Gli1-Mediated Regulation of Sox2 Facilitates Self-Renewal of Stem-Like Cells and Confers Resistance to EGFR Inhibitors in Non–Small Cell Lung Cancer

Namrata Bora-Singhal, Deepak Perumal2, Jonathan Nguyen and Srikumar Chellappan

Department of Tumor Biology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL, 33612, USA

Abstract

Non–small cell lung cancer (NSCLC) patients have very low survival rates because the current therapeutic strategies are not fully effective. Although EGFR tyrosine kinase inhibitors are effective for NSCLC patients harboring EGFR mutations, patients invariably develop resistance to these agents. Alterations in multiple signaling cascades have been associated with the development of resistance to EGFR inhibitors. Sonic Hedgehog and associated Gli transcription factors play a major role in embryonic development and have recently been found to be reactivated in NSCLC, and elevated Gli1 levels correlate with poor prognosis. The Hedgehog pathway has been implicated in the functions of cancer stem cells, although the underlying molecular mechanisms are not clear. In this context, we demonstrate that Gli1 is a strong regulator of embryonic stem cell transcription factor Sox2. Depletion of Gli1 or inhibition of the Hedgehog signaling significantly abrogated the self-renewal of stem-like side-population cells from NSCLCs as well as vascular mimicry of such cells. Gli1 was found to transcriptionally regulate Sox2 through its promoter region, and Gli1 could be detected on the Sox2 promoter. Inhibition of Hedgehog signaling appeared to work cooperatively with EGFR inhibitors in markedly reducing the viability of NSCLC cells as well as the self-renewal of stem-like cells. Thus, our study demonstrates a cooperative functioning of the EGFR signaling and Hedgehog pathways in governing the stem-like functions of NSCLC cancer stem cells and presents a novel therapeutic strategy to combat NSCLC harboring EGFR mutations.

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Introduction

Lung cancer is the leading cause of cancer related deaths in the United States [1]. Although non–small cell lung cancer (NSCLC) patients with early-stage disease are treated by surgery, about 30% to 60% develop recurrent tumors, which result in mortality [2,3]. Chemo-therapeutic agents like gemcitabine, platinum compounds, and taxanes improve survival to a limited extent, but overall survival rates remain low because of recurrence of more aggressive, drug-resistant tumors [4,5]. NSCLC in non-smokers show predominantly mutations in EGFR [6]; such patients respond well to EGFR inhibitors like erlotinib but eventually develop resistance and succumb to the disease [7]. In all the cases, the recurrence can be local or metastatic, and commonly occur after a period of clinical dormancy [2]. Resistance to EGFR inhibitors occurs through various mechanisms, including the appearance of the T790M gatekeeper mutation, expression of c-Met gene, or activation of alternate signaling pathways [8,9]. Development of strategies to combat resistance to EGFR inhibitors in NSCLC will be of immense benefit to a large number of patients [10].

Address all correspondence to: Srikumar Chellappan, H. Lee Moffitt Cancer Center and Research Institute, Department of Tumor Biology, 12902 Magnolia Drive, Mail Code: SRB3, Tampa, FL, 33612, United States.
E-mails: Srikumar.Chellappan@moffitt.org, Chellappan@aol.com

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Cancer stem cells (CSCs), a subpopulation of cells within the tumor, have been proposed to be responsible for the initiation and progression of a variety of cancers, including NSCLC [11–13]. CSCs from NSCLC cell lines, tumor samples, and mouse models have been isolated based on various markers including ALDH1, side-population phenotype, and CD133 positivity [14–16]. CSCs are slow-dividing cells that are highly drug resistant, and it has become clear that targeting such cell population would be imperative to combat NSCLC. The absence of effective therapy is related to the complexity of CSCs, and therefore better understanding of the biology of CSCs is a requisite.

The developmental pathways associated with lung including the Hedgehog (Hh) signaling pathway have been shown to promote the genesis and progression of human cancers [17]. Three Hh genes exist in mammals, namely, Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh); of these, Shh is the most widely expressed [17–19]. Elucidation of the Hh signaling pathway showed that secreted Shh binds to the receptor Patched (Ptch) present on the cell membrane, releasing the Ptch-mediated repression of Smoothen, which is a seven-pass transmembrane spanning protein essential for the transduction of Hh signaling [17,20]. Smoothened facilitates the interaction of different Hh downstream effectors resulting in the activation of the Gli transcription factors. In humans, the three Gli proteins, Gli1, Gli2, and Gli3, coordinate specific Hh responses in the cell by modulating gene expression [17,18,20,21]. Genes of the Hh pathway including Gli1 and Ptch1 are targets of Gli, therefore representing a feedback loop; furthermore, Gli3 is thought to repress Gli1-mediated transcription, while Gli2 is thought to upregulate Gli1 function [20,21].

