RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway–Dependent Tumors

Xuesong Zhao\textsuperscript{1,2}, Tatyana Ponomaryov\textsuperscript{1,2,3}, Kimberly J. Ornelli\textsuperscript{1,2}, Pengcheng Zhou\textsuperscript{1,2}, Sukriti K. Dabral\textsuperscript{1,2}, Ekaterina Pak\textsuperscript{1,2}, Wei Li\textsuperscript{4}, Scott X. Atwood\textsuperscript{5}, Ramon J. Whitson\textsuperscript{5}, Anne Lynn S. Chang\textsuperscript{5}, Jiang Li\textsuperscript{5}, Anthony E. Oro\textsuperscript{5}, Jennifer A. Chan\textsuperscript{6}, Joseph F. Kelleher\textsuperscript{7}, and Rosalind A. Segal\textsuperscript{1,2}

Abstract

Aberrant Shh signaling promotes tumor growth in diverse cancers. The importance of Shh signaling is particularly evident in medulloblastoma and basal cell carcinoma (BCC), where inhibitors targeting the Shh pathway component Smoothened (Smo) show great therapeutic promise. However, the emergence of drug resistance limits long-term efficacy, and the mechanisms of resistance remain poorly understood. Using new medulloblastoma models, we identify two distinct paradigms of resistance to Smo inhibition. Sufu mutations lead to maintenance of the Shh pathway in the presence of Smo inhibitors. Alternatively activation of the RAS–MAPK pathway circumvents Shh pathway dependency, drives tumor growth, and enhances metastatic behavior. Strikingly, in BCC patients treated with Smo inhibitor, squamous cell cancers with RAS/MAPK activation emerged from the antecedent BCC tumors. Together, these findings reveal a critical role of the RAS–MAPK pathway in drug resistance and tumor evolution of Shh pathway–dependent tumors.

Introduction

Sonic Hedgehog (Shh) signaling plays a critical role in growth and patterning during development, and aberrant activation of Shh signaling is implicated in several cancers (1). Germline mutations that activate Shh signaling predispose to basal cell carcinoma (BCC) and medulloblastoma in humans and mice, whereas somatic mutations in Shh components are frequently observed in such tumors (2, 3).

Signaling is initiated when Shh, a secreted protein, binds its receptor, Patched (Ptch). In the absence of Shh, Ptch suppresses activity of Smoothened (Smo). When Shh binds Ptch, Smo initiates a signaling cascade, which inactivates the tumor-suppressor Suppressors of Fused (Sufu), and activates Gli transcription factors. Gli target genes include Gli1, Pch, Nmyc, and Cyclin D1 (Cyclin D1). Small-molecule antagonists of Smo, including Vismodegib (GDC-0449; Roche), Sonidegib (LDE225; Novartis), and XL-139 (BMS/Exelixis), provide promising targeted therapy for BCC and medulloblastoma (1–4), and are in clinical trials or in use for these indications. Despite initial success of Smo inhibitors, long-term efficacy is limited by pre-existing or acquired drug resistance (8, 9). Studies of other cancers indicate that both cell-autonomous mutations and microenvironment-derived factors contribute to therapeutic resistance (10). Amplification of Gli2 and point mutations in Smo that prevent drug binding have been reported to cause resistance in preclinical and clinical studies (4, 5, 11). Increased activation of PI3K, aPKC-\mu, or cell-cycle components may also contribute to resistance (5, 12, 13). Additional mechanisms of resistance are likely to arise in clinical practice, and must be understood to develop more effective therapeutic strategies for Shh-dependent tumors.

To date, the absence of reliable in vitro systems for growing and maintaining Shh-dependent tumors has been a major impediment for studying these cancers (14). Here, we report an approach for generating stable medulloblastoma cell lines that are tumorigenic and retain key characteristics of Shh-subtype medulloblastoma. Using these models, we identify two paradigms of resistance to Smo inhibitors. Loss of Sufu reactivates the Shh pathway downstream of Smo and thereby causes acquired therapeutic resistance. In a second scenario, activation of the RAS–MAPK pathway overrides oncogenic addiction to Shh signaling and enables proliferation of resistant tumors with enhanced metastatic behavior. In human cancers, MAPK pathway activation is increased in metastatic medulloblastoma tumor cells. Strikingly,
the MAPK pathway also becomes activated after vismodegib treatment as Shh-dependent basal cell cancer transitions to squamous cell cancer resistant to Smo inhibitors. Together, these results indicate that reactivation of the Shh pathway or interactions between Shh and MAPK pathways can alter tumor behavior and therapeutic responses. Therefore, future treatments must consider these distinct mechanisms of tumor evolution.

Materials and Methods

Detailed description is given in Supplemental Materials.

Animals

All experimental procedures were done in accordance with the NIH guidelines and approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee. Pch+/− mice (The Jackson Laboratory; ref. 2), nu/nu mice (Charles River Laboratories).

