JNK Signaling Mediates EPHA2-Dependent Tumor Cell Proliferation, Motility, and Cancer Stem Cell–like Properties in Non–Small Cell Lung Cancer

Wenqiang Song1,2, Yufang Ma3, Jialiang Wang3,4, Dana Brantley-Sieders2, and Jin Chen1,2,4,5,6

Abstract

Recent genome-wide analyses in human lung cancer revealed that EPHA2 receptor tyrosine kinase is overexpressed in non–small cell lung cancer (NSCLC), and high levels of EPHA2 correlate with poor clinical outcome. However, the mechanistic basis for EPHA2-mediated tumor promotion in lung cancer remains poorly understood. Here, we show that the JNK/c-JUN signaling mediates EPHA2-dependent tumor cell proliferation and motility. A screen of phospho-kinase arrays revealed a decrease in phospho-c-JUN levels in EPHA2 knockdown cells. Knockdown of EPHA2 inhibited p-JNK and p-c-JUN levels in approximately 50% of NSCLC lines tested. Treatment of parental cells with SP600125, a c-JUN-NH2-kinase (JNK) inhibitor, recapitulated defects in EPHA2-deficient tumor cells, whereas constitutively activated JNK mutants were sufficient to rescue phenotypes. Knockdown of EPHA2 also inhibited tumor formation and progression in xenograft animal models in vivo. Furthermore, we investigated the role of EPHA2 in cancer stem–like cells (CSC). RNA interference-mediated depletion of EPHA2 in multiple NSCLC lines decreased the ALDH+ cancer stem–like population and tumor spheroid formation in suspension. Depletion of EPHA2 in sorted ALDH+ populations markedly inhibited tumorigenicity in nude mice. Furthermore, analysis of a human lung cancer tissue microarray revealed a significant, positive association between EPHA2 and ALDH expression, indicating an important role for EPHA2 in human lung CSCs. Collectively, these studies revealed a critical role of JNK signaling in EPHA2-dependent lung cancer cell proliferation and motility and a role for EPHA2 in CSC function, providing evidence for EPHA2 as a potential therapeutic target in NSCLC. Cancer Res; 1–11. ©2014 AACR.

Introduction

Recent technological advances in analyzing the human cancer genome permit the study of gene copy number, expression level, and mutation status in human tumor tissue. These studies uncovered previously unknown genetic alterations and aberrant expression in the genes encoding Eph receptor tyrosine kinases (RTK; refs. 1, 2). EPH receptors belong to a large family of RTKs. Since their discovery in the 90s, EPH molecules have been increasingly recognized as key regulators of both normal development and disease (reviewed in refs. 3–5). The EPH receptor family can be divided into two subclasses, EPHA and EPHB, based on sequence similarity and preference for binding either the GPI-anchored EPHRIN-A ligands or the transmembrane EPHRIN-B ligands. Both A and B class EP receptors are single transmembrane-spanning domain receptors with distinct domains for ligand binding, receptor clustering, and signaling. Binding of EPHRINS to EPH receptors induces receptor clustering and activation. In addition to ligand-induced receptor activities, EPH receptors can also be activated by other cell-surface receptors, such as EGF receptor and ERBB2 (6, 7). Multiple intracellular signaling pathways have been linked to EPH receptors, including RAS/RAF/MAPK, PI3K/AKT/mTOR, SRC, ABL, and RHO/RAC/CDC42 (reviewed in refs. 3–5).

Analyses of the lung cancer genome revealed that both mRNA and protein levels of EPHA2 are elevated and high levels of EPHA2 correlate with smoking, K-RAS mutation, brain metastasis, disease relapse, and poor patient survival in several studies with large numbers of human tumor samples (reviewed in refs. 1, 8–10). In addition, a gain-of-function EPHA2 mutation has also been identified in human squamous lung cancer specimens (10), suggesting an oncogenic role for EPHA2 in lung cancer. Overexpression of EPHA2 in BEAS-2B immortalized lung epithelial cell line increased colony formation in soft agar, decreased FAS ligand-mediated apoptosis, and increased invasion in Transwell assays (10). In a separate study, EPHRIN-A1 ligand stimulation in multiple NSCLC lines reduced colony size in clonogenic growth assay and decreased motility in a wound...
closure assay, suggesting that tumor-promoting function of EPHA2 receptor in lung cancer is ligand independent (11). Although these early studies established a role for EPHA2 in tumor promotion in cultured lung cancer cells, the in vivo role and mechanistic basis of EPHA2 in these cancer cells remain poorly understood. In addition, the potential role of EPHA2 in lung cancer stem–like cells (CSC) has not been investigated.

