NEOPLASIA
www.neoplasia.com

Volume 17 Number 7 July 2015 pp. 538–551 538

Gli1-Mediated Regulation of Sox2 Facilitates Self-Renewal of Stem-Like Cells and Confers Resistance to EGFR Inhibitors in Non–Small Cell Lung Cancer[1]

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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.

Neoplasia (2015) 17, 538–551

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]. Chemotherapeutic 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].

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[1] This study was partially supported by the grant CA139612 from the National Cancer Institute. Support of the Shared Resources at Moffitt Cancer Center is gratefully acknowledged.

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Received 21 October 2014; Revised 22 June 2015; Accepted 2 July 2015

1476-5586
http://dx.doi.org/10.1016/j.neo.2015.07.001

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 Smoothened, 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 1μM erlotinib, and PC-9-GR was maintained in culture media with 1μM gefitinib. All the cultures were maintained at 5% CO_2 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. Gefitinib (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 ALDH^{high} 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 1×10^6 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 ALDH^{high} cells as per manufacturer's protocol. In brief, cells were suspended in Aldefluor assay buffer containing Aldefluor substrate (1 μM) at 1×10^6 cells/ml density and incubated at 37°C for 45 minutes. To sort the Aldh^{high} and Aldh^{low} 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 Aldh^{high}) 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 (1×) (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

Observer Z.1 inverted microscope, and images were acquired using the AxioCam MRm3 CCD camera and Axiovision version 4.7 (Carl Zeiss Inc., Germany). The numbers of spheres greater than or equal to 50 μm were counted. To study the effect of the drugs on the self-renewal ability of SP cells, the appropriate concentrations were added to the respective wells on Day 0 and Day 5, and the size and number of the spheres were analyzed on Day 10. The sphere formation assays were performed twice with triplicates of each treatment in every assay.

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 calculated by $2^{-\Delta\Delta Ct}$ method. The primers are as follows:

Gli1 FP 5'-CCCAATCACAAGTCAGGTTCCT-3',
Gli1 RP 5'-CCTATGTGAAGCCCTATTTGCC-3',
Gli2 FP 5'-TGCACCAAGGAGTACGACAC-3',
Gli2 RP 5'-AGCATGTACTGCGCCTTGAA-3',
Gli2 FP 5'-TGCACCAAGGAGTACGACAC-3',
Gli2 RP 5'-AGCATGTACTGCGCCTTGAA-3',
FoxM1 FP 5'-TTTATCAGTGCTGCTAGCTGAGG-3',
FoxM1 RP 5'-TCTGAACTGGAAGCAAAGGAGA-3',
ABCG2 FP 5'-CACAAGGAAACACCAATGGCT-3',
ABCG2 RP 5'-ACAGCTCCTTCAGTAAATGCCTTC-3',
ALDH1 FP 5'-CCGCAAGACAGGCTTTTCAG-3',
ALDH1 RP 5'-CATTGTCGCCAGCAGCAGA-3',
Sox2 FP 5'-GGGAAATGGGAGGGGTGCAAAAGA-3',
Sox2 RP 5'-TTGCGTGAGTGTGGATGGGATTGG-3',
Oct4 FP 5'-ACATCAAAGCTCTGCAGAAAGAACT-3',
Oct4 RP 5 -CTG AAT ACC TTC CCAAAT AGA ACC C-3',
Nanog FP 5'-AGAAGGCCTCAGCACCTA-3',
Nanog RP 5'-GGCCTGATTGTTCCAGGATT-3',
GAPDH FP 5'-GGTGGTCTCCTCTGACTTCAACA-3',
GAPDH RP 5'-GTTGCTGTAGCCAAATTCGTTGT-3'

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://bioconductor.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 *P* 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) 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 Addgene [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'-TCCTGATTCCAGTTTGCCTC-3',
RP 5'-GGGAGAGGAGGAGGGGAG-3'

Cell Viability Assay

Cell viability was measured with thiazolyl blue tetrazolium bromide (MTT) after 48 hours of mentioned 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. $Aldh^{high}$ 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 1*G*). Also, the size of the spheres that formed was significantly smaller compared with the control siRNA transfected cells (Figure 1*E*). 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 1*F*). 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 ($P = .4$) 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 can 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 3*C*). 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