Human studies

All human subjects work was reviewed by the Institutional Review Board Committees of Brigham and Women’s Hospital and Dana-Farber Cancer Institute, University of Calgary, and Stanford University for appropriate use, that informed consent was obtained from all subjects when required, and appropriate waiver of consent requirements was obtained for minimal risk studies.

SMB cell culture

Shh-subtype medulloblastoma (SMB) cells were cultured as neurospheres in DMEM/F12 media (2% B27, 1% Pen/Strep). SMB(GF) cells were generated by culturing parental SMB cells for ≥3 weeks with the above media supplemented with EGF, bFGF (20 ng/mL each), 0.2% heparin.

Cell survival assays

SMB cells in 96-well plates (3 × 10^4 cells/well) were incubated for 72 hours in LDE225, vismodegib, LEQ506 or ATO, or for 120 hours in BKM120, BEZ235, PD325901, or CI-1040. Viability was measured using CellTiter 96 Aqueous One Solution (Promega), and calculated as the percentage of control (DMSO treated).

Gene copy number analysis

Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen). Genomic copy number for Sufu was determined by qPCR with custom-designed primers using 5 ng of genomic DNA/reaction. Copy number was calculated as described in Supplemental Information.

Immunohistochemistry, immunocytochemistry, and immunoblotting

Human medulloblastoma and matched metastases were stained with hematoxylin and eosin (H&E), or with anti-pERK1/2 (Cell Signaling Technology; 1:400). Visualized using the Envision Plus Detection Kit (DAKO). Human skin tumors were immunostained with: anti-Keratin14(ab7800; Abcam); anti-Gli1 (C-18; Santa Cruz Biotechnology); anti-pERK (#9101; Cell Signaling Technology). Immunoreactivity was visualized with Alexa-Fluor secondary antibodies and confocal microscopy (Leica SP8). Staining antibodies: V5-tag (Invitrogen; 1:1,000), GFP (Aves Labs; 1:1,000), and Zic (made in house, 1:400; ref. 15). Immunoblot antibodies: pAKT (S473), AKT, pERK1/2 (T202/Y204), ERK1/2, pS6, S6, pan-Ras, Gli1, Sufu, p53, cleaved caspase-3, Nmyc, Flag tag (Cell Signaling Technology; 1:1,000), actin (Sigma; 1:10,000), HA-tag (Millipore; 1:1,000), Gli2 (Aviva; 1:1,000), c-MYC (Santa Cruz Biotechnology; 1:1,000), V5-tag (Invitrogen; 1:1,000).

Transplantation and in vivo treatment

A total of 5 × 10^6 cells in 100 μL were injected s.c. in flank of nu/nu mice (6–8 weeks old). Tumor volumes (V = 0.5 × A × B^2) were measured twice/week. When tumors reached 150 mm^3, animals were randomly grouped for treatment with vehicle or LDE225 (diphosphatase salt in 0.5% methylcellulose, 0.5% Tween 80, at 80 mg/kg by oral gavage once daily). Mice with tumors ≥2,000 mm^3 were euthanized. For orthotopic tumors, 1 × 10^6 cells in 2 μL were injected into cerebella of nu/nu mice (6–8 weeks old). Animals were sacrificed when symptomatic.

Skin tumor sequencing

Sequencing of clinical samples was performed under Institutional Review Board approval at Stanford University. Medically qualified patients 18 years or older with advanced BCCs were enrolled and informed consent was obtained for tumor sequencing (protocol #18325). Tissue samples were stored in RNALater at −80°C (Ambion). DNA was isolated using the DNeasy Blood & Tissue Kit. Capture libraries were constructed from 2 μg DNA from BCC and normal skin using the Agilent SureSelect XT Human All Exon V4 Kit. Enriched exome libraries were multiplexed and sequenced on Illumina HiSeq 2500 platform to generate 100-bp paired-end reads. Sequencing reads were aligned to human reference genome sequence (hg19) using Burrows-Wheeler Aligner. SAM to BAM conversion and marking of PCR duplicates were performed using Picard tools (version 1.86), followed by local realignment around indels and base quality score recalibration using the Genome Analysis Toolkit (GATK; v2.3.9). Mean target coverage was 114X over coding regions. Somatic SNVs and indels were called using GATK. Variants were annotated for standard quality metrics and for presence in dbSNP138.

Accession numbers

The microarray data accession number is GSE69359.

Results

SMB cell lines maintain features of Shh-subtype medulloblastoma

Studies of Shh-dependent tumors have been limited by the lack of appropriate tumor cell lines. To establish new Shh-dependent tumor cell lines, we propagated tumor cells from spontaneous medulloblastoma of Pch−/− mice as neurospheres (2). To retain Shh pathway dependency, we omitted EGF and bFGF from the media as these growth factors (GF) can promote differentiation of granule cell precursors (GCP). cells of origin for Shh-subtype medulloblastoma (16, 17). Several tumor samples tested (7 of 35, or 20%) grew for many passages and were designated SMB lines. The three most stable lines, SMB21, SMB55, and SMB56, were used in this study.