In this study, we sought to establish the mechanism of action for EPHA2-mediated tumor promotion in multiple NSCLC lines. We used antibody arrays to identify EPHA2 downstream targets, and a combination of short hairpin RNA (shRNA)-mediated gene silencing, small-molecule kinase inhibitor, and rescue of the knockdown phenotype by expressing constitutively activated mutants for target validation. Furthermore, we analyzed the role of EPHA2 in lung cancer stem–like population. Collectively, these studies established mechanistic basis for EPHA2-dependent lung cancer cell proliferation, motility, and CSC function, and provided evidence for EPHA2 as a potential therapeutic target in NSCLC.

Materials and Methods

Cell lines, plasmids, and reagents

Human NSCLC cell lines were provided by the Specialized Programs of Research Excellence (SPORE) in lung cancer at Vanderbilt-Ingram Cancer Center (Nashville, TN). The cell lines were validated with DNA finger printing through the Vanderbilt Technology for Advanced Genomics Core facility in the fall of 2013. They were cultured in RPMI-1640 medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human EPHA2 cDNA was obtained from Open Biosystems and subcloned into pCLXSN retroviral vector containing Neomycin gene for G418 selection. Human EPHA2 cDNA and constitutively activated c-JUN-NH2-kinase (JNK)-1 and JNK2 were obtained from Addgene and subcloned into pCLXSN retroviral vector. Hairpin shRNAs targeting human EPHA2 were purchased from Open Biosystems. JNK inhibitor SP600125 was purchased from Cell Signaling Technology. Human Phospho-kinase antibody array and lung cancer tissue microarray (TMA) were purchased from R&D Systems and US Biomax, respectively.

Lentiviral shRNA knockdown and retroviral overexpression experiments

Short hairpin RNA (shRNA) construct in the pLKO.1 lentiviral vector containing the following EPHA2 targeting sequence was used: 5'-CGGACAGACATATGGGATATT-3'. Vector control (pLKO.1) or EPHA2 shRNA lentiviral particles were produced by cotransfection of HEK 293T cells with targeting plasmids and packaging vectors, psPAX2 and pMD2.G, using lipofectamine 2000 (Invitrogen and Life Technologies). Viral supernatants were collected by centrifugation and were used to infect NSCLC cells for 24 hours. Cells were changed to new growth medium for another 24 hours, followed by puromycin selection (2 μg/mL Sigma-Aldrich) for 3 to 5 days.

Retroviruses carrying vector (pCLXSN), pCLXSN-EPHA2, pCLXSN-JNK-CA, or pCLXSN-c-JUN were produced by cotransfection of HEK293T cells with overexpression plasmids and packaging vector, pCLAmpho. Viral supernatants were used to infect NSCLC cells, followed by selection of 800 μg/mL G418 (Sigma-Aldrich) for 10 days.

Cell growth assays

Cell growth was measured by MTT, colony formation, and bromodeoxyuridine (BrdUrd) assays. For MTT assay, 2.5 × 104 cells were plated into each well of 96-well plate in 100 μL of complete growth medium. JNK inhibitor was added on the second day after cell attachment. Cell viability was measured by incubating cells with 20 μL of 5 μg/mL tetrazolium salt MITT (Sigma-Aldrich) and quantified by reading absorbance at 590 nm using Microplate reader (Bio-Tek). For colony formation assay, 200 or 400 cells in complete growth medium were plated into each well of a 12-well plate. Cells were growing for 10 to 14 days, and the medium was changed every 3 days. At the end of the experiment, cell colonies were stained with crystal violet (Sigma-Aldrich) and the foci were photographed. For BrdUrd incorporation assay, 2 × 104 cells per well in complete growth medium were plated onto Matrigel-coated 2-well Lab-TekII chamber slide. Cells were starved for 20 hours, followed by 10 μg/mL BrdUrd labeling in the presence of 0.5% FBS for 16 hours. BrdUrd detection was performed using BrdUrd Staining Kit (Invitrogen and Life Technologies). BrdUrd+ cells were enumerated in four random fields, at ×40 magnification per chamber, and proliferation index was calculated as the percentage of BrdUrd+ nuclei/total nuclei.

Apoptosis assay

Tumor cells were serum starved for 48 hours and apoptosis was measured by Annexin V-FLUOS Staining Kit (Roche) as per the manufacturer’s instruction. Briefly, cells were gently trypsinized and washed once with serum containing medium. Cell suspensions were incubated with Annexin V-Fluorescein and propidium iodide to detect phosphoserine on the outer leaflet of apoptotic cell membranes and to differentiate from necrotic cells, respectively. Annexin V fluorescein-labeled cells were detected by fluorescence-activated cell sorting (FACS) analysis. For tumor xenografts, apoptosis was measured by TUNEL assay on tumor sections, as described previously (12).

