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A crucial requirement for Hedgehog signaling in small cell lung cancer

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AUTHOR CONTRIBUTIONS 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. J.K.C. edited the manuscript. M.R.M. and T.S. designed, performed and analyzed 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 cyclopamine. 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.Buonamici, 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.

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Abstract

Small-cell lung cancer (SCLC) is an aggressive neuroendocrine subtype of lung cancer for which there is no effective treatment^{1,2}. Using a mouse model in which deletion of *Rb1* and *Trp53* in the lung epithelium of adult mice induces SCLC^{3,4}, 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 tumors^{5–8} 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 *Ptch1^{lacZ/+}* (*Ptch1* 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 *Ptch1^{lacZ/+}* 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 *Kras*¹² 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 SCLC^{13,14} (Fig. 1b and Supplementary Fig. 2a,b). Otherwise, only subsets of tracheal epithelial cells stained positive for LacZ activity (Supplementary Fig. 2c).

Rb1-Trp53-Ptch1^{lacZ/+} 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-Ptch1^{lacZ/+}* 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⁺/LSL-SmoM2-YFP* (*Rosa26* is also known as *Gt(ROSA)26Sor*) mice carrying a conditional, constitutively 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^{lox/lox}; Trp53^{lox/lox}; Rosa26⁺/LSL-SmoM2-YFP* (*Rb1-Trp53-SmoM2*) and *Rb1^{lox/lox}; Trp53^{lox/lox}* (*Rb1-Trp53*) mice for 8 months. All *Rb1-Trp53-SmoM2* tumors analyzed expressed *YFP*, and analysis of *Gli1* mRNA levels was indicative of a physiological activation of the Hedgehog pathway in these tumors (Supplementary Fig. 4). *Rb1-Trp53-SmoM2* mice developed more mSCLCs than did their *Rb1-Trp53* littermates (these mSCLCs were also associated with a greater tumor volume and higher mitotic index) but had comparable apoptotic cell death levels (Fig. 2d–f and Supplementary Fig. 5). We also determined that Hedgehog pathway activation could not replace loss of *Rb1* or *Trp53* using *Rb1^{+/lox}; Trp53^{lox/lox}; Rosa26⁺/LSL-SmoM2-YFP* or *Rb1^{lox/lox}; Trp53^{+/lox}; Rosa26⁺/LSL-SmoM2-YFP* mice because one wild-type *Trp53* allele was sufficient to prevent tumor development for up to 8–9 months after Ad-Cre exposure (data not shown), whereas retention of a wild-type *Rb1* allele produced features of lung adeno-carcinoma but not SCLC (Supplementary Fig. 6 and data not shown). The inability of *SmoM2* alone to initiate tumors in lung epithelium may be because of its weak activity and/or the ability of *Trp53* to normally restrict full Hedgehog signaling activation¹⁹.

In determining whether Hedgehog signaling was required for the expansion of SCLC tumor cells, we found that treatment with cyclopamine, a Smo inhibitor²⁰, 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 cyclopamine¹⁰, we treated mSCLC cell lines with HPI-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 SCLC2 (Fig. 3d). 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 *Rb1* accelerates SCLC development⁴. We analyzed cohorts of *Rb1^{lox/lox}; Trp53^{lox/lox}; Rb12^{lox/lox}; Smo^{+/+}*, *Rb1^{lox/lox}; Trp53^{lox/lox}; Rb12^{lox/lox}; Smo^{+/lox}* and *Rb1^{lox/lox}; Trp53^{lox/lox}; Rb12^{lox/lox}; Smo^{lox/lox}* mice 6 months after Ad-Cre exposure (Fig. 3e). *Rb1-Trp53-Rb12* triple-knockout mice with mutated *Smo* developed fewer and smaller tumors than did their littermates that had wild-type *Smo* and those that were 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 development⁴. We analyzed cohorts of *Rbl1*^{lox/lox}; *Trp53*^{lox/lox}; *Rbl2*^{lox/lox}; *Smo*^{+/+}, *Rbl1*^{lox/lox}; *Trp53*^{lox/lox}; *Rbl2*^{lox/lox}; *Smo*^{+/lox} and *Rbl1*^{lox/lox}; *Trp53*^{lox/lox}; *Rbl2*^{lox/lox}; *Smo*^{lox/lox} mice 6 months after Ad-Cre exposure (Fig. 3e). *Rbl1-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–h 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 LX22CL cells (derived from the primary xenograft line LX22)²⁵. Inhibition of Hedgehog signaling resulted in fewer colonies, whereas activation of Smo signaling increased the cells' long-term ability to grow in a clonogenic assay²⁶ (Fig. 4a). LX22CL cells surviving a single carboplatin and etoposide treatment showed an increased sensitivity to manipulation of ligand-dependent Smo signaling in the same assay (Fig. 4a). In contrast to what we observed with mSCLC cells, only a small fraction of human chemonaive SCLC cells (<1%) expressed a primary cilium (data not shown). However, three rounds of treatment with carboplatin and etoposide produced a strong increase in the number of LX22CL cells with a primary cilium (~20%). We were able to modify Smo localization in the primary cilia of these cells by manipulating the Hedgehog pathway (Fig. 4b,c). Furthermore, LX22 primary SCLC xenografts had a marked increase in the expression of Sonic hedgehog and nuclear Gli2 following chemotherapy (Supplementary Fig. 8a,b). Although we did not detect cilia in chemo-naive xenografts (data not shown), approximately 10% of chemo-resistant LX22 tumor cells had primary cilia (Supplementary Fig. 8c). Thus, the increased sensitivity of chemoresistant SCLC cells to the inhibition of Smo signaling is directly correlated with Smo localization in the primary cilia of these cells, which is a sign of activation of the Hedgehog pathway.

