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## SOX2 expression is an early event in a murine model of EGFR mutant lung cancer and promotes proliferation of a subset of EGFR mutant lung adenocarcinoma cell lines

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### Abstract

**Objectives**—Primary and acquired resistance to EGFR TKIs in EGFR mutant lung cancer occurs primarily through secondary mutations in EGFR or Met amplification. Drug resistance can also be mediated by expression of pluripotency transcription factors, such as OCT4, SOX2 and NANOG that decrease terminal differentiation. In this study, we investigated the expression and role of SOX2 in model systems of EGFR mutant tumors.

**Materials and Methods**—Immunoblotting or immunohistochemistry was used to assess expression of pluripotency transcription factors in lungs of transgenic mice or in human NSCLC cell lines. Expression of SOX2 was reduced by shRNA knockdown, and response to erlotinib and cellular proliferation were assessed.

**Results and Conclusion**—Induction of mutant EGFR in transgenic CCSP-rtTA/TetO-EGFR<sup>L858R/T790M</sup> mice correlated with increased OCT4 and SOX2 expression in lung tissue prior to tumor development. Established lung tumors retained SOX2 expression. To assess a role for SOX2 in tumorigenesis, a panel of NSCLC cell lines with activating EGFR mutations was assessed for SOX2 expression. Two of six cell lines with mutant EGFR showed detectable SOX2 levels, suggesting SOX2 expression did not correlate with EGFR mutation status. To assess the role of SOX2 in these cell lines, HCC827 and H1975 cells were infected with lentivirus containing SOX2 shRNA. Knockdown of SOX2 decreased proliferation in both cell lines and increased sensitivity to erlotinib in HCC827 cells. Because constitutive activation of the PI3K/Akt pathway

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Conflict of interest statement.- SOX2 is expressed and promotes proliferation in a subset of EGFR mutant lung adenocarcinomas. None declared.

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is associated with EGFR TKI resistance, cells were treated with PI3K/AKT inhibitors and expression of SOX2 was examined. PI3K/Akt inhibitors decreased SOX2 expression in a time-dependent manner. These data suggest targeting SOX2 may provide therapeutic benefit in the subset of EGFR-mutant tumors with high constitutive levels of SOX2, and that until more direct means of inhibiting SOX2 are developed, PI3K/Akt inhibitors might be useful to inhibit SOX2 in EGFR TKI resistant tumors.

### Keywords

SOX2; non-small cell lung cancer; EGFR; Akt

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### Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide and new therapeutic strategies are needed [1]. In lung cancer, drugs that target tumor-specific mutations in EGFR, MET or ALK are effective in a subset of patients [2, 3]; however, acquired resistance develops in nearly all patients. Thus the identification and development of strategies to prevent resistance are needed to achieve further improvements in survival. The EGFR TKI erlotinib improves progression-free survival in patients with activating mutations in EGFR over chemotherapy [4], and was recently approved by the FDA as a first line treatment in these patients. Resistance mechanisms to EGFR TKIs include mutations in EGFR that change affinity for EGFR TKI (T790M) that occur in 50% of patients, and MET amplification that occurs in 20% of patients [5-7]. Additional mechanisms of resistance include EMT, histologic transformation from adenocarcinoma to small cell lung cancer (SCLC) [8] and activation of IGF1R signaling. Another mechanism of resistance believed to play a role in lung cancer is the acquisition of a dedifferentiated or 'cancer stem cell' (CSC) phenotype. The cancer stem cell hypothesis holds that only a rare subpopulation of tumor cells are capable of forming the full range of differentiated cells that make up a tumor. These cells can self renew, cycle slowly and exhibit drug resistance. In certain tumors the CSC population is believed fixed and a cellular hierarchy exists. In others, the phenotype appears to be plastic and may increase upon the acquisition of additional mutations or selective pressures in the tumor microenvironment.

Transcription factors such as SOX2, OCT4, NANOG, KLF4, LIN28 and c-MYC can reprogram somatic cells into pluripotent embryonic stem cells [9]. In lung cancer, overexpression of pluripotency genes contributes to drug resistance and poor prognosis [10-13].

The PI3K/Akt signaling pathway controls cell proliferation, survival, metabolism and drug resistance and is constitutively activated in a majority of human cancers, including NSCLC [14]. Activation of the PI3K/Akt pathway promotes resistance to EGFR TKI. Here, we investigate the role of pluripotency transcription factors and their relationship to Akt signaling and erlotinib sensitivity in mutant EGFR-driven lung cancer.

