AKT1 Activation is Obligatory for Spontaneous BCC Tumor Growth in a Murine Model that Mimics Some Features of Basal Cell Nevus Syndrome

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Abstract

Patients with basal cell nevus syndrome (BCNS), also known as Gorlin syndrome, develop numerous basal cell carcinomas (BCC). Hedgehog (Hh) signaling, Sonic hedgehog (SHH), regulates segment polarity as well as a wide range of biological activities, from establishing left–right body symmetry and limb patterning to eye and central nervous system development. The other two Hh ligands, Desert hedgehog (DHH) and Indian hedgehog (IHH), are mainly involved in the development of male germ cells and cartilage, respectively. In the absence of SHH, PTCH1, a tumor suppressor protein kinase, is intrinsically activated in keratinocytes derived from the skin of newborn Ptch1–/– mice in the absence of carcinogenic stimuli. Introducing Akt1 haplodefiency in Ptch1–/– mice (Akt1–/+Ptch1–/–) significantly abrogated BCC growth. Similarly, pharmacological inhibition of Akt with perifosine, an alkyl phospholipid Akt inhibitor, diminished the growth of spontaneous and UV-induced BCCs. Our data demonstrate an obligatory role for AKT1 in BCC growth, and targeting AKT may help reduce BCC tumor burden in BCNS patients. Cancer Prev Res. 9(10); 794–802. ©2016 AACR.

Introduction

Hedgehog (Hh) pathways are crucial for vertebrate fetal development (1). Of the three known mammalian Hh ligands, Sonic hedgehog (SHH) regulates segment polarity as well as a wide range of biological activities, from establishing left–right body symmetry and limb patterning to eye and central nervous system development. The other two Hh ligands, Desert hedgehog (DHH) and Indian hedgehog (IHH), are mainly involved in the development of male germ cells and cartilage, respectively (1). In the absence of SHH, PTCH1, a tumor suppressor and the Hh receptor, blocks Hh signaling by repressing a membrane-bound, G-protein–coupled, receptor-like protein known as smoothened (SMO) (1, 2). The binding of SHH to PTCH1 relieves SMO repression, triggering a canonical Hh response. Thereby SMO moves to the primary cilium, and activates the GLI family of transcription factors (3). GLI forms a cytoplasmic complex with several accessory modulators, including the serine–threonine kinase Fused (FU), Suppressor of Fused (SUFU), and costal2 (COS2), a kinesin-related protein that binds the GLI-containing complex to microtubules. It is believed that SMO activity favors dissociation of these complexes and translocation of an active form of GLI from the cytoplasm to the nucleus, where it promotes the transcription of Hh target genes, including PTCH1, GLI, CCND1, bone morphogenetic proteins (BMPs), and a member of the TGFβ superfamily (1, 2, 4).

The Hh pathway while highly active during human fetal development normally shuts down soon after birth. However, aberrant activation in adults drives the development and/or maintenance of numerous types of human malignancies, including cancers of the pancreas, prostate, and brain. It also regulates the proliferation of cancer stem cells (CSC), tumor progression, and metastases and may also hasten tumor relapse by augmenting multidrug resistance (MDR) pathways (2, 5). While most human tumors do not harbor somatic mutations in the Hh signaling pathway and demonstrate ligand-dependent Hh pathway activation (6), ligand-independent Hh pathway activation underlies the development of basal cell carcinoma (BCC), the most common type of human malignancy worldwide, due to loss-of-function mutations in PTCH1, gain-of-function mutations in SMO, as well as missense mutations in GLI1 and GLI3 (7). Germ-line mutations in PTCH1 cause basal cell nevus syndrome (BCNS) or Gorlin syndrome (8). Individuals affected with BCNS typically develop large numbers of BCCs often beginning in early childhood and are at substantially increased risk for additional neoplasms such as medulloblastomas and rhabdomyosarcomas. The clinical utility of targeting aberrant Hh signaling in BCNS patients is illustrated by the results of our phase II clinical trial that showed that the orally administered SMO inhibitor vismodegib had remarkable efficacy in ablating BCCs (9). While these and other successes led to FDA approval of vismodegib for treating advanced/inoperable and metastatic BCCs, subsequent studies revealed significant tumor recurrence and acquired clinical resistance to vismodegib. This occurs during therapy primarily through secondary mutations in SMO (for example, D473G, D473Y, Q477E, and G497W) and, to a lesser extent, through concurrent copy number changes in SUFU and GLI2, thereby impairing drug binding and/or...
reactivating Hh pathways, confirming the importance of the Hh pathway in BCC growth (10–13). Several lines of evidence, however, demonstrate that additional pathways may synergistically contribute to BCC tumorigenesis. For example, there is a high risk of BCC in patients with cartilage-hair hypoplasia (CHH), an inherited disorder due to mutations in the RMRP (RNA component of mitochondrial RNA-processing endoribonuclease), and in patients with xeroderma pigmentosum (XP), an autosomal recessive disorder with defective nuclear excision DNA repair (14).