SMB lines are tumorigenic and retain key characteristics of in vivo Shh-subtype medulloblastoma. SMB cells are small (~5 μm in diameter) and exhibit a morphology similar to cerebellar GCPs. These tumor-derived cells express medulloblastoma markers, including stem cell/progenitor marker Nestin, neuronal marker...
Tuj1, cerebellar marker Zic1, cell proliferation marker Ki67, and Math1(Atoh1), a hallmark of Shh-subtype medulloblastoma (Fig. 1A and B). Importantly, SMB cells exhibit constitutively activated Shh signaling, as Shh signaling components and target genes Gli1, Gli2, Boc, Ccnd1, Nmyc, and SFRP1, are highly expressed in SMB cells and in primary medulloblastoma from Pch+/− mice (Fig. 1B). To compare SMB cells with human medulloblastoma, we performed gene-expression analysis of SMB cells, in vivo primary medulloblastoma, and cerebellum of P6 and adult mice. We compared these profiles with signature profiles of human medulloblastomas (WNT, SHH, group C, group D). Similarity to each subtype for each sample defined by “signature score” and normalized by normal mouse samples. D, quantitative RT-PCR analysis of Gli1 in SMB cells treated with DMSO or 1 μmol/L LDE225 for 24 hours; mean ± SD; n = 3; ***, P < 0.0001, Student t test. E, dose-dependent inhibition of Shh signaling by LDE225 (0, 5, 50, 500 nmol/L, 48 hours). Shh signaling assessed by immunoblot for Gli1; apoptosis assessed by cleaved caspase-3. F, survival analysis of SMB cells treated with indicated LDE225 concentrations (72 hours; mean ± SEM, n = 6).
medulloblastoma (WNT, SHH, group C, group D; ref. 18). Similarity to each subtype for each sample is defined by the "signature score," which quantitatively measures similarity of gene-expression patterns to predefined signature genes. Subtype signature score indicates that SMB cells and in vivo primary medulloblastoma both closely resembled Shh-subtype medulloblastoma (Fig. 1C). We used a second algorithm, agreement of differential expression (AGDEX; ref. 19), to compare SMB cells with human medulloblastoma. AGDEX analysis also indicates that both Ptc+/− primary medulloblastoma and SMB cells exhibit the highest agreement with human SHH subtype (Supplementary Fig. S1F).

Notably, activated Shh signaling in SMB cells is exquisitely sensitive to Smo inhibitors, as demonstrated by reduced Gli1 mRNA and protein following treatment with Smo inhibitor LDE225 (Fig. 1D and E). Importantly, proliferation and survival of SMB cells also depend on active Shh signaling as demonstrated in cell survival assays with three different Smo inhibitors LDE225, vismodegib, and LEQ506 (20). These inhibitors reduce cell number in all SMB lines, and increase apoptosis as assessed by activated caspase-3 (Fig. 1E and F; Supplementary Fig. S1A).

SMB cells, even after more than 20 passages in culture, initiate tumors in vivo when transplanted into nude mice, either subcutaneously or as orthotopic xenografts in the brain (Supplementary Fig. S1B). Transplanted SMB cells exhibit typical Shh-subtype histology (Supplementary Fig. S1C–S1E). Recent sequencing studies of large cohorts of medulloblastoma patients revealed that p53 is among the most frequently mutated genes in Shh-subtype medulloblastoma (10%–20%; refs. 21, 22). To evaluate p53 status in our SMB cells, we first sequenced coding exons of p53. Y233C and C138R point mutations were detected in SMB21 and SMB55 cells, respectively, but no mutations were detected in SMB56 cells. However, other alterations can impinge on p53 activity. All three SMB lines showed elevated levels of p53 protein compared with wild-type murine neural progenitor cells, and p53 expression in SMB cells did not change in response to gamma irradiation (Supplementary Fig. S1G), indicating that the p53 signaling axis is dysregulated in all SMB lines.

Together, these data indicate that our culturing protocol can establish Shh-subtype cell lines with dysregulated p53, and these lines can be used to study Shh-subtype medulloblastoma in vitro and in vivo and provide a platform for rapid, large-scale functional study and drug screening.

**SMB cell lines provide a model to study drug resistance**

A major concern with Smo inhibitors or other targeted therapies is the emergence of drug resistance. To determine whether SMB cell lines can help address this challenge, we asked whether the effects of known resistance mechanisms, such as Smo mutants (D477G, L225R, and S391N) and Gli2 overexpression, can be recapitated in SMB cells. To achieve stable expression of exogenous genes, we adapted the piggyBac transposon system to integrate exogenous genes into the SMB genome (Supplementary Fig. S2A). SMB21 cells stably expressing GFP, Smo(WT), Smo (D477G), Smo(L225R), Smo(S391N), Gli2, or Gli2AN, constitutively activate Gli2 lacking the amino-terminal repressor domain (23), were tested for sensitivity to LDE225 in a cell survival assay. Although cells expressing GFP or Smo(WT) remain sensitive to Smo inhibitors LDE225, LEQ506, or vismodegib, cells expressing Smo mutants, Gli2, or Gli2AN are resistant to Smo inhibitors (Fig. 2A; Supplementary Fig. S2B–S2D). Consistent with previous studies, cells expressing Smo mutants or Gli2AN exhibit constitutively activated Shh signaling even in the presence of LDE225, as demonstrated by Gli1 expression (Fig. 2B).

