolated from the skin of E18.5 embryos as described by Hager et al. (Hager et al., 1999). Proliferative basal cells were selected via rapid attachment (10 minutes) onto vitrogen/fibronectin-coated 60 mm dishes and cultured in keratinocyte growth media (EMEM, 8% chelex-treated fetal bovine serum, 4 ng/ml epidermal growth factor (EGF) and 50% fibroblast conditioned media), with media changes every second day. After 4 weeks of incubation, cells were fixed in 100% ethanol at –20°C for 20 minutes, and stained with 1% crystal violet.

Results

Ectopic expression of Shh results in perinatal lethality and a range of developmental phenotypes

Shh was expressed in mouse epithelial cells from E9.5 under the control of the hK14 promoter. Due to perinatal lethality

associated with the embryonic phenotypes, transgenic embryos were produced via independent pronuclear injections and thus establishment of transgenic lines was not possible. We analysed 27 independent *Shh* transgenic embryos at E17.5 or E18.5 and although embryonic phenotypes varied significantly in their severity, transgenic embryos could be categorised into four groups according to skin phenotype (Table 1). The first group of *hK14-Shh* transgenics (10/27) exhibited no overt epidermal anomalies and were indistinguishable from wild-type embryos with respect to skin phenotype (herein referred to as $Shh^{Regular}$) (Fig. 1A). The only anomaly observed in $Shh^{Regular}$ embryos was the presence of a postaxial (posterior) nubbin of digit V (Fig. 1A), often in association with duplication of digit I (Fig. 1B,G) or II. The second group of *hK14-Shh* transgenics (8/27) displayed a wrinkled skin phenotype (herein referred to as $Shh^{Wrinkled}$). $Shh^{Wrinkled}$ embryos were larger than wild-type littermates, developed pre-axial polysyndactyly (Fig. 1C,H) and severe soft-tissue syndactyly. Although the skin of $Shh^{Wrinkled}$ embryos maintained the opacity of wild-type skin

Table 1. Number of skin phenotypes produced from each hedgehog-transgenic construct

	Phenotype			
	Regular	*Wrinkled*	*Taut*	*Translucent*
hK14-Shh	10 (7)*	8 (3)*	2 (4)*	7 (4)*
hK14-Dhh	11	4	4	4
hK14-Ihh	28	0	0	0

*Numbers in brackets represent the number of skin phenotypes produced by Shh overexpression that were unavailable for further analysis due to sample degradation.

it was substantially wrinkled, exhibiting excessive skin folds (Fig. 1K and Fig. 3B). In addition, blistering of the skin was evident over varying degrees of spina bifida and exposed fore-, mid- and hindbrain regions (Fig. 1L). A number of *hK14-Shh* transgenics (2/27), referred to as Shh^{Taut}, displayed pre-axial polysyndactyly and taut (lacking in skin folds) opaque skin with irregularly spaced blisters (Fig. 3C: black arrows). The phenotype of Shh^{Taut} embryos paralleled that described in the previously published report of *K14-Shh* transgenic mice (Oro et al., 1997). The remaining *hK14-Shh* transgenics (7/27) were referred to as $Shh^{Translucent}$ and exhibited the most severe developmental dysmorphologies (Fig. 1J,K). The limbs from 4/7 $Shh^{Translucent}$ embryos displayed polysyndactyly (Fig. 1L,M), with a marked shortening of the proximal-distal axis. The remaining 3/7 $Shh^{Translucent}$ embryos presented with paddle-shaped limb buds that appeared developmentally similar to E11.5-12.5 wild-type limbs (Fig. 1N) and no obvious cartilage or digit formation (Fig. 1O). Craniofacial structures of the $Shh^{Translucent}$ embryos displayed severe developmental defects including cleft palate, fronto-nasal bossing, clefting of the lower jaw (Fig. 1J) and complete lack of cranial and vertebral closure (Fig. 1K). The skin of $Shh^{Translucent}$ embryos was entirely taut, shiny and translucent, with minimal epidermal thickening or keratinisation, reminiscent of early embryonic epidermis (E14.5-15.5). The $Shh^{Translucent}$ epidermal phenotype closely resembles that of the *p63* null mouse, which has been suggested to model the loss of epidermal stem cell renewal (Yang et al., 1999). Further description of *hK14-Shh* transgenics will be restricted to the two novel and uncharacterised skin phenotypes, $Shh^{Wrinkled}$ and $Shh^{Translucent}$.