Recently, a significant fraction of human BCCs was shown to carry mutations in various cancer-related genes that regulate a variety of cellular processes, including cell growth, differentiation, mitotic cycle, and oncogenic transformation (15). These data implicate involvement of multiple tumor driver pathways in BCC pathogenesis and suggest that identification of molecular targets distinct from SMO could help ameliorate the limitations of currently available SMO inhibitors and improve therapeutic index. One such molecular target that we have identified is AKT1, the serine/threonine protein kinase that regulates cell survival and is known to be dysregulated in numerous types of human cancer (16–20). Using SKH1-1-Pch1⁻/⁻ mice that we recently described as a susceptible model for BCCs development, this study demonstrates that AKT1 is obligatory for BCC tumorigenesis, and that genetic and pharmacological inhibition of AKT prevents BCC growth.

Materials and Methods

Cells and reagents

Adult normal human epidermal keratinocytes (Lonza) and murine ASZ2001 BCC cells (a gift from Dr. E. Epstein Jr; ref. 21) were cultured in Medium 154CF, supplemented with Human Keratinocyte Growth Supplement (Thermo Fisher). ASZ2001 cells display cellular morphology similar to that of human BCCs, express BCC markers, and are sensitive to SMO inhibition (22, 23). The authors did not authenticate the cell lines. pUSEamp-myrAkt1 was obtained from Millipore. Perifosine was purchased from Selleckchem, edelfosine from Sigma-Aldrich, and MK-2206 from ChemieTek.

Western blotting, immunohistochemistry, and immunofluorescence

The experiments were performed as previously described (24–26). Antibodies against AKT1, AKT2, AKT3, p-AKT1 (S473), Gli1, and Gli2 were purchased from Cell Signaling Technology, SOX9 from Abcam, and β-actin from Sigma-Aldrich. Fluorescent images were acquired using the Zeiss LSM 5 Exciter confocal microscope with 40× oil immersion objective (Carl Zeiss) and analyzed using ImageJ software (NIH).

Proliferation assay

Proliferation was assessed using BrdUrd kit III (Roche Diagnostics Corp.), and Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher) according to the manufacturers’ instructions.

Colony formation assay

Primary human keratinocytes were transduced with the viral construct expressing myrAkt1 (pUSEamp-myrAkt1) or pUSEamp vector (Millipore) at 10 μg using lipofectamine 2000 (Invitrogen) and stable colonies were selected in the presence of G418 (Sigma-Aldrich). Virally transduced cells were then irradiated with UV (30 mJ/cm²). Cells were then resuspended in top agar (0.7% agarose in keratinocyte media) and placed with the base layer prepared in 0.8% agar in keratinocyte media with supplements. After 3 weeks, cells were stained with Crystal Violet, and the number of colonies was counted. The experiment was repeated twice independently.