## Materials and Methods

### Cell culture

Human NSCLC cell lines with activating EGFR mutations, PC9 (exon 19 deletion), HCC827 and HCC827 GR5 (E746\_A750 deletion), H820 (L858R) and H1975 (L858R) were used. H1975 and H820 cells also contain T790M mutation at exon 20 of the *EGFR* gene. HCC827 GR5 cells are EGFR TKI resistant and have *MET* amplification [7]. All cells were maintained in RPMI 1640 containing 5% or 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere.

### SOX2 shRNA knockdown

pLKO.1 lentivirus containing constitutive or doxycycline-inducible shRNA against Sox2 and scrambled control construct (Sigma) were packaged in 293FT cells using PsPAX2 and pMDG.2 packaging plasmids from Addgene. Cells were grown to 70–80% confluence in a T25 flask and transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY). Media was changed following overnight infection, and viral supernatants were collected after 24h and stored at -80°C for future infections.

### Immunoblotting

Cells were plated at a density of  $5 \times 10^5$  per well in six-well plates. The following day, cells were treated with drug or equal volume of DMSO for the indicated times. Cell extracts were prepared by adding 2× Laemmli sample buffer supplemented with a phosphatase and protease inhibitor cocktail [15]. Lysates were sonicated and the protein concentration was quantified using the bicinchoninic acid protein assay. Equivalent protein was loaded and proteins were separated by SDS-PAGE then transferred to 0.45-μm nitrocellulose membranes. Equivalent loading was confirmed by staining membranes with fast green as previously described [15]. Membranes were blocked for 1 h in blocking buffer (5% milk, 1× TBS, 0.1% Tween 20) and placed in primary antibody (5% bovine serum albumin, 1× TBS, 0.1% Tween 20) overnight at 4°C. The following day, membranes were washed thrice in wash buffer (1× TBS, 0.1% Tween 20). Primary antibodies against P-Akt, Oct4 (#2788), P-EGFR (Y1068) (#3777), total EGFR (#2232), EGFR L858R (#3197), P-stat3 Y705 (#9145), SOX2 (#3728) from Cell Signaling (Danvers, MA) and an alpha-tubulin antibody #T5168 from Sigma (St. Louis, MO) were detected using horseradish peroxidase–linked secondary antibodies and visualized with the enhanced chemiluminescent detection system (GE Healthcare Biosciences, Pittsburgh, PA). Immunoblot experiments were performed at least thrice.

### Immunohistochemistry

Immunohistochemistry was performed on lungs harvested from Dox- treated mice. Lung tissues were fixed in formalin, embedded in paraffin blocks and sectioned to slides. Briefly, slides were heated at 65°C and then placed through xylene and ethanol washes. Antigen retrieval was performed using target retrieval solution (Dako, Carpinteria, CA) and a decloaking chamber. Sections were incubated with (1:50) SOX2 antibody (Cell Signaling

#3728) overnight at 4°C. Detection was done using a VECTASTAIN Elite ABC kit (Vector Laboratories).

### Proliferation assay

The effect of erlotinib on proliferation was determined using the sulforhodamine B (SRB) assay. Cells were plated at a density of 5,000 per well in 96-well plates in RPMI 1640 + 5% FBS and allowed to grow for 24 h in a humidified 5% CO<sub>2</sub> incubator at 37°C. Cells were incubated with drugs for 48h. Following drug treatments cells were fixed with 10% trichloroacetic acid for 1h at 4°C. Cells then were washed 5 times with distilled and de-ionized water. After air drying, cells were stained with a 0.4% (w/v) solution of SRB in 1% acetic acid. Cells were then washed with 1% acetic acid 5 times to remove the unbound dye. To redissolve the dye, 10 mM Tris solution was added and the absorbance was read at 515 nm. Cell survival was calculated as a percentage of the value of DMSO-treated controls minus the value of untreated cells on day 0. Experiments were done in triplicate and each drug concentration was evaluated in sextuplet wells for any given experiment.