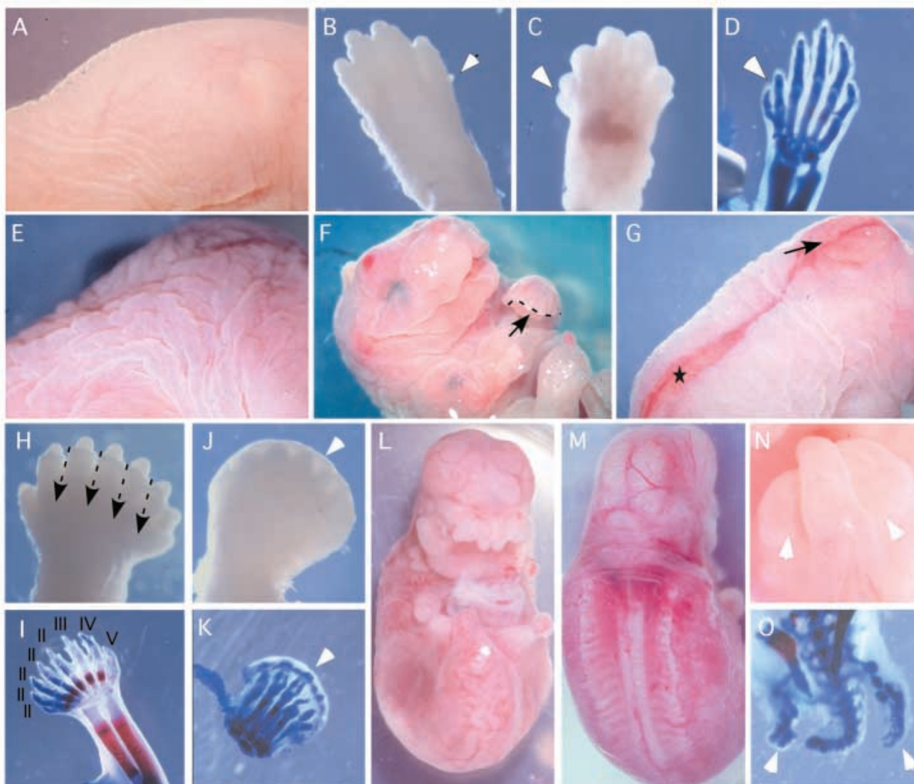

Fig. 1. Range of developmental phenotypes resulting from ectopic hedgehog signalling activity. Shh, Dhh and Ihh overexpression gave rise to *Regular* transgenic embryos that present with skin indistinguishable from wild-type epidermis (A), and mild limb phenotypes such as a post-axial nubbin (B, arrowhead) and/or digit duplication (C, arrowhead; D, alcian blue staining of digit duplication). A subset of *Shh* and *Dhh* transgenics presented with a *Wrinkled* skin phenotype which presented with grossly wrinkled skin (E,F) and blisters over regions of exposed brain (G, arrow) and spinal cord (G, star), pre-axial polydactyly (H-K), complete inter-digital soft tissue webbing (H, arrows), excessive soft-tissue (J, arrow) and calcification at the distal tip (K, arrowhead). Shh and Dhh overexpression also gave rise to *Translucent* embryos [displayed severe developmental defects (L,M)] and *Translucent* limbs often lacked proximal distal growth, presenting as limb buds (N,O, arrowheads).

Shh pathway activity promotes basal cell hyperplasia and BCC formation

Keratin 14 is a marker of epidermal basal cells and is normally expressed in a single layer of interfollicular basal cells and the outer root sheath (ORS) cells of the hair follicle in wild-type epidermis (Fig. 2A,D). In *Shh*^{Wrinkled} transgenic epidermis we observed disorganised *K14* mRNA (Fig. 2B) and protein (Fig. 2E) expression in up to 12 cell layers. Hyperplasia (Fig. 3E) was restricted to the basal cell compartment as confirmed by the appropriate number of stratified layers (loricrin, Fig. 2K and keratin 10, data not shown). The basal cells of *Shh*^{Wrinkled} epidermis were found to express high levels of keratin 6 (K6, a hyperproliferative marker, Fig. 2H), Ki67 (a mitotic activity marker, data not shown) and the proliferating cell nuclear antigen (PCNA) marker (Fig. 2N), indicating an increased number of basal cells undergoing proliferative activity. In wild-type skin, epidermal stratification begins, and hence the terminal differentiation markers K10 and loricrin are expressed, as soon as the basal cells detach from the basement membrane (Fig. 2J). In *Shh*^{Wrinkled} skin we note the late onset of terminal differentiation markers in up to ten layers from the basement membrane (loricrin, Fig. 2K and keratin 10, data not shown). These results suggest that Shh promotes epidermal hyperplasia by inhibiting the onset of epidermal stratification, thereby acting to maintain epidermal basal cells in a proliferative state. The embryonic epidermis of *Shh*^{Wrinkled} transgenics also displayed a variety of skin lesions some of which resembled human BCCs (Fig. 3G-I). The embryonic BCC-like lesions observed in *Shh*^{Wrinkled} mice displayed characteristics of human BCCs including palisading cells at the tumour border and retraction sites where the tumour epithelium is separated from the mesenchyme (clefting) (Fig. 3G,I, arrows), and antibody profiles paralleled that of human BCCs: K14-positive, K6-positive overlying epidermis, terminal differentiation marker (loricrin)-negative, and a decrease in basement membrane proteins (BPAG2 and laminin5) (data not shown). In order to determine the tumorigenic potential of *Shh*^{Wrinkled} epidermis, embryonic back skin was grafted onto SCID recipient mice; tumour growths were visible one month after grafting, and ulceration was evident in several affected grafts (Fig. 3M,N). Adult *Shh*^{Wrinkled} graft skin displayed a variety of skin lesions including epidermal hyperplasia (Fig. 3P), sebaceous hyperplasia (Fig. 3P), basaloid follicular harmatomas (BFHs) (Fig. 3T), regions resembling micro nodular BCCs (Fig.

