Correlations between the Sonic Hedgehog Pathway and basal cell carcinoma

Omar Lupi, MD, PhD

From the Universidade Federal do Rio de Janeiro (UFRJ) and Instituto de Dermatologia Prof Rubem Azulay/Santa Casa RJ, Rio de Janeiro, Brazil

Correspondence
Omar Lupi, MD, PhD
Rua Frei Leandro, 16/501
Lagoa
Zip Code: 22.470-210
Rio de Janeiro/RJ
Brazil
E-mail: omarlupi@globo.com

Abstract
The Hedgehog (HH) family of intercellular signaling proteins has some essential functions in patterning both invertebrate and vertebrate embryos. Identified as an important regulator of segment polarity and tissue organization in flies, the HH pathway can also play a significant role in human development and in cutaneous carcinogenesis. The family received their name because when the D. melanogaster HH protein malfunctions the mutant fly ends up looking like a small prickly ball, similar to a curled-up hedgehog.

The Sonic hedgehog (SHH) pathway is implicated in the etiology of the most common human cancer, the basal cell carcinoma (BCC). Mutations in the receptor of SHH, the patched gene (PTCH), have been characterized in sporadic BCCs as well as those from patients with the rare genetic syndrome nevoid BCC. Human PTCH is mutated in sporadic as well as hereditary BCCs, and inactivation of this gene is probably a necessary if not sufficient step for tumorigenesis. Delineation of the biochemical pathway in which PTCH functions may lead to rational medical therapy for skin cancer and possibly other tumors.

Introduction

The Hedgehog (HH) family of intercellular signaling proteins plays certain essential roles in patterning both invertebrate and vertebrate embryos. Identified as an important regulator of segment polarity and tissue organization in Drosophila melanogaster, the HH pathway can also play a significant role in human development and in cutaneous carcinogenesis.

Fruit flies have a single HH gene, whereas vertebrates have evolved three different types of homolog (the Sonic, Desert, and Indian types), probably by duplication of the single ancestral gene. Their name was coined because, when the D. melanogaster HH protein malfunctions, the mutant fly looks like a small prickly ball, similar to a curled-up hedgehog.

The Sonic Hedgehog (SHH) pathway is implicated in the etiology of the most common human cancer in the white population, basal cell carcinoma (BCC). Mutations in the receptor of SHH, the patched gene (PTCH), have been characterized in sporadic BCCs, as well as those from patients with the rare genetic syndromes nevoid BCC and xeroderma pigmentosum (XP).

Genetic studies in D. melanogaster have shown that PTCH is a crucial part of the SHH pathway, important in determining embryonic patterning and cell fate in multiple structures of the developing embryo. Human PTCH is mutated in sporadic as well as hereditary BCCs, and inactivation of this gene is probably a necessary, if not sufficient, step for tumor formation. Delineation of the biochemical pathway in which PTCH functions may lead to rational medical therapy for skin cancer and possibly other tumors.

The SHH pathway

The vertebrate Sonic-type gene (SHH) shows almost the same expression in every animal so far examined, with little variation in sequence, implying that it is an important gene. Desert and Indian-type genes, however, have different patterns of expression in different classes of animal. The C-terminal peptide diffuses from the cell, whereas the N-terminal peptide remains associated with the cell surface. The HH proteins are also secreted, in either cleaved or uncleaved form, to mediate signaling to other cells. This signaling is activated by the binding of the SHH protein to the membrane receptor PTCH, which is a human tumor suppressor protein that regulates growth and patterning in embryos. PTCH represses the expression of HH target genes, such as glioma transcription factor-1 (GLI-1) and PTCH-1, important in the regulation of limb development.

The third component of the SHH pathway is the transmembrane protein called Smoothed (SMO). In the absence of HH, PTCH inhibits SMO, thereby blocking the expression of target genes. Binding of HH to PTCH suspends the inhibitory effect of PTCH on its signaling partner SMO.

