A crucial requirement for Hedgehog signaling in small cell lung cancer

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Small-cell lung cancer (SCLC) is an aggressive neuroendocrine subtype of lung cancer for which there is no effective treatment1,2. Using a mouse model in which deletion of Rb1 and Trp53 in the lung epithelium of adult mice induces SCLC3,4, we found that the Hedgehog signaling pathway is activated in SCLC cells independently of the lung microenvironment. Constitutive activation of the Hedgehog signaling molecule Smoothened (Smo) promoted the clonogenicity of human SCLC in vitro and the initiation and progression of mouse SCLC in vivo. Reciprocally, deletion of Smo in Rb1 and Trp53-mutant lung epithelial cells strongly suppressed SCLC initiation and progression in mice. Furthermore, pharmacological blockade of Hedgehog signaling inhibited the growth of mouse and human SCLC, most notably following chemotherapy. These findings show a crucial cell-intrinsic role for Hedgehog signaling in the development and maintenance of SCLC and identify Hedgehog pathway inhibition as a therapeutic strategy to slow the progression of disease and delay cancer recurrence in individuals with SCLC.

Activation of Hedgehog signaling has been reported in a subset of human SCLC cell lines and tumors5–8 without changes in Hedgehog pathway gene copy numbers9. Furthermore, we sequenced exons from 16 Hedgehog pathway genes in human SCLC and found no evidence for recurrent mutations (Supplementary Table 1). These observations raised questions about the potential roles of Hedgehog signaling in SCLC development as well as the relative contributions of cell-autonomous and non–cell-autonomous Hedgehog activity10. To investigate the Hedgehog pathway in SCLC in vivo, we first crossed mice carrying conditional alleles of Rb1 and Trp53 to Ptc1lacZ/+ (Ptc1 is also known as Ptc1) mice in which LacZ activity parallels Hedgehog pathway activation11. X-gal staining revealed that the vast majority of mouse SCLC (mSCLC) stained positive for lacZ activity in vivo 9–12 months after exposure to adenovirus expressing Cre (Ad-Cre) (Fig. 1a). The X-gal staining intensity was similar to that found in the cerebellum of Ptc1lacZ/+ mice (Supplementary Fig. 1a,b). We found no recurrent copy number changes in Hedgehog pathway genes in mouse tumors (Supplementary Table 2), which argues against mutations directly activating Hedgehog signaling in this model. Non-SCLC mouse lung tumors induced by oncogenic Kras12 were largely negative for LacZ activity (Supplementary Fig. 1c,d). A subset of stromal cells underlying the bronchiolar epithelium also stained positive for X-gal, both in wild-type and Rb1- and Trp53-mutant mice, indicating that this result was not caused by tumor-suppressor loss (Fig. 1a, Supplementary Fig. 2 and data not shown). We found reporter activity in ~60% of the small lesions and weak X-gal staining in ~35–40% of neuroendocrine cells, which are considered candidates for the cell of origin of SCLC13,14 (Fig. 1b and Supplementary Fig. 2a,b). Otherwise, only subsets of tracheal epithelial cells stained positive for LacZ activity (Supplementary Fig. 2c).

Rb1-Trp53-Ptc1lacZ/+ tumor cells expressed lacZ in culture (Fig. 1c) and in allografts (Fig. 1c), and seven of eight single-cell subclonal cultures derived from Rb1-Trp53-Ptc1lacZ/+ tumors retained LacZ activity (Fig. 1c and data not shown). These subclones expressed components of the Hedgehog pathway (Fig. 1d and Supplementary Fig. 3a,b). Thus, Rb1-Trp53 mutant mSCLC cells maintain Hedgehog activity cell autonomously and independently of the lung cellular microenvironment. Shh-LIGHT2 reporter cells, in which the luciferase reporter is induced when the Hedgehog pathway is active15, were
cultured with conditioned medium from mSCLC cells but showed no induction of reporter activity (data not shown). However, culture of the reporter cells with the mSCLC cells resulted in mild luciferase induction (Fig. 1e), suggesting active Hedgehog ligands that may be retained in close proximity to the producing cells. Accordingly, immunohistochemistry analysis showed that mSCLC cells expressed Hedgehog ligands in vivo (Fig. 1f). Appropriate Hedgehog signaling depends on a functional primary cilium.\(^{16,17}\) We found that ~12% of mSCLC spheres in culture and subsets of neuroendocrine tumor cells in vivo (Fig. 1f) had a primary cilium. Moreover, addition of conditioned medium containing active Sonic hedgehog to mSCLC cells grown in low serum enhanced their survival and increased expression of the Hedgehog pathway member and target Gli1 (Fig. 2a,b). Together, these data suggest that the Hedgehog pathway is active in mSCLC cells through an autocrine-juxtacrine loop and that one function of the pathway is to enhance survival.