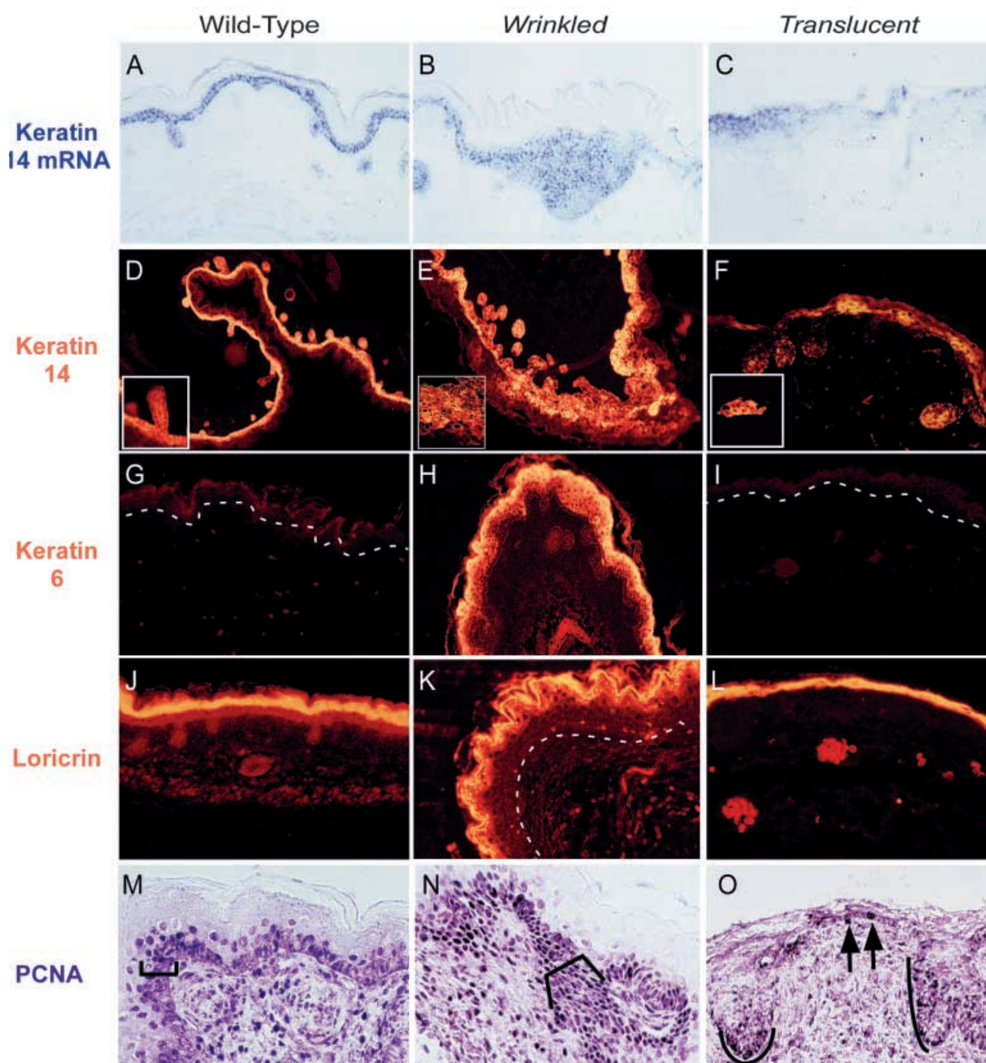

Fig. 2. Shh and Dhh overexpression results in epidermal basal cell anomalies. (A,D) Wild-type skin is characterised by a single-cell layer of basal cells. (B,E) *Wrinkled* transgenic skin on the other hand presents with an increase in the number of basal cell layers, (H,N) the epidermis exists in a hyperproliferative state and (K) exhibits a delay in terminal differentiation. (C,F) *Translucent* skin lacks a substantial amount of basal cells and (L) presents as a bilayer of differentiated cells. (O) Few proliferative cells are observed within the basal layer of *Translucent* skin (arrows), with proliferation mainly restricted to basaloid lesion invaginating into the dermis (unbroken lines).

3O,R) and sclerosing BCCs (Fig. 3Q,S). In situ hybridisation analysis of $Shh^{Wrinkled}$ skin revealed ectopic suprabasal expression of Shh, (Fig. 4A) and Ptc1 (Fig. 4B), which is normally restricted to the basal cells of wild-type skin. Despite this suprabasal expression, there is a lack of stratified epithelium phenotype, suggesting that hedgehog pathway activity has no effect on differentiated keratinocytes. High levels of *Gli1* (Fig. 4C) and *Gli2* mRNA were present in tumour cells, but not in the overlying epidermis. No change in Gli3 expression was observed. Interestingly there is a complete ablation of Ptc1 expression within the basal cells of $Shh^{Wrinkled}$ epidermis (Fig. 4B, arrow), suggesting that Shh promotes basal cell hyperplasia by inhibiting Ptc attenuation and negative feedback inhibition of the Shh signal.

Shh overexpression can deplete the epidermis of proliferating basal cells while maintaining tumorigenic potential

$Shh^{Translucent}$ embryos showed very little epidermal thickening or keratinisation and displayed a complete lack of hair follicle or sebaceous structures (Fig. 3F). Instead, the skin consisted of an epidermal bilayer with a thin and dispersed dermis. The epidermal bilayer of $Shh^{Translucent}$ embryos did not express keratin 18 (a periderm marker, data not shown), and expressed the terminal differentiation markers K10 (data not shown) and loricrin (Fig. 2L), indicating that the process of terminal differentiation remained unaffected. In contrast to the basal cell hyperplasia present in $Shh^{Wrinkled}$ embryos, we observed a substantial (approximately 70%) loss of basal cells, with only