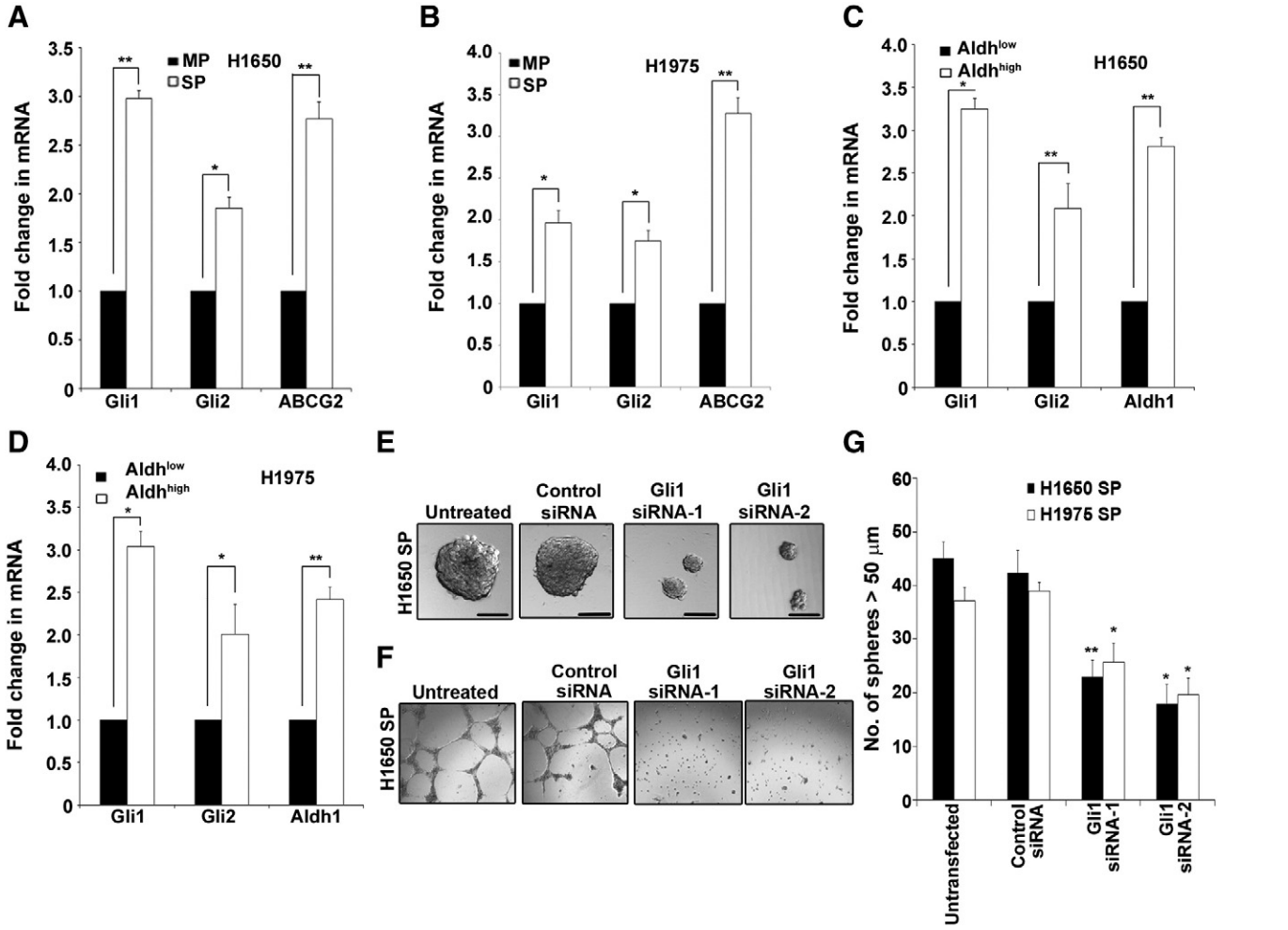

Figure 1. Expression of Gli1 and Gli2 in CSCs from NSCLC.(A-B) Real-time PCR analysis of mRNA from SP and main-population cells isolated from H1650 (A) and H1975 (B) cell lines reveals higher levels of *Gli1* and *Gli2* mRNA in SP cells. *ABCG2* mRNA expression is used as positive control for SP cell phenotype. (C-D) Higher levels of *Gli1* and *Gli2* mRNA are observed in isolated Aldh^{high} cells from H1650 (C) and H1975 (D) cells. The mRNA expression of *Aldehyde dehydrogenase-1* (*Aldh1*) is used as a positive control. (E) SP cells isolated from H1650 cells transfected with two different *Gli1* siRNAs form smaller spheres as compared with nontarget control siRNA. (F) *Gli1* siRNA treated H1650 SP cells show abrogation of formation of angiogenic tubule-like structures when grown on endothelial growth medium as compared with control siRNA–treated H1650 SP cells. (G) The quantitation of number of spheres generated shows fewer spheres in SP cells isolated from *Gli1* siRNA–treated H1650 and H1975 cell lines as compared with the control siRNA– treated cells.

transcription factors on *Sox2* proximal promoter region (−528 to +328) using a specific primer pair [37] (Figure 4*A*). The results show that Gli1 as well as Gli2 can bind to the *Sox2* proximal promoter region (Figure 4*B*). 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 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 4*E*). 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 4*F*) or H1975 cells (Figure 4*G*) 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