We next crossed Rb1–Trp53 conditional mutant mice to Rosa26\(_{\text{LSL-SmoM2-YFP}}\) (Rosa26 is also known as Gt(ROSA)26Sor) mice carrying a constitutive, conditionally active mutant allele of Smo (SmoM2) fused to YFP (also known as Tg(Thy1-YFP)16Jrs)\(^{18}\) (Fig. 2c). We aged cohorts of Ad-Cre–infected Rb1\(^+/+\), Trp53\(^+/+\), Ptch1\(^{lacZ}\) mice infected with Ad-Cre. Arrowheads point to a LacZ\(^+/+\)-Smo\(^{+}\)-stromal cell. (b) Quantification of X-gal staining in Syp\(^+\) cells, lesions and tumors. ND, not detected. (c) Shown at the top left and in the center are X-gal staining and immunostaining for the neuroendocrine marker CGRP of a mouse SCLC sphere in culture. The asterisk indicates a stromal cell. Shown at the bottom is an X-gal staining of representative clones (1 and 2) derived from parental SCLC cell lines (1 and 2). We used Gapdh as a loading control. (d) Luciferase activity in Shh-LIGHT2 reporter cells co-cultured with mouse SCLC cells (n \(\geq 3\)). We used conditioned media from either 293 cells (Con-CM) or 293 cells secreting active Sonic hedgehog (ShhN-CM) as controls. Data are relative to Con-CM values. (e) Relative luciferase activity of mSCLC cells. **P < 0.01, *P < 0.001.

In determining whether Hedgehog signaling was required for the expansion of SCLC tumor cells, we found that treatment with cyclopamine, a Smo inhibitor\(^{20}\), decreased the survival of SCLC cells in low serum and also decreased Gli1 mRNA levels; a structural analog of cyclopamine, tomatidine, which does not inhibit Smo function, had minimal effects (Supplementary Fig. 7a,b). To rule out nonspecific activities of cyclopamine,\(^{10}\) we treated mSCLC cell lines with HIP-1 (ref. 21) and GANT-61 (ref. 22), two inhibitors of Gli proteins; this treatment reduced Gli1 levels and cell survival compared to vehicle treatment (Fig. 3a,b and Supplementary Fig. 7c,d). We observed similar effects with the Smo inhibitor NVP-LDE225 (refs. 23,24) (Supplementary Fig. 7e). We observed decreased proliferation and increased apoptosis in mSCLC tumors that we treated short term with cyclopamine in vivo (Fig. 3c,d and Supplementary Fig. 7f); these levels were comparable to those seen with cisplatin, a platinum-based drug used to treat individuals with SCLC\(^{2}\) (Fig. 3d). Staining for the
vasculature marker PECAM-1 was similar in vehicle- and cyclopamine-treated tumors (data not shown). These observations suggest that the Hedgehog pathway is required for the maintenance of SCLC.

To further test whether inhibition of Hedgehog signaling intrinsically suppresses SCLC development, we used a mouse model in which loss of Rbl2 accelerates SCLC development. We analyzed cohorts of Rb1lox/lox; Trp53lox/lox, Rb1lox/lox, SmoM2+/+, Rb1lox/lox; Trp53lox/lox, Rh1lox/lox, SmoM2+/+, SmoM2+/+, and Rb1lox/lox; Trp53lox/lox, Rb1lox/lox, SmoM2lox/lox mice 6 months after Ad-Cre exposure (Fig. 3e). Rb1-Trp53-Rbl2 triple-knockout mice with mutated Smo developed fewer and smaller tumors than did their littermates that had wild-type Smo and those that were heterozygous for Smo (Fig. 3f and data not shown). Histopathological analysis confirmed that all tumors had features of SCLC (Fig. 3g and data not shown). This decrease in tumor number was associated with a lower mitotic index but not with a change in cell death levels at this time point (Fig. 3i). Thus, the Hedgehog pathway contributes to the maintenance of mSCLC tumors and participates in their initial development in vivo.