The Hh pathway has also been implicated in regulation of CSCs in various cancers and is known to increase tumor invasiveness [22–24]. Our earlier studies have shown that side-population (SP) cells isolated by Hoechst 33342 exclusion from multiple NSCLC cell lines and human tumor explants have CSC-like properties [25,26]. SP cells could self-renew and form spheres in low-adherence plates and initiate tumors in mice; furthermore, a gene expression profile derived from these stem-like cells correlated with poor prognosis [27]. The self-renewal properties of stem-like SP cells were driven mainly by the embryonic stem cell transcription factor Sox2, whereas Oct4 and Nanog appeared to play a lesser role [26]. We have found that Sox2 levels were regulated by EGFR signaling cascades; inhibition of EGFR significantly reduced the expression of Sox2 and abrogated self-renewal of SP cells [26]. Our earlier work also showed that Sox2 levels were elevated in progressed, metastatic adenocarcinomas; such a correlation was not found in squamous cell carcinomas [26]. Other groups have shown that Hh and EGFR can cooperatively regulate multiple genes including Sox2 in basal cell carcinoma [28]. Given the importance of Sox2 in facilitating stem-like properties of CSCs, the present study attempts to investigate the Hh pathway-mediated regulation of Sox2 expression in NSCLC cells, especially in stem-like cells. Components of the Hh pathway are altered in human NSCLC, and their expression predicts poor prognosis. Our experiments also show that inhibition of the Hh pathway using the Smoothened inhibitors GDC-0449 or BMS-833923 (XL139) abolished the self-renewal of SP cells. Depletion of Gli1, the major mediator of Hh function, led to a reduction in the levels of Sox2 and significantly abrogated the self-renewal of SP cells as well as their ability to form angiogenic tubules that represent vascular mimicry. Interestingly, Hh pathway inhibitors appeared to enhance the growth-suppressive properties of EGFR inhibitors and depletion of Gli1-sensitized NSCLC cells to erlotinib and gefitinib. We believe that these studies will shed light on novel mechanisms underlying the genesis of NSCLC and will lead to the identification of novel therapeutic modalities to combat NSCLC by overcoming resistance to EGFR inhibitors.

**Materials and Methods**

**Cells and Reagents**

All the four human NSCLC cell lines, H1650, HCC827, PC-9, and H1975, were purchased from ATCC and maintained in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Atlas Biologicals). The H1650, HCC827, and PC-9 cell lines harbor deletion ΔE746-A750 in the exon 19 of EGFR tyrosine kinase receptor. The H1975 harbors L858R mutation and additionally T790M mutation in the EGFR receptor. The drug-resistant HCC827-ER cell line was maintained in culture media with 1 μM erlotinib, and PC-9-GR was maintained in culture media with 1 μM gefitinib. All the cultures were maintained at 5% CO2 at 37°C. Human EGF (E9644-2MG) was purchased from Sigma-Aldrich. Recombinant Human SHH N-Terminus (1845-SH-025) was purchased from R&D Systems. Gli1 inhibitor (G4408), erlotinib (E-4007), and BIBW (A-8644) were purchased from LC Laboratories. GDC-0449 (S1082) and BMS-833923 (S7138) were purchased from Selleck Chemicals.

**Antibodies**

The antibodies used are as follows: Sox2 (#3579), Oct4 (# 2750), Nanog (#4903), acetylated histone H3 Lysine 9 (# 9671), and Gli1 (#2643) were from Cell Signaling Technologies; mouse monoclonal antibody to actin was from Sigma-Aldrich; Gli2 (SC-271786) was purchased from Santa Cruz Biotechnologies; and Gli3 antibody (Ab55437) was from Abcam.

**Isolation of SP Cells and ALDHhigh Cells**

For isolation of SP cells, asynchronously growing cells were harvested using Accutase reagent (Sigma Aldrich), washed once with PBS, and resuspended in Dulbecco’s modified Eagle’s medium: F12K medium (Gibco, Life Technologies) with 2% FBS at 1 × 10⁶ cells/ml density. Cells were then incubated with 4 μg/ml of Hoechst 33342 dye (Life Technologies) for 90 minutes at 37°C in the presence or absence of 1 μM Fumitremorgin C (Sigma Aldrich) [15,25,26].

The Aldefluor assay kit (Stem cell Technologies) was used to isolate ALDHhigh cells as per manufacturer’s protocol. In brief, cells were suspended in Aldefluor assay buffer containing Aldefluor substrate (1 μM) at 1 × 10⁶ cells/ml density and incubated at 37°C for 45 minutes. To sort the ALDHhigh and ALDHlow population, the gates were set relative to baseline fluorescence determined by addition of Aldh-specific inhibitor diethylaminobenzaldehyde from the assay kit. Both Hoechst- and Aldefluor-stained cells were sorted using FACS Vantage (BD FACSDiVa) cell sorter as described in previous publications [25,26]. Data analyses were done using the FlowJo software (Tree Star).

