Induction of basal cell carcinoma features in transgenic human skin expressing Sonic Hedgehog

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Hedgehog (HH) signaling proteins mediate inductive events during animal development\(^6\). Mutation of the only known HH receptor gene, Patched (PTC), has recently been implicated in inherited and sporadic forms of the most common human cancer, basal cell carcinoma (BCC)\(^6\). In *Drosophila*, HH acts by inactivating PTC function\(^7\), raising the possibility that overexpression of Sonic Hedgehog (SHH) in human epidermis might have a tumorigenic effect equivalent to loss of PTC function. We used retroviral transduction of normal human keratinocytes to constitutively express SHH. SHH-expressing cells demonstrated increased expression of both the known HH target, BMP-2B, as well as bcl-2, a protein prominently expressed by keratinocytes in BCCs. These keratinocytes were then used to regenerate human skin transgenic for long terminal repeat-driven SHH (LTR-SHH) on immune-deficient mice. LTR-SHH human skin consistently displays the abnormal specific histologic features seen in BCCs, including downgrowth of epithelial buds into the dermis, basal cell palisading and separation of epidermis from the underlying dermis. In addition, LTR-SHH skin displays the gene expression abnormalities previously described for human BCCs, including decreased BP180/BPAG2 and laminin 5 adhesion proteins and expression of basal epidermal keratins. These data indicate that expression of SHH in human skin recapitates features of human BCC in vivo, suggest that activation of this conserved signaling pathway contributes to the development of epithelial neoplasia and describe a new transgenic human tissue model of neoplasia.

The Hedgehog (HH) signaling pathway, first identified in *Drosophila* as necessary for segment polarity, impacts cellular proliferation via effectors such as Wnt and transforming growth factor-β (TGFβ) family proteins\(^8\). Patched (PTC) is a repressive component of this pathway, and HH proteins must overcome PTC-mediated repression to activate HH target genes. The recent identification of mutations in the human PTC gene in basal cell carcinoma (BCC)\(^9\) suggests that HH signaling may be important in human cutaneous carcinogenesis. Transgenic mice constitutively expressing SHH in skin develop abnormal hair follicles, which proliferate into BCC-like tumors (A.E.O. et al., in press), however, perinatal lethality and the considerable species differences between murine and human skin limit the usefulness of this model. Keratin promoter-targeted Sonic Hedgehog (SHH) expression in these transgenic mice carries further limitations, because SHH is expressed in developing ectoderm from 9.5 days post coitus and impacts development of other tissues which may in turn affect developing skin. In addition, major differences between murine and human species in epidermal tissue constrain the relevance of purely murine tissue models to human neoplasia. These include dramatic discrepancies in epidermal thickness, hair follicle density, epidermal differentiation kinetics and tissue susceptibility to neoplastic transformation. The fact that disruption of murine genes homologous to human disease genes can fail to exactly replicate the corresponding disease phenotype in mice, as seen in the case of knockout mice generated in attempted models of Lesch-Nyhan disease\(^9\) and cystic fibrosis\(^9\), underscores potential limitations of murine models of human disease.

To circumvent these problems, we used the skin-regenerating ability of human keratinocytes to determine whether SHH is sufficient to induce BCC features in post-natal human epidermis in the absence of developing hair follicular structures. Retroviral expression vectors were produced for high-level constitutive SHH expression driven by the retroviral long terminal repeat (LTR) (Fig. 1a). Available antibodies to human SHH do not function with cultured cells so a marker gene was used to verify successful transfer of the LTR-driven SHH expression construct to keratinocytes. The green fluorescent protein (GFP) from *Aequorea victoria* was expressed on the same vector as SHH via an internal ribosome entry site (IRES). Fluorescence analysis of transduced and untransduced control keratinocytes verified efficient transfer and activity of the LTR-SHH vector (Fig. 1b). SHH is not normally expressed in mature post-natal human keratinocytes grown in vitro (Fig. 2, a and b, and data not shown). Keratinocytes transduced with LTR-SHH vector express the active 19-kDa N-terminal cleavage product of SHH (ref. 19–20) (Fig. 2a). In order to verify that expressed SHH is active in inducing target gene transcription, messenger RNA from LTR-SHH transduced keratinocytes was analyzed for expression of the TGFβ family member BMP-2B, a known target gene of SHH in mammals\(^\)\(^9\). In addition to producing SHH mRNA, LTR-SHH keratinocytes induce expression of BMP-2B over the minimal levels seen in controls (Fig. 2b), indicating the presence of functional SHH protein. Keratinocytes in human BCCs express markedly elevated levels of bcl-2, in contrast to the other common epidermal malignancy, squamous cell carcinoma (SCC)\(^9\). Bcl-2 expression was found to be increased in SHH-expressing keratinocytes (Fig. 2c), consistent with a potential role for induction of BCC features by SHH.

