

EphA2 in the Early Pathogenesis and Progression of Non–Small Cell Lung Cancer

Jennifer M. Brannan,^{1,4} Banibrata Sen,¹ Babita Saigal,¹ Ludmila Prudkin,² Carmen Behrens,² Luisa Solis,² Wenli Dong,³ B. Nebiyu Bekele,³ Ignacio Wistuba^{1,2} and Faye M. Johnson^{1,4}

Abstract

Overexpression of the receptor tyrosine kinase EphA2 occurs in non–small cell lung cancer (NSCLC) and a number of other human cancers. This overexpression correlates with a poor prognosis, smoking, and the presence of Kirsten rat sarcoma (*K-Ras*) mutations in NSCLC. In other cancers, EphA2 has been implicated in migration and metastasis. To determine if EphA2 can promote NSCLC progression, we examined the relationship of EphA2 with proliferation and migration in cell lines and with metastases in patient tumors. We also examined potential mechanisms involving AKT, Src, focal adhesion kinase, Rho guanosine triphosphatases (GTPase), and extracellular signal–regulated kinase (ERK)-1/2. Knockdown of EphA2 in NSCLC cell lines decreased proliferation (colony size) by 20% to 70% in four of five cell lines ($P < 0.04$) and cell migration by 7% to 75% in five of six cell lines ($P < 0.03$). ERK1/2 activation correlated with effects on proliferation, and inhibition of ERK1/2 activation also suppressed proliferation. In accordance with the *in vitro* data, high tumor expression of EphA2 was an independent prognostic factor in time to recurrence ($P = 0.057$) and time to metastases ($P = 0.046$) of NSCLC patients. We also examined EphA2 expression in the putative premalignant lung lesion, atypical adenomatous hyperplasia, and the noninvasive bronchioloalveolar component of adenocarcinoma because *K-Ras* mutations occur in atypical adenomatous hyperplasia and are common in lung adenocarcinomas. Both preinvasive lesion types expressed EphA2, showing its expression in the early pathogenesis of lung adenocarcinoma. Our data suggest that EphA2 may be a promising target for treating and preventing NSCLC.

Lung cancer is the leading cause of cancer death worldwide and in the United States, where it kills approximately 160,000 people every year (1). The 5-year survival rate of all lung cancer patients is only approximately 15% (2). Roughly 90% of all cancer deaths are caused by metastatic disease, which is already present in the majority of non–small cell lung cancer (NSCLC) patients at diagnosis. There is no standard screening or prevention strategy for former smokers, who remain at a high risk for NSCLC. We recently discovered that the receptor tyrosine kinase EphA2 is expressed in more than 90% of NSCLC but not significantly in normal lung tissue, suggesting that it may be a molecular target for treatment or prevention.

The Eph family of receptors is the largest group of receptor tyrosine kinases identified to date, having 14 family members in mammals. Eph receptors and their ligands regulate diverse cellular processes including axon guidance, angiogenesis, and embryonic patterning (3, 4). Eph receptors are classified into two subfamilies, A and B, based on sequence similarity and ligand affinity. Like the Eph receptors, their ligands, called ephrins (Eph-receptor family interacting proteins), are membrane bound and divided into two subfamilies based on how they are attached to the membrane. The A-type ephrins are attached to cell membranes via a glycosylphosphatidylinositol anchor, and the B-type ephrins contain a transmembrane domain. Receptor–ligand interactions occur primarily, but not exclusively, within the same class. Eph receptors are expressed in numerous tissue types (5), but their expression tends to be highest in the nervous system and is higher in embryos than in adults. Eph receptors have distinct albeit frequently overlapping patterns of expression, suggesting a level of redundancy within the family (6).