**Sphere Formation Assay for Self-Renewal**

The sorted cells (SP or ALDHhigh) were plated in ultra-low attachment 96-well plate (Corning Inc.) at a density of 10,000 cells/ml (1000 cells/100 μl/well) in stem cell selective medium [Dulbecco’s modified Eagle’s medium: F12K (1:1) supplemented with N2 supplement (1x) (Invitrogen), 10 ng/ml EGF, and 10 ng/ml bFGF (Sigma Aldrich)] at 37°C for 10 days [25,26]. The spheres were observed using an automated Zeiss...
calculated by 2
performed using Bio-Rad CFX96 Real-Time system. Data were
(Bio-Rad). Levels of mRNA were analyzed using qRT-PCR that was
of RNA was converted into cDNA using iScript cDNA synthesis kit

RNA Isolation and Quantitative Reverse Transcription
Polymerase Chain Reaction (qRT-PCR) Analysis
Total RNA was isolated from the cells by RNeasy Miniprep kit
from Qiagen following the manufacturer’s protocol. One microgram
of RNA was converted into cDNA using iScript cDNA synthesis kit
(Bio-Rad). Levels of mRNA were analyzed using qRT-PCR that was
performed using Bio-Rad CFX96 Real-Time system. Data were
normalized using GAPDH as an internal control, and fold change was
analyzed on Day 10. The sphere formation assays were performed twice
with triplicates of each treatment in every assay.

Data Set of Lung Adenocarcinoma
Analysis of Gli1 Expression in Publicly Available Microarray
files were analyzed using the software package Bioconductor (http://
biocomputing.org). Expression values were normalized using MAS5.0 in R.
Statistical analyses were done using R package (http://www.r-project.org/).
Kaplan-Meier and log-rank tests (for
value) were conducted to
determine whether the gene expression correlates with prognosis. A
P value of less than .05 was considered to indicate statistical significance,
and all tests were two-tailed. All the analyses were performed with
packages in R unless otherwise specified.

Analysis of Gli1 Expression in Publicly Available Microarray
Data Set of Lung Adenocarcinoma
Gene expression profiles analyzed in this study include 22,283 probes
quantified with Affymetrix HG-U133A on 360 lung adenocarcinoma
samples from [29]. The Harvard data from the NCI Director’s Challenge
set was an outlier for our analysis, and hence we removed 82 samples from
the total 442 samples. Raw signal intensities for each probe set in the CEL
files were analyzed using the software package Bioconductor (http://
biocomputing.org). Expression values were normalized using MAS5.0 in R.
Statistical analyses were done using R package (http://www.r-project.org/).
Kaplan-Meier and log-rank tests (for
value) were conducted to
determine whether the gene expression correlates with prognosis. A
P value of less than .05 was considered to indicate statistical significance,
and all tests were two-tailed. All the analyses were performed with
packages in R unless otherwise specified.

siRNA Transfection
Two different chemically synthesized double-stranded siRNAs for
Gli1 were purchased from Santa Cruz Biotechnologies (sc-37911)
and Ambion (107670), respectively. The siRNAs were transfected at
a concentration of 100 pmol each into the cells using Oligofectamine reagent (Invitrogen) as per manufacturer’s protocol. A non-target
siRNA (AM4635, Ambion) was used as a control for all the
transfection experiments. The cells were harvested 48 hours
posttransfection for different assays. All the siRNA experiments
were performed thrice.

Lysate Preparation and Western Blot Analysis
The cells were washed twice with ice-cold PBS, scraped and centrifuged
at 800 g, and lysed using M2 lysis buffer (20 mM Tris–HCl pH 6.0,
0.5% NP-40, 250 mM NaCl, 3 mM EGTA, and 3 mM EDTA)
containing protease inhibitors as described previously [30]. The protein
content was quantitated by Bradford assay (Bio-Rad). Equal amounts of
proteins (50 μg) were separated using SDS-PAGE, transferred to
nitrocellulose membranes (Bio-Rad Transblot Semi-dry), blocked with
5% nonfat dry milk in PBS with 0.1% Tween-20, and incubated with
appropriate primary antibodies: 1:3000 diluted HRP-conjugated
secondary antibodies (Pierce Biotechnology) were used and signals
were detected using ECL (GE Healthcare).

Angiogenic Tubule Formation Assay
The sorted cells were allowed to differentiate on Matrigel (BD Biosciences)
for 10 days to form tubule-like structures. One hundred microliters of
thawed Matrigel was layered on the wells of 96-well tissue culture plates
followed by incubation for 60 minutes at 37°C to allow polymerization.
Sorted cells were layered (12,000 cells/100 μl of Matrigel) on the gels and
incubated overnight at 37°C. Tubule formation was assessed in bright
field using EVOS FL microscope system, and images were acquired with
EVOS software (Life Technologies Inc., USA).

Transfections and Luciferase Assays
Sox2 proximal promoter-luciferase construct was kindly provided by
Dr. Angel G. Martin (Inbiomed, Spain) [31]. Bacterial expression
plasmid for Gli1 (TCH1003) was purchased from Transomic and further
subcloned into pcDNA3 vector. Expression plasmid for Gli2 (# 17648)
was purchased from Ambion [32]. The cells were transiently transfected
using FugeneHD (Promega) according to the manufacturer’s protocol.
Luciferase assays were carried out 48 hours posttransfection using the
dual-luciferase assay system (Promega) according to manufacturer’s
instructions. Luciferase activity was measured using a luminometer (Turner
Luminometer). For each experiment, the relative luciferase activity was measured as the ratio of the
Firefly luciferase to Renilla luciferase, and the fold changes were calculated compared with the control
luciferase vector alone from at least three independent experiments.