Human skin was regenerated on immune-deficient mice\(^9\) to produce unmodified epidermis, epidermis transgenic for LTR-driven SHH-GFP (LTR-SHH) or epidermis transgenic for an LTR-driven GFP control vector. In this approach, transduced keratinocytes were seeded on devitalized human dermal substrate, grown in vitro for 7 days then grafted to the back fascia of
nude mice. Skin was analyzed at 4, 6 and 8 weeks postoperatively. LTR-SHH human skin is somewhat abnormal in texture during this time period, but regenerates skin with kinetics similar to that of unmodified controls or controls treated with GFP alone. At all time points LTR-SHH skin exhibits well-established histological features of BCC (ref. 24–26) including epidermal budding, peripheral palisading, and epidermal–dermal separation (fig. 3 and Table 1); the latter is also seen in areas without prominent bud formation. These three major histologic findings are not restricted to certain focal areas, but are consistently present throughout all regenerated LTR-SHH skin. BCC features are seen to varying degrees in all stepwise sections through all tissue blocks spanning the entire 1.5 cm of a typical graft. Epithelial downgrowth into the dermis in LTR-SHH skin ranged from small epidermal bud formation to more deeply penetrating epithelial islands (Fig. 3, b, c, e and f). In unmodified regenerated human skin, as well as in GFP transgenic controls, none of these changes were observed in multiple stepwise sections (Fig. 3, a and d, and Table 1). No hair follicle structures were observed in any regenerated human skin sections.

Basal cell carcinoma is currently defined mainly by histologic criteria, and no identifying markers entirely specific for this tumor have yet been identified. It has been shown recently, however, that two key molecules involved in epidermal adhesion to the underlying basement membrane, BP180 (BPAG2, type XVII collagen) and laminin 5, are expressed at lower levels in human BCCs in vivo27; this decrease may account for the characteristic epidermal–dermal separation and pattern of dermal invasion seen in the tumors. To determine whether the histologic features seen in our LTR-SHH human epidermis are accompanied by the expected abnormalities in protein expression, we used human species-specific monoclonal antibodies that recognize human BP180 and laminin 5. Immunostained LTR-SHH transgenic human skin has decreased amounts of both Laminin 5 (Fig. 4a) and BP180 (Fig. 4b), decreases not observed in control regenerated skin (Fig. 4c and d). The background dermal staining visible in the figure is due to recognition by the anti-mouse fluorescein isothiocyanate (FITC)-labeled secondary antibody of low levels of murine dermal immunoglobulin deposition in the nude mouse (data not shown). Epithelial tissue seen penetrating LTR-SHH dermis also stains strongly with a monoclonal antibody to the keratin 14 basal marker and lacks suprabasal keratin expression (Fig. 4f and data not shown).
In addition to our findings, the physiologic relevance of SHH activation in human epithelium is supported by a number of important recent observations. First, the potent inhibitor of SHH pathway function, PTI, is mutated in spontaneous human BCCs and in the human basal cell nevus syndrome. Second, murine epidermis transgenic for SHH demonstrates basaloid proliferations consistent with our findings. Third, conserved mutations in a single residue in SHH that are not found in normal human tissue have been identified in multiple human BCCs in a picture reminiscent of conserved activating Ras mutations. It is unlikely that additional genetic events are necessary to produce the observed changes in the absence of deliberate mutagenesis in the short time course of our experiments; this is further supported by the fact that SHH-induced mutagenesis in other systems has never been observed. In combination with the current work demonstrating rapid induction of BCC features in postnatal human tissue, we may argue from these findings that SHH pathway gain of function is sufficient to induce the major elements of BCC.