A major issue in the management of individuals with SCLC is disease recurrence following chemotherapy. We tested the possibility that signaling downstream of Smo has a role in this process by manipulating the activity of the Hedgehog pathway in suspension cultures of human

Figure 2 Constitutive Hedgehog signaling is sufficient to promote SCLC in mice. (a) Cell viability for two mouse SCLC cell lines (mSCLC1 and mSCLC2) treated with conditioned media from 293 cells (Con-CM) or 293 cells secreting active N-terminal Sonic hedgehog (ShhN-CM) for 4 days (n ≥ 3). (b) Quantitative RT-PCR analysis for GlI1 levels after 24 h of treatment (n ≥ 3). (c) Strategy to constitutively activate Smo (SmoM2) in Rb1-Trp53 mutant lung cells. (d) Whole-mount images of tumors (Tu) and immunostaining for synaptophysin (Syp) (red) counterstained with DAPI (blue). (e) We quantified tumor number and area in mice from both genotypes (n = 8 for Rb1-Trp53 and n = 9 for Rb1-Trp53-SmoM2 mice). (f) Quantification of cell proliferation and cell death by immunostaining for phospho histone 3 (PH3) and cleaved caspase 3 (CC3) in tumors. Mean ± s.e.m. are shown. NS, not significant. ∗P < 0.01, ∗∗P < 0.001.

Figure 3 Hedgehog pathway activity is necessary for the growth of mouse SCLC cells. (a, b) Cell viability (after 4 d) (a) and RT-quantitative PCR analysis of GlI1 levels (after 24 h) (b) for three independent SCLC cell lines treated with HPI-1 (10 μM) or vehicle control (Con) (n = 3). (c) The treatment protocol of mice having tumors with vehicle (n = 4 mice), cyclopamine (Cyc) (n = 4) or cisplatin (Cis) (n = 2). The arrow indicates the experimental treatment, and the arrowhead indicates the vehicle. (d) Quantification of PH3+ and CC3+ cells on tumor sections. (e) The experimental strategy to test the effects of deleting Smo in Rb1-Trp53-Rbl2 mutant lung cells. (f) Lung sections from mice infected with Ad-Cre and aged for 6 months before analysis. The counterstain is H&E, and the tumors appear dark purple. (g) Synaptophysin (Syp) immunostaining on tumors (Tu) counterstained with DAPI (blue). (h) Tumor numbers and tumor area in mutant mice (n = 6 for Rb1-Trp53-Rbl2 mice with wild-type Smo and n = 3 for Rb1-Trp53-Rbl2 mice with mutated Smo). (i) Quantification of cell proliferation and apoptotic cell death by immunostaining for phospho histone 3 (PH3) and cleaved caspase 3 (CC3) in tumors. Mean ± s.e.m. are shown. NS, not significant. ∗P < 0.01.
LDE225 or vehicle (orange triangle); transfection with a vector expressing shRNA molecules targeting human Smo or control shRNA (red triangle); or 5 μM of recombinant human Sonic hedgehog or vehicle (green square); 100 nM NVP-LDE225 (80 mg per kg per day once a day, orange triangle), etoposide (12 mg per kg per day intraperitoneally on days 1, 2, 3 and 15 after the start of treatment) and carboplatin (E/C) (60 mg per kg per day intravenously on days 1, 8 and 15 after the start of treatment) alone (green diamond) or followed by NVP-LDE225 (red square) as indicated in the figure. The tumor volume (n = 8, two independent experiments) is shown. Mean ± s.e.m. are shown. **P < 0.001 compared to E/C then vehicle.

Figure 4  Hedgehog signaling is crucial for the growth of chemoresistant human SCLC cells. (a) Colony formation in chemonaive and chemosurviving LX22CL cells assessed by serial passage in methycellulose (n = 4). The clonal capacity following each treatment is shown relative to its respective control, to which we assigned a value of 1. Treatments and matching controls were as follows: infection with adenovirally expressed SmoM2 (AdSmoM2) or adenovirally expressed iGai (AdiGai) (green diamond); 0.2 μg ml⁻¹ of recombinant human Sonic hedgehog or vehicle (green square); 100 nM NVP-LDE225 or vehicle (orange triangle); transfection with a vector expressing shRNA molecules targeting human Smo or control shRNA (red triangle); or 5 μM of recombinant human Sonic hedgehog-neutralizing monoclonal antibody 5E1 or mouse IgG1 (brown circle). (b) Smo localization (green) in the primary cilia (AcT, red) of chemoresistant LX22CL cells counterstained with DAPI. (c) Smo expression in the primary cilia of chemoresistant LX22CL cells treated in vitro with recombinant Sonic hedgehog (rShh) (1 mg ml⁻¹), the Smo agonist SAG (200 nM), cyclopamine (Cyc, 3 μM) or NVP-LDE225 200 nM (n = 3). (d) We treated nude mice subcutaneously implanted with LX22 tumors with vehicle (control, white square), NVP-LDE225 (80 mg per kg per day once a day, orange triangle), etoposide (12 mg per kg per day intraperitoneally on days 1, 2, 3 and 15 after the start of treatment) and carboplatin (E/C) (60 mg per kg per day intravenously on days 1, 8 and 15 after the start of treatment) alone (green diamond) or followed by NVP-LDE225 (red square) as indicated in the figure. The tumor volume (n = 8, two independent experiments) is shown. Mean ± s.e.m. are shown. **P < 0.001 compared to E/C then vehicle.