To test resistance of engineered SMB cells in vivo, SMB21 parental, Smo(WT)-expressing, and Gli2AN generate tumors resistant to LDE225 (Fig. 2C). Together, these results indicate that mutations that confer clinical resistance to LDE225 are effective in SMB cells, demonstrating that these lines provide attractive systems for studying drug resistance.

**Identification of novel routes for circumventing Smo inhibition**

To identify novel routes through which medulloblastoma cells can escape Smo inhibition, we used SMB cells to test three groups of candidates. The first group includes key Shh pathway components downstream of Smo: Sufu, Gli1, Gli3, and Nmyc. We used the piggyBac transposon system to overexpress Gli1, Gli3, or Nmyc, or used shRNA to knockdown Sufu. Unlike Gli2, expression of Gli1, Gli3, or Nmyc in SMB cells cannot bypass Smo inhibition, reactivate Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F). In contrast, shRNA knockdown of Sufu reactivates Shh signaling and confers robust resistance to LDE225 in SMB cells (Fig. 3A and B). Consistent with the key role of Sufu, several of the resistant tumors that arose spontaneously from subcutaneously implanted SMB cells following treatment with LDE225 showed drastic reduction of Sufu protein levels compared with sensitive tumors not exposed to LDE225 (Fig. 3C), and many exhibited genomic loss of Sufu (Fig. 3D). To identify possible treatment for tumors that are resistant due to Sufu loss, we tested arsenic trioxide (ATO), an FDA-approved drug shown to antagonize Gli action (24). ATO inhibits proliferation of SMB cells and Sufu knockdown cells with an effective dose similar to previous studies (Fig. 3E; ref. 24). Together, these data indicate that Gli inhibitors can treat intrinsic Shh pathway activation.

In addition to Shh pathway–specific components, we tested molecules that are key drivers of WNT and group 3 (MYC) medulloblastoma subtypes (25). Neither expression of wild-type nor constitutively activated form of CTNNB1, CTNNB1(S33Y), nor overexpression of stabilized MYC, MYC(T58A), confer resistance to Smo inhibitors (Supplementary Fig. S2F–S2I). Thus, oncogenic mutations critical for other medulloblastoma subtypes cannot alter subtype identity, and thereby confer resistance. Recent studies indicate that WNT and Shh-subtype medulloblastoma have distinct cellular origins, as Shh-subtype medulloblastoma originate from GCPs and WNT subtype from dorsal brainstem cells (26). Thus, the cellular context may explain why signaling pathways driving other medulloblastoma subtypes fail to confer Smo-inhibitor resistance.

The third group of candidates tested encompassed genes involved in RTK/RAS signaling, as RTK/RAS signaling is implicated in development of normal cerebellar GCPs and medulloblastoma (27, 28). Expression of HRAS(G12V) or BRAF(V600E), but not PIK3CA(H1047R) or AKT(Myristoylated), induced resistance to LDE225 in SMB cells (Fig. 4A–C; Supplementary Fig. S3A and S3C). As expected, both HRAS(G12V) and BRAF(V600E) activate the MAPK pathway in SMB cells, as demonstrated by increased phosphorylation of ERK (Fig. 4A).
Supplementary Fig. S3B and S3D). HRAS(G12V) and BRAF(V600E) also cause resistance to other Smo inhibitors LEQ506 and vismodegib, indicating that this effect is generalizable for Smo antagonists (Supplementary Fig. S3E and S3F). SMB(HRAS) cells subcutaneously transplanted in nude mice were resistant to treatment with Smo inhibitors (Fig. 4D). Furthermore, MAPK activation is greater in SMB tumors that spontaneously develop resistance to Smo inhibitors following treatment with LDE225 than in vehicle treated, sensitive tumors (Fig. 4E). Taken together, these data indicate that activation of RAS/MAPK provides a novel way for cells to evade Smo inhibition.

Surprisingly, HRAS(G12V) does not confer resistance by reactivating Shh signaling downstream of Smo (Fig. 5A). Instead, HRAS(G12V) suppresses Shh signaling in SMB cells, as expression of multiple Shh pathway targets and components are downregulated in SMB(HRAS) cells (Fig. 5A–C; Supplementary Fig. S4A–S4D). Notably, Math1(Atoh1), a hallmark of Shh-subtype medulloblastoma that is not a direct target of SHH signaling, is decreased in SMB(HRAS) cells. These results suggest that HRAS renders SMB cells independent of Shh-signaling for growth, and thereby causes resistance.