There are in fact two PTCH genes, namely PTCH-1 and PTCH-2. PTCH-1 is critical for embryonic development, and its loss is linked to tumorigenesis. Germ line inactivation of
one copy of PTCH-1 predisposes to BCC and medulloblastoma in mice and humans. In many cases, medulloblastoma arising from perturbations of PTCH-1 function leads to a concomitant up-regulation of PTCH-2. As increased expression of PTCH-2 is associated with medulloblastoma and other tumors, Lee et al. investigated the role of PTCH-2 in tumor suppression by generating PTCH-2-deficient mice. In contrast with PTCH-1−/− mice, PTCH-2−/− animals were born alive, showed no obvious defects, and were not cancer prone; however, the loss of PTCH-2 markedly affected tumor formation in combination with PTCH-1 haploinsufficiency. Therefore, PTCH-2 seems to modulate tumorigenesis associated with PTCH-1 haploinsufficiency. Rahnama et al. analyzed the biological properties of several PTCH-2 splice variants. PTCH-2 promoter regulation assays demonstrated that only one of the PTCH-2 variants could inhibit the activity of SHH, whereas none was capable of inhibiting the activated form of SMO (SMO-M2); this contrasts with PTCH-1. Despite the fact that the PTCH-2 isoforms lacked the ability to inhibit SMO-M2 activity, all PTCH-2 variants, as well as PTCH-1, on cotransfection with SMO, were able to change SMO localization from being largely dispersed in the cytoplasm to the juxtanuclear region. Using PTCH-1−/− mouse cells, it has been shown that the PTCH-2 variants and PTCH-1 differentially act to reconstitute not only the SHH but also the Desert HH-dependent transcriptional response. A novel component in the vertebrate signaling pathway, name HIP (for Hedgehog interacting protein), has been identified, and seems to encode a membrane glycoprotein that binds to the SHH protein with an affinity comparable with that of PTCH-1. HIP was found to bind to SHH directly and attenuate SHH signaling, as does PTCH, whereas its expression was induced by SHH signals. Overexpression of HIP in cartilage leads to a shortened skeleton, which resembles that seen when HH function is lost.

Tojo et al. examined the expression patterns of HIP, SHH and PTCH gene mRNA by human BCCs, in comparison with those by normal human skin and various skin tumors. It was found that mRNA expression of both HIP and PTCH genes was enhanced in all BCC samples, whereas none of the other skin tumors tested exhibited an increased level of such mRNAs when compared with normal skin. The transcription of the SHH gene, however, was at a baseline level in most BCCs. These results indicated that both HIP and PTCH gene expression are specifically involved in the development of BCCs, and that the production of HIP is linked with the expression of the PTCH gene but not the SHH gene. All of these findings support a model in which these general transcriptional targets (HH protein, PTCH, SMO, and HIP) are able to modulate the response to the HH signal by binding to HH proteins in a negative regulatory feedback loop. It is also possible that new components of the SHH pathway will be described in the near future.

**SHH and BCC**

The recent identification of mutations in the human PTCH gene, in both humans with basal cell nevus syndrome (BCNS) and in sporadic BCC, suggests that HH signaling may be important in human cutaneous carcinogenesis. During the past few years, it has become evident that mutations in PTCH or in one of the components of the SHH signaling pathway contribute to the formation of BCC, and also to odontogenic keratocysts, medulloblastomas, and breast carcinomas.

Gene regulatory relationships defined in *D. melanogaster* suggest that the overproduction of SHH in mouse skin will mimic the loss of PTCH function seen in human BCCs. SHH has mitogenic effects in several tissues, including presomitic mesoderm, retina, and cerebellum. Transgenic mice overexpressing SHH in the skin develop many features of BCNS, demonstrating that SHH is sufficient to induce BCC in mice.

SHH and PTCH RNA accumulate in follicular, but not interfollicular, skin in normal mice. The distribution of the former is regular in the ectoderm during skin embryogenesis; each spot of SHH signal overlies the mesenchymal condensation of a presumptive follicle, suggesting that SHH can interfere with hair development. Hardy suggests that epithelial invaginations require a combination of both mesenchymal and epidermal signals. Fan et al., however, observed that established hair follicle structures are not required for BCC development. Initiating events in the epidermis, such as the SHH pathway, can induce BCC features without any inductive influence from supporting mesenchymal cells.