LX22CL cells (derived from the primary xenograft line LX22) are sensitive to Hedgehog pathway inhibition, largely in a cell-autonomous manner. Such a mode of action for Hedgehog signaling may be relevant to several cancer types30–37 (Supplementary Discussion). Our study also reveals a previously unsuspected role for the Hedgehog pathway during SCLC initiation in addition to its role in the maintenance of tumors, suggesting that both early and advanced SCLC lesions may be responsive to Hedgehog pathway inhibitors. Future experiments should investigate the exact mode of action of Hedgehog molecules on SCLC cells, including juxtacrine and autocrine mechanisms. The cell-intrinsic activation of Hedgehog signaling in SCLC raises the possibility that metastasis of SCLC cells is largely independent of their microenvironment34. Finally, our data indicate that treatment of individuals with SCLC with Hedgehog pathway inhibitors may cooperate with chemotherapy and/or radiation therapy regimens to inhibit the growth of primary and metastatic SCLC and to reduce tumor recurrence in affected individuals.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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The histopathology of these xenografts indicated in the figure. The tumor volume (n = 8, two independent experiments) is shown. Mean ± s.e.m. are shown. **P < 0.001 compared to E/C then vehicle.
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A.N.K. analyzed the histopathology of all mouse lung tumors and edited the manuscript. A.S. performed and analyzed the immunohistochemistry. D.N.W. and L.G.M. edited the manuscript and analyzed the immunohistochemistry. L.G.M. performed experiments with Smo inhibitors and chemotherapy in culture and in xenografts. C.A.O and J.K.C. generated HPI-1 for cell culture experiments. K.-S.P. and J.S. designed the experiments involved mouse cells. K.-S.P. and J.S. designed and performed the experiments related to the primary cilia in mouse cells. M.P., M.L.S. and R.K.T. designed and performed the experiments related to the genomic analysis of mouse and human tumors. K.-S.P. and K.B. quantified the proliferation and survival phenotypes in tumors treated with cycloamine. K.-S.P. and J.F.C. analyzed gene and protein expression levels in tumor cells. K.-S.P. performed all the other experiments involving mouse cells. K.-S.P. and J.S. designed the experiments for the analysis of mouse SCLC cells in culture and in vivo, and generated the corresponding figures. C.D.P designed and analyzed the research performed by A.M. and W.L.D. on the human SCLC cells in vitro, J.F., S.Buonomici, S. Bennett, J.Y., R.G., B.O., M.D., A.M., W.L.D. and T.J.B. designed and performed in vivo xenograft experiments and analyzed the data. K.-S.P., J.S., C.D.P. and S.B. wrote and edited the manuscript.

ONLINE METHODS

Mice and xenograft assays. Trp53lox/lox, Ptc1lox/lox, Rosa26Lox-LSL-SmoM2, YFP, Rb1lox/lox, Rb2lox/lox and Smo1lox/lox mice were described previously.1,4,11,18,38,39 Ad-Cre (Baylor College of Medicine) infections were performed as described.1 Mice were maintained on a mixed genetic background (129/SvJ, 129/Ola and C57BL/6). Independent litters were analyzed to control for background effects. Equal numbers of males and females were used. LX222 tumors were serially passaged as fragments (3 × 3 × 3 mm³) in nude mice. All mouse lines were maintained according to practices prescribed by the NIH at the Stanford Research Animal Facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care; the animal studies carried out at Novartis were in accordance with the Novartis Guide for the Care and Use of Laboratory Animals.

RNA and DNA analyses. RNA and DNA analyses are described in the Supplementary Methods.