RAS is normally regulated by upstream GFs and receptor tyrosine kinases, and so receptor activation might mimic the effects of HRAS in SMB cells. During normal cerebellar GCP development, GF, such as bFGF, antagonize Shh pathway activity and promote differentiation (29, 30). When SMB cells were exposed to GFs that are common components of stem cell media (bFGF and EGF; 20 ng/mL) both PI3K–AKT and RAS–RAF-MAPK pathways were activated, Shh signaling was suppressed, and SMB cells became resistant to LDE225 (Fig. 5D–G). We tested the GFs individually, and found that bFGF, not EGF, suppresses Shh signaling and causes resistance (Supplementary Fig. S4E and S4F). Together, these results demonstrate that sustained activation of FGF/RAS/RAF signaling enables Shh-subtype medulloblastoma to grow in a Shh pathway–independent manner.

**Figure 2.** SMB cells provide a model to study resistance to Smo inhibition. A, survival analysis of SMB21 cells expressing GFP (control), Smo mutants, or Gli2ΔN treated with indicated LDE225 concentrations (72 hours; mean ± SEM; n = 4). B, Shh signaling was analyzed by immunoblot for Gli1 in SMB21 cells expressing GFP, HA-tagged wild-type or mutant Smo, treated with DMSO or 1 μmol/L LDE225 for 24 hours. C, SMB21 or SMB21 cells expressing Smo(WT) retain LDE225 responsiveness in vivo; SMB21 cells expressing Smo(D477G) or Gli2ΔN initiate resistant tumors; tumor volume over time; mean ± SEM; n = 5.
developed resistance after 40 days. Sufu immunoblot of vehicle (subcutaneously grafted SMB21 cells initially responded to LDE225, but DMSO or 1-knockdown of Sufu causes constitutive activation of Shh signaling; indicated LDE225 concentrations (72 hours; mean
SMB21, SMB55, SMB56 with shRNA knockdown of Sufu treated with
Loss of Sufu confers resistance to Smo inhibition. A, relative survival for
Figure 3. Zhao et al.
RAS/MAPK activation alters characteristics of Shh pathway–dependent tumors
Morphologically, SMB(HRAS) cells exhibit a dramatically different appearance from SMB parental cells. SMB parental cells are small, with little cytoplasmic material, whereas SMB(HRAS) cells appear larger with extended cellular processes (Supplementary Fig. S7). Immunohistochemical characterization revealed that SMB(HRAS) cells are proliferative, as indicated by Ki67, and poorly differentiated, as indicated by Nestin, a stem cell/progenitor marker (Fig. 7A).

The striking morphologic differences in SMB(HRAS) cells suggests that they may be more motile than parental cells. Indeed these cells were more invasive when tested in a Matrigel invasion assay (Fig. 7B). Interestingly, when SMB(HRAS) cells were s.c. injected in nude mice, they initiated resistant tumors, and also generated lung metastases in 2 of 9 mice. Metastases, were never found in 8 mice injected with SMB cells (Fig. 7C and D). Together, these data indicate that activation of RAS/MAPK increases tumor invasiveness.

Clinical observations from a rare set of matched primary and metastatic lesions from an individual patient provide additional evidence that RAS activation promotes metastasis in medulloblastoma, as the primary lesion exhibits low level of MAPK activation, whereas the metastatic frontal lobe lesion exhibits robust MAPK activation (Fig. 7E and F). In human primary Shh-subtype medulloblastoma, most areas are predominantly negative for MAPK activation (Supplementary Fig. S8, Table S2); however, in one tumor, cancer cells in the perivascular niche were strikingly positive for ERK phosphorylation (Supplementary Fig. S8D) whereas in a desmoplastic/nodular medulloblastoma, ERK phosphorylation was elevated in perinodular regions (Supplementary Fig. S8B). Both tumors exhibited regions with high FGF immunostaining (Supplementary Fig. S8B and S8D). Cells with MAPK activation within human Shh-subtype medulloblastomas may generate resistant tumors when challenged by Smo inhibitors.

Vismodegib treatment of BCC engenders RAS/MAPK–dependent tumors
Smo inhibitor vismodegib is approved for clinical use for patients with advanced BCC, but is not yet approved for

FGF/RAS-mediated resistance to Smo inhibitors is reversible
To investigate whether FGF/RAS/MAPK signaling is required for SMB cells to both develop and maintain resistance to Smo inhibitors, we removed oncogenic HRAS from SMB(HRAS) cells using lentiviral delivered Fp to cleave FRT sites within the transposon (Supplementary Fig. S5A and S5B). Removal of RAS decreases Erk phosphorylation and restores Shh signaling activity and susceptibility to Smo inhibitors (Fig. 6A–C). Similarly, cells resistant to Smo inhibitors due to prolonged bFGF treatment regained Shh signaling activity and susceptibility to Smo inhibitors when bFGF was removed from media for prolonged time periods (Fig. 6D and E). Together, these data indicate that prolonged activation of FGF/RAS/MAPK signaling both initiates and maintains Shh-signaling independence and resistance to Smo inhibitors.