### Animal study

Animal studies were conducted under a protocol approved by the Animal Care and Use Committee of the National Cancer Institute. The generation of bi-transgenic mice harboring the *CCSP-rtTA* and TetO-regulated *EGFR<sup>L858R+T790M</sup>* transgenes has been previously described [16]. To induce expression of mutant EGFR, mice were fed doxycycline-impregnated food pellets (NIH-31 with 650 ppm doxycycline, Harlan-Teklad, Madison, WI). Beginning at 6 weeks of age, groups of 3 mice received doxycycline (DOX) food or regular cereal diet. Mice were sacrificed after 3 or 9 weeks. At sacrifice, lungs were removed and inflated with 10% neutral-buffered formalin. Lungs were embedded in paraffin and sectioned for immunohistochemistry.

### Results

To determine whether pluripotency genes play a role in mutant EGFR-driven lung tumorigenesis, we examined the expression of OCT4 and SOX2 in lungs of *CCSP-rtTA/TetO-EGFR<sup>L858R+T790M</sup>* mice by immunoblotting analysis. Mice fed doxycycline (dox) in their diet displayed increased expression of phospho- and mutant-EGFR in whole lung lysates compared to mice that received a normal diet (Figure 1A). Doxycycline also induced lung tumors after 3 weeks of treatment, whereas mice on a regular diet had normal lung phenotypes (data not shown). The levels of phosphorylated AKT and FOXO were evaluated as readouts of EGFR signaling and were increased in the groups that received doxycycline. Furthermore, SOX2 and OCT4 expression increased with dox administration compared to the control diet. To investigate if EGFR mutant lung tumors expressed SOX2, lungs of mice that received dox were stained for SOX2 by immunohistochemistry (IHC). IHC of mouse lungs showed that tumors displayed both nuclear and cytoplasmic SOX2 (Figure 1B). In addition, cells lining bronchioles stained positive for SOX2, whereas alveolar cells were negative. This finding is consistent with SOX2 expression in clara, goblet and ciliated cells, as previously reported [17]. To determine if SOX2 and OCT4 expression were maintained in tumors when EGFR signaling was decreased, mice were given doxycycline to induce

tumors, then switched to a normal diet for 2 and 5 days. EGFR expression and activation were rapidly reduced when dox was withdrawn, which correlated with decreased SOX2 and OCT4 expression (data not shown). These data show that EGFR signaling correlates with SOX2 expression in lung tumors.

To examine the relationship of SOX2 to EGFR mutation status, SOX2 levels were analyzed in a panel of human lung cancer cells with activating EGFR mutations that are either EGFR TKI sensitive (PC9, HCC827) or resistant (H1975, H820, HCC827 GR5, H1650) (Figure 2). (A549 and H522 are Kras mutant NSCLC cell lines that were used as positive controls.) Of the six EGFR mutant cell lines, only H1975 and HCC827 cells had detectable levels of SOX2 by immunoblotting analysis. Therefore, SOX2 expression did not appear to correlate with EGFR mutation status or TKI sensitivity in these cell lines.

To examine the effect of EGFR signaling on SOX2 in human cell lines, EGFR mutant cell lines were treated with erlotinib (Figure 3A). At 3 days, erlotinib decreased p-EGFR and p-Akt levels in HCC827 cells, and this correlated with a decrease in SOX2, OCT4, and NANOG expression. In contrast, erlotinib had a minimal or no impact on p-EGFR and p-Akt in H1975 cells, likely due to the presence of a T790M mutation. In H1975 cells, expression of the three pluripotency markers increased with 5  $\mu$ M erlotinib. At 25  $\mu$ M, expression of p-EGFR and SOX2 were decreased. Phospho-Akt was not decreased by erlotinib in H1975 cells. These experiments show that erlotinib can decrease p-EGFR and SOX2 in EGFR TKI resistant and sensitive cells in a dose dependent manner.

The PI3K/AKT pathway increases cell survival and therapeutic resistance in cancer [18], and Akt has been shown to phosphorylate and stabilize SOX2 [19]. To determine if SOX2 is under the control of the PI3K/AKT pathway in these cells, H1975 and HCC827 cells were treated with a PI3K inhibitor (LY294002) or an AKT inhibitor (triciribine) (Figure 3B). Phosphorylation of AKT and FOXO decreased with inhibitor treatment, which correlated with decreased SOX2 expression. This suggests that SOX2 is under the control of PI3K/AKT pathway in these cells.