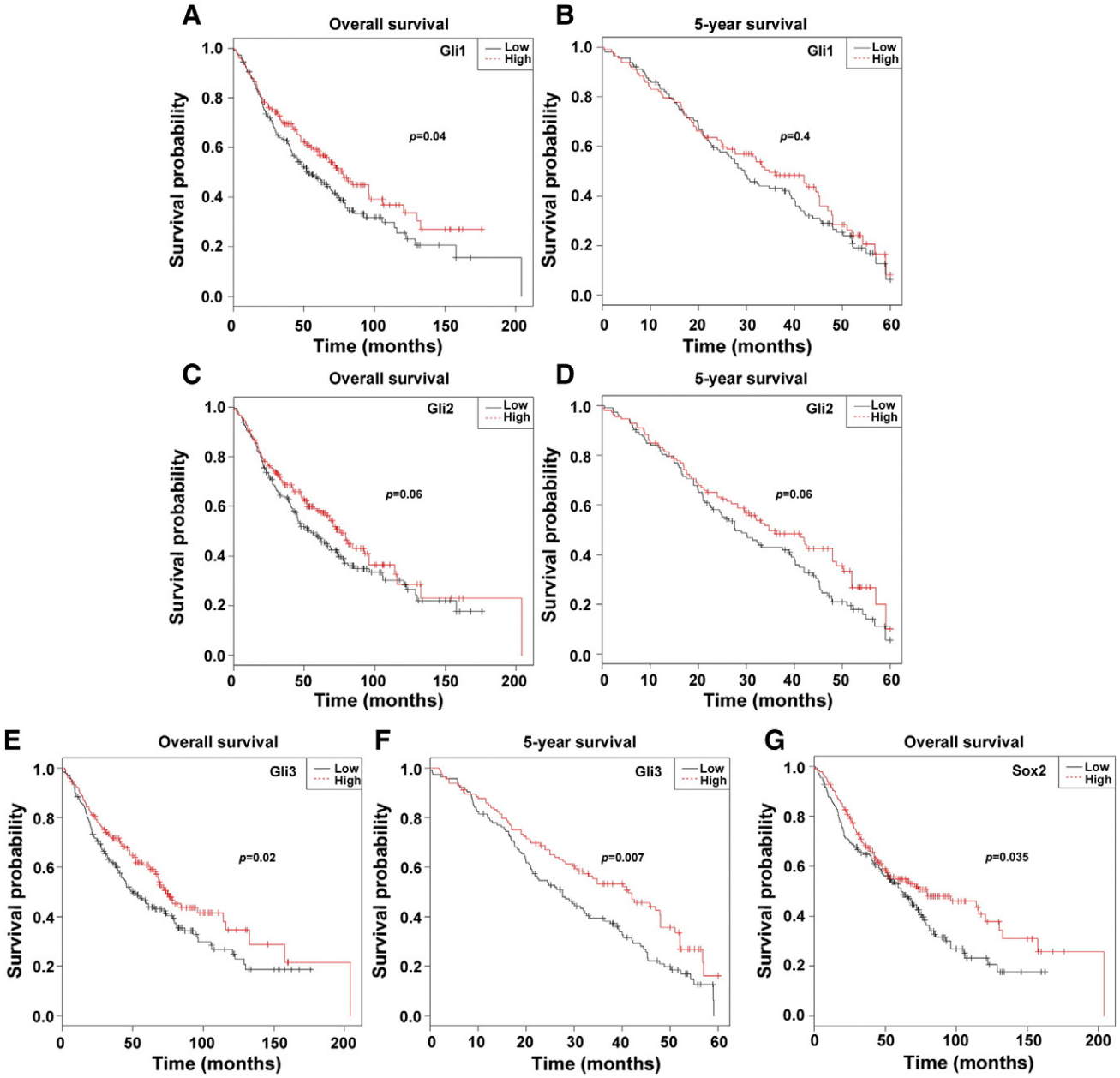

Figure 2. Kaplan-Meir survival curves for Gli family of transcription factors using the NCI Director's Challenge set.(A-F) Kaplan-Meier survival analysis (overall and 5-year) on the NCI Director's Challenge data set shows poor prognosis in patients with higher mRNA expression of Gli transcription factors. 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.