Generation of SKH1-Pch1⁻/⁻ and SKH1-Akt1⁻/⁻ Pch1⁻/⁻ mice and assessment of microscopic lesions

All animal experiments were performed in accordance with guidelines of our approved Columbia University Institutional Animal Care and Use Committee (IACUC) protocol. SKH1-Pch1⁻/⁻ mice were generated by crossing B6-Pch1⁺/⁺ (27) with SKH1 hairless mice (Charles River Laboratories). The resulting progeny were genotyped and characterized for BCC phenotype and number. The experimental group received B6.129P2-Akt1tm1Mbb/J mice (Jackson Lab) with SKH1-Pch1⁻/⁻ mice. The resulting F1 generation was crossed with B6.129P2-Akt1tm1Mbb/J mice to generate SKH1-Akt1⁻/⁻ Pch1⁻/⁻ mice.

Tumor protocol in Pch1⁻/⁻ and Akt1⁻/⁻ Pch1⁻/⁻ mice

UV study: Pch1⁻/⁻ and Akt1⁻/⁻ Pch1⁻/⁻ and their wild-type Pch1⁺/⁺ littermates were irradiated with UV (180 mJ/cm²) twice per week for 30 weeks. Itraconazole study: Pch1⁻/⁻ mice (n = 20) were UV-irradiated (180 mJ/cm²) twice per week for 30 weeks to induce BCCs. Irradiation was terminated. Mice were then treated with 2-hydroxypropyl-b-cyclodextrin (n = 10) or itraconazole (n = 10) (Sigma-Aldrich) (twice daily, 100 mg/kg, i.p.) for 24 days. The size of tumors was measured as previously described (28). For the perifosine study, control group received 0.9% NaCl, gavage, twice a week, n = 8) 30 minutes prior to each UV irradiation. All animals were irradiated with 180 mJ/cm² UV twice weekly for 32 weeks. Tumor number and tumor volume were recorded once a week and plotted in terms of weeks on test.

All experiments were performed in accordance with guidelines of our approved Columbia University Institutional Animal Care and Use Committee (IACUC) protocol.
SKH1- and 4), but not in primary keratinocytes isolated from postnatal day 2 WT in hairless SKH1-
spontaneous BCCs (b, d) in

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Statistical analyses

Statistical analyses were performed using the Student t test (two-tailed): P < 0.05 was considered statistically significant.

Results

AKT1 is intrinsically activated in Pch1+/− keratinocytes and spontaneous BCCs

We recently showed that the growth of BCC is greatly enhanced in hairless SKH1-Pch1+/− mice (26). Although the original introduction of Pch1+/− into the C57BL/6 strain (B6-Pch1+/−; ref. 27) rendered these animals susceptible to BCC following skin exposure to ionizing or UV radiation (29), these tumors grow quite slowly in these mice—perhaps due to their derivation from the C57BL/6 genetic background that is known to be inherently tumor resistant. In contrast, SKH1 hairless mice are highly susceptible to the growth of squamous cell carcinomas (SCC) following chronic UV exposure (25, 30). Chronic UV irradiation of SKH1-Pch1+/− wild-type (WT) mice results in the sequential growth of benign papillomas and SCCs in a pattern closely mimicking that of human SCCs, while these animals never develop BCCs. By introducing Pch1 heterozygosity onto the tumor-susceptible SKH1 hairless background, we generated SKH1-Pch1+/− (hereafter referred to as Pch1+/−) mice. These animals exhibit the spontaneous growth of BCCs that characterizes patients with BCNS (31). AKT activation typically occurs in response to extracellular stimuli, and its phosphorylation at S473 is necessary for the full activation of the PI3K–AKT pathway (32). We detected S473 phosphorylation in BCCs that developed spontaneously in Pch1+/− mice and in human BCCs (Fig. 1A and B). AKT S473 phosphorylation was present in tumor cells but was undetectable in tumor stroma (Fig. 1C). Moreover, AKT S473 phosphorylation was detectable in primary keratinocytes isolated from newborn day 2 WT Pch1+/− mice (lanes 3 and 4), but not in primary keratinocytes isolated from postnatal day 2 WT Pch1+/− mice (lanes 1 and 2). E, increased colony-forming capability of normal human keratinocytes expressing constitutively active AKT (pSEAnpm-Akt1, myrAkt1). *P < 0.005, compared with control. Normal primary human keratinocytes were transduced either with a viral construct expressing myr-Akt1 (myr-Akt1) or vector (−, pBAP) only.

