Basal Cell Carcinomas in Mice Overexpressing Sonic Hedgehog

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Mutations in the tumor suppressor gene PATCHED (PTC) are found in human patients with the basal cell nevus syndrome, a disease causing developmental defects and tumors, including basal cell carcinomas. Gene regulatory relationships defined in the fruit fly Drosophila suggest that overproduction of Sonic hedgehog (SHH), the ligand for PTC, will mimic loss of ptc function. It is shown here that transgenic mice overexpressing SHH in the skin develop many features of basal cell nevus syndrome, demonstrating that SHH is sufficient to induce basal cell carcinomas in mice. These data suggest that SHH may have a role in human tumorigenesis.

A large body of evidence supports the idea that multiple genetic events are required to transform normal epithelium into benign growths and then into metastatic tumors (1). Some types of tumors rarely show complete progression: For example, basal cell carcinomas (BCCs) of the skin—the most common tumors in Caucasians, with about 750,000 new cases annually in the United States—are generally only locally invasive (2). The lack of a mouse model of BCCs and the difficulty in culturing human BCCs has slowed progress in understanding the mechanisms underlying BCC biology.

Basal cell nevus syndrome (BCNS) is an autosomal dominant disease characterized by developmental defects and a predisposition to certain tumors (3). The most common morphologic abnormalities are skeletal defects such as polydactyly, jaw and rib defects, and spina bifida; the most common tumors are BCCs, medulloblastomas, and meningiomas. The defective gene is ptc (4), a gene on chromosome 9q, first identified in Drosophila as a regulator of embryonic pattern formation. Numerous sporadic BCCs also have 9q loss and ptc mutations, suggesting that many BCCs unrelated to BCNS arise from somatic damage to both copies of ptc (4, 5).

The ptc gene encodes a transmembrane receptor that represses transcription of genes encoding transforming growth factor–β and Wnt class signaling proteins and ptc itself (6). One vertebrate PTC ligand is the secreted protein SHH, which binds to PTC in cultured cells and frog oocytes. The fly homolog of SHH, Hedgehog (HH), is believed to inactivate PTC function, suggesting that HH proteins induce target gene transcription by inactivating their receptor’s function.

The COOH-terminal part of SHH is an autoprotease and cholesterol transferase that cleaves the SHH precursor into two fragments and adds a cholesterol moiety to the NH₂-terminal fragment (6). The latter fragment is sufficient for all known signaling events and contains a zinc hydroxalase-like domain that may act as a peptide (6, 7). Thus, in addition to binding to PTC, SHH may cleave an unknown target molecule, although no catalytic activity has yet been detected. In Drosophila, ptc represses its target genes except where ptc function is inactivated by Hh, and this relationship appears to be conserved in vertebrates. Excess Hh function has an effect similar to loss of ptc function (8). This genetic relationship means that overexpression of Shh in mouse skin might mimic the loss of ptc function seen in human BCCs.

In normal mice, Shh and ptc RNA accumulate in follicular but not interfollicular skin. Initial expression of Shh and ptc in skin occurs in Hardy stage 1 hair follicles. In 14.5-day postcoital (dpc) skin, Shh RNA accumulates at regularly spaced intervals in the ectoderm. Each spot of Shh signal overlies the mesenchymal condensation of a presumptive follicle (9) (Fig. 1A). High levels of ptc RNA accumulate in each underlying mesenchymal condensation and at slightly lower levels in the Shh-expressing ectodermal cells (Fig. 1B), presumably because of induction of ptc transcription by SHH (10).

To examine in vivo the effect of excess SHH signaling, we generated transgenic mice that overexpress SHH specifically in the skin. We fused Shh to the keratin 14 (K14) promoter (Fig. 1) (11), which drives expression as early as 9.5 dpc in the ectoderm and at later stages in both the follicular and interfollicular epithelium (12). In total, 26 transgenic mice derived from pronuclear injection were examined as embryos or neonates; lines could not be established because of perinatal lethality. In transgenic embryos, high levels of Shh RNA and ptc RNA accumulated in the basal layer of the epidermis, both in the follicular and the interfollicular epithelium (Fig. 1, C and D). The heightened ptc expression confirmed that functional SHH was present and capable of inducing the ptc target gene in epidermal cells. The ptc transcripts were also present in the mesenchyme underlying the ectoderm of transgenics, presumably resulting from movement of SHH into these cells.