reporter. 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

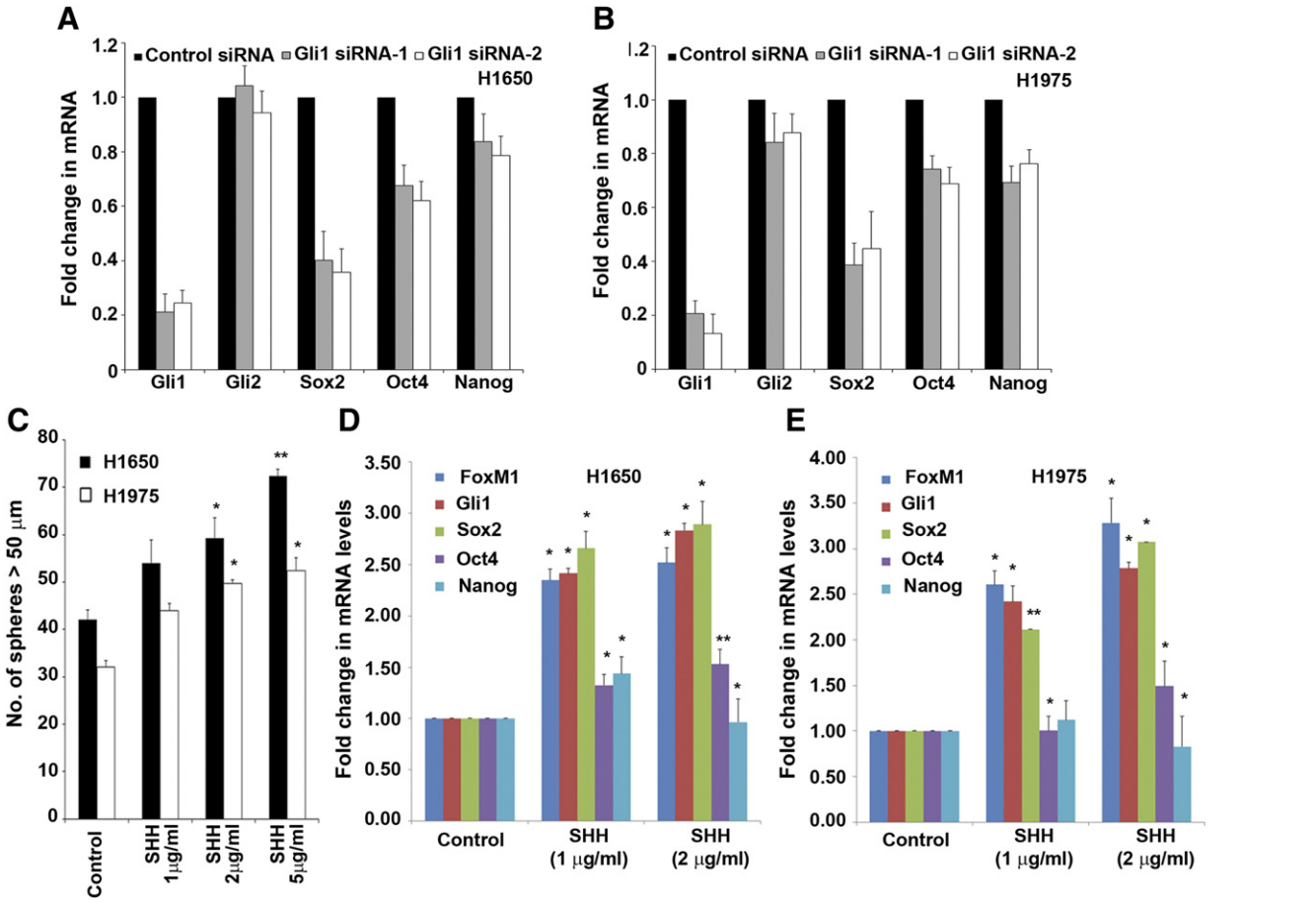

Figure 3. NSCLC EGFR mutant cells are responsive to exogenous SHH.(A-B) Real-time PCR analysis of two different *Gli1* siRNA–transfected H1650 (A) and H1975 (B) cells shows decrease in *Sox2*, *Oct4*, and *Nanog* mRNA expression. (C) A higher number of spheres are formed in the presence of increasing concentration of recombinant SHH in H1650 and H1975 SP cells as compared with untreated control SP cells in both cell lines. (D) Real-time PCR analysis showing that recombinant SHH protein treatment increases the levels of *Sox2, Oct4*, and *Nanog* mRNA expression. *FoxM1*, a known target of Hh pathway, is used as a positive control.