Oro evaluated the effects of excess SHH signaling using transgenic mice that overexpress the protein specifically in the skin (K14-SHH mice). These mice exhibited skeletal and skin abnormalities reminiscent of those commonly seen in BCNS patients, such as polydactyly, spina bifida, and BCC-like epidermal proliferations throughout the skin surface after only the first few days of skin development. The histologic features of human BCCs, such as the proliferation and formation of peripheral “palisades” by keratinocytes and the separation of the epidermis from the underlying dermis, were all found in the epidermal lesions of K14-SHH transgenic mice. Immunohistochemical evaluation of the mice lesions also showed a pattern very similar to the BCC profile, with the presence of basal keratins (K14), keratin 6, and laminin. Fan et al. transfected normal human keratinocytes from one single donor, freshly isolated from cutaneous sites not previously exposed to sunlight, with a retrovirus containing the SHH gene. This transgenic human skin was seeded on devitalized human dermis and grafted to four nude mice. These areas were analyzed at 4, 6, and 8 weeks and exhibited well-established histologic features of BCC, not restricted to focal areas, but consistently present throughout all
regenerated skin.\textsuperscript{17} It is very interesting to note that DNA repair-defective XP knockout mice can require up to 20 weeks of daily irradiation with mutagenic doses of ultraviolet-B (UV-B) to develop a single skin lesion with neoplastic features.\textsuperscript{18}

In contrast, human epidermis transgenic for \textit{SHH} developed widespread BCC changes within 4 weeks in the absence of UV radiation or other carcinogenic stimuli.\textsuperscript{19} Fan and Khavari\textsuperscript{19} suggested that \textit{SHH}-induced epidermal hyperplasia is accompanied by continued cell proliferation in normally growth-arrested suprabasal cells \textit{in vivo}. Cell cycle exit before outward migration and terminal differentiation is the classical pattern of normal stratified epithelium. \textit{SHH}-expressing cells fail to exit the S and G2/M phases of mitosis, which occurs in normal keratinocytes exposed to elevated calcium concentrations, and also resist exhaustion of replicative growth capacity.\textsuperscript{19} These observations, together with the ability of \textit{SHH} to resist apoptosis by the induction of \textit{bcl-2},\textsuperscript{15} may provide a plausible model for \textit{SHH} induction of epithelial neoplasia.

Couve-Privat \textit{et al.}\textsuperscript{1} evaluated the role of \textit{UV} in the deregulation of the \textit{SHH} pathway, analyzing the alterations of \textit{SMO}, the transmembrane signaling component regulated by \textit{PTCH}, in both BCCs and squamous cell carcinomas from UV-hypersensitive XP patients. The authors detected \textit{UV}-specific \textit{SMO} mutations in 30% of XP BCCs, three times higher than that in sporadic BCCs in the white population, thus confirming the high rate of \textit{UV}-induced mutations in DNA repair-deficient XP patients. No alteration was found in XP squamous cell carcinomas, indicating the involvement of \textit{SMO} specifically in the development of BCC.

Hutchin \textit{et al.}\textsuperscript{20} used mice engineered to conditionally express the \textit{HH} effector GLI-2, and showed that continued HH signaling is required for the growth of established BCCs. Transgene inactivation led to BCC regression, accompanied by reduced tumor cell proliferation and increased apoptosis, leaving behind a small subset of nonproliferative cells that could form tumors on transgene reactivation. Nearly all BCCs arose from hair follicles, which harbor cutaneous epithelial stem cells, and reconstitution of regressing tumor cells with an inductive mesenchyme led to multilineage differentiation and hair follicle formation.\textsuperscript{20} Hutchin \textit{et al.}\textsuperscript{20} believe that continued \textit{HH} signaling is probably required for proliferation and survival of established BCCs; the tumors represent an aberrant form of follicle organogenesis, creating an opportunity to treat BCCs using HH pathway inhibitors.

Trichoepitheliomas, benign follicular tumors with a histopathologic pattern very similar to BCCs, also contain somatic mutations in the \textit{PTCH} gene, reinforcing the importance of the \textit{SHH} pathway in skin tumorigenesis.\textsuperscript{24} The \textit{PTCH} gene is located on chromosome 9q (9q22–3),\textsuperscript{14} and its deletion or inactivated mutation is observed in more than 50% of human BCCs.\textsuperscript{24} \textit{SHH} seems to be a proto-oncogene and \textit{PTCH} acts as a tumor suppressor gene in BCC pathogenesis.\textsuperscript{7} Zedan \textit{et al.}\textsuperscript{24} detected allelic loss in the \textit{PTCH} gene from patients with BCNS by polymerase chain reaction (PCR). This result allows a practical identification of the mutation and will be useful in the prenatal diagnosis of BCNS. It may also be useful in the differential diagnosis between atypical cases of BCC and other types of epidermal neoplasias without these same mutated alleles.