To identify therapeutic approaches for treating SMB cells resistant to Smo inhibitors, we tested PI3K and MEK pathway inhibitors. Although HRAS cells showed similar sensitivity as SMB parental cells to PI3K inhibitors BEZ235 and BKM120, they were much more sensitive to MEK inhibitors CI-1040 and PD325901. Thus, MEK inhibitors provide therapeutic treatment for resistant tumors driven by activation of FGF/RAS/MAPK signaling (Fig. 6F and G; Supplementary Figs. SSC–SS6).

Loss of Sufu confers resistance to Smo inhibition. A, relative survival for SMB21, SMB55, SMB56 with shRNA knockdown of Sufu treated with indicated LDE225 concentrations (72 hours; mean ± SEM, n = 3). B, shRNA knockdown of Sufu causes constitutive activation of Shh signaling; DMSO or 1 μmol/L LDE225 for 24 hours; immunoblot for Glil. C, subcutaneously grafted SMB21 cells initially responded to LDE225, but developed resistance after 40 days. Sufu immunoblot of vehicle (n = 4) and LDE225 (n = 5)-treated tumors showed drastically reduced Sufu protein in resistant tumors (#1, #3, #4, 5). D, genomic copy number of Sufu in sensitive and resistant tumors determined by quantitative PCR. E, relative survival for SMB21 and Sufu knockdown cells treated with ATO (72 hours; mean ± SEM, n = 3).
Figure 4.
RAS/MAPK signaling confers resistance to Smo inhibitor. A–C, relative survival for SMB21 cells expressing candidate genes treated with LDE225 (72 hours). HRAS(G12V), BRAF(V600E), not PIK3CA(H1047R) or myristoylated AKT, confer resistance; mean ± SEM; n > 5. A', elevated phospho-Erk in SMB21 cells expressing HRAS(G12V) or BRAF(V600E). B' and C', elevated phospho-AKT in SMB21 cells expressing PIK3CA(H1047R) or AKT(Myr). D, SMB21(HRAS) cells initiate resistant tumors in vivo; tumor volume over time; mean ± SEM; n = 5. (Experiments were performed concurrently with Fig. 2C, the same SMB21 control shown here and Fig. 2C.) E, phospho-Erk in sensitive and resistant tumors from engrafted SMB21 cells mean ± SEM; *, P < 0.05, unpaired Student t test.
medulloblastoma. Among BCC patients, approximately 21% that initially respond subsequently develop tumors resistant to this inhibitor (31). In some cases, posttreatment resistant tumors exhibit characteristics of squamous cell carcinoma (SCC; refs. 32–36). We analyzed three patients who developed SCC at the site of the antecedent BCC tumor following treatment with vismodegib. Posttreatment resistant tumors display low level of Gli1 and high level of phospho-ERK, suggesting upregulated RAS/MAPK and downregulated Shh signaling (Fig. 7G). To determine whether SCCs that develop following vismodegib are derived from the antecedent BCC in the same location, we analyzed patient-matched normal tissue or blood, and pre- and post-relapse tumor samples by exome sequencing with germline and dbSNP variants removed. Tumors we assayed initially responded to vismodegib before acquiring resistance to the inhibitor. Strikingly, 91% of genetic variations \( n = 1248 \) in the SCC sample were shared between pretreatment BCC and posttreatment SCC, whereas only 3% and 6% of somatic genetic variations \( n = 43 \) and \( n = 84 \) were unique to the SCC or shared with patient-matched normal sample, respectively, suggesting that the SCC arose from the BCC (Fig. 7H). These results suggest that tumors can evolve from Shh pathway–dependent BCC to the RAS–MAPK pathway–dependent SCCs, and thereby develop resistance to Smo inhibitors.

Figure 5.
FGF/RAS/RAF signaling suppresses Shh-signaling. A–C, Shh-pathway targets and components decrease in SMB(HRAS) cells, by immunoblot, qRT-PCR, and microarrays; mean ± SD; \( n = 3; \), \( P < 0.0005 \), Student \( t \) test. D, GFs induce Shh-signaling independence in SMB cells. SMB cells cultured with or without GF for 2 weeks, then treated with LDE225 (72 hours; relative survival; mean ± SEM; \( n = 3 ~ 8 \)). E, GF treatment activates AKT and MAPK signaling. F and G, GF treatment suppresses Shh-pathway targets and components; qRT-PCR and microarray analysis of SMB and SMB(GF) cells; means ± SD; \( n = 3; \), \( P < 0.05 \), Student \( t \) test.
Discussion

The studies presented here introduce a set of Shh pathway–dependent medulloblastoma cell lines (SMB) and identify two distinct mechanisms of therapeutic resistance to Smo inhibitors. Loss of Sufu drives resistance to Smo inhibition by activating downstream Shh signaling. Alternatively, activation of RAS/MAPK signaling, either due to new mutations or to
microenvironmental factors, constitutes a novel mechanism of resistance that circumvents Shh pathway dependence in a growing tumor.