Transwell migration assay

Tumor cell migration was assessed by a modified Boyden chamber assay using 8 mm pore size Transwell (Costar). The Transwell inserts were pretreated with 1% bovine serum albumin (BSA) in Opti-MEMI medium for 30 minutes. Tumor cells (1 × 106 of H1975, 2 × 105 of A549, or 3 × 104 of H1650 for 24-hour migration, and 2 × 105 of H1975 or 4 × 104 of A549 for 6-hour migration) were plated on top of the Transwell. Growth medium was added to the lower chamber and cells were allowed to migrate for 6 or 24 hours. Cells that passed through the Transwell membrane and stayed on the bottom of membrane were fixed with 4% paraformaldehyde and stained with crystal violet, photographed, and counted.

Screen of phospho-kinase array

Human phospho-kinase antibody arrays were purchased from R&D system (Catalog # ARY003). Capture antibodies
were presotted in duplicate on nitrocellulose membranes by the manufacturer. Membranes were incubated with blocking buffer for 1 hour at room temperature. Cell lysates derived from control and EPHA2 knockdown H1975 cells (500 µg protein) were mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the array overnight at 4°C, followed by three washes in washing buffer. Streptavidin-horseradish peroxidase and chemiluminescent detection reagents were subsequently added, and chemiluminescence was detected in the same manner as a Western blot analysis. Densitometry was performed on scanned blots using NIH ImageJ software version 1.45 to quantify the average pixel density/spotted area after normalization to the pixel density of the background controls. Data are representation of two independent experiments.

**Tumor sphere assay**

Cells (0.5 × 10^5 per well for A549, 1 × 10^5 per well for H1975) were plated into 6-well plates with ultralow attachment surfaces in serum-free medium DMEM/F12 (Gibco and Life Technologies) supplemented with commercial hormone mix B27 (Invitrogen and Life Technologies), EGF (20 ng/mL, R&D Systems), basic fibroblast growth factor (bFGF; 20 ng/mL, R&D Systems), and heparin (2 µg/mL, Stemcell Technologies). Floating sphere cultures were kept for 7 to 9 days. EGF and bFGF were replenished every 3 days. Plates were scanned and numbers of tumor sphere per well were enumerated automatically using The GeCount system (Oxford Optronix).

**Antibodies, Western blot analysis, and immunofluorescence**

Antibodies against the following proteins were used: EPHA2 (1:1000, D7, Millipore); EPHA2 (1:1000, c-20), ACTIN (1:1000, I-19; Santa Cruz Biotechnology); pThr183/Tyr185 EPHA2 (1:1000, c-20), ACTIN (1:1000, I-19; Santa Cruz Biotechnology); pThr202/Tyr204 ERK, ERK, pSer235/236 pS6, S6, SAPK/JNK, SAPK/JNK, pSer63 c-JUN, c-JUN, pSer473 AKT, AKT, pThr202/Tyr204 ERK, ERK, pSer233/236 pS6, S6, NOTCH3, SOX2 (1:1000, Cell Signaling Technology); ALDH (1:5000, BD Transduction Laboratories). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega. Western blot analyses were performed by standard methods using indicated antibodies. Densitometry was performed on scanned blots using NIH ImageJ software version 1.45 to quantify the average pixel density/spot area after normalization to the pixel density of the corresponding loading controls. Data are representation of three to four independent experiments.

For immunofluorescence studies, 2 × 10^4 cells were plated onto Matrigel-coated 2-well LabTek II chamber slide. Cells were fixed next day with 4% paraformaldehyde, permeabilized, and blocked with 0.3% Triton X-100, 3% BSA in PBS, followed by incubation with anti–p-c-JUN (1:100; Cell Signaling Technology) overnight at 4°C. The primary antibody was detected by incubation with the Alexa-conjugated secondary antibody (1:500, Invitrogen and Life Technologies) for 1 hour at room temperature. The slides were mounted with ProLong Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen and Life Technologies) and photographed.

**Immunohistochemistry**

Lung cancer TMA (US Biomax) or xenograft tumor sections from A549 and H1975 cells were stained by immunohistochemistry as described previously (13). Briefly, rehydrated paraffin sections were subjected to antigen retrieval in citrated buffer (2 mmol/L citric acid, 10 mmol/L sodium citrated, pH 6.0) using the PickCell Laboratories 2100 Retriever. Endogenous peroxidases were blocked by 3% H₂O₂ for 30 minutes. Sections were incubated with blocking solution and stained with primary antibodies (anti–EPHA2 1:25, anti–p-c-JUN 1:100, anti–ALDH 1:100) overnight at 4°C. Sections were subsequently washed and stained with biotinylated secondary antibodies, followed by avidin-peroxidase reagent and 3,3'-diaminobenzidine substrate. After hematoxylin counterstain, sections were mounted and photographed on an Olympus BX60 microscope using a digital camera and NIH Scion Image software.