Figure 1.

AKT1 is intrinsically activated in Pch1+/− keratinocytes and spontaneous BCCs. A, the levels of p-AKT1 (S473) in spontaneous murine BCCs developed in SKH1-Pch1+/−(Pch1−/−) mice and human BCCs. Western blotting assessed three representative BCCs from three different animals or three separate patients. β-Actin was used as an internal control. B, densitometric scanning of A. C, immunohistochemical assessment of AKT1 and p-AKT1 (S473) in tumor adjacent skin (a, c) and spontaneous BCCs (b, d) in Pch1−/−. Scale bar, 100 μm. Histogram represents percent p-AKT1 (S473)-positive cells in tumor-adjacent skins and BCCs in Pch1−/− mice. Three fields were counted. *, P < 0.05. D, p-AKT S473 levels are increased in primary keratinocytes isolated from postnatal day 2 WT Pch1+/− mice (lanes 3 and 4), but not in primary keratinocytes isolated from postnatal day 2 WT Pch1+/− mice (lanes 1 and 2). E, increased colony-forming capability of normal human keratinocytes expressing constitutively active AKT (pSEAnpm-Akt1, myrAkt1). *P < 0.005, compared with control. Normal primary human keratinocytes were transduced either with a viral construct expressing myr-Akt1 (myr-Akt1) or vector (−, pBAP) only.
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Figure 2.
Akt1 haploinsufficiency is sufficient to prevent the growth of spontaneous microscopic BCCs. A, representative H&E staining of skin sections from Akt1+/- Ptch1+/- mice and Ptch1+/- littermates. Scale bar, 200 µm. B, assessment of size and number of spontaneous BCCs in Akt1+/- Ptch1+/- and Ptch1+/- mice. Three skin strips (average 1.5 cm × 0.1 cm) were analyzed for each mouse. Each dot represents data from one mouse. C, the levels of AKT isoforms in Akt1+/- Ptch1+/- mice and Ptch1+/- littermates, assessed by Western blotting. 50 µg protein per lane, actin as an internal control. Brain, brain extracts from Akt1+/- Ptch1+/-.

Genetic ablation of Akt1 prevents the spontaneous growth of BCCs in Akt1+/- Ptch1+/- mice

There are three known Akt isoforms, including Akt1, Akt2, and Akt3. Akt1-null mice manifest growth retardation (33), whereas Akt2-null mice display an insulin-resistant, diabetic-like syndrome (34), and Akt3 null mice show impaired brain development (35). Because Akt1 and Akt2, but not Akt3, are expressed in the skin, and because mouse embryo fibroblasts lacking Akt1 (Akt1 +/- MEF) are sensitive to UV-induced apoptosis (33), we next assessed the effects of Akt1 deletion in Ptch1+/- mice. This study utilized Akt1 haploinsufficient Ptch1+/- mice, as Akt1+/- littermates rarely survive. The few that do show severe growth retardation and high perinatal mortality (data not shown). Similar to BCNS patients where BCCs develop spontaneously even in sun-protected areas, spontaneous microscopic BCCs were detectable in our Ptch1+/- mice starting as early as 8 weeks of age with 100% tumor incidence (ref. 26; Fig. 2). Figure 2A shows representative pictures of histological sections of skin from Ptch1+/- and Akt1 +/- Ptch1+/- mice at 12 months of age. Ptch1+/- mice carry the insertion of a promoterless lacZ-neo fusion gene, thereby deleting a portion of exon 1 and all of exon 2 of Ptch1. Therefore, lacZ activity, mimicking an expression pattern of endogenous Hh target genes (e.g., Ptch1), serves as an accurate indicator of Hh pathway activation. In Ptch1+/- mice, BCCs were detected by β-gal staining (red arrowheads) with an inset showing a magnified view of an area with multiple BCCs (Fig. 2A, b’). Akt1 haploinsufficiency resulted in substantial reductions in the tumor burden (in both size and number) of spontaneous BCCs in Akt1 +/- Ptch1+/- mice as compared with their Akt1 WT Ptch1+/- littermates (Fig. 2B). Despite isoform-specific functions, it has been shown that Akt1 knockdown can, in some instances, upregulate and activate AKT2, which in turn compensates for Akt1 loss (36). Our analysis of Akt1+/- Ptch1+/- mice shows that AKT2 levels are no different than those in Ptch1+/- mice (Fig. 2C), indicating non-overlapping roles for this AKT isoform and that AKT1 is crucial for the development of spontaneous BCCs in Ptch1+/- mice.