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 6*D*, 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 6*D*) 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

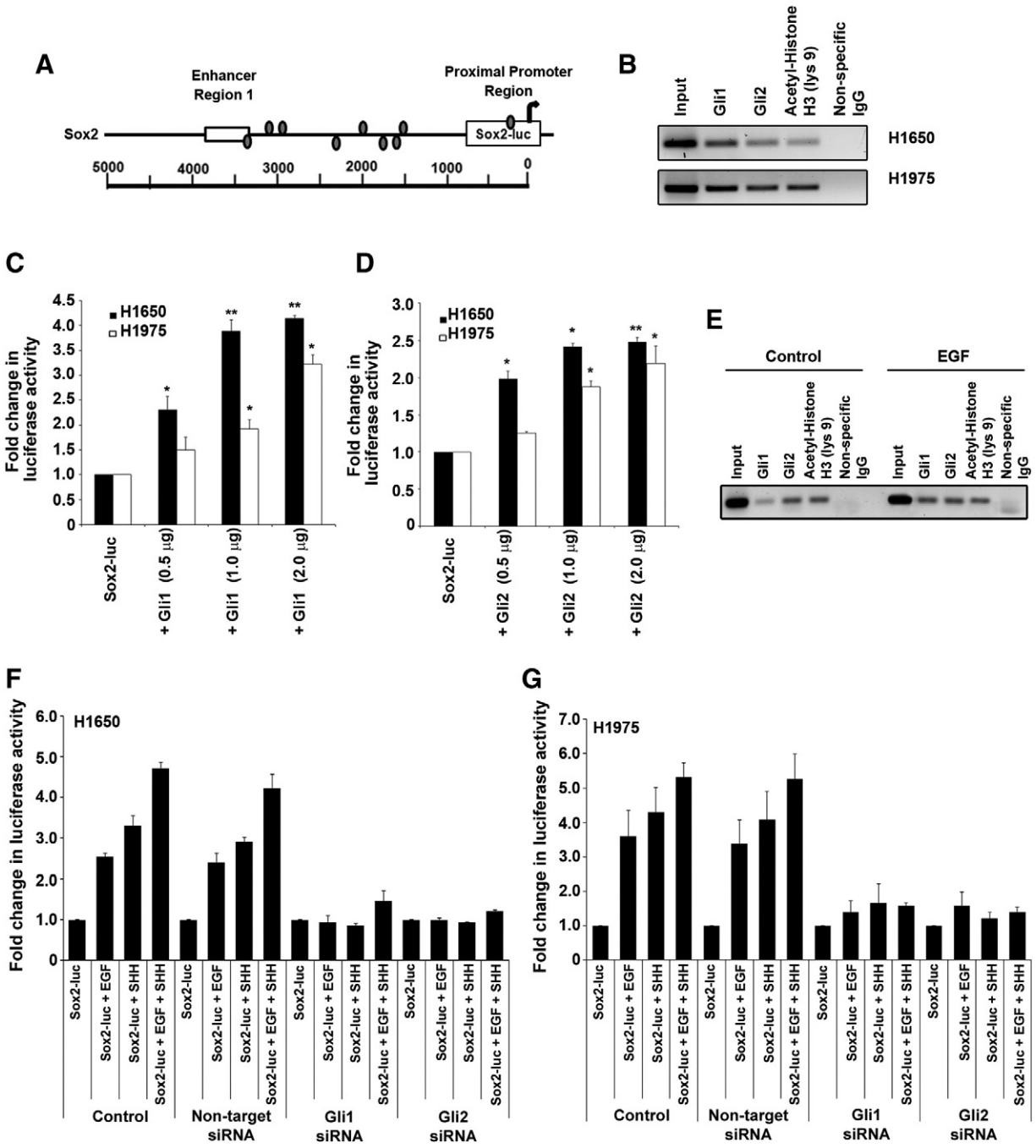

Figure 4. Sox2 gene expression is regulated by Gli1 and Gli2 transcription factors.(A) A schematic representing the *Sox2* proximal promoter region and the enhancer region with potential Gli binding sites as shown by filled ellipses. (B) ChIP assays performed on H1650 and H1975 show presence of Gli1 and Gli2 on *Sox2* proximal promoter region. Acetylated histone H3 (Lys9) was used as positive control and nonspecific IgG was used as the negative control for immunoprecipitation. (C-D) The luciferase reporter assay with *Sox2* proximal promoter reporter (Sox2-luc) co-transfected Gli1 (C) and Gli2 (D) expression vector shows an increase in luciferase activity in both H1650 and H1975 cells. (E) ChIP analysis conducted on EGF-treated cells after serum starvation shows an increase in Gli1 binding to the *Sox2* proximal promoter region. Serum-starved cells were used as control here. (F-G) Decrease in *Sox2* proximal promoter luciferase activity (Sox2-luc) is observed in H1650 (F) and H1975 (G) cells treated with *Gli1* and *Gli2* siRNA followed by EGF, recombinant SHH treatment, or a combination of the two; treatment of untransfected cells or those transfected with nontargeting siRNA was used as control.

suppress self-renewal (Figure 6*E*). 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