To more directly evaluate the role of SOX2 expression in EGFR mutant lung cancer, cells that had high SOX2 levels were infected with lentivirus containing doxycycline-inducible shRNA to SOX2 or dox-inducible scrambled shRNA. Following antibiotic selection, cell proliferation was evaluated by counting cells grown in the presence or absence of dox over time (Figure 4A). In the presence of dox, the shSOX2 RNA infected cells grew more slowly, but cells infected with the scrambled shRNA grew at the same rate, indicating SOX2 plays a role in proliferation. Next, we tested the erlotinib sensitivity of NSCLC cells infected with lentivirus to scrambled (scr) or SOX2 shRNA. Stable SOX2 knockdown increased erlotinib sensitivity in HCC827, but had little impact on growth inhibition by erlotinib in H1975 cells (Figure 4B). This shows that the ability of SOX2 to control sensitivity to erlotinib is context dependent. This may be relevant to H1975 cells since they also have Met amplification as well as a T790M mutation in EGFR. Collectively, our data suggest that SOX2 is under the control of the PI3K/Akt pathway in EGFR mutant lung cancer cells and contributes to proliferation in EGFR mutant lung tumors with high SOX2 levels.

## Discussion

Tumor genotype is an important determinant of lung cancer initiating cells [20]. Although the cell of origin for Kras mutant lung tumors has been proposed [21], the cell or origin for EGFR mutant lung cancer remains poorly defined [20]. Here we investigated the expression of pluripotency transcription factors in a mouse model of mutant EGFR-driven lung cancer. We show that SOX2 and OCT4 expression correlate with EGFR signaling in lung tissue of mutant CCSP-rtTA/TetO-EGFR<sup>L858R/T790M</sup> mice, and that mutant EGFR-driven lung tumors in this model express SOX2. In human NSCLC cell lines, EGFR mutation status or TKI sensitivity did not correlate with SOX2 expression. For example, whereas HCC827 cells had high SOX2 expression, MET-amplified HCC827 GR5 cells that were made resistant to EGFR TKIs had low levels of SOX2. Interestingly, MET expression has been inversely correlated with SOX2 expression in human EGFR mutant lung cancers [22]. Therefore, acquisition of MET amplification might confer SOX2 independence and might be a negative predictor of SOX2 expression in EGFR mutant NSCLC.

In this study, knockdown of SOX2 slowed the proliferation of HCC827 and H1975 cells suggesting that SOX2 plays a role in proliferation. This finding is similar to the role of SOX2 in other histologic types of lung cancer. For example, SOX2 is amplified and overexpressed in 23% of squamous cell carcinomas and 27% of small cell lung cancer [23-26]; in both subtypes SOX2 promotes proliferation, whereas in squamous cell carcinoma SOX2 overexpression also promotes migration and anchorage-independence [23]. Because SOX2 expression has been linked to drug resistance [27-29], we examined the effect of SOX2 expression on erlotinib sensitivity in these cells. SOX2 knockdown increased the sensitivity to erlotinib in EGFR TKI sensitive HCC827 cells, but had a much smaller effect on erlotinib sensitivity in EGFR TKI resistant H1975 cells.

What mechanisms could explain the context dependency and complexity of regulation of SOX2 by EGFR? In cancer SOX2 is regulated by androgen, TGF beta, chemotherapy or hypoxia [30-33], and there is evidence that EGFR and SOX2 can regulate each other. In neural progenitor cells, EGFR signaling causes SOX2 to bind to the EGFR promoter that drives its expression [34] and EGFR-Hedgehog/Gli signaling induces SOX2 expression in pancreatic cancer initiating cells [35]. In NSCLC, Singh *et al* showed that EGFR is upstream of SOX2 in the side population [36], and several studies have suggested SOX2 expression plays a role in lung tumor-initiating cells [37] [12] [38, 39]. However, it is unclear if EGFR controls SOX2 in differentiated tumor cells, since in our study only 2 cell lines with constitutively active EGFR had high SOX2 expression. Because PC9, H820, H1650 all had low SOX2 expression, it is possible that mutations in other pathways related to pluripotency may drive the increased SOX2 expression in HCC827 and H1975 cells. According to the Cancer Cell line encyclopedia [40], these cell lines harbor mutations in members of the notch, sonic hedgehog and nuclear receptor pathways; (HCC827-mutations in NCOA3, MAML3, PTCH1 and H1975-mutations in NCOA3 [41], GLI1, Notch4 and MAML2+3).