The K14-Shh transgenic mice exhibited skeletal and skin abnormalities reminiscent of those seen in BCNS. The most frequent abnormality was polydactyly of both the fore- and hind limbs, some of which had eight digits (Fig. 1E). Each digit looked similar to the normal central digits, as in the chick talpid2 mutant (13). Distal phalanges were often missing, giving rise to a shortened but wider limb. Distal cartilage bifurcations, ectopic sites of cartilage formation between the digits, and a distal rim of persistent ossification were apparent. Spina bifida, a failure to close the neural tube, was also frequently observed in the transgenics. This defect always affected the caudal portion of the spine and, in severe mutants, extended to the thoracic spine. Skeletal preparations revealed that spinal processes that normally enclose the spinal cord failed to form dorsally (Fig. 1F) (14). These effects on skeletal development suggest that SHH penetrates internal tissues, as does the mesenchymal ectopic ptc expression.

The K14-Shh transgenic mice had multiple BCC-like epidermal proliferations throughout their skin surface after only the first few days of skin development. Dead perinatal embryos invariably had erosions that destroyed much of the skin surface. The skin lacked normal folds and was translucent and friable (Fig. 1H). Skin histology revealed massive proliferations of cells associated with primordial invaginating hair follicles, which were hyperchromatic but cytologically normal. At 18.5 dpc, the epidermal proliferations often involved most of the epidermal surface (Fig. 2, B and C). In mildly affected embryos, one or two epidermal growths were interspersed with six to eight follicles that appeared normal (Fig. 2D). In human BCCs, epidermal cells pro-
literate and form peripheral "palisades," a columnar epithelium resembling the basal keratinocyte layer. BCCs lack cell adhesion molecules that normally attach basal cells to the basement membrane zone, resulting in clefts between the basement membrane and tumor (15). These histological features of human BCCs were also found in the epidermal proliferations of K14-Shh transgenics (Fig. 2E).

The profile of marker proteins in the murine growths paralleled that of human BCCs. Human BCCs express basal keratins such as K14, do not express markers of differentiating stratified epithelium such as loricrin (16), and produce keratins associated with hyperproliferation, such as keratin 6, in the overlying epidermis (16, 17). Basement membrane proteins such as laminin 5 (Lam5) and bullous pemphigoid antigen 2 (BPAg2) are expressed at reduced levels in BCCs (18). In each of these respects, the K14-Shh–induced skin growths resembled BCCs. At 18.5 dpc, the growths were K14-positive (Fig. 3B) and did not express suprabasal differentiation markers (Fig. 3D). The interfollicular stratifying epithelium appeared to differentiate normally (Fig. 3, C and D), with hyperproliferation in overlying epidermis as revealed by keratin 6 expression (Fig. 3F). Both Lam5 (Fig. 3H) and BPAg2 (Fig. 3J) production was lower in K14-Shh proliferations than in control epidermis, as in BCCs (18). Excess Shh therefore has little effect on stratifying epidermis but causes growth of invaginating hair follicles into BCC-like tumors.

BCCs depend on the surrounding stroma for continued growth. Malignant tumors metastasize upon transplantation into mice, but transplanted BCCs undergo growth arrest or differentiation (19). Donor skin from 18.5-dpc K14-Shh transgenic embryos, marked with black or agouti hair, was transplanted to the dorsum of scid mice with white hair and examined 3, 5, and 10 weeks later (20) (Fig. 2, G to J). In each of 16 control grafts, the epithelium matured normally into wild-type hair follicles (Fig. 2G). In each of eight transgenic grafts, markedly reduced numbers of pigmented hairs were observed (Fig. 2H), showing that Shh can interfere with hair development. Transplanted transgenic skin growths did not enlarge and were partially differentiated. Epithelial growths in five grafts showed signs of mature hair follicle differentiation, including multiple mature hair shafts, sebaceous glands, and cysts in the invagination (Fig. 3, I and J). There was no evidence of metastasis. Thus, as with human BCCs, continued growth of tumors in the transgenics requires the proper tissue context.