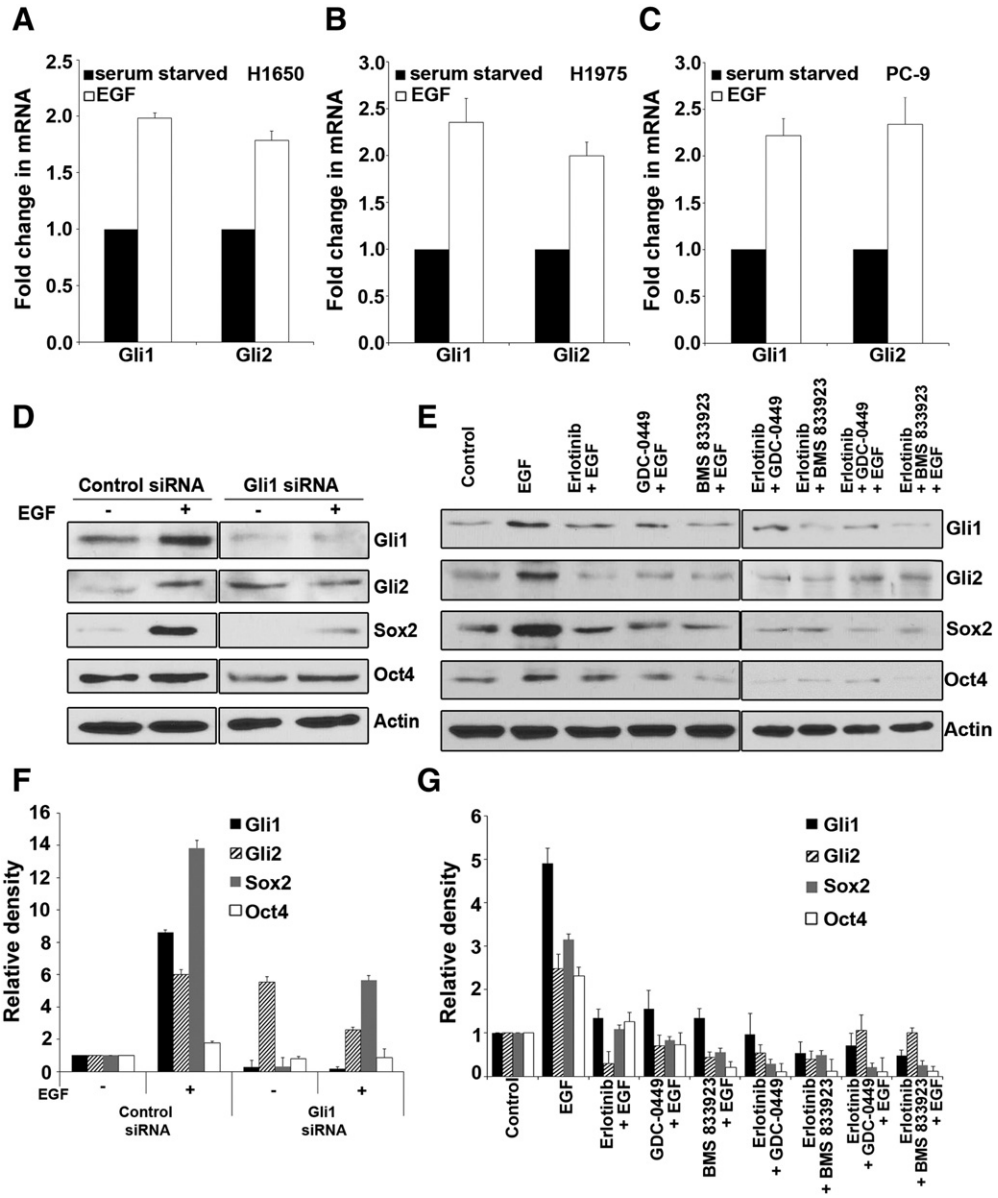

Figure 5. Cooperative induction of Sox2 expression by EGFR signaling and Gli proteins.(A-C) Real-time PCR analysis in serum-starved or EGF-treated H1650 (A), H1975 (B), and PC-9 (C) cells shows increase in *Gli1* and *Gli2* mRNA expression in EGF-treated cells as compared with serum-starved control cells. (D) Western blot analysis with lysates from H1650 cells treated with Gli1 siRNA followed by EGF stimulation showed a decrease in Sox2 and Oct4 protein expression as compared with EGF stimulation in control siRNA treatment. (E) Western blot analysis on lysates from H1650 cells pretreated with either EGFR inhibitor erlotinib or the Smoothened inhibitors GDC-0449 or BMS-833923, or a combination of the two, for 3 hours followed by EGF induction shows lower expression of Sox2 and Oct4 proteins as compared with EGF stimulation without any pretreatment. (F-G) Quantitative analysis of the Western blot in panel D (F) and panel E (G) using ImageJ software. The data are represented as relative density calculated over the loading control actin.