Although our experiments were performed using pre-clinical models that may not be representative of human disease, our results suggest SOX2 may be a therapeutic target in EGFR mutant lung cancers that overexpress SOX2. Drugs designed to target transcription



factors are rare, and blocking upstream signaling pathways that regulate them might be a practical approach to reduce their activity. In this regard, our results demonstrate that the PI3K/AKT pathway regulates SOX2 and that inhibitors of this pathway may be useful to decrease SOX2 expression in EGFR TKI sensitive or resistant NSCLC.

## Acknowledgments

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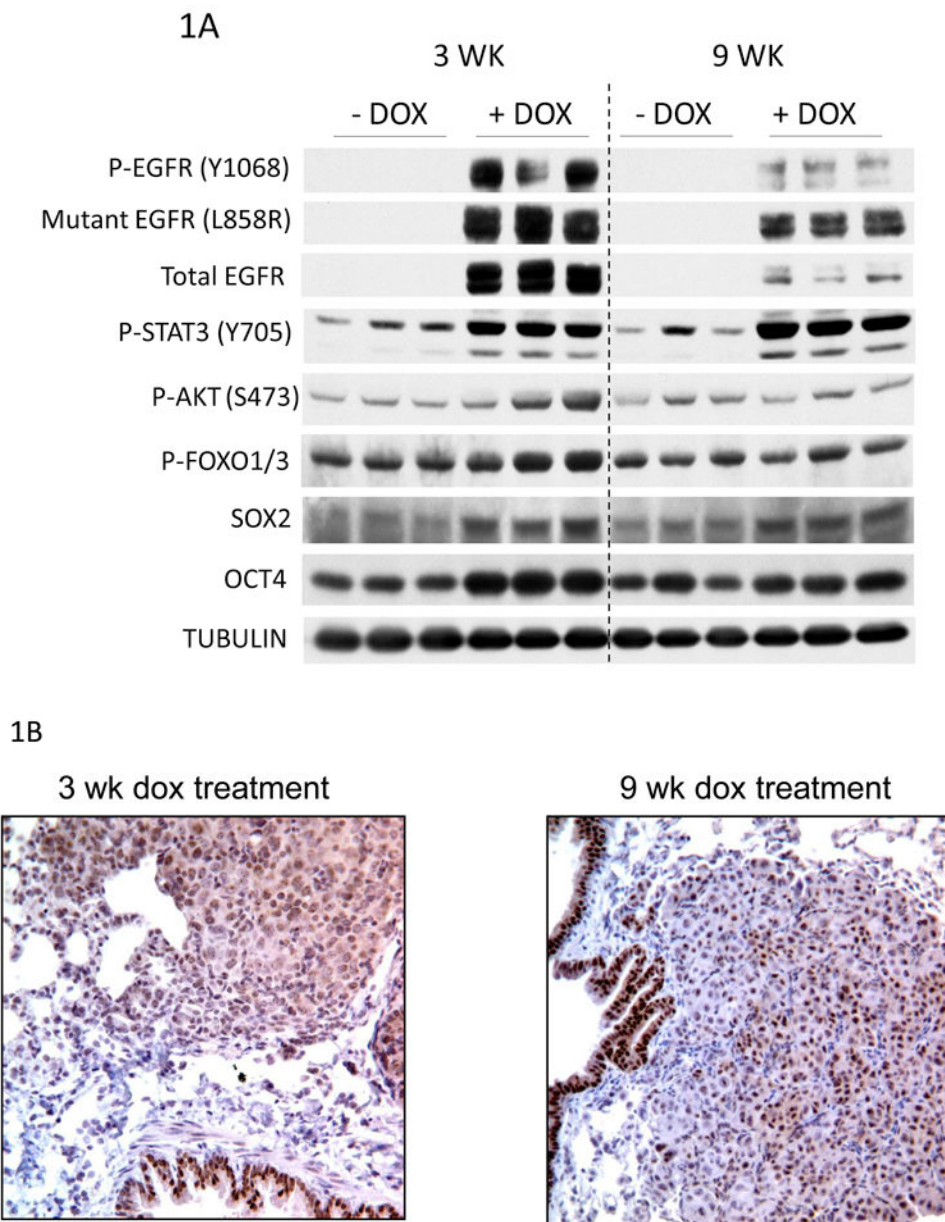