SMB cells as a model for Shh-subtype medulloblastoma

Cell lines that faithfully model the cancer from which they are derived provide important tools for studying disease mechanism.

Figure 7.

RAS activation alters characteristics of Shh pathway–dependent tumors. A, SMB and SMB(HRAS) cells stained with anti-Nestin, Ki67; DAPI, blue; scale bar, 50 μm. B, invasion of SMB(HRAS) or SMB cells assessed using Matrigel-coated Transwell apparatus. Invasion measured at 18 hours and normalized to SMB cells; mean ± SD; n = 3; *, P < 0.05, Student t test. C and D, H&E-stained lung sections from animals engrafted with SMB (C) or SMB (HRAS) cells (D); arrow, metastasis. Metastases detected in 2 of 9 HRAS mice; 0 of 8 SMB parental mice; scale bar, 50 μm. E and F, phospho-ERK in primary and metastatic samples from human Shh-subtype medulloblastoma. Phospho-ERK high in metastatic, frontal lobe tumor (F), but not in matched primary cerebellar tumor (E); scale bar, 50 μm. G, pretreatment BCC with characteristic H&E, keratin14, and Gli1 with an absence of phospho-ERK immunoreactivity. SCC tumor that developed at the same location after vismodegib displayed a spindle-like morphology characteristic of SCC and lacked keratin14 and Gli1 immunostaining, but was positive for phospho-ERK; scale bar, 100 μm. H, sequencing suggests shared lineage for posttreatment-resistant SCC and pretreatment BCC. BCC and SCC share most genetic variations (n = 1248; ratio = 0.91); SCC-normal share (n = 84; ratio = 0.06); SCC unique (n = 43; ratio = 0.03).
and discovering novel therapies. Investigation of medulloblastoma biology has been limited by the lack of stable lines that are tumorigenic and Shh pathway dependent. Most established medulloblastoma lines, are adherent cell cultures maintained in serum-containing media, and do not depend on the Shh pathway for growth and survival (6, 14). Current protocols can only culture freshly isolated tumor cells for a short time before key characteristics of Shh-subtype medulloblastoma are lost. Here, we present Shh pathway–dependent medulloblastoma lines (SMB) that can be used as effective and faithful in vitro models to study Shh-subtype medulloblastoma.

The protocol that enabled development of SMB lines is a modified version of neural stem cell culture methods. Key aspects include growing cells as nonadherent spheres and eliminating serum, EGF and bFGF from media. Generation of tumor spheres is commonly used to enrich for cancer stem-like cells (37). Indeed, high-grade gliomas can be perpetuated as neurospheres by maintaining cells in neural stem cell media with EGF and bFGF (37, 38). However, these conditions do not maintain tumorigenicity and Shh pathway dependency of medulloblastoma cells (39), as FGFl signaling has an antagonistic effect on Shh signaling (29, 30, 40). Instead, medulloblastoma neurospheres from Pch+/−/− mice cultured without exogenous EGF or bFGF generate Shh-subtype medulloblastoma cell lines (SMB) that are tumorigenic, maintain markers of Shh-subtype medulloblastoma and remain dependent on Shh pathway activity even after many passages in vitro.

A previous study isolated rare lines from medulloblastomas of Pch+/−/−/− mice (41). We observe distinct modes of p53 signaling dysregulation in each SMB line. Because all three lines are derived from Pch+/−/−/− mice, mutations or inactivation of p53 signaling may have developed during primary medulloblastoma tumorigenesis in each individual animal. In human medulloblastoma, the importance of p53 has become increasingly apparent. Among human medulloblastoma, p53 mutations were detected in 13% to 21% of Shh-subtype MBs (21, 22). We conclude that SMB cells offer a faithful model for investigating the Shh pathway in medulloblastoma, and facilitating the MAPK cascade (49). Thus, paracrine PlGF-mediated RAS/MAPK signaling could also attenuate efficacy of Smo inhibitors. Cross-talk between Shh and FGFR/RAS signaling has been widely recognized during organogenesis in multiple tissues (50, 51). Depending on biologic context, interactions between FGFR/RAS and Shh pathways can be synergistic or antagonistic. In cerebellar GCPs and Pch+/−/−/− medulloblastoma cells, acute bFGF treatment suppresses Shh signaling and proliferation, and concomitantly promotes cell differentiation (29, 30). Similarly, oncogenic RAS can block Shh signaling in NIH3T3 cells and pancreatic cancer models (52). Here, we again observe an antagonistic relationship between Shh and FGFR/RAS signaling in SMB cells. Importantly, however, this process does not promote terminal differentiation; instead tumor cells remain proliferative and tumorigenic.