### Forkhead box (FOX) proteins and BCC

FOX proteins have also been shown to play an important role in regulating the expression of the genes involved in cell growth, proliferation, differentiation, longevity, and transformation.\textsuperscript{43} The functional importance of this gene family in normal human skin physiology and disease processes is not well understood. Recent studies\textsuperscript{44–46} have established that some FOX genes are downstream targets of \textit{SHH} signaling.

Members of the FOX subfamilies A–G, I–L, and Q are grouped into class 1 FOX proteins, whereas members of FOX subfamilies H and M–P are grouped into class 2 FOX proteins.\textsuperscript{43} The C-terminal basic region within the FOX domain is the common feature of class 1 FOX proteins. FOXH-1 and FOXO-1 mRNAs are expressed in human embryonic stem cells. The FOXA-1 gene is amplified and overexpressed in esophageal and lung cancer. The FOXM-1 gene is upregulated in pancreatic cancer and BCC as a result of transcriptional regulation by the \textit{SHH} pathway. The FOXO-1 gene is related to rhabdomyosarcomas and the FOXO-3 and FOXO-4 genes are associated with some hematologic malignancies.\textsuperscript{43}

Branccacio \textit{et al.}\textsuperscript{26} established FOXE-1 as a downstream target of the \textit{SHH/GLI} pathway in hair follicle morphogenesis, and as a crucial player for correct hair follicle orientation into the dermis and subcutaneous tissue. Teh \textit{et al.}\textsuperscript{27} investigated the role of FOX proteins in transducing \textit{SHH} effects in human skin using degenerate PCR to identify FOX genes differentially expressed in BCCs. All three known FOXM-1 isoforms (a, b, and c) were detected in human skin and cultured keratinocytes, and the transcriptionally active FOXM-1b isoform was found to be upregulated in BCCs. Real-time quantitative PCR (RT-PCR) showed that the increase in FOXM-1 mRNA levels was specific for BCCs and not a reflection of increased cell proliferation, in that no upregulation was seen in squamous cell carcinomas or proliferating primary human keratinocyte cultures.\textsuperscript{53} Immunostaining studies showed intense nuclear and cytoplasmic staining throughout BCC tumor islands and not confined to the peripheral regions of the tumor in which proliferating Ki-67-immunopositive cells are predominantly localized. Expression of the \textit{SHH} target GLI-1 in primary keratinocytes and other cell lines caused a significant elevation of the FOXM-1 mRNA level and transcriptional activity, indicating that FOXM-1 is a downstream target of GLI-1. The activation of \textit{SHH} signaling via GLI-1 is an important...
determinant of FOXM-1 expression in mammalian cells.\textsuperscript{53,54} Given the role of FOXM-1 in cell proliferation, the upregulation of FOXM-1 in BCCs may be one of the mechanisms whereby SHH signaling exerts its mitogenic effect on basal keratinocytes, leading to the development of BCC.\textsuperscript{53,54}

### Chemoprevention of BCCs

The development of novel, pathogenesis-based therapies requires a better knowledge of the molecular mechanisms leading to the development of these tumors.\textsuperscript{55,56} BCCs are characterized by aberrant activation of SHH signaling as a result of mutations in the PTCH or SMO genes.\textsuperscript{57} In addition, about 50% of cases carry mutations in the p53 tumor suppressor gene. Recently, specific inhibitors of the SHH signaling pathway have been developed, and have shown promising results in preclinical studies on experimental BCCs.\textsuperscript{28,29}