Overexpression of EphA2 has now been reported in NSCLC and a number of other human cancers (7, 8). Increased EphA2 expression frequently correlates with a poor prognosis and likely contributes to the development of the malignant phenotype (9–14). Two mechanisms by which EphA2 may promote cancer progression are via effects on proliferation and migration. Treatment of breast or bladder cancer cells with the dimeric

Authors' Affiliations: Departments of ¹Thoracic/Head and Neck Medical Oncology, ²Pathology, and ³Biostatistics, The University of Texas M.D. Anderson Cancer Center, and ⁴The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas
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Requests for reprints: Faye M. Johnson, Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: 713-792-6363; Fax: 713-792-1220; E-mail: fmjohns@mdanderson.org.

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EphA2 ligand ephrin-A1-Fc (EA1-Fc) led to decreases in total EphA2 and cell proliferation (15, 16). Likewise, EphA2 depletion in pancreatic cancer and glioma cells leads to decreased invasion and migration (17, 18). EphA2 also may act as a tumor suppressor. Stimulation of EphA2 inhibits the proliferation of nonmalignant epithelial and endothelial cells (19). The effects of EphA2 modulation in NSCLC have never been studied previously.

One mechanism by which EphA2 may affect cancer cell progression is via its interactions with the rat sarcoma (Ras)/extracellular signal-related kinase (ERK) pathway. EphA2 has been shown to both positively and negatively regulate ERK (19–22). Miao et al. (19) observed that treatment with EA1-Fc resulted in significant and sustained inhibition of phospho (p)-ERK1/2 in a variety of cells *in vitro*. Concurrently, they showed that the decrease in ERK activation correlated with a decrease in cell proliferation in a clonogenic growth assay. Likewise, Macrae et al. described inhibition of growth factor-induced activation of ERK1/2 by EA1-Fc. Conversely, Pratt and Kinch found in a number of cell lines that ligand activation of EphA2 activated the Ras/ERK pathway within the same time frame that inhibition occurred in the Miao study (21). Interactions between EphA2 and a number of proteins involved in cell migration, including Src, focal adhesion kinase (FAK), the Rho guanosine triphosphatases (GTPase), and AKT, have also been observed (18). Overexpression of EphA2 in human mammary epithelial cells destabilized cell-cell adhesion via a RhoA-dependent mechanism (23). In prostate carcinoma cells, EA1 activated Src and its downstream effector, FAK, which led to Rho-dependent contractility (24).

Defining the role of EphA2 in NSCLC is particularly important because EphA2 expression is common in NSCLC and correlates with a smoking history and activating mutations of the Kirsten rat sarcoma (*K-Ras*) gene (7). Recent advances in targeted NSCLC therapy have benefited nonsmokers with adenocarcinoma and mutations in the epidermal growth factor receptor (*EGFR*), whereas treatment options for smokers with *K-Ras* mutations are very limited. *K-Ras* mutations also occur in atypical adenomatous hyperplasia (AAH), the putative premalignant lesions of lung adenocarcinoma (25–28), leading us to hypothesize that increases in EphA2 may occur in premalignant lung lesions. This hypothesis has never been studied in relation to NSCLC and has been addressed only indirectly in relation to other cancers. In relation to breast cancer, for exam-

ple, EphA2 overexpression was found to be sufficient to transform mammary epithelial cells (29). In contrast, *EphA2* knockout mice were more susceptible to chemical-induced skin carcinogenesis than were wild-type mice (30), showing again that the role of EphA2 is not well defined and may depend on the cell type.

In the present study, we examined the role of EphA2 (*a*) in the proliferation and migration of NSCLC and thus its potential as a therapeutic target and (*b*) in the preinvasive lung lesions AAH and the bronchioloalveolar component of adenocarcinoma and thus its potential as a prevention target. We also examined potential mechanisms of these effects involving AKT, Src, FAK, Rho GTPases, and ERK1/2.