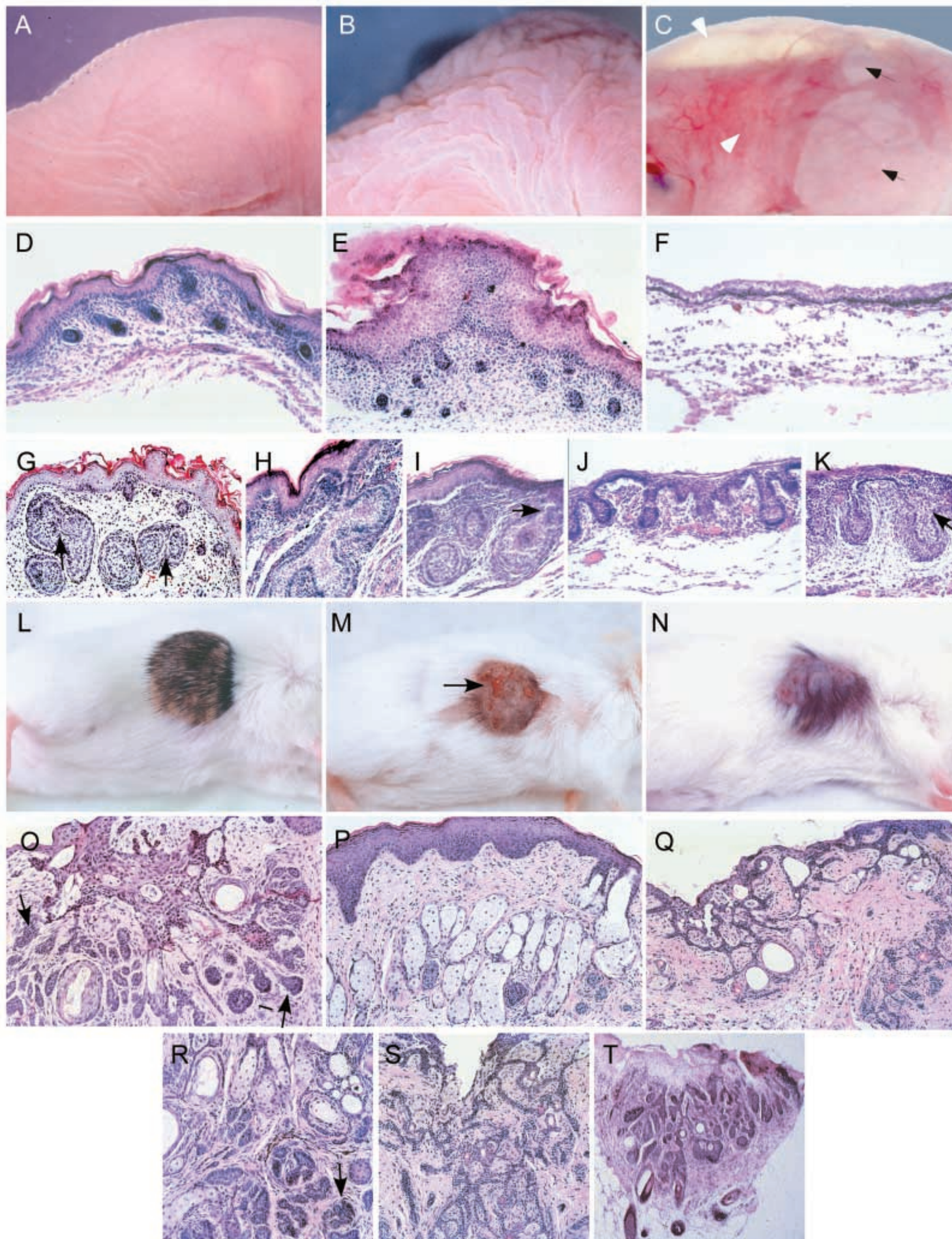

Fig. 3. Histological analysis of hedgehog-induced skin lesions. (A) Wild-type skin is opaque in colour and (D) presents with regularly spaced skin folds and hair follicles. *Wrinkled* transgenic skin on the other hand presents with excessive skin folds (B) and is characterised by epidermal hyperplasia (E). (C) *Translucent* transgenic skin is macroscopically thin and fragile (arrowheads) with regions of opaque blisters (arrows) and (F) presents as an epidermal bilayer that lacks any HF or sebaceous structures. (G-K) Basal cell lesions were evident from E17.5 in both *Wrinkled* (G-I) and *Translucent* epidermis (J,K). Lesions displayed clefting (G,I,K, arrows) and palisading cells, characteristic of human BCCs. (L-M) Gross morphology of wild-type (L) and *Wrinkled* (M,N) skin grafts two months post-graft. (M) Grafts from *Wrinkled* embryonic skin displayed thick tumour masses and ulceration was often evident (arrow). (P) Histological analysis of *Wrinkled* graft skin identified epidermal hyperplasia, sebaceous hyperplasia, and (O,T,Q-S) basaloid growths such as basaloid follicular harmatomas (BFHs) (T) and regions resembling micronodular (O,R, arrows) and sclerosing BCCs (Q,S).

sporadic clusters of keratin 14 mRNA (Fig. 2C) and protein (Fig. 2F) detectable along the epidermis in *Shh*^{Translucent} embryos. Upon further examination, all K14-expressing cells were found to express the differentiation marker K10, characterising them as committed early differentiating (PMD) cells. The absence of any immature K14 basal cells shows that ectopic Shh pathway activity can lead to the depletion of epidermal progenitor cells. Consistent with the observation that *Shh*^{Translucent} epidermis consists of only committed epidermal cells, we observed no K6 expression (Fig. 2I) and negligible interfollicular proliferative activity (Ki67, data not shown, and PCNA, Fig. 2O). *Shh*^{Translucent} embryonic epidermis also displayed basaloid invaginations (Fig. 3J,K) which were classified as BCC-like based on their histological analysis, the expression of Gli1, Gli2 and Ptc1 and the observation that they displayed the same antibody profile as observed in human BCCs. Expression of the proliferation markers Ki67 (data not shown) and PCNA (Fig. 2O, arrows) was mainly restricted to within these basaloid lesions. The tumorigenic potential of *Shh*^{Translucent} skin could not be analysed, given 0/14 successful skin grafts (compared to 11/12 for the *Shh*^{Wrinkled} phenotype). The extent of Shh pathway perturbation was difficult to discern due to the substantial loss of epidermal basal cells, alongside poor epidermal stratification and cell morphology, however, we did observe regions of intense Shh (Fig. 4D), Ptc1 (Fig. 4E) and Gli1 (Fig. 4F) expression within the interfollicular PMD cells and basal lesions of *Shh*^{Translucent} skin, indicating ectopic hedgehog pathway activation.