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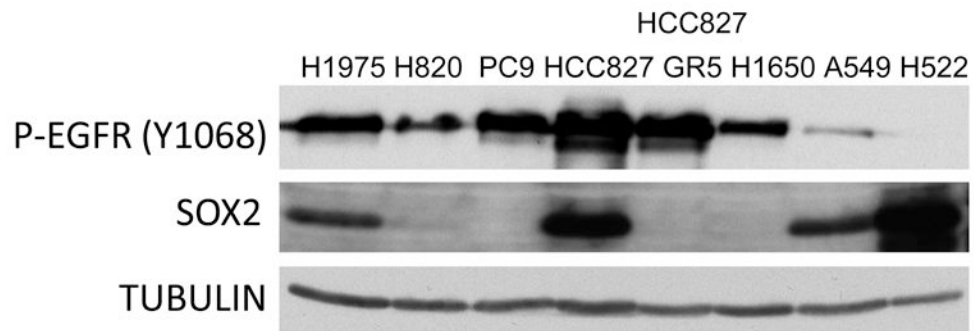
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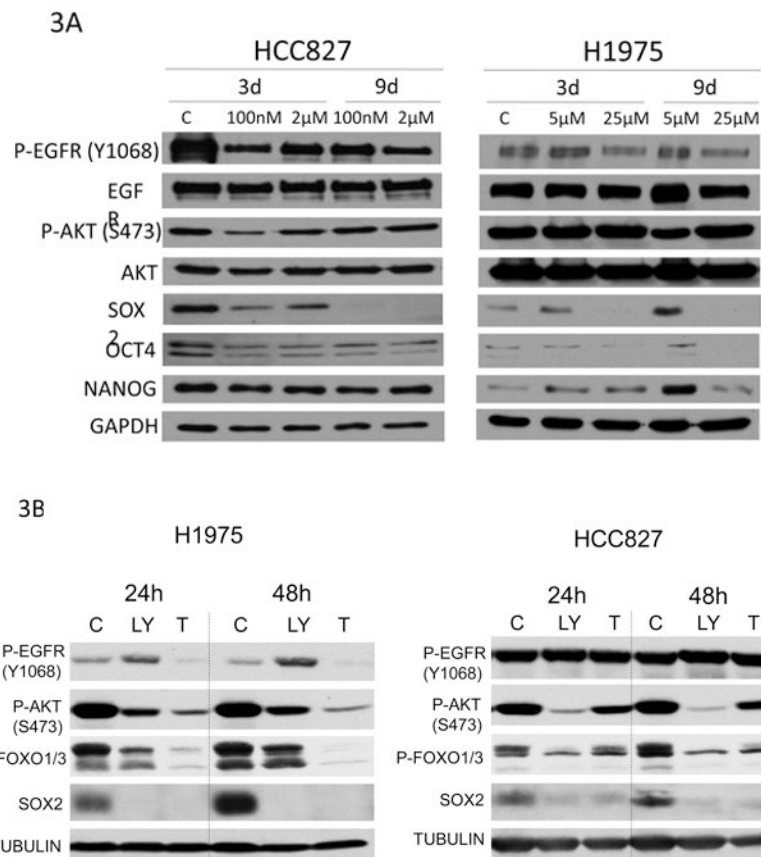
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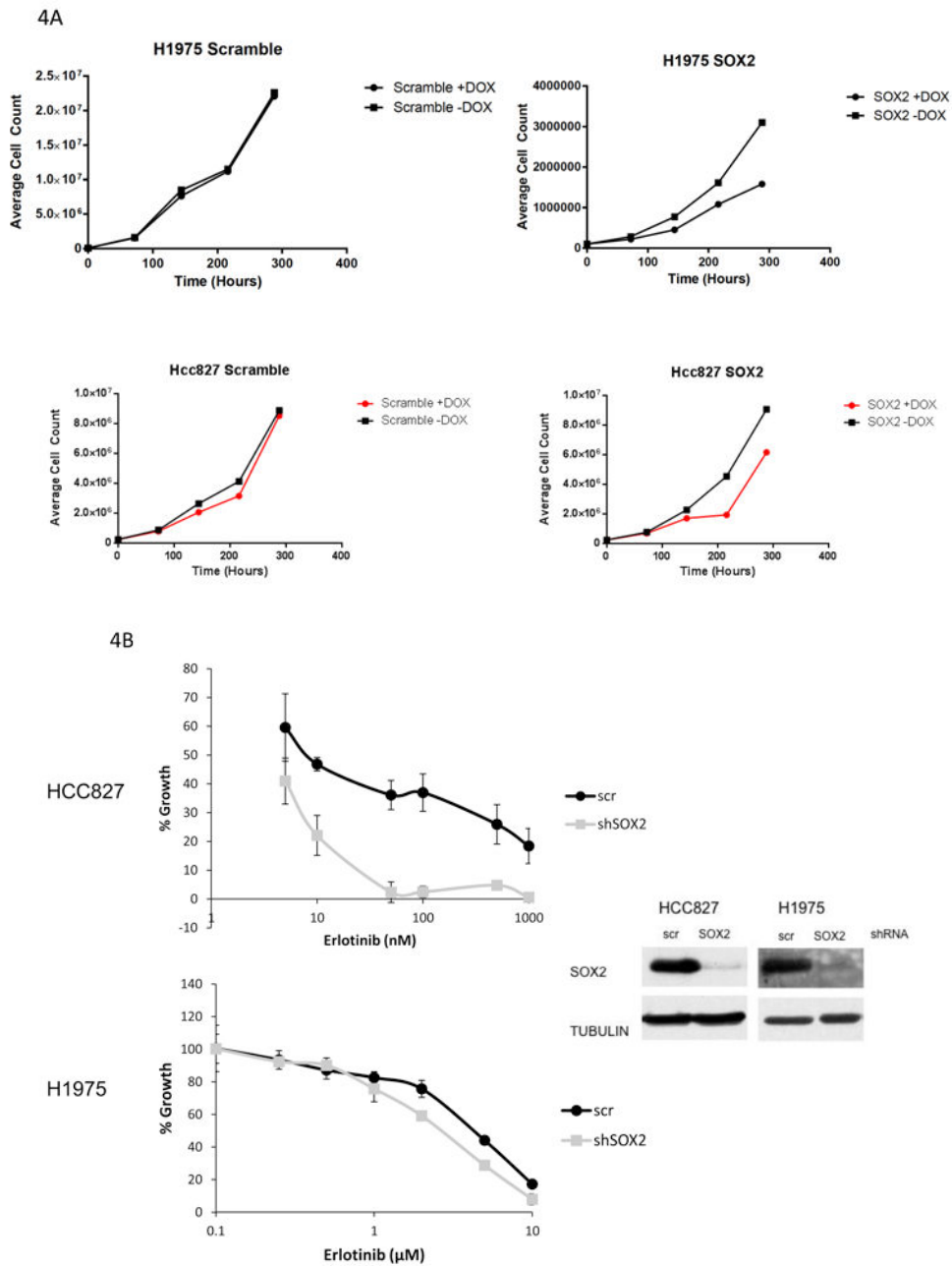
**Figure 1.** Elevated EGFR signaling correlates with SOX2 expression in lung tissues *in vivo*. A) Immunoblots of whole lung lysates of CCSP-rtTA/TetO- EGFR<sup>L858R+T790M</sup> mutant mice treated with DOX for 3 and 9 weeks. B) Immunohistochemical staining of CCSP-rtTA/TetO-egfr<sup>L858R+T790m</sup> mouse lungs show SOX2 expression in tumors after 3 and 9 weeks of DOX administration.



**Figure 2.** Basal levels of SOX2 vary in human NSCLC cell lines. Immunoblot analysis of human NSCLC cell lines with activating mutations in EGFR (H1975, H820, PC9, HCC827, HCC827 GR5, H1650) or Kras (A549, H522).

**Figure 3.**

A) Erlotinib alters the expression of SOX2 and OCT4 in EGFR mutant NSCLC cell lines. Immunoblot analysis of H1975 and HCC827 cells treated with varying doses of erlotinib for 3 and 9 days. C=vehicle control. B) PI3K/AKT pathway inhibitors decrease SOX2 expression in NSCLC cell lines. Immunoblot analysis of H1975 (left) and HCC827 (right) cells treated with DMSO (C), 10μM LY294002 (LY) or 10μM tricibine (T) for the indicated times.



**Figure 4.** Effect of SOX2 knockdown in EGFR mutant lung cancer cell lines. A) Sox2 knockdown decreases proliferation in HCC827 and H1975 cells. B) Sox2 knockdown increases sensitivity to erlotinib in HCC827 cells.