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 7*A*, 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 7*A*). Similar results were obtained in H1975 cells, which are less sensitive to gefitinib and erlotinib (Figure 7*B*); the

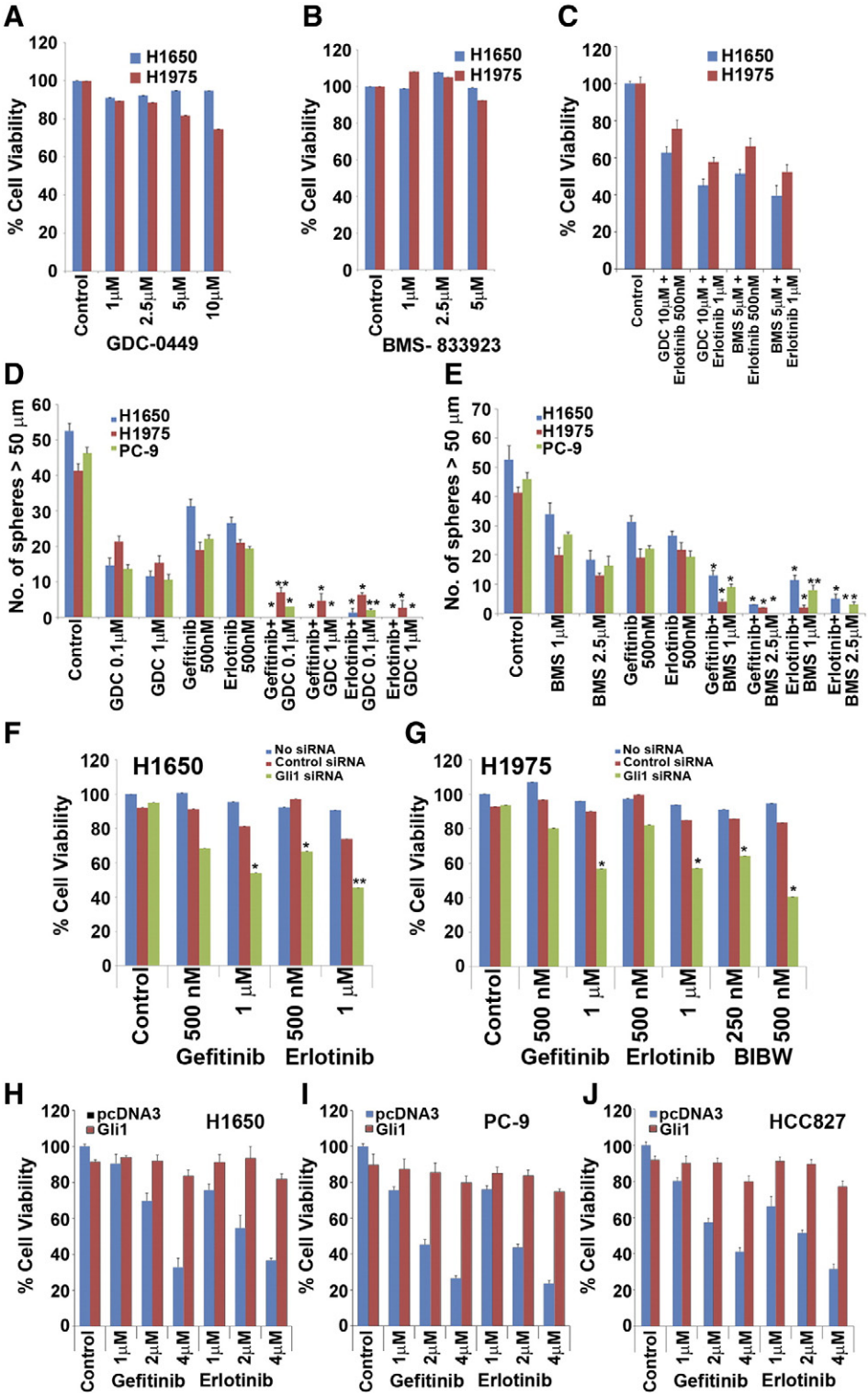

Figure 6. EGFR and Hh pathways cooperate to modulate self-renewal.(A-B) Cell viability assays performed on H1650 and H1975 cells with various concentrations of GDC-0449 (A) and BMS-833923 (B). (C) Cell viability assay performed on both H1650 and H1975 cells with a combination of GDC-0449 and erlotinib 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 7*B*). 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 Oct4 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 Smoothened (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 Mcl1 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 [19–21,23]. 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