SMO inhibition suppresses AKT1 signaling in vitro and in vivo

Itraconazole is an FDA-approved azole antifungal drug recently shown to be a potent, and specific inhibitor of Hh signaling (37). It is thought to reduce SMO translocation to the cilium (38). Treatment of murine ASZ001 cells (derived from BCCs induced in B6-Ptch1+/-) with itraconazole (1–30 µmol/L) dose dependently inhibited the growth of these cells (data not shown) and decreased the levels of the Hh components GLI1, GLI2, and cyclin D1 (Fig. 3A). Interestingly, itraconazole treatment decreased S473 phosphorylation (Fig. 3A). Cyclopamine, a natural compound known to block Hh signaling by binding to SMO, also suppressed
AKT phosphorylation (data not shown). Oral administration of itraconazole (100 mg/kg twice daily for 24 days) suppressed the growth of existing UV-induced microscopic BCCs in Ptc1+/− mice (Fig. 3B) and decreased Hh signaling, AKT phosphorylation, and the number of p-AKT-positive cells in tumors harvested from these animals (Fig. 3C and D). Taken together, these data indicate that AKT signaling is downstream of the Hh pathway.

Akt1 haploinsufficiency prevents UV-induced BCC growth in Ptc1+/− mice

UV exposure is the major known risk factor for the induction of BCCs, both sporadically in the general population and in BCNS patients (39). Chronic UV irradiation of WT Ptc1+/+ mice induces the growth of SCCs (Fig. 4A, WT, red arrowheads; Fig. 4B), and these animals were largely resistant to the development of BCCs, as indicated by the absence of β-gal staining (Fig. 4A, WT). In Ptc1+/− skin, chronic UV exposure resulted in extensive epidermal hyperplasia (Ptc1+/− in Fig. 2A vs. Fig. 4A) and greatly enhanced the number and size of BCCs (Fig. 4A, Ptc1+/−, black arrowheads). Akt1 haploinsufficiency, however, substantially reduced the development of UV-induced BCCs in Ptc1+/− mice (Fig. 4C). Extensive colocalization of p-AKT1 S473 with other known BCC markers, GLI1 and SOX9, indicated their concurrent activation in Ptc1+/− BCCs (Fig. 4D, d and g), compared with apparent reductions in AKT S473 phosphorylation and colocalization in Akt1+/+ Ptc1+/− BCCs (Fig. 4D, d, l, i, and o). These data, together with substantial decreases in Ki67, a cell proliferation marker, in Akt1+/+ Ptc1+/− BCCs (Fig. 4D, c vs. k) further confirmed the importance of AKT1 in BCC growth.