Shh pathway activity regulates the fate of epidermal progenitor cells

In order to further characterise the proliferative potential of *Shh*^{Wrinkled} and *Shh*^{Translucent} epidermis, we analysed the expression of the transcription factor p63 which is normally expressed in a subset of embryonic epidermal basal cells (Fig. 5A), and is thought to maintain the proliferative potential of basal cells (Koster and Roop, 2004; McKeon, 2004). p63 expression can therefore be used to mark epidermal progenitor cells. We observed a substantial increase in the number of basal cells expressing p63 in *Shh*^{Wrinkled} epidermis, with expanded p63 expression detected in up to four basal cell layers (Fig. 5C). In order to determine whether Shh activity augments epidermal proliferative activity by directly increasing the pool of epidermal progenitor cells, we analysed the clonogenic growth potential of *Shh*^{Wrinkled} keratinocytes using a well-characterised in vitro cell culture assay system (Bickenbach and Chism, 1998; Kaur and Li, 2000). Primary keratinocytes from E18.5 *Shh*^{Wrinkled} transgenic embryos and wild-type littermate controls were plated at 10^6 per 60 mm dish and the epidermal progenitor population enriched for by rapid attachment (10 minutes) to collagenI/fibronectin-coated dishes. Cell cultures were analysed following 4 weeks of cultivation. The results from three independent experiments revealed that Shh^{Wrinkled} keratinocytes consistently gave rise to greater numbers of colonies compared to wild-type controls, i.e. 151±19 versus 41±4, respectively, reflecting a three-fold increase in colony numbers from Shh^{Wrinkled} skin. In addition, Shh^{Wrinkled} keratinocyte cultures displayed an increase in colony density with respect to wild-type controls (represented in Fig. 6C and Fig. 6B, respectively), thereby supporting our observation that Shh^{Wrinkled} keratinocytes are hyperproliferative. These data therefore show that Shh can induce epidermal progenitor cell hyperplasia and that Shh promotes the proliferation of epidermal stem cells and their immediate progeny. In contrast, *Shh*^{Translucent} embryos exhibited a complete absence of epidermal p63 expression (Fig. 5D). We therefore performed in vitro keratinocyte analyses in order to determine the growth potential of *Shh*^{Translucent} epidermis. Consistent with the observation that *Shh*^{Translucent} skin exhibits a severely depleted basal compartment we obtained a limited (1×10^3) number of primary keratinocytes, which failed to attach, survive or grow, under the same conditions used to culture wild-type and *Shh*^{Wrinkled} keratinocytes. The inability of *Shh*^{Translucent} embryonic back skin to graft onto SCID mice (0/14) also supports a lack of epidermal regeneration capacity. We also speculate that epidermal stem cells of the bulge are absent in *Shh*^{Translucent} skin, given the absence of any hair follicle structures in these mice. Definitive evidence of the absence of follicular stem cells requires the identification of appropriate markers for these cells. Although CD34 has recently been shown to be a marker for the adult murine hair follicle bulge region (Trempus et al., 2003), and although we could detect the expression of CD34 in wild-type adult epidermis, CD34 was not expressed in embryonic (E18.5) epidermis (data not shown), suggesting that it is not

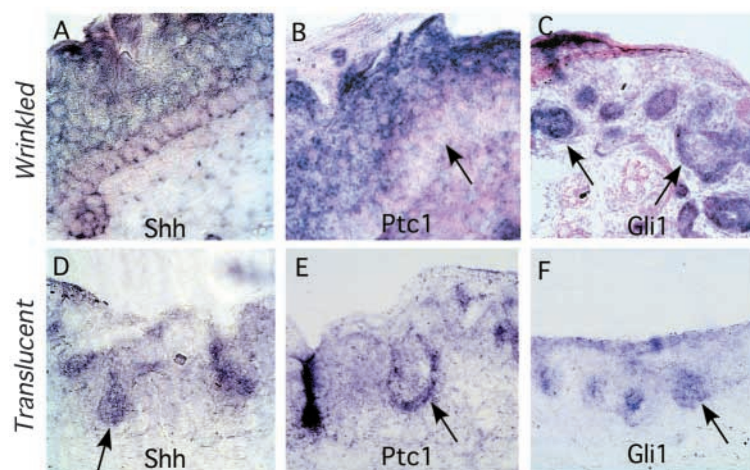

Fig. 4. Hedgehog pathway activity is perturbed in embryonic *Wrinkled* and *Translucent* skin phenotypes. (A) Shh expression is evident in both the basal and suprabasal layers of E18.5 *Wrinkled* epidermis. (B) Although Ptc1 expression is evident in stratified epithelia of *Wrinkled* skin, endogenous Ptc1 expression is completely ablated within the basal cell compartment (arrow). (D,E) High levels of Shh (D) and Ptc1 (E) expression are present within the PMD cells and basaloid lesions of E18.5 *Translucent* epidermis. We also observed high levels of Gli1 within the basaloid lesions of *Wrinkled* (D) and *Translucent* (J) skin.

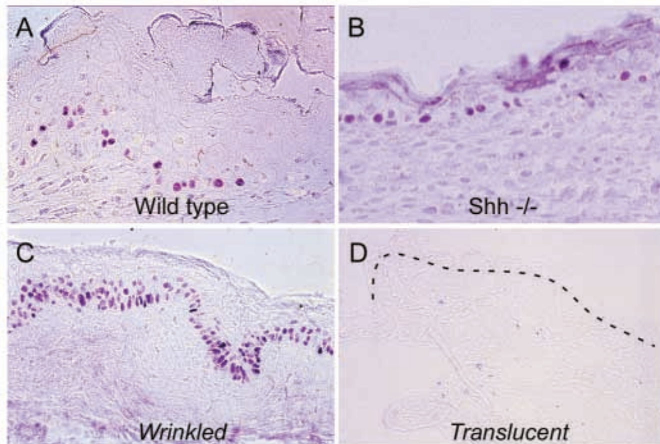

Fig. 5. Analysis of p63 expression in E18.5 *Shh* mouse models. (A) Within wild-type epidermis, p63 is expressed within a subset of epidermal basal cells. (B) Loss of Shh activity (*Shh* null embryos) does not alter the expression of p63 within the basal cell compartment. (C) *Wrinkled* epidermis displays a marked increase in the number of epidermal cells expressing p63 whereas (D) *Translucent* skin displays a complete ablation of p63 expression.

a suitable marker for the embryonic follicular stem cell compartment.

To identify the impact that loss of Shh pathway activity has on the epidermal proliferative compartment, we analysed the expression of p63 in the skin of E17.5 and E18.5 *Shh* null embryos and consistently observed a modest decrease in the number of p63-positive epidermal cells (Fig. 5B). Thus although epidermal homeostasis remains largely unaffected in the absence of Shh activity, ectopic Shh expression can result in epidermal progenitor cell hyperplasia or hypoplasia.