Mechanisms of resistance to Smo inhibition

Several Smo inhibitors show promise in preclinical and clinical studies. Vismodegib was the first drug of this class approved by the FDA to treat BCCs. In one study, 6 of 28 BCC patients treated with vismodegib developed resistance to Smo inhibitors (31). Here, we demonstrate that Sufu mutation can occur after treatment with Smo inhibitors, and cause secondary resistance by activating Shh signaling downstream of Smo. As loss of Sufu generates medulloblastomas that never respond to Smo inhibition (21, 42), this finding provides proof of principle that clinically relevant resistance mechanisms can be studied in SMB cells.

Data that overexpression of Gli1 or Nmyc does not confer resistance to Smo inhibition may seem surprising. However, Gli2 is the primary activator of the Shh signaling pathway in GCP development and medulloblastoma, whereas Gli1 is not essential (43, 44). In preclinical and clinical settings, amplification and constitutive activation of Gli2 generates Shh-subtype medulloblastoma resistant to Smo inhibition (5, 12, 21). In a recent study of human Shh-subtype medulloblastoma (n = 133), 10 cases of Gli2 amplification were identified, but no cases of Gli1 amplification were seen (21). Therefore, our results with overexpression of Gli2 transcription factors are consistent with clinical observations. In contrast, Nmyc amplification is reported in Shh-subtype medulloblastoma that do not respond to Smo inhibition (21). Although we cannot exclude the possibility that higher expression achieved by other means might confer resistance, our results suggest that other genetic changes may be needed in conjunction with Nmyc for tumors to grow in the presence of Smo inhibitors.

An important finding of this study is identification of a Shh pathway–independent mechanism of resistance. RAS-mediated resistance involves shifting oncogenic addiction to a second oncogenic pathway. De novo mutations or a microenvironment with abundant GF can stimulate RAS/MAPK signaling, eliminate Shh pathway dependency, and cause resistance in medulloblastoma. Indeed, in human medulloblastoma, components of the RTK–RAS–MAPK pathway are often overexpressed (27), and epigenetic inactivation of RAS association domain family 1A (RASSF1A) tumor-suppressor gene is frequently observed (45, 46). Although de novo RAS/RAF mutations have not been detected in primary human medulloblastoma, such mutations might be favored following treatment with Smo inhibitors (28), as mutations that confer resistance are often only detected following treatment with targeted therapies (11, 47). Strikingly in our studies, xenografts with spontaneous resistance to Smo inhibitors display activation of ERK signaling in vitro. Thus, our data indicate that GF stimulation, genetic or epigenetic changes affecting the RAS–RAF–MEK pathway should be assessed in patients that develop resistance to Smo inhibitors. In addition to intrinsic mutations in tumor cells, tumor microenvironment may alter drug efficacy (48). Our study suggests that microenvironments with abundant FGF could provide protective niches for cells exposed to Smo inhibitors. We show that in human medulloblastoma, ERK activation occurs in locations adjacent to blood vessels or in perinodular spaces. Recent work suggested that stromal production of placental GF (PlGF) in human medulloblastoma promotes cancer cell survival by activating the MAPK cascade (49). Thus, paracrine PlGF-mediated RAS/MAPK signaling could also attenuate efficacy of Smo inhibitors.
and more metastatic in vivo. Comparison of Shh-subtype primary medulloblastoma and metastatic metastatic lesions from the same person, reveal high levels of phosphorylated ERK1/2 in metastases. Consistent with our findings, ectopic expression of Eras (embryonic stem cell–expressed Ras), which is structurally similar to RAS oncogene (53), increases leptomenigeal metastases in models of Shh-subtype medulloblastoma, and these metastatic cells differ genetically and epigenetically from primary tumor cells (54, 55). Together, these data indicate that RAS activation results in resistance to Smo inhibitors and alters tumor characteristics.

Clinical studies of resistant BCC also suggest a role for RAS/MAPK in tumor evolution. Several studies have reported occurrences of SCCs during treatment of BCC with vismodegib (32–36). Sequencing of matched pre- and posttreatment skin tumor samples supports the hypothesis that BCC tumors activate RTK/RAS/MAPK signaling and generate SCCs under selective pressure by Smo inhibition (56). Thus, our findings indicate a novel scenario of resistance by which Shh pathway–dependent tumor cells evolve and escape Shh signaling dependence. Future studies are required to assess the prevalence of this resistance mechanism in patients.

Disclosure of Potential Conflicts of Interest

A.L.S. Chang reports receiving other commercial research support and is a consultant/advisory board member for Genentech. A.E. Orlo reports receiving a commercial research grant from Novartis and Genentech. J.F. Kelleher has ownership interest (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

References

RAS Drives Drug Resistance and Tumor Evolution in Shh Tumors


34. Lee J, Kotliarov S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391–403.

RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway–Dependent Tumors

Xuesong Zhao, Tatyana Ponomaryov, Kimberly J. Ornell, et al.


Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-2999-T

Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2015/06/30/0008-5472.CAN-14-2999-T.DC1

This article cites 56 articles, 18 of which you can access for free at: http://cancerres.aacrjournals.org/content/75/17/3623.full#ref-list-1

This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/75/17/3623.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/75/17/3623.

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.