ChIP Assays
ChIP assays were conducted on asynchronous H1650 and H1975
as previously described using indicated antibodies [30]. The
interactions at the promoter were analyzed using PCR. Each ChIP
assay was performed twice. The sequences of the ChIP PCR primers
are as follows:

FP 5’-TCCTGATTCTTCAAGTTCTC-3’,
RP 5’-GGGAGAGGAGGAGGAGGAGG-3’

Cell Viability Assay
Cell viability was measured with thiazolyl blue tetrazolium
bromide (MTT) after 48 hours of indicated treatment. Briefly,
cells were plated in 96-well plates at a density of 5000 cells/well in triplicates. After treatments, they were incubated with the 1mg/ml MTT solution at 37°C for 2 hours. The reaction was terminated with DMSO that solubilizes the formazan product formed. Absorbance at 590 nm was recorded using a plate reader.

For overexpressing Gli1 in H1650, PC-9, and HCC827 cells, they were transiently transfected with pcDNA3-Gli1 using FugeneHD reagent (Promega) according to the manufacturer’s protocol. The cells were then treated with different concentrations of gefitinib and erlotinib for 48 hours after which cell viability was tested with MTT reagent.

**Statistical Analysis**

The data are presented here with ± standard deviation values unless otherwise stated. The statistical comparisons between the groups were carried out by unpaired two-tailed Student’s t test to calculate the P value for statistical significance. *P < .05 and **P < .01.

**Results**

**Higher expression of Gli1 and Gli2 in cancer stem-like SP cells**

Because the Hh pathway has been shown to play a significant role in NSCLC progression and has been implicated in proliferation and maintenance of tumor-initiating cells [17,18,22,23], we examined the levels of the Gli transcription factors in cancer stem-like SP cells isolated from NSCLC cell lines. A qRT-PCR showed that the levels of Gli1 (two-fold or more) and Gli2 (1.5- to 2-fold) were significantly higher in SP cells as compared with the nonstemlike main-population cells from EGFR mutant H1650 and H1975 cell lines (Figure 1, A and B). ABCG2 expression was used as a positive control, as it confers the SP phenotype (Figure 1, A and B). Similar results were obtained when cancer stem-like cells isolated based on aldehyde dehydrogenase activity were analyzed by qRT-PCR. Aldhhigh cells from H1650 and H1975 cells showed significantly higher expression of Gli1 and Gli2 in both the cell lines (Figure 1, C and D). Aldehyde dehydrogenase (Aldh1) was used as the positive control (Figure 1, C and D).

Gli1 expression is known to mediate the induction of Hh pathway targets, and we examined whether Gli1 expression contributes to stem-like functions of SP cells. Towards this purpose, Gli1 was depleted in H1650 cells using two different siRNAs; self-renewal of SP cells was assessed by sphere-formation assays on low-adherence plates, following established protocols [26]. Depletion of Gli1 by siRNAs reduced the ability of SP cells to form spheres by 50%, compared with cells transfected with a non-targeting control siRNA (Figure 1G). Also, the size of the spheres that formed was significantly smaller compared with the control siRNA transfected cells (Figure 1F). These results suggested that Gli1 might be playing a distinct role in self-renewal of NSCLC CSCs.

Cancer stem cells from glioblastoma as well as SP cells from NSCLC cell lines have been reported to show vascular mimicry, a process in which they can differentiate to form CD31 positive tubular structures in Matrigel [26,33–35]. Depletion of Gli1 using two different siRNAs abrogated the ability of SP cells to form angiogenic tubules (Figure 1F). Thus, it appears that Gli1 plays a role in multiple facets of SP cell function, including self-renewal and vascular mimicry.

**Correlation of Gli expression with poor patient prognosis**

Given the role of Gli1 in regulating self-renewal of NSCLC stem-like cells, we examined whether the expression of Gli genes predicts the prognosis of lung adenocarcinoma patients. The Kaplan-Meier survival analysis was conducted on a subset of the data from the Director’s Challenge set for Gli1, Gli2, and Gli3. It was found that high levels of Gli1 expression correlated with poor overall survival in patients (P = .04); however, the 5-year survival rate (P = .A) was not significant (Figure 2, A and B). At the same time, increased expression of Gli2 (P = .06 (overall survival) and P = .06 (5-year survival)) did not show a significant correlation with prognosis (Figure 2, C and D). This could be reflective of the fact that Gli2 might be functioning through Gli1 and plays a secondary role in transcriptional regulation. Increased Gli3 levels (P = .02 (overall survival) and P = .007 (5-year survival)) also correlated with poor disease prognosis (Figure 2, E and F) in patients. Similar Kaplan-Meier analysis also showed that Sox2 expression predicted poor prognosis in lung adenocarcinoma when overall survival was examined (P = .035; data not shown). These results indicate that Gli family of transcription factors and Sox2 might be playing an important role in NSCLC disease progression and survival of patients.