The level of ectopic Shh activity directs epithelial cell response

The observation that *hK14-Shh* transgenics present with a range of skin and developmental phenotypes that appeared to increase in severity from $Shh^{Regular}$, to $Shh^{Wrinkled}$, to Shh^{Taut} and finally $Shh^{Translucent}$, suggests that each phenotype may result from increasing levels of hedgehog transgene activity. RNA in situ analysis showed an increase in transgene expression from $Shh^{Regular}$, through $Shh^{Wrinkled}$, to Shh^{Taut} (Fig. 7A-C), as determined by a transgene-specific RNA probe. However, transgene analysis was hindered in the $Shh^{Translucent}$ phenotype due to the severe depletion of basal cells in which transgene expression is directed (Fig. 7D). Genomic PCR analyses were used in order to determine the relative copy number associated with each skin phenotype. PCR analyses indicate an increase in transgene copy number from $Shh^{Regular}$, to $Shh^{Wrinkled}$, to Shh^{Taut}, thereby supporting our RNA in situ expression level data. In addition, genomic copy number analyses indicate that $Shh^{Translucent}$ embryos have a consistently higher transgene copy number than other phenotypes (Fig. 7E).

Dhh, but not Ihh, can regulate epidermal homeostasis and induce tumour formation

In order to address the question of functional redundancy of three mammalian hedgehog molecules during skin morphogenesis and tumour formation, we overexpressed both Dhh and Ihh using the hK14 promoter. Dhh overexpression in the basal cells of murine epithelial tissues resulted in 23 independent *hK14-Dhh* transgenic embryos with phenotypes that are indistinguishable from Shh overexpression. We observed 11/23 $Dhh^{Regular}$ (cf 10/27 $Shh^{Regular}$) transgenics that displayed no epidermal anomalies. A small number of Shh^{Taut} (2/27) and Dhh^{Taut} (4/23) transgenics parallelled the previously published report of Shh overexpression (Oro et al., 1997). We also observed 4/23 $Dhh^{Wrinkled}$ (cf 8/27 $Shh^{Wrinkled}$) transgenic embryos with excessive wrinkling of the skin, characterised by the excess proliferation of suppernumerary progenitor cells. The remaining 4/23 $Dhh^{Translucent}$ (cf 7/27 $Shh^{Translucent}$) transgenics exhibited a translucent skin phenotype, characterised by an apparent loss of epidermal tissue maintenance. These data demonstrate that although Dhh is not normally expressed in the skin, it can act as a functional homologue of Shh in regulating epidermal regeneration and stem cell homeostasis. In addition, the observation that $Dhh^{Wrinkled}$, Dhh^{Taut} and $Dhh^{Translucent}$ epidermis develop a variety of skin tumours, including BCC-like lesions, indicate for the first time that Dhh has tumorigenic potential.

Twenty-eight independent *Ihh* transgenic embryos were produced and no overt HF, sebaceous or interfollicular epidermal phenotypes were observed (hence 28 $Ihh^{Regular}$ transgenic). Although Ihh overexpression did not result in an epidermal phenotype, it gave rise to a mild embryonic limb phenotype (refer to Fig. 1B-D). Previously published data have shown that although both Shh and Dhh have strong polarising activity in the developing limb bud, Ihh (even at high concentrations) gives rise to only minor digit duplications of the chick wing (Pathi et al., 2001). These results support our observations that Ihh, although capable of inducing the same phenotype as low levels of Shh activity, is not homologous with the ability of Shh to induce strong polarising effects in the limb. In support of Ihh functional activity we observed high levels of *Ihh* transgene (Fig. 8A) and Ihh protein (Fig. 8B) expression within the basal layer of $Ihh^{Regular}$ transgenic epidermis. In addition, we observed high levels of endogenous Shh (Fig. 8C) and Ptc1 (Fig. 8D)

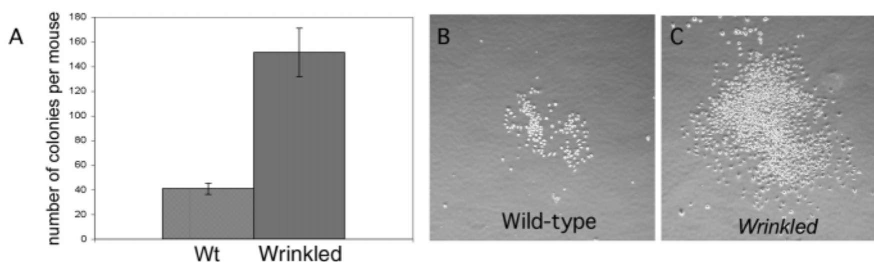

Fig. 6. Shh pathway activity can increase keratinocyte growth potential. (A) E18.5 wild-type epidermis contains an average of 41 keratinocyte colonies per mouse whereas *Wrinkled* epidermis contains over 150 keratinocyte colonies per mouse. (B,C) A typical representation of wild-type colony density (B) and *Wrinkled* colony density (C). *Translucent* keratinocytes lacked colony-forming efficiency.

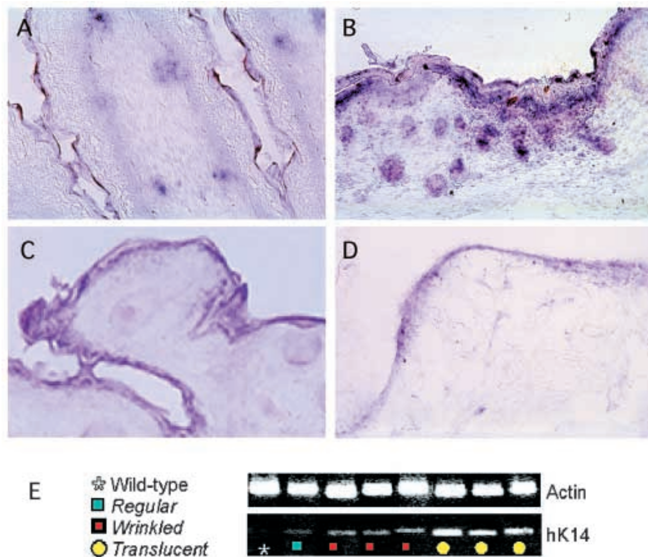

Fig. 7. The level of hedgehog expression increases with the severity of the skin phenotype. (A-C) RNA in situ analysis of transgene expression showed an increase in transgene expression from *Regular* (A), to *Wrinkled* (B) and to *Taut* (C). (D) Detection of transgene expression within *Translucent* skin was hindered by the decrease in basal cells. (E) Genomic PCR analyses indicate that the relative level of transgene copy number is *Translucent* > *Wrinkled* > *Regular*. This suggests that differences between transgenic phenotypes may be related to the level of transgene copy number, and an increase in embryonic phenotype severity correlates with a significant increase in copy number.