Human BCCs do not behave as a classic malignancy in that they rarely metastasize, fail to readily grow in culture and exhibit few features of malignant cells in conventional assay systems, so they may represent a condition intermediate on the spectrum between reactive hyperproliferation and a well-established malignancy characterized by many genetic lesions. DNA repair-defective XPA and XPC knockout mice can require up to 20 weeks of daily irradiation with mutagenic doses of ultraviolet-B radiation to develop a single skin region with neoplastic features. In contrast, LTR-SHH human epidermis develops widespread BCC changes within 4 weeks in the absence of carcinogenic stimuli. Given the fact that human cells are much more resistant to neoplastic transformation than mice, these findings suggest that SHH may act dominantly to trigger BCC changes independent of a need for additional genetic events. The fact that the vast majority of human BCCs are composed of diploid cells and that, unlike squamous cell carcinoma of the skin, BCCs arise de novo without precursor lesions supports this possibility. An increase in HH signaling in such a...

These observations are consistent with the pattern seen in human BCC (ref. 24-26).

Recent studies have underscored the fact that signaling pathways utilized in embryonic development may also have a major impact on the control of cellular proliferation and the development of neoplasia in postnatal mammalian tissues. Here we show that human skin transgenic for LTR-driven SHH displays both tissue architectural features of BCC and the gene expression profile found in human neoplasm. There are no cell markers solely specific for BCC, and there remains a lack of absolute certainty concerning identification and classification of BCC subtypes; however, the changes seen in LTR-SHH skin changes fulfill all the major histologic and gene expression pattern criteria currently available to identify this tumor. Of all the transgenic models of cutaneous epithelial neoplasia yet generated, none have produced the specific histologic and gene expression features of BCC demonstrated here. All of the many oncoproteins and growth regulators previously misexpressed in the skin have caused growth abnormalities more consistent with the squamous hyperplasia characteristic of early squamous cell carcinoma (SCC), a form of skin cancer completely distinct from BCC both in its histology and in marker gene expression. The fact that every aspect of our findings fit with BCC but not with SCC suggests that SHH action specifically induces BCC features, rather than serving as a nonspecific inducer of epithelial hyperplasia.
Table 1 Summary of histologic findings in LTR-SHH skin

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<tr>
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<th>Control</th>
<th>LTR-SHH</th>
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<tr>
<td>Basal cell parakeratosis</td>
<td>0/23 (0)</td>
<td>27/31 (87)</td>
</tr>
<tr>
<td>Epidermal-dermal separation</td>
<td>0/23 (0)</td>
<td>28/31 (90)</td>
</tr>
<tr>
<td>Epithelial budding and downgrowth</td>
<td>0/23 (0)</td>
<td>31/31 (100)</td>
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Multiple 5-μm sections were obtained in a stepwise fashion through the tissue biopsies that spanned the full 1.5 cm human skin region from each regenerated human skin graft specimen. Histologic features of BCC were defined by standard criteria. Briefly, a, basal cell parakeratosis keratinocytes with elongated nuclei closely juxtaposed in parallel at the periphery of epithelial aggregates; b, epidermal-dermal separation; retraction of epithelium from underlying dermis; c, epithelial budding and downgrowth; focal areas of epithelial tissue penetrating into underlying dermal tissue to a depth of twice or more the thickness of the surrounding epidermis. For controls (GFP and untransduced) a total of 23 sections were analyzed and for LTR-SHH a total of 31 sections were analyzed. The percent of tissue sections demonstrating histologic abnormalities is noted in parentheses. Four mice were grouped per vector.

setting could be due to overactivity of SHH itself or one of its downstream targets, such as homologues of the Smoothered gene.

The transgenic human tissue approach used here offers understanding and opportunities distinct from murine models of human cancer. Classic heterotopic transplant experiments demonstrate that epithelial invaginations require a combination of multiple mesenchymal and epidermal signals. Our data suggest that established hair follicle structures are not required for BCC development in humans. Our findings also indicate that the development of BCC features can be accomplished largely, perhaps entirely, by inactivating events in the epidermis in a process that does not require a primary inductive influence from supporting mesenchymal cells.

Progress toward therapies for BCC has been impeded by the lack of accurate model systems and the difficulty of growing human BCCs in vitro. The current approach may be a method of use in this regard; to our knowledge, this is the first in vitro transgenic human tissue model of neoplasia. Use of such transgenic human tissue models for skin and visceral tissues may assist future efforts to understand and to develop novel therapies for BCC and other human cancers. Our results highlight the importance of conserved mechanisms of embryonic organization in the control of cellular proliferation and offer a human tissue model for study and intervention in the molecular events in human cutaneous carcinogenesis.