Materials and Methods

Cell lines and reagents

NSCLC cells (Table 1) were obtained from the American Type Culture Collection and grown as previously described (7). Antibodies used included EphA2 (clone D7, Millipore); pEGFR (Y1086), EGFR, pERK1/2, ERK1/2, pFAK (Y379), pFAK (Y576/Y577), FAK, pSrc (Y416), c-Src, pEphA2 (Y594), and RhoA (Cell Signaling Technology); calnexin (BD Biosciences); and EphA2 antibody (for immunohistochemistry; Santa Cruz Biotechnology). PD98059 was purchased from Calbiochem. EA1-Fc and control Fc were purchased from Sigma, rehydrated in sterile PBS, and used at 1 µg/mL for all experiments. Rho inhibitor was purchased from Cytoskeleton.

Tissue microarray and immunohistochemistry

Tissue microarrays were constructed from 279 surgically resected NSCLC, 22 AAH, and 18 noninvasive bronchioloalveolar specimens using triplicate 1-mm-diameter cores per tumor of tissue obtained from central, intermediate, and peripheral tumor areas as previously described (7). The tissue microarrays were stained for expression of EphA2 using a standard two-step indirect immunohistochemistry protocol as previously described (7). We also used this staining protocol on whole tissue sections from 22 AAHs and 22 adenocarcinomas with noninvasive bronchioloalveolar patterns (18 cases). Scores for EphA2 staining were calculated by multiplying the staining intensity (0, below the level of detection; 1, weak; 2, moderate; and 3, strong) by the percentage of cells staining at each intensity level (0–100%) to generate a final score that ranged from 0 to 300.