The sufficiency of Shh for inducing tumors led us to search for human Shh mutations. A preliminary screen of human tumors revealed a Shh mutation in 1 of 43 BCCs, 1 of 14 medulloblastomas, and 1 of 6 breast carcinomas. These mutations were not detected in blood samples from the same patients (21) nor in blood DNA from 100 normal individuals (Fig. 4, A and B) (22). Remarkably, the three tumors contained the same mutation, a change of His332 to Tyr. Human His332, equivalent to His334 in the mouse sequence, is located on the surface of Shh that may interact with a substrate for the hypothetical peptidease reaction (Fig. 4C) (7). The independent occurrence of the same mutation three times in tumors raises the possibility that human His332 to Tyr is a gain-of-function mutation of Shh. No biochemical assay is yet available for the putative zinc hydrolase activity, but in vivo tests may reveal altered activities of the changed signaling molecule.

Normally Shh is expressed in localized areas to control pattern formation. Ubiquitous epithelial expression would reduce or erase the asymmetry, preventing normal digit fates and interfering with formation of dorsal neural tube–derived structures.

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**Fig. 1.** Expression and phenotypes of normal and transgenic mouse skin. 35S-labeled (red) antiserum Shh and ptc probes were used to detect expression patterns by in situ hybridization (S2). (A) Focal ectodermal (e) Shh RNA accumulation (red grains) in regularly spaced arrays in a presumptive follicle of 14.5-dpc trunk skin. (B) Accumulation of ptc in both the ectodermal cells (e) where Shh RNA is expressed and the underlying mesenchymal cells (m). (C and D) Sections of 18.5-dpc transgenic (trans) skin growths hybridized with (C) Shh or (D) ptc probes. The highest levels of Shh are in the transgenic epithelium (e), with ptc RNA accumulation in epithelial and neighboring dermal (d) cells. (E through H) Transgenic animals display marked skeletal and skin phenotypes. (E) Transgenic forepaw stained with alizarin red (calcified bone) and alcian blue (cartilage) provides an example of the polydactyly that is frequently observed. There are ectopic centers of cartilage between digits (d), terminal phalangeal bifurcations (b), and a persistent rim of ossification at the distal rim (p, phalanx). (F) An 18.5-dpc transgenic embryo that failed to form dorsal spinal processes. This defect leads to a lack of closure of the spinal canal (arrows) and spina bifida. (G) Skin from a wild-type 18.5-dpc embryonic mouse showing regularly spaced skin folds. (H) Skin from a transgenic mouse showing translucent plaques and a lack of normal folds. (I) Schematic of the K14-Shh transgenic, including the K14 promoter fragment, the β-globin 5’ intron, and the K14 polyadenylation sites (12). The bar in (A) represents 40 μm, for (A) through (D). Skeletal preparations were prepared as in (33).
In BCNS patients, the haploinsufficiency of ptc may sensitize anterior limb or dorsal neural tube tissues to normal levels of Shh, resulting in polydactyly or spina bifida. That a twofold change in PTC dose may cause such changes in humans suggests that a precise balance between SHH and PTC is required.

The expression of Shh in basal keratinocytes is sufficient to induce mouse skin tumors that are indistinguishable from human BCCs. This effect of Shh may be completely unrelated to its normal function in skin development; Shh targets induced at high levels could create novel cell types and growth properties. However, the patterns of Shh and ptc transcription in the developing follicle are consistent with a normal role in controlling the proliferation of basal cells in the follicular epithelium. The decrease in mature hairs in the grafts along with the presence of abortive hair follicles in differentiating tumor buds suggests that Shh stimulates the growth of pluripotent follicular epithelium at the expense of differentiation. Such a role is supported by recent studies in chick feather bud development, where Shh induces feather bud outgrowth (24). Juxtaposition to a novel tissue environment appears to inhibit Shh activity, resulting in follicular differentiation.