**Response of EGFR mutant NSCLC cells to exogenous Shh protein**

Embryonic stem (ES) cell transcription factors Sox2, Oct4, and Nanog are known to play a role in maintaining stemness of CSCs derived from various tumor types [36]. Hence, we examined whether depletion of Gli1 affects stem-like functions of SP cells by modulating the expression of these core ES cell transcription factors. Towards this purpose, qRT-PCR analysis was conducted on H1650 and H1975 cells transfected two different siRNAs to Gli1 or a control, non-targeting siRNA. It was found that the levels of all three transcription factors were reduced upon Gli1 depletion; interestingly, the maximal reduction was observed for Sox2 in both the cell lines (Figure 3, A and B). Because studies from our laboratory had shown that Sox2 plays a major role in the self-renewal properties of stem-like SP cells [26], this result raises the possibility that Gli1 is affecting the stem-like functions of these cells by regulating the expression of Sox2 and other factors.

Because depletion of Hh pathway effector Gli1 abrogated the self-renewal properties of CSCs from EGFR mutant NSCLC cell lines, we investigated if exogenous addition of SHH protein could increase their stem-like properties. Addition of recombinant SHH protein to sorted SP cells from H1650 and H1975 cell lines increased their ability to form spheres in stem cell–specific media in a dose-dependent manner (Figure 3C). Furthermore, stimulation of the cells with recombinant SHH induced the expression of Sox2 in both H1650 and H1975 cells, as seen by qRT-PCR (Figure 3, D and E). Expression of Gli1 as well as FoxM1, which is a known target of Shh signaling, also increased with the treatment; the effect on Oct4 and Nanog was minimal (Figure 3, D and E). These results confirmed that the EGFR mutant NSCLC cell lines are sensitive to Hh signaling pathway and that Sox2 may be a common target gene of the EGFR and Hh signaling pathway.

**Regulation of Sox2 expression by Gli proteins**

Because depletion of Gli1 transcription factor reduced the expression of Sox2 mRNA, experiments were conducted to assess whether Gli1 could transcriptionally induce Sox2. An examination of the Sox2 upstream regulatory region using the Genomatix MatInspector program showed the presence of multiple Gli binding sites in Sox2 promoter/enhancer region. ChIP assays were conducted on asynchronous H1650 and H1975 cells to assess the presence of Gli
transcription factors on Sox2 proximal promoter region (−528 to +328) using a specific primer pair [37] (Figure 4A). The results show that Gli1 as well as Gli2 can bind to the Sox2 proximal promoter region (Figure 4B). Next, we carried out transient transfection assays on H1650 and H1975 cells using a Sox2 proximal promoter luciferase reporter construct (Sox2-luc). It was found that both Gli1 and Gli2 could efficiently induce the Sox2-luc in a dose-dependent manner in both H1650 and H1975 cell lines; Gli1 was the stronger inducer of Sox2 in both cell lines (Figure 4, C and D). This result, combined with the siRNA experiment, strongly suggests that Gli1 and perhaps Gli2 can transcriptionally induce the Sox2 gene.

Our earlier studies had shown that EGFR signaling induced Sox2 expression in EGFR mutant cell lines [26]. Given this background, we next investigated if EGF can increase the binding of the Gli transcription factors on the Sox2 proximal promoter region. To examine this, H1650 cells were serum starved for 24 hours and then stimulated with 100 ng/ml EGF for 18 hours. ChIP assays showed that there was a detectable amount of Gli1 and Gli2 associated with the promoter in serum-starved cells. Interestingly, stimulation with EGF enhanced the binding of Gli1 to the promoter; there was no significant increase in the binding of Gli2 to the promoter (Figure 4E). Based on these results, we next examined if Sox2-luc could be induced by EGF or recombinant SHH or a combination of both EGF and SHH in H1650 and H1975, and whether such an induction required the presence of Gli1 or Gli2. Towards this, we transfected H1650 cells (Figure 4F) or H1975 cells (Figure 4G) with control siRNA or siRNAs to Gli1 or Gli2 followed by transfection with Sox2-luc. Cells were serum starved for 24 hours and then stimulated with EGF or SHH or the combination for 18 hours. Luciferase assays showed that stimulation with EGF or SHH induced the Sox2-luc activity in both the cells lines; the induction was significant in cells transfected with a control siRNA as well. A combination treatment with EGF and SHH has an additive effect in the Sox2-luc activity in untransfected as well as control siRNA treated cells. Interestingly, depletion of Gli1 or Gli2 significantly abrogated the EGF- or SHH-mediated induction of Sox2 proximal promoter
This raises the likelihood that Gli1 and possibly Gli2 is mediating the induction of Sox2 downstream of both EGFR and SHH in these NSCLC cell lines.