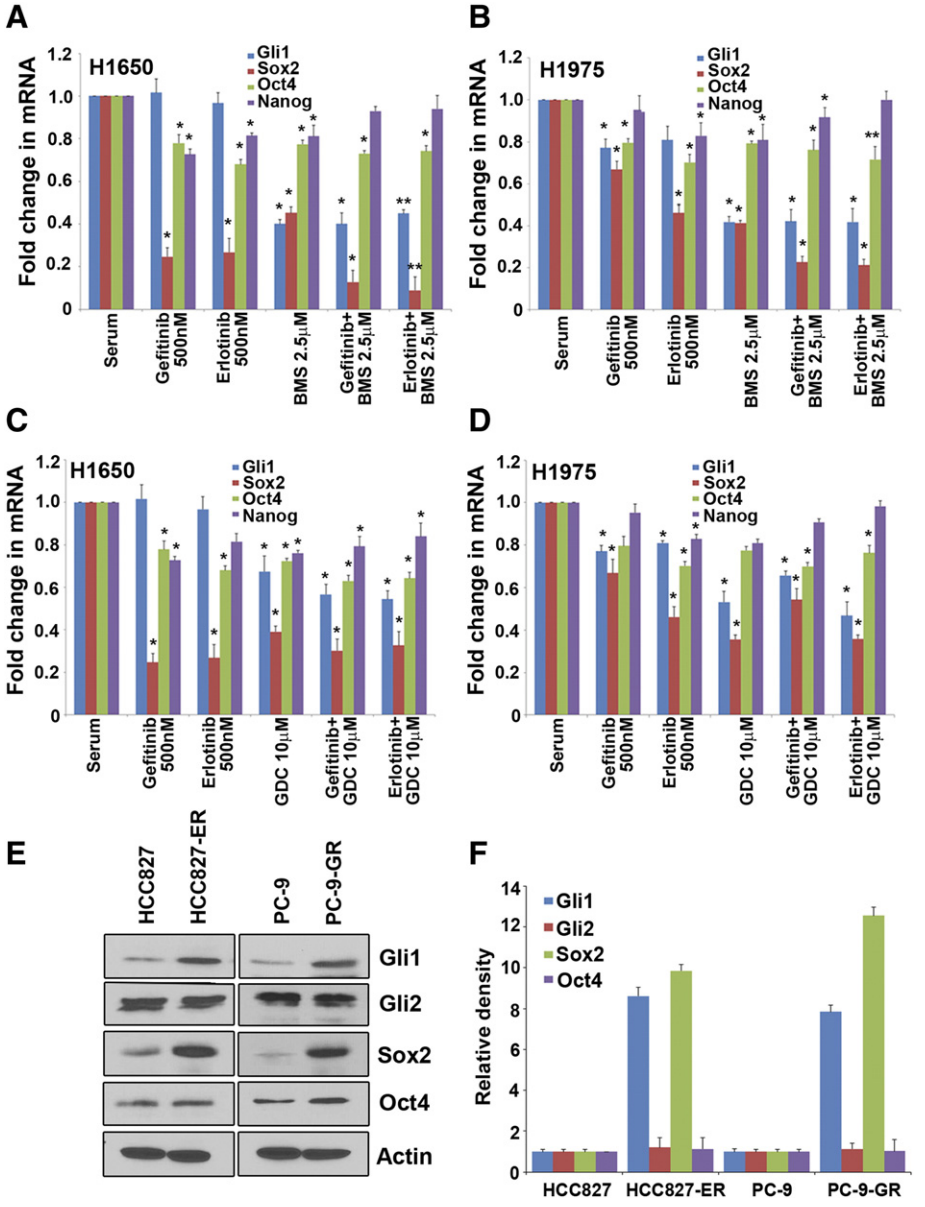

Figure 7. Decrease in stem cell transcription factors with combined inhibition of EGFR signaling and Hh pathway.(A-B) Real-time PCR analysis of mRNA expression of stem cell transcription factors like *Sox2*, *Oct4*, and *Nanog* shows a marked decrease in their levels in both H1650 (A) and H1975 (B) cells when treated with combination of EGFR inhibitors gefitinib and erlotinib with Smoothened inhibitor BMS-833923. (C-D) Real-time PCR analysis of mRNA expression in H1650 (C) and H1975 (D) cells shows a similar effect on *Sox2, Oct4*, and *Nanog* mRNA levels when the cells are treated with gefitinib or erlotinib in combination with Smoothened inhibitor GDC-0449. (E-F) Western blot analysis with erlotinib-resistant HCC827 (HCC827-ER) and gefitinib-resistant PC-9 (PC-9-GR) shows increase in Gli1 and Sox2 protein expression as compared with the parental cells (E). A quantitative analysis of the Western blot performed using the ImageJ software (F). The data are represented as relative density calculated over the loading control actin.

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.

Acknowledgements

We thank Eric Haura and Fumi Kinose for providing the *in vitro*–developed drug-resistant cell lines HCC827-ER and PC-9-GR. We thank Smitha Pillai for helpful discussions and Tyler Keeley and Rebecca Swearingen for experimental assistance. This study was

partially supported by the grant CA139612 from the National Cancer Institute. Support of the Shared Resources at Moffitt Cancer Center is gratefully acknowledged.

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