Pharmacologic inhibition of AKT inhibits UV-induced BCC growth

We next utilized a battery of AKT inhibitors that are currently in active clinical trials for various other human cancers to assess pharmacologic effects in ASZ001 murine BCC cells. These include the alkyl phospholipid perifosine and MK-2206, an allosteric inhibitor that binds to and inhibits AKT in a non-ATP competitive manner. Perifosine, in particular, has been shown to be relatively nontoxic and well tolerated in phase I/II clinical trials in patients with head and neck SCCs (40–42). Our results indicate that perifosine has a lower IC50 for AKT (4 μmol/L) in ASZ001 cells as compared with other inhibitors, which had IC50 values ranging up to 50 μmol/L (data not shown). Accordingly, we selected a concentration of 4 μmol/L for further
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Figure 4. Haplodeficient Akt1 suppresses UV-induced BCC tumorigenesis in \textit{Ptch1}\textsuperscript{+/–} mice. \textit{Ptch1}\textsuperscript{+/–} (\(n=6\)) and Akt1\textsuperscript{+/–} \textit{Ptch1}\textsuperscript{+/–} (\(n=5\)), and their WT littermates (\(n=6\)) were irradiated with UV (380 mJ/cm\(^2\), twice weekly) for 30 weeks. \(\Delta\), representative H&E staining of skin sections harvested at week 30. Akt1 deficiency suppresses the growth of UV-induced skin tumors (\(\Delta\)). Akt1\textsuperscript{+/–} \textit{Ptch1}\textsuperscript{+/–} mice were treated with perifosine or edelfosine orally (125 mg/kg, twice weekly) to chronically reduce levels of p-AKT, and their WT littermates (\(\Delta\)) were treated with placebo. Akt1\textsuperscript{+/–} \textit{Ptch1}\textsuperscript{+/–} mice were treated with perifosine or edelfosine orally (125 mg/kg, twice weekly) to chronically reduce levels of p-AKT, and their WT littermates (\(\Delta\)) were treated with placebo. Akt1\textsuperscript{+/–} \textit{Ptch1}\textsuperscript{+/–} mice were treated with perifosine or edelfosine orally (125 mg/kg, twice weekly) to chronically reduce levels of p-AKT, and their WT littermates (\(\Delta\)) were treated with placebo.

Discussion

In our previously published investigator-originated clinical trial, we showed that oral administration of the Hh pathway inhibitor vismodegib led to a dramatic reduction in the number of BCCs in BCNS patients, confirming the importance of Hh signaling in driving BCC pathogenesis. However, it soon became apparent in these patients that many of the BCCs that were no longer visible clinically and histologically rapidly recurred after drug discontinuation (9). These results while disappointing do raise intriguing questions regarding the source of the recurrent tumor cells and the mechanisms underlying their regrowth. Moreover, it is known that tumor resistance to vismodegib usually results from SMO mutations that block drug binding and/or reactivate Hh signaling (11, 12). These data collectively suggest that treatment with the currently available inhibitors of Hh signaling is unlikely to permanently eradicate BCCs, and that identification of additional tumor driver pathways distinct from the Hh pathway could lead to the development of innovative therapeutic approaches and could improve clinical outcomes.

Aberrant Akt activation is known to occur frequently in numerous types of human tumors, including those of the skin (16, 18, 19). BCNS patients develop a few to hundreds of BCCs, which in many cases grow aggressively, and may require multiple mutilating surgical procedures. In particular, spontaneous development of multiple BCCs in sun-protected skin is decidedly uncommon in the general human population, but is often a characteristic feature of BCNS patients. Except for the Hh pathway, the mechanisms underlying spontaneous tumor growth are largely unexplored, in...
Pharmacological inhibition of AKT signaling inhibits the proliferation of ASZ001 cells and suppresses UV-induced BCC growth. **A**, alkyl phospholipids reduce AKT phosphorylation and induce apoptosis in ASZ001 cells. ASZ001 cells were treated with 4 μmol/L of each AKT inhibitor, including alkyl phospholipids, and MK-2206, an allosteric AKT inhibitor, for 24 hours. The levels of AKT phosphorylation at S473 and T308 and cleaved caspase-3 were assessed by Western blotting. β-Actin serves as an internal control. **B and C**, AKT inhibition reduces proliferation of ASZ001 cells. Assessment of BrdUrd incorporation 24 hours (**B**) and EdU incorporation 2 or 5 days (**C**) after treatment with 4 μmol/L of each AKT inhibitor. Error bars, SD; ***, P < 0.001.**