Methods

Vectors and gene transfer. A SHH-RES-GFP retroviral expression vector was generated as an aid to rapid confirmation of transduction efficiency. The full-length human SHH CDNA (ref. 35) without 3' polyadenylation signals (HindIII to XbaI fragment) and 610 bp IRS sequence (XbaI and SalI fragment from pGEMST7+, generous gift of C. Nolan) were first subcloned into HindIII and Sall sites of pEGFP1 (Clontech, Palo Alto, CA). The pSHH-RES-GFP fragment was digested with NotI and SalI and subcloned into the BamHI and NotI sites of LZR5 (ref. 36), an MFG-based retroviral expression plasmid. A control vector expressing only the green fluorescence protein (GFP) was produced by subcloning the BamHI and NotI fragment of EGFP into pEGFP into the BamHI and NotI sites of LZR5 (Fig. 1c). Amphotropic retrovirus was produced as previously described. Human keratinocytes were freshly isolated from sites of normal human skin not exposed to sunlight, plated on a Matrigel-treated fibroblast feeder layer then grown to passage 1 and transduced as previously described. Keratinocytes were utilized from the same patient for each series of experiments to control for possible donor tissue variations and were either untreated or transduced with retroviral expression vectors for SHH-RES-GFP or GFP as previously described. Fluorescence and phase-contrast microscopy was performed with a Zeiss axiophot fluorescence microscope (Zeiss, Jena, Germany) to examine gene transfer efficiency to keratinocytes in vitro with the SHH-RES-GFP retroviral expression vector.

Analysis of gene expression in vitro. Whole-cell extracts were made from transduced and control keratinocytes and analyzed simultaneously with antibodies to human SHH (gift of T. Jessell) as well as to BRG1 (ref. 39); similar blotting was performed with antibody to β-actin (Dako, Carpinteria, CA). A total of 20 μg of cell extract was loaded per lane. For examination of gene expression, mRNA was purified and digested with deoxyribonuclease I (DNase I; Gibco BRL, Gaithersburg, MD) to remove any contaminating genomic DNA. Reverse transcriptase polymerase chain reaction analysis was then performed, internally controlled for expression of keratin 5 by inclusion of keratin 5 primers in all reactions. Primers included the following: SHH primers [5'-GTC ATCCATCTCCGAGG CGAG-3' and 5'-GTCG GCA GCCATCTCGTG-3'] priming 422 bp. BNP-28 primers [5'-CAGCAT CAGCTCGTGAAC and 5'-TCTCCA CAGTCTCCTCCTG-3'] priming 390 bp and keratin 5 primers [5'-GTTGACCCGT GACGCCAAGGTGGATCA-3' and 5'-GCCCAGTCCGCTGACTGACGAC-3'] priming 700 bp.

Transgenic human skin. Transduced keratinocytes were seeded on devarilized human dermis and grafted to nude mice. Briefly, 5 × 10^5 keratinocytes that were either untreated or transfected with the SHH-RES-GFP or GFP alone expression vectors were seeded on 1.5 cm pieces of devarilized human dermis and grown in vitro for 7 days in keratinocyte growth media containing DMEM/F12 (3:1 ratio; Gibco BRL), fetal bovine serum (10%), penicillin-streptomycin (100 IU/ml), adenosine (1.8 × 10^-5 M), cholera toxin (1 × 10^-5 M), EGF (10 ng/ml), hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), and trichloroacetic acid (2 × 10^-4 M). These composite grafts were grafted to hairless condition to back flanks of nu/nu mice. Four mice were grafted per vector group (Fig. 1b); a lacZ vector and untransduced keratinocyte groups were utilized as additional controls. At 4, 6 and 8 weeks biopsy was performed and frozen tissue sections prepared. The human species origin of regenerated epithelium was confirmed in tissue sections using species-specific antibodies to human involunin and filagrin (BTI Inc., Steubenville, OH). Immunostained sections were then stained with hematoxylin and eosin for analysis of histologic features. Immunofluorescence analysis of tissue sections was performed using monoclonal antibodies to human BP180 (gift of G. Giudicini), [3] chain of laminin 5 (gift of P. Marinkovich) and keratin 14 (ref. 44) (Harlan Sera-lab Inc., Sussex, UK).

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