The rapid and frequent appearance of Shh-induced tumors suggests that disruption of the SHH-PTC pathway is sufficient to create BCCs. The mouse BCCs appear within the first 4 days of skin development, unlike mouse squamous neoplasia, where tumors arise 1 to 12 months after oncogene expression (25). The K14-Shh tumor kinetics are consistent with previous clinical and epidemiologic data, which suggest that BCCs, in contrast to melanomas and squamous cell carcinomas, lack precursor or intermediate cellular phenotypes (2).

The gene ptc joins APC in a class of

![Fig. 2.](image-url)

(A through E) Skin histology of K14-Shh transgenic skin. Hematoxylin-eosin-stained sections of wild-type (wt) and transgenic (trans) mouse skin reveal a marked proliferation of the follicular epithelium. (A) Wild-type 17.5-dpc embryo showing normal keratinization and hair bud formation. Note spacing of hair follicles (HF) and wild-type basal (B), squamous (S), and horny (H) layers of stratifying epithelium. (B) Sagittal sections of transgenic 17.5-dpc trunk skin showing a massive downward growth (Gr) of an apparent hair follicle. Note the large size of the invagination compared to adjacent, apparently wild-type hair follicles (hF). (C) Marked proliferation of cells in a 17.5-day, strongly affected mutant embryo. Note the extensive epidermal growth pattern, which encompasses most of the basal cells (outlined by white arrows). (D) Lower power view of sections from mildly affected skin showing the regular number and spacing of many primary hair follicles (HF) with intermittent follicles forming downward growths (Gr). (E) High-power view of cells showing the artificial clefing around proliferating cells (C), and peripheral palisading of columnar cells (P), characteristic of BCCs. (F) Paraffin-embedded section of human nodular BCC showing the above characteristics: T, tumor tissue; D, dermis. (G and H) Photographs of 3-week post-graft skin from B6C3F1 wild-type (G) and transgenic (H) skin grafted onto CB-17 scid/scid recipients (20). Note the dramatic reduction in pigmented hair in the graft. (I) Low- and (J) high-power view of haematoxylin-eosin stain of paraffin-embedded skin showing the ongoing differentiation of BCCs into mature hair follicle epithelium. Note the lack of clefing and the presence of multiple mature hair shafts (arrowheads), HS, and sebaceous glands (S) in each tumor bud. Skin grafts performed as in (27). Bar in (J) represents 32 μm for (E) and (J), 80 μm for (A) through (C), (F), and (I), and 200 μm for (D).
Fig. 3. Expression of BCC marker proteins in mouse tumors. Immunologic characterization of 18.5-dpc wild-type (wt) and transgenic (trans) skin. For a normal bright-field image, see Fig. 2A. (A and B) Staining of antibodies to K14 reveals that the epidermal proliferations express basal keratins. (C and D) Loricin (Lor) shows normal granular staining (G), which is consistent with the normal differentiation pattern of stratified epithelium. (E and F) Expression of keratin 6 (K6) increases in transgenic skin, consistent with hyperproliferation of the epithelium. Note the lack of induced staining in the epidermal growths, consistent with previous studies on human BCCs. (G and H) Antibodies to LAM5 reveal decreased expression in transgenic epithelium. Note the reduction in expression throughout the epithelium (arrows). (I and J) Antibodies to BAP2 reveal a marked decrease of BAP2 expression in invaginating epithelium (arrows) compared to basal epithelium (arrowheads). Immunohistochemistry of skin was performed as in (34). Bar in (A) represents 62 µm for (A) through (H) and 25 µm for (I) and (J). Fixed sections were treated with rabbit primary antibodies, followed by anti-rabbit rhodamine-conjugated secondary antibodies, and were visualized with the use of a Bio-Rad confocal microscope. B, basal layer; G, granular layer.