According to Chen et al.,\textsuperscript{30} the steroidal alkaloid cyclopamine, isolated from the plant \textit{Veratrum californicum}, has both teratogenic and antitumor activities arising from its ability to specifically block cellular responses to the vertebrate HH signaling pathway. It has been shown that this inhibitory effect is mediated by direct binding of cyclopamine to the heptahelical bundle of SMO.\textsuperscript{31} Cyclopamine can also reverse the retention of partially misfolded SMO in the endoplasmic reticulum, presumably through binding-mediated effects on protein conformation.\textsuperscript{32,33} These observations have revealed the mechanism of cyclopamine’s teratogenic and antitumor activities, and further suggest a role for small molecules in the physiologic regulation of SMO. McFerren\textsuperscript{17} and Yanai et al.\textsuperscript{14} recently proposed that cyclopamine could be effective in the induction of the differentiation and apoptosis of BCCs, and in gastric cancer. Cyclopamine suppressed the growth of gastric cancer cells in vitro.\textsuperscript{34} Chemoprevention of BCCs and also a possible useful effect against other hyperproliferative cutaneous diseases, such as psoriasis, are also possible, based on cyclopamine-induced HH inhibition.\textsuperscript{26,28,35}

### Conclusions

The correlation between BCC and sunlight exposure is clear, as 95% of cases occur in sun-exposed areas in the white population.\textsuperscript{17} DNA lesions induced by UV radiation are considered to be the most important etiologic features in BCC pathogenesis.\textsuperscript{17,18} The SHH/PTCH pathway helps to explain how single somatic mutations can produce BCCs in both sporadic cases\textsuperscript{13–15} and in BCNS patients.\textsuperscript{13,14}

It is important to point out, however, that, according to Reifenberger et al.,\textsuperscript{31} several BCC-associated genes have already been described from the SHH/PTCH pathway. They screened for mutations in the SHH pathway genes in 42 skin tumors; single-strand conformational polymorphism analysis, followed by DNA sequencing, was used to screen for mutations in PTCH, SMO, and GLI-1 genes, as well as in the p53 tumor suppressor gene, and in the proto-oncogenes NRAS, KRAS, HRAS, BRAF, and CTNNB1. Microsatellite markers flanking the PTCH, SUFUH and p53 loci at 9q22, 10q24, and 17p13, respectively, were studied for loss of heterozygosity (LOH).\textsuperscript{14} The authors found PTCH mutations in 28 of 42 tumors (67%).\textsuperscript{14} Microsatellite analysis revealed LOH on 9q22 in 20 of the 38 tumors investigated (53%), including 14 tumors with and six tumors without PTCH mutations.\textsuperscript{34} SMO mutations were identified in four of the 42 BCCs (10%). Seventeen BCCs (40%) carried p53 mutations, with only three tumors showing evidence of biallelic p53 inactivation. Interestingly, 72% of the p53 alterations were presumably UV-induced transition mutations. In contrast, only 40% of the PTCH and SMO alterations corresponded to UV signature mutations. No mutations were identified in GLI-1, NRAS, KRAS, HRAS, BRAF, or CTNNB1, suggesting that PTCH, SMO, and p53 mutations are critically important in the pathogenesis of sporadic BCCs.\textsuperscript{14} Salto-Tellez et al.\textsuperscript{31} analyzed the expression of RUNX-3, a nuclear effector of the bone morphogenetic protein, and found that the RUNX-3 protein was uniformly overexpressed in the nuclei of BCC cells, containing no mutation in the coding region, implicating RUNX-3 as a putative new oncogene in certain human BCCs.\textsuperscript{35} This observation indicates that RUNX-3 appears to be a universal downstream mediator of a constitutively active SHH pathway in BCC.\textsuperscript{35}

Another important consideration is that the SHH-induced neoplasias appear to lack the aggressiveness of other malignancies characterized by multiple genetic alterations.\textsuperscript{19} This may be useful to explain the lack of markedly invasive histologic features and metastasis in the natural evolution of BCCs, as well as the slow rate of growth observed in most BCCs.

Basic research on BCC has been impeded by the lack of accurate model systems, practical animal models, and the difficulty of growing human BCC cells in vitro.\textsuperscript{12} The SHH model may be very useful to understand each step of BCC carcinogenesis, and may provide a basis for the molecular target-based chemoprevention and therapeutic management of sporadic BCCs and the cutaneous lesions of BCNS.\textsuperscript{9}

### References

3. Couve-Privat S, Bouadjar B, Avril MF, \textit{et al.} Significantly high levels of ultraviolet-specific mutations in the smoothened gene in basal cell carcinomas from DNA...