For quantification of lung cancer TMAs, relative expression was scored using a continuous scale as follows: 0 = 0% to 10% positive tumor epithelium, 1 = 10% to 25% positive tumor epithelium, 2 = 25% to 50% positive tumor epithelium, and 3 = >50% positive tumor epithelium/core. TMA cores were scored blind by three independent individuals, the average of which was reported here. Differential expression between tissue samples and correlation between EPHA2 and ALDH expression were quantified and statistically analyzed using the χ² analysis.

**In vivo tumor studies**

Eight-week-old nude mice were purchased from Harlan–Sprague–Dawley and used for in vivo tumor evaluation. The animals were housed under pathogen-free conditions, and experiments were performed in accordance with the Association and Accreditation of Laboratory Animal Care International guidelines and with approval by the Vanderbilt University (Nashville, TN) Institutional Animal Care and Use Committee. To evaluate the role of EPHA2 on tumor growth, 2.5 × 10⁶ EPHA2 knockdown cells were injected subcutaneously into the flank of the nude mice, and the same numbers of vector control cells were injected contralaterally. Tumor progression was monitored by palpation and tumor size measured by a digital caliper. At the end of the 4 weeks, tumor volume was calculated using the following formula: volume = length × width² × 0.52.

**Flow cytometric analysis**

The cell profile of ALDH was determined by flow cytometric analysis using the Aldefluor Assay Kit (Stem Cell Technologies) as per the manufacturer’s instruction. Briefly, cells were incubated in Aldefluor assay buffer containing efllux inhibitor and the ALDH substrate. Cells that could catalyze substrate to its fluorescent product were considered ALDH⁺. Sorting gates for FACS were drawn relative to cell baseline fluorescence, which was determined by the addition of the ALDH-specific inhibitor, DEAB, during the incubation and DEAB-treated samples as negative controls. Nonviable cells were identified by 7-aminoactinomycin D inclusion. Flow cytometric analyses were performed on
FACScan or FACSCalibur flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Tree Star).

**Statistical analysis**

For comparisons between two groups, a Student t test was used, and for analysis with multiple comparisons, ANOVA or Kruskal–Wallis tests were used, using Prism software (GraphPad Software). χ² analysis was used for categorical outcomes. All tests of statistical significance were two sided, and the exact tests used for each experiment were listed in the text and in the figure legend. P values less than 0.05 were considered to be statistically significant.

**Results**

**Phosphorylation of JNK and c-JUN is regulated by EPHA2 in lung cancer cells**

Previous studies showed that knockdown of EPHA2 receptor in cultured lung cancer cells inhibited proliferation (11). Conversely, expression of EPHA2 in immortalized BEAS-2B bronchial epithelial cells increased cell survival and invasion (10). Consistent with these observations, shRNA-mediated stable knockdown of EPHA2 in non–small cell lung cancer (NSCLC) lines inhibited tumor cell proliferation and motility (Fig. 1 and Supplementary Fig. S1). To identify signaling pathways downstream of EPHA2 receptor that mediate tumor-promoting effects in cancer cells, cell lysates derived from vector-control or EPHA2 shRNA knockdown H1975 cells were used to screen two human phospho-kinase antibody arrays (human phospho-kinase antibody array and human phospho-RTK antibody array). As shown in Fig. 2A, phosphorylation at Ser63 residue of c-JUN is decreased in EPHA2 knockdown cells, relative to vector control cells, in two independent experiments, suggesting that EPHA2 may regulate the JNK/c-JUN pathway in lung cancer cells.

To determine whether depletion of EPHA2 affects JNK/c-JUN signaling in response to serum stimulation, H1975 or A549 cells were serum starved overnight and stimulated with 10% FBS following a time course, and phosphorylated levels of JNK and c-JUN were determined by Western blot analysis. We found that p-JNK/JNK and p-c-JUN/c-JUN levels were consistently decreased in EPHA2 knockdown cells in four independent experiments (Fig. 2B). Interestingly, we also found that total c-JUN levels seem to be decreased in EPHA2-deficient cells. Next, we sought to investigate whether phosphorylation of c-JUN and JNK are altered in other EPHA2-depleted NSCLC cells. Consistent with observations in H1975 and A549 cells, knockdown of EPHA2 resulted in decrease of p-c-JUN and p-JNK levels in four and five out of 10 NSCLC lines tested, respectively (Supplementary Fig. S2C).