Orally administered perifosine prevents the growth of UV-induced skin tumors (**D**) and microscopic BCCs (**E**) in Ptch1<sup>+/−</sup> mice. Mice were irradiated with UV (180 mJ/cm<sup>2</sup>, twice weekly) and received either 0.9% NaCl (n = 8) or perifosine (125 mg/kg of body weight, n = 8), twice weekly for 32 weeks. Each dot represents data from one mouse. Three skin strips (avg. 1.5 cm x 0.1 cm) were analyzed for each mouse.
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part due to the lack of suitable animal models. SKH1-Ptch1+/− mice are uniquely susceptible to the development of both spontaneous and UV-induced BCCs, thereby providing an animal model with features that closely resemble those of patients with BCNS. This study directly assessed the effects of Akt1 loss by introducing Akt1 haplodeficiency in SKH1-Ptch1+/− mice, and demonstrated that Akt1 is essential for spontaneous BCC tumorigenesis. The absence of AKT1 phosphorylation, indicative of the lack of its activity, was confirmed in Akt1+/−/Ptch1+/− mice. The concomitant decreased levels of Ki67, a marker of proliferation, and tumor burden in these mice further emphasize the importance of Akt1 function in cell survival and proliferation. UV and ionizing radiation are known to exacerbate BCC tumor burden in BCNS patients. The remarkable preclinical efficacy against UV-induced BCCs in Ptch1−/+ and Ptch1−/− mice of the Akt inhibitor perifosine, selected over other inhibitors based on our in vitro studies, indicated that Akt could be an alternative viable target for reducing tumor burden in patients with BCNS.

Akt activation is posttranslational and is mediated through the upstream kinase cascade (e.g., phosphoinositide-dependent kinase 1 [PDK1]) in response to various growth factors and external stimuli. With respect to the Hh pathway, the activation of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and their downstream effectors (e.g., RAS/RAF/MEK/ERK and PI3K/AKT) has been shown to enhance GLI stability and transcriptional activity in various cancers (e.g., chronic lymphocytic leukemia, gastric cancer, and melanoma; refs. 43–47). Akt-mediated GLI activation has also been linked to drug resistance in breast cancer cells (48). While these data position AKT activation upstream of external stimuli. With respect to the Hh pathway, the activation of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and their downstream effectors (e.g., RAS/RAF/MEK/ERK and PI3K/AKT) has been shown to enhance GLI stability and transcriptional activity in various cancers (e.g., chronic lymphocytic leukemia, gastric cancer, and melanoma; refs. 43–47). Akt-mediated GLI activation has also been linked to drug resistance in breast cancer cells (48). While these data position AKT activation upstream of GLI has been shown to transcriptionally regulate AKT1 expression, and GLI-mediated Akt and c-MET phosphorylation was recently shown to promote migration and invasion of thyroid tumor cells (50). Our data, demonstrating in vitro and in vitro inhibition of AKT phosphorylation by the SMO inhibitor irtraconazole, also imply that Akt acts downstream of Hh in BCC. In addition, despite the absence of external stimuli, Akt1 phosphorylation levels were significantly elevated in postnatal Ptch1−/+ keratinocytes compared withPtch1−/− keratinocytes, suggesting the existence of intrinsic mechanisms driving AKT phosphorylation. Elevated EGFR levels were previously reported in human BCCs, and forced expression of EGFR and GLI was shown to be critical for oncogenic transformation of spontaneously transformed, non-tumorigenic HaCaT keratinocytes (51, 52). However, EGFR levels, as well as PDGFR, were barely detectable, and no apparent differences were observed in p-MEK1/2 levels, regardless of Ptch1 status (data not shown). These results strongly suggest the lack of involvement of EGFR signaling in Akt phosphorylation in Ptch1−/+ keratinocytes; however, the exact mechanisms await further investigation. In addition, while GLI1 levels remained somewhat similar in UV-induced BCCs, irrespective of Akt1 status, the apparent reduction in SOX9 levels in Akt1−/+ BCCs raises interesting questions with regard to the targets of AKT1 and its relevance to Hh signaling and BCC pathogenesis. Integration of the Akt and Hh pathways is undoubtedly under the influence of multiple regulatory pathways, which may account for the specificity and tumorigenic susceptibility of different target cells. Further investigation will likely reveal additional novel mechanism-driven targets for the chemoprevention and treatment of this most common type of human malignancy.

Disclosure of Potential Conflicts of Interest
D.R. Bickers is a consultant/advisory board member for SC Johnson Co. and has received an expert testimony from Goodwin Proctor LLP. No potential conflicts of interest were disclosed by the other authors.

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