Drug treatments. Mouse tumors were identified using an eXplore RS microCT Scanner and the imaging software MicroView (GE Healthcare) at the Stanford Small Animal Imaging facility. Cyclopamine28 was administered daily by oral gavage at 25 mg per kg body weight. Cisplatin (Calbiochem) was administered intraperitoneally at 10 mg per kg body weight. Human SCLC xenografts were measured twice a week and their volume was calculated using the ellipsoid formula (length × width²) / 2. NVP-LDE225 (refs. 24,25) was provided daily (once a day) by oral gavage. Carboplatin (Sigma-Aldrich) and etoposide (BIOQUANT Image Analysis Corp.) were administered by intravenous injection and intraperitoneal injection, respectively.

Histopathological analyses. We used 5 µm paraffin sections for H&E staining or immunostaining. For immunofluorescence, sections were dewaxed and hydrated using Trilogy (Cell Marque). The antibodies used were to the following proteins: calcitonin gene related peptide (CGRP) (Sigma-Aldrich, C8198), synaptophysin (Neomarkers, RB-1461-P1), Clara cell secretory protein (CCSP) (Santa Cruz Biotechnology, sc-9772), phosphohistone 3 (Upstate, 06-570), cleaved caspase 3 (Cell signaling, D175), Ki-67 (BD, 550609) and YFP (Invitrogen, A11122), surfactant protein C (Biomol) and one-way analysis of variance using the GraphPad Prism software.

Immunoblot analysis. Protein levels for Sufu, Ptc1 and Gli3 were analyzed as previously described.40

Cell culture and in vitro assays. Mouse SCLC cells were grown as described.41 N-terminal Sonic hedgehog–conditioned media was prepared in DMEM with 0.5% serum. For co-culture experiments, 2 × 10⁵ SCLC cells per well in 24-well plates were plated onto confluent Shh-LIGHT2 cells in 0.5% serum DMEM for 48 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega). For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Roche), mouse cells were seeded at 2 × 10³ per well in 96-well plates and incubated for 4 days in conditioned media or media with 0.5% serum. Cyclopamine was extracted from the plant Veratrum californicum at Infinity Pharmaceuticals. Cyclopamine, HPI-1 and GANT-61 were produced and used as described.22,23 NVP-LDE225 was used at 400 nM for experiments in culture; the vehicle control consisted of cells treated with ethanol. The AlamarBlue Cell Viability Assay (Invitrogen) was used to measure viability using a FLUOstar OPTIMA Multidetection Microplate Reader (BMG Labtech). Advanced RPMI was used with 0.5% or 10% newborn calf serum.

To analyze primary cilia in mouse cells, cultured cells and frozen sections were fixed in either 100% ice-cold methanol at -20 °C or 4% PFA and PBS at room temperature for 10 min, depending on the antigen, and incubated with a monoclonal antibody to mouse polyglutamylated tubulin (GT335). Alexa-dye–conjugated secondary antibodies (Invitrogen) were used to visualize the immunostaining.

Human LX222CL cells were cultured as described.46 Clonogenic assays are described in the Supplementary Methods section. For experiments with human SCLC cells and primary cilia, the Smo agonist SAG (Calbiochem) was used at 200 ng ml⁻¹, cyclopamine was used at 3 µM, and NVP-LDE225 was used at 200 nM. The total number of ciliated cells analyzed per experiment was at least 200. Only those cells clearly showing Smo localization along the axoneme or at tip of the cilium were counted as positive for Smo ciliary localization. Cells were incubated with cyclopamine and NVP-LDE225 after starvation for 24 h. Then, recombinant Sonic hedgehog was added to each well, except for the wells with the SAG-treated cells. SAG was added at the same time as recombinant Sonic hedgehog. After 6 h of treatment, primary cilia were stained in fixed cells using primary antibody to α-acetylated tubulin and antibody to γ-tubulin (Sigma-Aldrich, C7604) conjugated with AlexaFluor-568 and AlexaFluor-647 fluorescent dyes, respectively, using the Mix-n-Stain labeling kit (Biotium). Smo ciliary localization was analyzed using antibodies to Smo (LifeSpan) conjugated with AlexaFluor-488A using Mix-n-Stain (Biotium). All primary antibodies dilutions were made in Odyssey buffer (LiCor).

Statistical analysis. Statistical significance was assayed using a Student’s t test and one-way analysis of variance using the GraphPad Prism software.

Additional methods. Detailed methodology is described in the Supplementary Methods.