Once phosphorylated, c-JUN enters the nucleus and regulates transcription (14, 15). Accordingly, we assessed nuclear c-JUN expression by immunofluorescence. The number of cells expressing nuclear c-JUN is markedly decreased in EPHA2 knockdown cells relative to vector control cells (Fig. 2C), indicating that EPHA2 regulates c-JUN activity. Collectively, p-JNK and p-c-JUN levels were reproducibly and significantly decreased in approximately 50% of EPHA2 knockdown cell lines tested, whereas phosphorylation levels of AKT and extracellular signal-regulated kinase (ERK) were not changed in most cell lines (Supplementary Fig. 2).
JNK Signaling Mediates EPHA2-Dependent Tumor Malignancy

JNK was originally shown to mediate cell apoptosis in response to a variety of stress signals (14, 15). However, emerging evidence suggests that JNKs play a critical role in cell growth and survival in tumor cells (14–17). Indeed, JNK is activated in NSCLC and promotes neoplastic transformation in human bronchial epithelial cells (18). JNK2-α seems to be the major JNK isoform that plays a critical role in lung cancer (19). Therefore, the JNK/c-JUN pathway may, at least in part, mediate EPHA2-dependent regulation of tumor cell behavior. To investigate whether the JNK/c-JUN pathway is necessary and sufficient for cell proliferation and motility, we used two complementary approaches: a JNK kinase inhibitor, SP600125, and two constitutively activated JNK mutants (JNK1-CA and JNK2-CA).

Treatment of either A549 or H1975 cells with JNK inhibitor SP600125 inhibited p-JNK and p-c-JUN levels (Supplementary Fig. S3A) and reduced cell viability, colony formation, and tumor cell motility in a dose-dependent manner (Fig. 3A and B and Supplementary Fig. S3B), suggesting that the JNK/c-JUN pathway is required in lung cancer cells. To investigate whether the JNK/c-JUN pathway mediates EPHA2 receptor signaling, we utilized previously characterized constitutively activated MKK7-JNK1 and MKK7-JNK2 fusion mutants (hereafter termed JNK-CA; ref. 20). Reexpression of wild-type EPHA2 receptor in EPHA2 knockdown cells rescued p-JNK and p-c-JUN levels, colony formation, and cell motility (Fig. 3C–E), demonstrating that phenotypes in EPHA2 knockdown cells are not due to off-target effects. Expression of JNK1-CA, JNK2-CA, or c-JUN rescued phenotypes in the EPHA2 knockdown cells to the comparable level as wild-type EPHA2 (Fig. 3C–E), indicating that activated JNK1/2 or overexpression of c-JUN is sufficient to restore function in these cells. Collectively, these data suggest that the JNK/c-JUN pathway mediates EPHA2-dependent tumor cell proliferation and motility.

Loss of EPHA2 inhibits JNK/c-JUN signaling and suppresses tumor formation and tumor growth in vivo

To directly determine the in vivo role of EPHA2 in lung cancer, EPHA2 knockdown A549 or H1975 cells were injected subcutaneously into the dorsal flank of a nude mouse, and control cells carrying the empty vector were injected contralaterally in the same mouse. Loss of EPHA2 in knockdown tumors significantly inhibited tumor volume in the xenograft animal models (mean A549 tumor volume: vector control 419 ± 166 mm³ vs. EPHA2 shRNA 225 ± 90 mm³; mean H1975 tumor volume: vector control 623 ± 310 mm³ vs. EPHA2 shRNA 324 ± 166 mm³, P < 0.05, paired t test; Fig. 4A). Tumors were harvested at the end of the studies and tumor sections were subjected to Ki-67 immunohistochemistry and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay for assessing proliferation and apoptosis, respectively. Consistent with in vitro data, we found that tumor cell proliferation was decreased in EPHA2 knockdown tumors as compared with vector control tumors, whereas apoptosis levels were not significantly changed between the two groups (Fig. 4B and C). Analysis of tumor lysates revealed that EPHA2 was stably knocked down in tumors after grown in vivo for 30 days. Both phosphorylation and total levels of c-JUN were decreased in EPHA2-deficient H1975 tumors, whereas there was a predominant reduction in total JNK and c-JUN levels in both EPHA2-deficient H1975 and A549 tumors (Fig. 4D). Expression of EPHA2 and p-c-JUN was further analyzed in adjacent tumor sections by immunohistochemistry. As shown...
in Fig. 4E, EPHA2 receptor and p-c-JUN were coexpressed in the control tumors, whereas nuclear staining of p-c-JUN is markedly decreased in EPHA2 knockdown tumors. Taken together, these results show that EPHA2 promotes tumor growth of NSCLC, such that depletion of EPHA2 limited progression of this aggressive NSCLC xenografts model.