Fig. 4. Mutations of Shh in human tumor tissues. (A) Direct sequencing from PCR products reveals a mutation in the human Shh sequence at His133 (mouse His134) in BCC tumor tissue but not in the blood of the same individual. The particular nucleotide changed was the same for each tumor type. (B) The mutation was confirmed by restriction fragment length polymorphism. The mutation in tumor tissue (T) eliminates an Mscl site in Shh (arrow) present in blood (B) or normal (N) controls. (C) Molecular model of murine SHH (7) reveals the position of mouse His134 (white atom; equivalent to human His135). Putative catalytic (green) and zinc coordinating (red) residues are located near the charged residues. Molecular modeling was performed with RasMac. M, marker; MED, medulloblastoma; Breast, breast carcinoma.

REFERENCES AND NOTES
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10. The accumulation of Shh RNA is seen ectodermally later in development and in the anagen hair matrix, with ptc RNA accumulating in adjacent cells.
11. The Eco-Rf-Sacl fragment of the mouse Shh cDNA, which includes the entire cDNA and the first 420 base pairs (bp) of the 3' untranslated region, was placed into the keratin β-globin promoter cassette (12). The cassette was microinjected into the pronuclei of B10C2F2 hybrids, and transgenic embryos were identified by standard techniques.
14. Its abnormalities, such as abnormal fusion at the
Integration of What and Where in the Primate Prefrontal Cortex

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The visual system separates processing of an object’s form and color (“what”) from its spatial location (“where”). In order to direct action to objects, the identity and location of those objects must somehow be integrated. To examine whether this process occurs within the prefrontal (PF) cortex, the activity of 195 PF neurons was recorded during a task that engaged both what and where working memory. Some neurons showed either object-tuned (what) or location-tuned (where) delay activity. However, over half (52 percent, or 64/123) of the PF neurons with delay activity showed both what and where tuning. These neurons may contribute to the linking of object information with the spatial information needed to guide behavior.

Anatomical segregation of processing is an important principle of neural organization. Even within a modality, largely separate pathways process different attributes of the same stimulus. Perhaps the best explored example of segregation is in the visual system, where the analysis of visual scenes is carried out by at least two pathways. A “ventral pathway” through inferior temporal (IT) cortex processes information about features that identify objects, such as shape and color (object, or “what”) information, and a “dorsal pathway” through posterior parietal (PP) cortex processes information about location and spatial relations among objects (spatial, or “where”) information (1). This example raises the question of where and how information about object identity is integrated with information about object location. One region that may play a role in integration is the prefrontal (PF) cortex, which receives inputs from virtually all of the brain’s sensory systems (2) and has long been thought to be an area where diverse signals are integrated to serve higher order cognitive functions.

A major contribution of the PF cortex to cognition is the active maintenance of behaviorally relevant information “online,” a process known as working memory (3). Working memory is typically studied in tasks in which an animal must remember a cue stimulus over a delay period and then make a behavioral response based on the cue. Physiological studies in monkeys have revealed that many PF neurons are highly active during the delay of such tasks (4). The activity is often cue-specific, suggesting that this “delay activity” is the neural correlate of the working memory trace. Given its central role in cognition, PF neurons that contribute to working memory are obvious candidates for integrating diverse signals. However, the extent to which different types of information, such as what and where, are integrated within the PF cortex is not well understood. Highly processed spatial information from the PF cortex and object information from the IT cortex are received by separate regions of the PF cortex, the dorsolateral (areas 46 and 9) and the ventrolateral (area 12) PF cortex, respectively (5), but there are interconnections between these regions that could bring what and where together (2, 6).

Physiological studies have found that different neurons and even different regions of the PF cortex convey either object information (in the ventrolateral PF cortex) or spatial information (in the dorsolateral PF cortex), but no neurons have been reported to convey both (7). In previous studies, however, working memory for what and where was examined in two separate tasks: an object task and a spatial task. This separation rarely occurs in the real world and it raises the possibility that the apparent segregation of what and where working memory reflected an artificial behavioral segregation. Thus, to investigate whether object and spatial information is integrated by in-