Cooperation of EGFR signaling and Gli1 transcription factors to induce Sox2 expression

EGFR and Hh pathways have been reported to converge in certain types of cancer [28,38], and the above results suggested that they might be cooperating in the induction of Sox2 in NSCLC cells as well. Hence, we conducted experiments to assess whether EGF can induce Gli expression in NSCLC cells. Towards this purpose, H1650 cells were serum starved for 24 hours and stimulated with 100 ng/ml of EGF for 18 hours, and the levels of Gli1 and Gli2 were assessed by qRT-PCR. It was found that EGF stimulation induced the levels of Gli1 and Gli2 (~two-fold) in H1650, H1975, and PC-9 cell lines (Figure 5, A–C). To investigate the possibility that Gli1 is involved in the induction of endogenous Sox2 by EGFR signaling, we depleted Gli1 using siRNA in H1650 cells and stimulated with 100 ng/ml EGF for 18 hours. Western blot analysis showed that Gli1 depletion significantly reduced the EGF-mediated induction of endogenous Sox2 and Oct4 transcription factors (Figure 5, D and F); the induction of Sox2 by EGF was almost fully abolished, whereas the effect on Oct4 was less pronounced. There was only a minimal effect on Nanog expression (data not shown). This result supports the data

Figure 2. Kaplan-Meier survival curves for Gli family of transcription factors using the NCI Director’s Challenge set. (A-F) Kaplan-Meier survival analysis (overall and 5-year) on the NCI Director’s Challenge data set shows poor prognosis in patients with higher mRNA expression of Gli transcription factors. A significant correlation of poor overall survival is observed in patients with higher expression of Gli1 transcription factor (A-B). A similar trend of poor survival is also observed for patients with higher expression of Gli2 (C-D) as well as Gli3 (E-F) mRNA.
obtained in transient transfection experiments and raises the possibility that EGFR and Hh signaling pathways may cooperate to promote the induction of Sox2. To examine this hypothesis, we pretreated H1650 cells with erlotinib that targets EGFR signaling, and GDC-0449 and BMS 833923, which target Smoothened and therefore the Hh pathway. Cells were pretreated with the drugs for 3 hours followed by 100 ng/ml of EGF stimulation for 18 hours. Pretreatment with combination of EGFR inhibitor as well as Hh inhibitor was also tested. Western blot analysis showed that treatment with EGFR inhibitor or Smoothened inhibitors could prevent the induction of Sox2 by EGF (Figure 5, E and G). These results further support the observation that EGFR and Hh signaling pathways cooperate to promote Sox2 induction and thus may be modulating the stem-like functions of SP cells. At the same time, the possibility exists that other molecules might also be involved in the EGFR-mediated induction of Sox2.

**Regulation of self-renewal by EGFR and Hh signaling pathways**

Given the above results, additional experiments were conducted to assess whether EGFR and Hh signaling might cooperate to promote stem-like functions. First, we conducted MTT assays to assess whether two Smoothened/Hh inhibitors, GDC-0449 and BMS-833923, affect the viability of H1650 and H1975 cells. As shown in Figure 6, A and B, 10 μM of GDC-0449 or 5 μM of BMS-833923 reduced the viability of cells only marginally, as seen by MTT assays. We also compared the combination of EGFR inhibitor erlotinib with the Hh pathway inhibitors GDC-0449 or BMS-833923; interestingly, a combination of the two reduced the viability more effectively than each drug alone (Figure 6 C). Because depletion of Gli1 abrogated self-renewal of CSCs, we next examined whether Hh inhibitors could affect the self-renewal of SP cells from the EGFR mutant cell lines H1650, PC-9, and H1975. The two cell lines H1650 and PC-9 harbor deletion in Exon 19 ΔE746-A750 in the EGFR tyrosine kinase receptor. The H1975 cell line harbor L858R mutation as well as the secondary T790M gatekeeper mutation in the EGFR receptor, which makes it more resistant to tyrosine kinase inhibitor (TKI) therapies. As shown in Figure 6D, 0.1 μM of GDC-0449 could inhibit the self-renewal of SP cells from all the three cell lines; 500 nM gefitinib or erlotinib also had inhibitory effect on self-renewal, but this was of a lesser magnitude compared with GDC-0449. Interestingly, combining 0.1 μM or 1 μM of GDC-0449 with 500 nM gefitinib or erlotinib almost completely eliminated self-renewal (Figure 6D) in all the three cell lines. Similar cooperative effects were also observed with BMS-833923 as well, where 1 μM BMS-833923 cooperated with 500 nM erlotinib to
suppress self-renewal (Figure 6E). This suggests that the Hh inhibitors cooperate with EGFR inhibitors to abrogate the self-renewal of SP cells. Because the above results suggested that Hh pathway might be contributing to survival and self-renewal functions mediated by EGFR signaling, we next examined whether depletion of Gli1 could sensitize the EGFR mutant NSCLC cells to EGFR inhibitors. Towards this purpose, H1650 or H1975 cells were transiently transfected with a non-targeting control siRNA or a Gli1 siRNA. Cells were subsequently treated with 500 nM erlotinib or gefitinib (500 nM BIBW in H1975 only), and cell viability was measured by an MTT assay. It was found that depleting Gli1 could enhance the sensitivity of both the cell lines to EGFR inhibitors (Figure 6, F and G).
We also investigated the effect of overexpressing Gli1 in three TKI-sensitive cell lines; Gli1 was transiently transfected into H1650, PC-9, and HCC827 and subsequently treated with various concentrations of gefitinib (1-4 μM) and erlotinib (1-4 μM) (Figure 6, H and I). The cell viability of the treated cells measured by MTT assays clearly demonstrated that Gli1-overexpressing cells were more viable even in the presence of a higher concentration (4 μM) of the drugs gefitinib and erlotinib (Figure 6, H and I). These results suggested that Hh signaling pathway plays a role in conferring resistance to these drugs and that targeting this pathway might be a viable method to combat erlotinib or other EGFR inhibitor resistance in NSCLC.