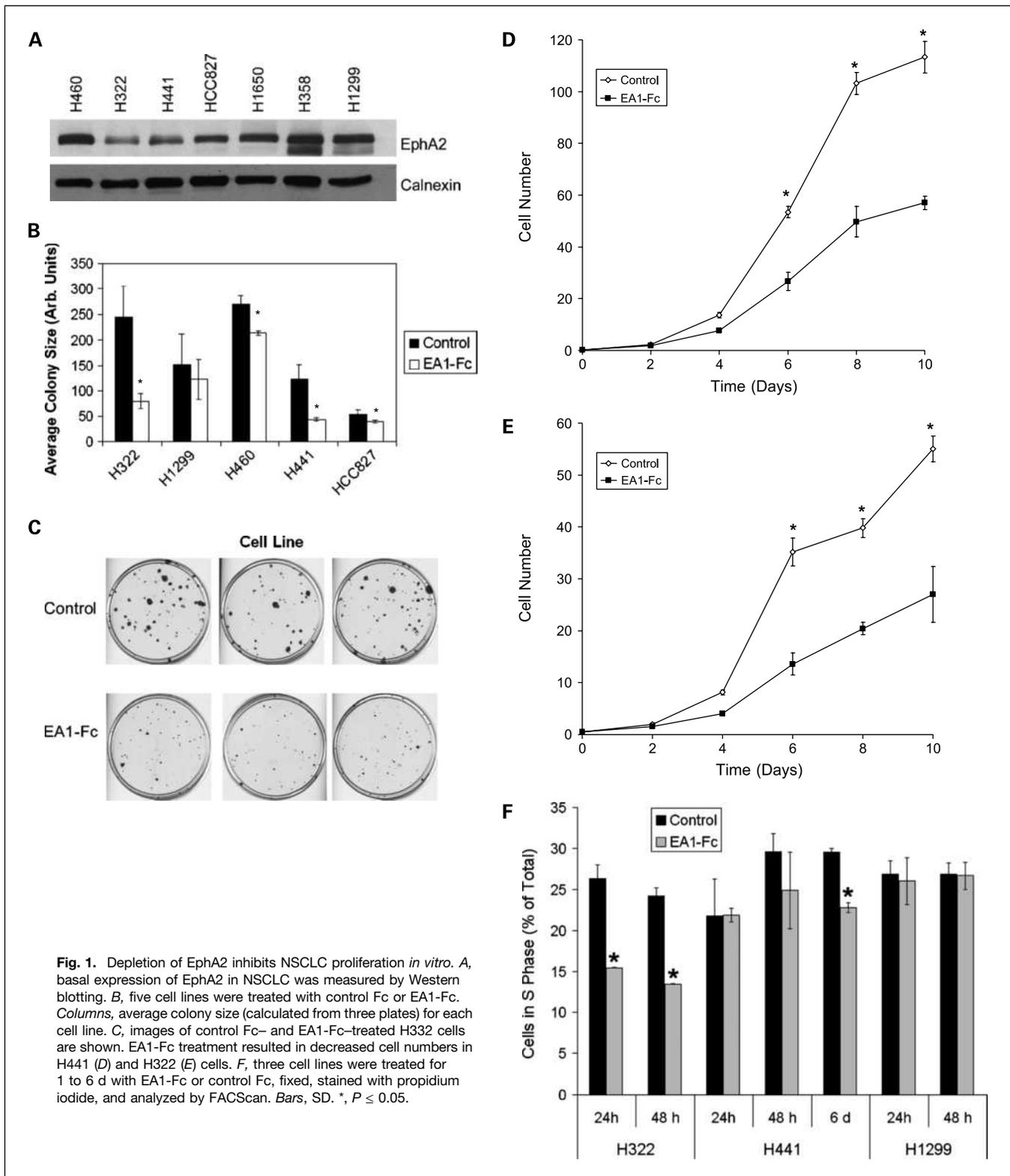
Transfection with small interfering RNA

On-Target Plus Smart Pool small interfering RNA (siRNA) targeting EphA2, nontargeting siRNA, and Dharmacon transfection reagent 2 were purchased from Dharmacon. H1299 and H441 cells were seeded

Table 1. NSCLC cell lines of this study and their characteristics

| Cell line | Histology | EGFR | K-Ras | p53 |
|-----------|-----------|---------------|----------|---------------------|
| H322 | AC | wt | wt | Codon 248 (R248L) |
| H1299 | LCC | wt | wt | Homozygous deletion |
| H460 | LCC | wt | Codon 61 | wt |
| H441 | AC | wt | Codon 12 | Codon 158 (R158L) |
| HCC827 | AC | DEL E746-A750 | wt | ND |
| H1650 | BAC | DEL E746-A750 | wt | wt |
| H358 | BAC | wt | Codon 12 | Homozygous deletion |

Abbreviations: AC, adenocarcinoma; wt, wild type; LCC, large cell carcinoma; BAC, bronchioloalveolar carcinoma; ND, no data.



into standard cell culture medium at a concentration of 1×10^5 /mL, 1 d before transfection. siRNA was used at a concentration of 100 nmol/L for all transfections. siRNA was diluted in serum-free medium, combined with the same volume of serum-free medium containing 2% Dharmacon 2 transfection reagent, and applied to the cells

along with fresh medium per manufacturer's instructions. Medium was changed 24 h after transfection, and cells were grown for the indicated time points. Controls included cells that were mock transfected (i.e., no siRNA) and those transfected with a nontargeting (scrambled) siRNA.