To further investigate whether loss of EPHA2 affects lung cancer formation in vivo, we knocked down EPHA2 in A549 and H1975 cells that express luciferase. EPHA2 depletion in these knockdown cells was confirmed by FACS and Western blot analysis (Supplementary Fig. S4). Serial diluted EPHA2 knockdown tumor cells (2.5 \times 10^5, 2.5 \times 10^4, and 2.5 \times 10^3) were injected subcutaneously into nude mice, and same numbers of control cells carrying the empty vector were injected contralaterally in the same mouse. Tumor formation was monitored by bioluminescence weekly. Vector control A549 and H1975 cells exhibited a greater frequency of tumor formation, particularly at low numbers of injected cells, compared with EPHA2 knockdown counterparts (Supplementary Fig. S4). Collectively, our tumor xenograft data suggest that EPHA2 receptor regulates both tumor formation and tumor growth in vivo.

**EPHA2 silencing suppresses ALDH^+ stem-like cell-enriched populations, tumor sphere formation, and tumorigenicity in vivo**

Recent evidence suggests that human lung cancers harbor CSCs or tumor-initiating/-propagating cells (TIC/TPC) that facilitate tumor cell heterogeneity, metastases, and therapeutic resistance (21, 22). Because CSCs/TICs/TPCs are intimately
linked with tumor initiation and knockdown of EPHA2 affects tumor formation in vivo, we tested whether EPHA2 deficiency affects CSC/TIC/TPC populations in lung cancer cells. ALDH has recently been identified as a marker for lung cancer cells with stem cell–like properties, and ALDH$^+$ cells were shown to be highly tumorigenic compared with ALDH$^-$ counterparts (23, 24). Accordingly, we used the Aldefluor assay to quantify the percentage of ALDH$^+$ cells in vector control and EPHA2 knockdown lung cancer cell lines. Cells were incubated in Aldefluor assay buffer containing efflux inhibitor and the ALDH substrate. Cells that could catalyze substrate to its fluorescent product were considered ALDH$^+$. Sorting gates for FACS were drawn relative to cell baseline fluorescence, which was determined by the addition of the ALDH-specific inhibitor DEAB during the incubation and DEAB-treated samples serve as negative controls. As shown in Fig. 5A, a subpopulation of ALDH$^+$ cells was detected by flow cytometry in control A549 and H1975 cells, and the ALDH$^+$ subpopulations significantly decreased in corresponding EPHA2 knockdown cells. Depletion of EPHA2 also reduced ALDH$^+$ populations in H23 and H1650 cells (Supplementary Fig. S5).
To relate the findings to human lung cancer, we analyzed the expression of EPHA2 and ALDH on adjacent sections of human lung cancer TMAs. Consistent with previous studies, we observed significantly elevated EPHA2 protein expression in human lung tumor samples relative to normal, adjacent control lung tissue (5/8 EPHA2+ normal lung tissue samples vs. 25/30 EPHA2+ lung cancer tissue samples; \( P < 0.05 \), \( \chi^2 \) analysis). Furthermore, we observed a positive correlation between EPHA2 expression and ALDH expression in a significant number of human lung cancer samples (21/30, 70%) relative to samples that were EPHA2 positive but ALDH negative (4/30, 13%; \( P < 0.05 \), \( \chi^2 \) analysis; Fig. 5B). Together, these data demonstrate the relevance of our studies in human cancer.

In addition to expressing stem cell markers, CSCs/TICs/TPCs are capable of propagation of isolated tumor cells as...
spheroids in suspension, and exhibit a high capacity for tumorigenic growth in mouse xenografts. We found that knockdown of EPHA2 significantly suppressed sphere-forming ability and in vitro self-renewal capacity of A549 and H1975 cells (Fig. 5C). To test the role of EPHA2 in tumorigenic capacity of CSCs, ALDH$^+$ populations in luciferase-expressing A549 cells were isolated by FACS sorting. EPHA2 expression was subsequently silenced by lentiviral shRNA transduction as determined by Western blot analysis. B, limiting dilutions of vector control or EPHA2 knockdown stem-like cell-enriched populations were injected into the left and right flanks of nude mice, respectively. Tumor formation was monitored weekly by palpation and luminescence imaging. Shown are bioluminescence images of mice at 4 weeks after injection (B). Tumor incidence is presented in Table 1.

Discussion

Recent efforts in analyzing cancer genome have identified EPHA2 as one of the genes that are elevated in human lung cancer, and high levels of EPHA2 are associated with poor clinical outcomes. However, mechanistic basis for EPHA2 in lung cancer promotion remains poorly understood. In this study, we found that EPHA2 regulates tumor cell proliferation and motility through the JNK-c-JUN pathway. In addition, we provide evidence that EPHA2 is important for CSC function. Collectively, these data identified JNK/c-JUN as a major signaling pathway that is activated by oncogenic EPHA2 receptor in NSCLC, and a critical role for EPHA2 in CSCs.