expression in the basal layer and high levels of Gli1 and Gli2 expression in the HFs of *Ihh*^{Regular} skin. Since Ihh overexpression had no effect on embryonic epidermal development, we grafted E18.5 back skin from 18 *Ihh*^{Regular} transgenic embryos onto SCID recipient mice, in order to determine whether ectopic Ihh activity had any effect on adult epidermal homeostasis. Grafts were monitored weekly for 6 months and both macroscopic and histological analysis revealed epidermal morphology indistinguishable from wild-type graft controls (data not shown). We have therefore shown that Ihh pathway activity does not induce an epidermal phenotype and it is therefore not functionally redundant with Shh. In addition, our data suggest that epidermal responsiveness to the hedgehog signal lies downstream of *Gli* gene transcriptional regulation.

Discussion

A novel role for the Shh pathway in maintaining the epidermal stem cell population

This report describes the characterisation of two novel hedgehog-induced skin phenotypes, *Translucent* and *Wrinkled*, in response to the ectopic activation of Shh and Dhh signalling in the basal cells of the skin, and shows that hedgehog pathway activity can directly regulate the number of epidermal basal cells with stem cell characteristics. Our analyses of *Shh/Dhh*^{Wrinkled} epidermis identified a marked increase in the number of epidermal progenitor cells in addition to elevated proliferation of epidermal progenitors in culture. The observation that Shh plays an important role in epidermal stem cell biology is consistent with recent observations that Shh signalling is required for the maintenance of adult neural progenitor cells (Machold et al., 2003), and promotes neocortex progenitor cell proliferation (Palma and Ruiz i Altaba, 2004). However unlike the telencephalon in which Shh has no effect on embryonic stem cell morphogenesis (Machold et al., 2003), we show that Shh pathway activity can promote embryonic progenitor cell hyperplasia. We have also shown that the Shh pathway can function as a negative regulator of epidermal stem cell fate. *Shh/Dhh*^{Translucent} transgenic skin was devoid of epidermal progenitor cell activity and consisted of a thin bilayer of committed epidermal cells. This is an unexpected and novel role for Shh signalling in epidermal stem cell biology. Interestingly, although loss of Shh activity in the neocortex results in a loss of epidermal stem cell maintenance, epidermal stem cell homeostasis remains largely unaffected in *Shh* null mice. We infer that an epidermal stem cell phenotype is not observed in the absence of Shh due to the presence of extensive compensatory mechanisms employed by the epidermis to maintain the functional attributes of skin. This interpretation is consistent with the rare incidence of genetic knockout mouse models that present with substantial loss of epidermal tissue, *p63* knockout mice and our *Shh/Dhh*^{Translucent} embryos being the only exceptions (Mills et al., 1999; Yang et al., 1999). The results presented here show that hedgehog is a key factor whose expression must be temporally regulated to ensure epidermal stem cell homeostasis. Although further evidence is required to elucidate the specific role that Shh and its downstream targets play within the epidermal stem cell niche, our data suggest that above a certain threshold of hedgehog pathway activation, Shh acts as a mitogen, thereby increasing the proliferative potential of epidermal basal cells. This hypothesis is consistent with the observation that Shh appears to induce proliferation at the onset of anagen, when

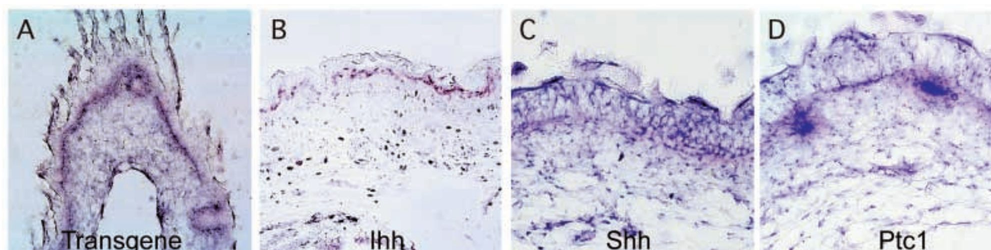

Fig. 8. Ihh overexpression perturbs epidermal hedgehog signalling activity. In response to high levels of Ihh transgene expression (A) and ectopic Ihh protein within the basal cells of mouse skin (B), we observed high levels of Shh (C) and Ptc1 (D) expression in both the basal and suprabasal layers of E18.5 *Ihh* transgenic epidermis.

the stem cells of the bulge are required to proliferate and give rise to a new hair shaft (Oro and Higgins, 2003).

Given the perinatal lethality associated with our embryonic phenotypes, the production of viable conditional Shh overexpression mouse model systems is required in order to analyse the progression/regression of the stem/progenitor cell population over time. An inducible conditional Shh overexpression system would also allow the documentation of precise timing of transgene expression onset, thus elucidating the role of heterochronic transgene expression in producing a given skin phenotype. We are currently in the process of transgenic animal production, which will allow us to further analyse the role of *Shh* and *Dhh* target genes in regulating epidermal basal cell homeostasis. However, the data presented here show a consistent trend between the level of Shh activity and the resulting skin phenotype, suggesting that the difference between the observed phenotypes is probably related to the level of transgene expression, with the *Translucent* phenotype resulting from the highest level of Shh expression. Such a mechanism would be consistent with the known dose dependency of Shh action in tissues such as the developing central nervous system. This is also consistent with the observation that different levels of hedgehog signalling activity give rise to different skin phenotypes and skin lesions (Grachtchouk et al., 2003). The loss of epidermal progenitor cells in $Shh^{Translucent}$ epidermis may be due to the exhaustion of the stem cell compartment; however, given that almost no basal cells remain, it is possible that high levels of ectopic Shh activity act to inhibit epidermal stem cell fate.