To test the idea that chemotherapy and Smo inhibition might cooperate, we treated mice bearing LX22 xenografts with carboplatin and etoposide chemotherapy followed by oral administration of NVPLDE225. NVP-LDE225 monotherapy in chemonaive tumors had little effect on tumor growth. Notably, however, NVP-LDE225 treatment was highly effective in preventing the recurrence of residual tumors following chemotherapy (Fig. 4d). The histopathology of these xenografts following treatment was unchanged (Supplementary Fig. 8d).

Direct mutations affecting key regulators of the Hedgehog signaling pathway are potent drivers of tumors^{8,27} that are sensitive to pharmacological inhibition of the Hedgehog pathway^{28,29}. SCLC cells do not carry recurrent mutations in Hedgehog pathway genes but are nevertheless 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 types^{30–37} (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/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cell-autonomous activation of the Hedgehog pathway in mouse SCLC. (a) X-gal staining and immunostaining for Ki-67 and the neuroendocrine marker synaptophysin (Syp) of lung tumors from *Rb1*^{lox/lox}; *Trp53*^{lox/lox}; *Ptch1*^{lacZ/+} mice infected with Ad-Cre. Arrowheads point to a LacZ+Ki-67⁻Syp⁻ stromal cell. (b) Quantification of X-gal staining in Syp⁺ cells, lesions and tumors. ND, not detected. 20 μ m 10 μ m (c) Shown at the top left and in the center are X-gal staining and immunostaining for the neuroendocrine Syp marker CGRP of a mouse SCLC sphere in culture. The asterisk indicates a stromal cell. Shown at the top right is an X-gal-stained subcutaneous allograft derived from *Rb1-Trp53* mutant *Ptch1*^{lacZ/+} tumor cells (the arrow indicates the skin of the recipient mouse). Shown at the bottom is an X-gal staining of representative clones (1 and 2) derived from parental *Rb1-Trp53*-mutant *Ptch1*^{lacZ/+} SCLC cells. (d) RT-PCR analysis for *Shh*, *Ptch1*, *Smo* and *Gli1* in two subclones (1 and 2) each from two independent parental SCLC cell lines (1 and 2). We used *Gapdh* as a loading control. (e) Luciferase activity in Shh-LIGHT2 reporter cells co-cultured with mouse SCLC cells ($n = 3$). 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. (f) Shown at the top is Sonic hedgehog (Shh) immunostaining (brown) on sections of mouse SCLC (Tu); the arrow indicates normal airway epithelial cells, the arrowhead indicates tumor-associated stromal cells and the counterstain used was hematoxylin (Hem). At the bottom, immunostaining for polyglutamylated tubulin (Tub, red) marks the primary cilium in a SCLC sphere (left), a single cell (inset) and a primary tumor (right). PNEC/NEB, pulmonary neuroendocrine cells including neuroepithelial bodies. Mean $\pm$ s.e.m. are shown. * $P < 0.01$, ** $P < 0.001$.

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 $\pm$ 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-Rb12* 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 (Syn) immunostaining on tumors (Tu) counterstained with DAPI (blue). (h) Tumor numbers and tumor area in mutant mice ($n = 6$ for *Rb1-Trp53-Rb12* mice with wild-type *Smo* and $n = 3$ for *Rb1-Trp53-Rb12* 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 $\pm$ s.e.m. are shown. NS, not significant. $*P < 0.01$.

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 β Gal (Ad β Gal) (green diamond); $0.2 \mu\text{g ml}^{-1}$ 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 $5 \mu\text{g ml}^{-1}$ of the 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, $3 \mu\text{M}$) or NVP-LDE225 200 nM ($n = 3$). (d) We treated nude mice subcutaneously implanted with LX22 tumors with vehicle (control, white square), NVP-LDE225 ($80 \text{ mg per kg per day}$ once a day, orange triangle), etoposide ($12 \text{ mg per kg per day}$ intraperitoneally on days 1, 2, 3 and 15 after the start of treatment) and carboplatin (E/C) ($60 \text{ mg per kg per day}$ 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 $\pm$ s.e.m. are shown. $**P < 0.001$ compared to E/C then vehicle.