Multiple intracellular signaling pathways have been linked to EPHA2 receptor in cancer cells (reviewed in refs. 2, 25). Ligand stimulation of EPHA2 has been shown to inhibit ERK and AKT phosphorylation, and tumor cell proliferation and motility in prostate and lung cancer cells, as well as in malignant glioma cells (11, 26, 27). Conversely, ligand-independent EPHA2 signaling and cross-talk with ERBB family receptors are linked to tumor cell proliferation and motility and upregulation of p-ERK and RHO-GTP levels (6, 7, 26) in breast cancer and malignant glioma cells. In lung cancer, although ephrins are expressed, the tumor promotion role of EPHA2 seems to be ligand independent, as we were unable to detect significant levels of EPHA2 receptor phosphorylation without exogenous EPHRIN-A1 stimulation, and ligand stimulation induced opposing phenotypes from EPHA2 receptor overexpression (11). It is currently unclear why ERK and AKT activities do not seem to be consistently affected by EPHA2 knockdown in NSCLC lines tested. Because of high mutation rates in lung cancer, it is possible that there are unidentified mutations in genes that act downstream of EPHA2 and affect ERK/AKT activities in these cell lines. Regardless of mechanisms, our data suggest that ERKs/AKTs are not direct targets of ligand-independent EPHA2 signaling in majority of the lung cancer cells.

JNK was originally shown to mediate cell apoptosis in response to a variety of stress signals (14, 15). However, emerging evidence suggests that JNKs play a critical role in

| Table 1. Tumor incidence in ALDH$^+$ populations |
|----------------------------------|-------------|------------|
| shRNA   | Cell number | Tumor incidence |
|         |             |             |
| VEC     | $1 \times 10^4$ | 5/5 (100%) |
|         | $1 \times 10^3$ | 5/5 (100%) |
|         | $1 \times 10^2$ | 4/5 (80%)  |
| KD      | $1 \times 10^4$ | 5/5 (100%) |
|         | $1 \times 10^3$ | 0/5 (0%)   |
|         | $1 \times 10^2$ | 0/5 (0%)   |

NOTE: ALDH$^+$ populations in A549 cells were isolated by FACS. Limiting dilutions of vector control (VEC) or EPHA2 knockdown (KD) stem-like cell-enriched populations were injected into nude mice and tumor formation was monitored by luminescence imaging 4 weeks after transplantation.
cell growth and survival in tumor cells (refs. 14, 15). For example, JNK activity is upregulated in colorectal cancer. Activation of JNK increases cell proliferation, probably through regulation of mTOR complex (mTORC)-1 signaling via phosphorylation of RAPTOR, as well as modulation of transcription of cyclin E via activation of c-JUN (28). In lung cancer, JNK is phosphorylated at Thr183/Tyr185 in NSCLC biopsy samples, and promotes tumor cell proliferation and motility (18, 19). However, it is not known what signal activates JNK in the lung cancer cells so far. Here, we provide evidence that JNK activity in lung cancer cells is regulated by EPHA2 receptor. First, knockdown of EPHA2 significantly inhibited phosphorylation levels of both JNK and c-JUN in multiple lung cancer cell lines (Fig. 2). Furthermore, treatment of JNK inhibitor recapitulates phenotypes in EPHA2 knockout cells, whereas overexpression of a constitutively activated JNK-CA rescued defects in EPHA2-deficient lung cancer cells (Fig. 3). Together, these data identified JNK/c-JUN as a previously unknown target of EPHA2 receptor signaling in cancer.

How EPHA2 regulates the JNK/c-JUN signaling pathway remains to be determined. JNK activity was reported to be regulated by RHO family small GTPases (29, 30). We and others have previously shown that EPHA2 regulates RHO activity in breast cancer and prostate cancer cells (7, 31–33), suggesting the possibility of RHO GTPases as candidates to link EPHA2 to JNK/c-JUN. In addition to regulation of JNK/c-JUN activities, EPHA2 also affects JNK/c-JUN protein levels. We found that depletion of EPHA2 leads to decreased total levels of JNK and c-JUN in some cell lines and tumor xenografts (Figs. 2 and 4 and Supplementary Fig. S2), whereas mRNA levels of JNK1, JNK2, or c-JUN are not significantly changed (Supplementary Fig. S2). In the case of c-JUN, phosphorylation by JNK at Ser63 and Ser73 is known to increase its activity and protect c-JUN from ubiquitination and subsequent degradation (34). Thus it is possible that decreased c-JUN phosphorylation in EPHA2 knockdown cells leads to reduced c-JUN protein stability. In addition, EPHA receptors are known to regulate mTORC1 activity in the nervous system and cancer cells (35, 36), and overexpression of EPHA2 in immortalized lung epithelial cells, BES-2B, increased phosphorylation of mTOR and S6K1(10). Indeed, we also observed a decrease of pS6 in several EPHA2 knockdown NSCLC lines (Supplementary Fig. S2). Because one of the major functions of mTORC1 is cap-dependent protein synthesis (37), it is also possible that EPHA2 regulates the protein synthesis of JNK and/or c-JUN through activation of mTORC1.