**Reduction in stem cell transcription factor expression with EGFR and Gli1 inhibitor combination treatment**

To further investigate the mechanisms by which Hh and EGFR inhibition abrogates the self-renewal of NSCLC CSCs, we examined how the inhibitors alone, or in combination, affected the expression of stem cell transcription factors. As shown in Figure 7A, 500 nM gefitinib or erlotinib could reduce the levels of Sox2 but had only marginal effect on Oct4 or Nanog in H1650 cells. BMS-833923 could reduce the levels of both Gli1 and Sox2 and in combination with EGFR inhibitors could almost completely eliminate the expression of Sox2 (Figure 7A). Similar results were obtained in H1975 cells, which are less sensitive to gefitinib and erlotinib (Figure 7B); the
Figure 6. EGFR and Hh pathways cooperate to modulate self-renewal. (A-B) Cell viability assays performed on H1650 and H1975 cells with various concentrations of GDC-0449 (A) and BMS-833923 (B). (C) Cell viability assay performed on both H1650 and H1975 cells with a combination of GDC-0449 and erlotinib or BMS-833923 and erlotinib. (D-E) Sphere assays conducted on SP cells isolated from H1650, H1975, and PC-9 cells showed a decrease in number of spheres when treated with combination of EGFR inhibitors gefitinib or erlotinib along with GDC-0449 (D) and BMS-833023 (E). (F-G) Cell viability assay in H1650 (F) and H1975 (G) cells treated with EGFR inhibitors after Gli1 depletion exhibits lower viability as compared with inhibitor treatments in nontargeting control siRNA–transfected cells. (H-J) Gli1 protein transiently overexpressed in H1650 (H), PC-9 (I), and HCC827 (J) and cell viability assay performed after treating the cells with various concentrations of gefitinib and erlotinib. Gli1 overexpression decreases the sensitivity of the cells to EGFR inhibitors and increases cell viability.
combination of BMS-833923 with EGFR inhibitors could significantly reduce the levels of Sox2 in these cells as well (Figure 7B). GDC-0449 could also cooperate efficiently with EGFR inhibitors in H1650 and H1975 cells (Figure 7, C and D). These observations are especially significant because H1975 cells are less sensitive to EGFR inhibitors but could be sensitized by inhibiting the Hh pathway.

We next investigated the expression of Sox2 as well as Gli family proteins Gli1 and Gli2 in HCC827 cells that were rendered resistant to erlotinib (HCC827-ER) and PC-9 cells that were rendered resistant to gefitinib (PC-GR), respectively [39,40], by exposure to the drugs. Western blot experiments clearly indicated that there was increased expression of Gli1 protein in drug-resistant cells compared with parental cells (Figure 7, E and F). However, such a change was not observed for Gli2 factor. Interestingly, Sox2 expression was also elevated in both drug-resistant HCC827-ER and PC-9-GR (Figure 7, E and F). There was no significant change in Ocr4 protein expression with in vitro–induced drug resistance in both HCC827 and PC-9 (Figure 7, E and F). These results therefore indicated that upregulation of Gli1 and Sox2 expression conferred resistance to EGFR TKIs in TKI-sensitive cells.

Discussion
The therapeutic strategies to treat NSCLC have multiple drawbacks including lower efficacy of treatment and resurgence of drug-resistant tumors [4,8]. In the past decade, the novel agents that have been used to target the genetic alterations of NSCLC were found to be effective in early stages of the disease, but the tumors recur by developing alternate survival pathways [9,41]. Indeed, the escape from drug sensitivity is one of the major hurdles facing the use of targeted therapeutic agents. A significant amount of work has been done to elucidate the molecular and biological mechanisms involved in drug resistance to various targeted agents, especially the resistance to EGFR inhibitors in the case of NSCLC [41,42]. The biopsies from NSCLC patients who acquire resistance show that, in addition to the original activating EGFR mutation, other mutations arose like EGFR T790M mutation that interferes with the binding of the drugs like gefitinib or erlotinib to the receptor, amplification of MET tyrosine kinase receptor driving cell growth [43]. Furthermore, mutations like amplification of EGFR or PIK3CA were also observed [43]. A small population with acquired resistance to EGFR inhibitors displays mutation in B-Raf gene (G469A ad V600E) [44]. Moreover, activation of Akt/mTOR survival pathway is also observed in patients with acquired resistance [41]. IGF-1R (insulin-like growth factor) that is ubiquitously expressed on cancer cell surface is also implicated in mediating resistance to EGFR inhibitor therapies [41]. In all the cases, alternate signaling pathways were found to adapt the tumors to flourish even in the presence of the targeted agents. Similarly, the therapeutic strategies against the Hh pathway involving the targeting of Smo (SMO) protein from the pathway resulted in drug resistance [45,46].