Hedgehog-induced proliferation is restricted to the epidermal basal cell compartment: the cellular origin of hedgehog-induced skin tumours

Previous overexpression studies have shown that elevated hedgehog pathway activity in the basal cells of the skin, using basal specific promoters (K5 and K14), gives rise to epidermal anomalies and BCC-like lesion formation (Grachtchouk et al., 2003; Oro et al., 1997; Sheng et al., 2002; Xie et al., 1998). However, we have recently shown that overexpression of hedgehog in only a subset of basal cells, using the human K1 promoter (hK1), results in the ablation of embryonic hair follicle development and does not give rise to BCC (Ellis et al., 2003). The overexpression of hedgehog via the hK1 promoter drives hedgehog activity in committed epidermal cells, thus only in a small subset (20-30%) of basal cells, the PMD-population. In contrast, the overexpression of hedgehog via the hK14 promoter drives Shh activity in all basal cell populations. The hedgehog-induced skin phenotype differences observed in *hK14-Shh* and *hK1-Hh* transgenic mice could be attributed to the timing at which hedgehog activity is induced by the keratin promoters (hK14: E9.5 versus hK1: E12.5). However, in view of the data presented here, it is a possibility that the major contributing factor is the activity of the hedgehog pathway in the epidermal progenitor compartment.

Although BCCs are the most common form of human skin cancer, much remains to be elucidated about the aetiology, including the cellular origin. The outer root sheath (ORS) cells of the hair follicles are strong candidates given that BCCs are seldom observed in hairless regions of the body and appear to invaginate from structures resembling HFs (Weedon, 1981). BCC also has an immature, stem cell-like appearance leading to the suggestion that BCC originates from aberrant Shh activity within the stem cell population of the interfollicular epidermis or those of the hair follicle bulge (Taipale and Beachy, 2001; Zhang and Kalderon, 2001). We observed *hK14-Shh* and *hK14-Dhh* embryonic skin either enriched or devoid of epidermal progenitor cell activity and/or hair follicles is equally competent to produce BCC. We hypothesise that hedgehog induces BCC transformation via promoting the proliferation and subsequent invagination of cells residing in the basal compartment. This is consistent with the proliferative effect of Shh on epidermal cells (Fan and Khavari, 1999; Fan et al., 1997; Morgan et al., 1998; Oro et al., 1997; Sato et al., 1999), and the role it plays during invagination of the developing hair follicle (Chiang et al., 1999; St-Jacques et al., 1998). Although we can not rule out the possibility that stem cells sustain genetic mutations that predispose their progeny to transformation, the results presented here suggest that neither stem cells nor ORS cells of the hair follicle are required for BCC formation. Although both stem cells and ORS cells reside in the basal compartment, and, according to our hypothesis can act as the cellular origin to hedgehog pathway-induced skin tumours, our data show that BCC transformation can occur in basal cells lacking inherent self-renewing potential. Consistent with this suggestion, only 26% of human BCCs exhibit p63 expression (Dellavalle et al., 2002), thus representing the subset of BCCs that arose within the epidermal progenitor compartment.

Dhh, but not Ihh, is a functional homologue of Shh signalling in the skin

In vitro biochemical evidence has shown that Shh, Dhh and Ihh have equal binding affinities for the Ptc1 receptor thereby suggesting that redundancy between hedgehog protein activity may exist (Carpenter et al., 1998). In vitro data have since identified a degree of functional homology amongst Shh and Ihh proteins in hypertrophic chondrocyte differentiation (Vortkamp et al., 1996) and osteoblastic differentiation (Kinto et al., 1997), and among Shh, Dhh and Ihh proteins during motor neuron induction and polarisation of the developing limb (Pathi et al., 2001). In order to further characterise the role of Shh signalling in maintaining the epidermal stem cell population and promoting basal cell proliferation, we overexpressed the mammalian homologues Dhh and Ihh in the basal cells of mouse skin and observed that Dhh, but not Ihh, is a functional homologue of Shh activity during epidermal stem cell homeostasis and skin tumour formation. Recent work by Watt and colleagues (Niemann et al., 2003) led to the hypothesis that Ihh plays a role in the proliferation of sebaceous cells, given the expression of Ihh in sebaceous tumours of *K14-ΔN-Lef1* mice. Our *K14-Ihh* transgenic epidermis directly tests this hypothesis, and despite ectopic expression of Ihh protein in the basal cells of $Ihh^{Regular}$ epidermis and activation of Shh and Ptc1 expression, there was no evidence of hair follicle or sebaceous lineage defects, nor any overt proliferative activity. These data suggest that the expression of Ihh within *K14-ΔN-Lef1* mice is not causative of sebaceous hyperplasia, but rather a consequence of inappropriate β-catenin signalling.

Our observation that Dhh overexpression gives rise to embryo phenotypes indistinguishable from Shh overexpression shows that Dhh is a functional homologue of Shh in epithelial

cells, and in particular, that ectopic Dhh pathway activation plays a role in regulating the proliferation of the epidermal basal cell compartment. Given that cancer can be seen as a disease of unregulated cell renewal, an understanding of the mechanisms underlying epidermal proliferative activity is fundamental to understanding how cancer cells obtain unlimited proliferative potential. The data presented here show that both Shh and Dhh signalling activity can result in epidermal hyperplasia or hypoplasia. Thus the elucidation of those targets common to both Shh and Dhh signalling may help identify the genes involved in epidermal cell renewal and those genes responsible for BCC formation. Our data also suggest that the current deficiencies in effective epidermal cell culture for tissue therapies might usefully be addressed by manipulation of the hedgehog pathway, such that the magnitude and timing of Shh activity can be used to drive basal cell proliferation, resulting in increased epidermal proliferation without disrupting epidermal stratification.

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