Although our studies suggest that JNK signaling mediates EPHA2-dependent tumor cell proliferation and motility in lung cancer, it cannot be excluded that other signaling pathways may also play a role downstream of EPHA2 receptor in subsets of lung cancer. Thus far, JNK/c-JUN activities are affected in five out of 10 cell lines tested (Supplementary Fig. S2). Other signaling pathways known to act downstream of EPHA2 receptor, such as the mTOR pathway, RHO family GTPases, STAT3 transcription factor, and FAK kinase, will be alternative avenues to pursue in subsets of lung cancer cells that JNK activities are not altered upon EPHA2 knockdown.

EPHA2 receptor has been implicated in stem cell biology previously. EPHA2 is highly expressed in embryonic stem cells (38, 39) and its expression is regulated by E-cadherin in embryonic stem cells (39). More recently, EPHA2 is also shown to drive self-renewal and tumorigenicity in human glioblastoma stem-like cells (40). Studies presented in this report demonstrated a critical role of EPHA2 in lung CSCs. EPHA2 expression is correlated with cancer stem cell marker ALDH expression (23, 24) in human lung cancer TMA (Fig. 5B). Knockdown of EPHA2 in lung cancer cells significantly reduced ALDH+ populations and the ability to form spheroids in suspension (Fig. 5A and C). Furthermore, RNA interference-mediated depletion of EPHA2 in stem-like cell-enriched sorted population inhibited tumorigenicity in vivo (Fig. 6 and Supplementary Fig. S6), suggesting an important role of EPHA2 in lung CSC function.

The identification of the JNK/c-JUN pathway as a downstream target of EPHA2 receptor in NSCLCs, as well as the role of EPHA2 in CSCs, opens up exciting opportunities for potential molecularly targeted therapies in lung cancer. Although early versions of JNK inhibitors displayed poor kinase selectivity and low efficacy, a new generation of more potent and selective irreversible JNK inhibitors has been identified (41) and may be developed for molecularly targeted therapy in NSCLC. In addition, a variety of strategies have been developed to target EPHA2, including activating monoclonal antibody, ephrin ligands, ligand-mimic peptides against EPHA2, and selective kinase inhibitors (reviewed in refs. 42, 43). As CSCs are often resistant to therapies and the fact that CSCs depend on EPHA2 for self-renewal in lung cancer, targeting EPHA2 may hold promise for treatment of drug-resistant lung cancer.

Disclosure of Potential Conflicts of Interest
J. Chen has received honoraria from the speakers’ bureaus of Ohio State University, Oklahoma Research Foundation, and Rutgers University. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: W. Song, J. Chen
Development of methodology: W. Song, Y. Ma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Song, Y. Ma, D. Brantley-Sieders
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Song, D. Brantley-Sieders, J. Chen
Writing, review, and/or revision of the manuscript: W. Song, J. Wang, D. Brantley-Sieders, J. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Song, J. Chen
Study supervision: J. Chen

Grant Support
This work was supported by Department of Veterans Affairs through a VA Merit Award (J. Chen), NIH RO1 grants CA95004 (J. Chen) and CA148934 (D. Brantley-Sieders), and pilot project grants from the VICC thoracic program (J. Chen). This work was also supported in part by the resources and the use of facilities at the VA Tennessee Valley Healthcare System and NCI Cancer Center Support Grant # P30 CA068485, utilizing the Translational Pathology, Flow Cytometry, and Small Animal Imaging Shared Resources.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 30, 2013; revised February 10, 2014; accepted February 17, 2014; published OnlineFirst March 7, 2014.
References


27. Fujisawa T, Aoki M, Taketo MM. JNK signaling promotes intestinal tumorigenesis through activation of mTOR complex 1 in APC(Δ716) mice. Gastroenterology 2011;140:1556–63.


JNK Signaling Mediates EPHA2-Dependent Tumor Cell Proliferation, Motility, and Cancer Stem Cell–like Properties in Non–Small Cell Lung Cancer

Wenqiang Song, Yufang Ma, Jialiang Wang, et al.

Cancer Res  Published OnlineFirst March 7, 2014.