It has also become clear that CSCs not only play a role in the initiation, dormancy, and metastasis of tumors but also contribute significantly to the drug resistance of a variety of tumors [47–49]. The expression of various drug transporters like ABCG molecules on the cell membrane, as well as the elevated levels of proteins including Mc11 and others, is thought to facilitate the survival of CSCs and contribute to drug resistance of the resultant tumors [25,50]. In this context, pathways that might affect the self-renewal as well as other stem-like properties of CSCs might be good targets for combating not only tumor growth but also drug resistance. Studies presented in this manuscript show that the Hh signaling pathway contributes to stem-like functions of NSCLC stem-like cells, and targeting this pathway confers sensitivity to EGFR inhibitors.

The Hh pathway was initially identified in Drosophila as a critical mediator of embryonic development, and it is highly conserved in higher organisms and very active in mammalian development [19–21]. It has been found that Hh pathway plays a crucial role in tumorigenesis when reactivated in adult mammalian tissues due to mutations or other mechanisms [18,21,51]. In this context, the results presented here show that high Gli1 expression predicts poor survival for lung adenocarcinoma patients, supporting the hypothesis that this pathway contributes to the genesis and drug resistance of this disease. Mechanistically, this could be explained by our results that show a selective induction of Sox2 gene by Gli1 compared with other ES cell transcription factors. Similar to Gli1, high Sox2 expression correlates with poor prognosis of lung adenocarcinoma patients and is known to play a clear role in stemness of lung adenocarcinoma as well as squamous carcinoma cells. A recent study has shown that Sox2 is amplified with PRKCI from chromosome 3q26 and enhances stemness in lung squamous cell carcinoma [52]. The study also showed that PKCi and Sox2 further activate Hh signaling to maintain stem-like features in lung squamous cell carcinoma [52]. Thus, there is a possibility of feed forward mechanism in Hh signaling mediated regulation of CSCs.

Vascular mimicry (also termed as vasculogenic mimicry) is a phenomenon by which tumor cells can acquire endothelial and vascular phenotype under oxidative stress to facilitate the supply of nutrients to the growing tumor [35,53–55]. SP cells from NSCLC as well as glioblastoma stem cells have been shown to have the ability to form angiogenic tubules on Matrigel, suggesting that the CSCs within the tumors might be facilitating this phenomenon [26,33,34,56]. Although our earlier results strongly suggest that Gli1 and the Hh pathway might be regulating self-renewal by modulating the expression of Sox2, it is not yet clear how Gli1 and the Hh pathway affect vascular mimicry. It is interesting that Gli1 depletion or inhibition could prevent this process; it remains to be determined the molecular mechanisms by which vasculogenic mimicry is regulated by the Hh pathway. It would be of significance if it turns out that Hh inhibitors, by their ability to inhibit vasculogenic mimicry, can also inhibit tumor angiogenesis and might synergize with antiangiogenic agents in therapeutic settings.

Certain recent studies in the past have suggested a cooperative interaction between EGFR and Hh signaling in cancers, promoting tumor growth and metastasis [28,38,57]. Our earlier studies had shown that EGFR-Src-Akt signaling pathway could transcriptionally induce Sox2 expression in SP cells; here we find that regulation of Sox2 by EGFR pathway may involve its cross talk with Hh signaling and Gli1 transcription factor [26]. EGFR-Pi3k/Akt pathway is also known to stabilize Gli proteins [38], and we have shown that stimulating the cells with EGF led to increased expression of Gli1 and Gli2 mRNA and protein. The present study also demonstrates a requirement of Hh pathway and Gli1 transcription factor in self-renewal of SP cells from EGFR–dependent NSCLC. Given this background, the results presented here show a clear rationale for combining Hh inhibitors with EGFR inhibitors to combat NSCLC. Our results show that combining these inhibitors or depletion of Gli1 decreases their viability as well as self-renewal. This is especially relevant in the case of H1975 cells, which harbor a T790M gatekeeper.
mutation and are generally refractory to EGFR inhibitors. Thus, targeting Hh pathway along with EGFR signaling could be a viable strategy to combat NSCLC, especially those that harbor EGFR mutations.

Conclusions
Although the Hh pathway has been implicated in stemness and lung cancer, the molecular mechanisms are still unclear. Our finding that Gli1 is a strong regulator of Sox2 transcription factor thus provides a mechanistic background to the role of Hh pathway especially in lung adenocarcinoma. Our study clearly demonstrates that Hh pathway and EGFR signaling cooperatively promote the stem-like functions of tumor-initiating cells of NSCLC through Sox2 transcription factor, and a combined therapeutic strategy may be beneficial in targeting the cancer stem-like cells and NSCLC.

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