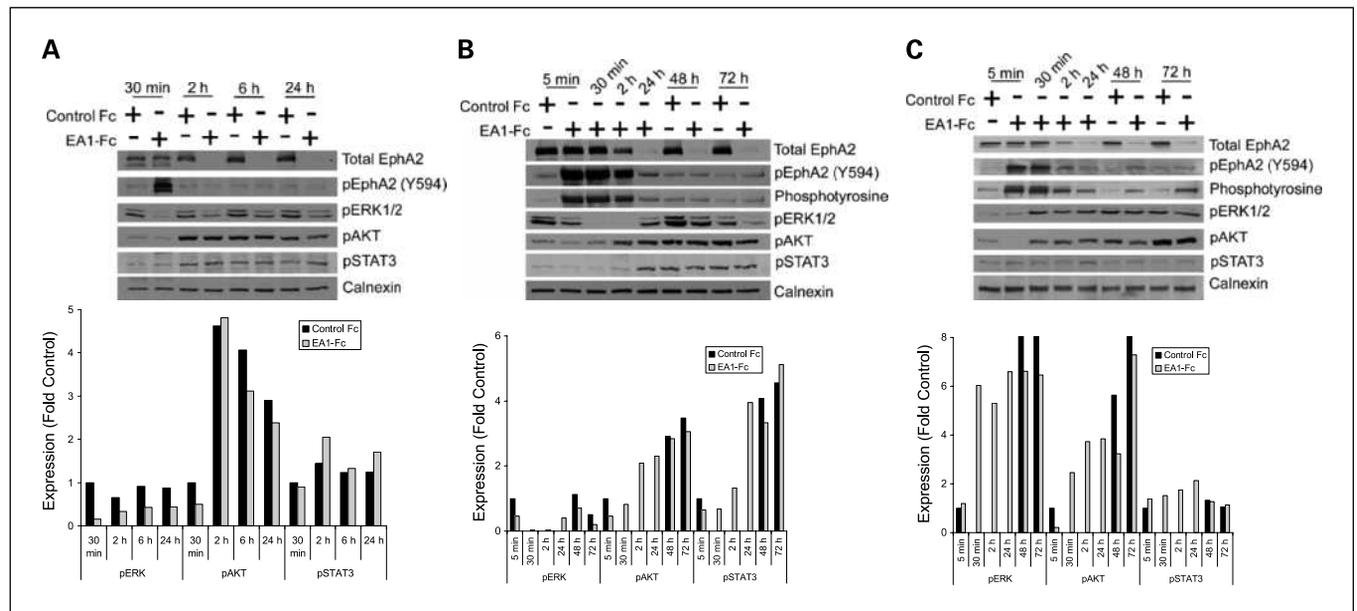


Fig. 2. Ligand treatment of NSCLC cell lines downregulates EphA2 and reduces ERK1/2 activity. H322 (A), H441 (B), and H1299 (C) cells were treated with EA1-Fc or control Fc for the indicated time points, and protein expression was examined by Western blot with the indicated antibodies. To measure total phospho-EphA2, cell lysates from treated cells were immunoprecipitated with EphA2 and blotted with an anti-phosphotyrosine antibody. The blots were quantified using ImageJ, and expression was corrected for the loading control (calnexin), ρ STAT3, phosphorylated signal transducer and activator of transcription 3.

Clonogenic growth assay

Cells were plated at a density of 20/mL (except HCC827 cells at 70/mL) 1 d before initiating incubation with 1 μ g/mL EA1-Fc or control Fc. Medium and ligand were replaced every 3 d. Cells were grown for 14 d, then washed with PBS, fixed with 1:10 formalin, and stained with crystal violet. The plates were then imaged, and colony size and quantity were analyzed using ImageJ (31). For cell counting analysis, cells were seeded, allowed to adhere overnight, and then treated with EA1-Fc or control Fc. At days 2, 4, 6, 8, and 10, cells were trypsinized and counted manually using a hemacytometer.

Cell cycle and apoptosis analysis

Subconfluent cells were treated with EA1-Fc or control Fc at 1 μ g/mL or transfected with siRNA as described above. At the indicated time points, cells were fixed and permeabilized, then analyzed using the APO-BrdU kit from Phoenix Flow Systems per manufacturer's instructions. Briefly, fixed cells were washed, labeled with bromodeoxyuridine, stained with FITC-anti-bromodeoxyuridine antibody, labeled with propidium iodide, and analyzed by flow cytometry at The University of Texas M.D. Anderson Cancer Center Flow Cytometry and Cellular Imaging Core Facility.

Western blotting and immunoprecipitation

Subconfluent cells were washed with ice-cold PBS and collected in lysis buffer (50 mmol/L Tris-HCl, 1% Triton-X100, 150 mmol/L NaCl, and 10 μ g/mL each protease and phosphatase inhibitor cocktail from Pierce). Lysates were held on ice for 15 min before clarification at 14,000 rpm for 10 min. Equal protein aliquots were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, probed with primary antibody, and detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reagent (Amersham Biosciences). For immunoprecipitation, 500 μ g of total protein were diluted in lysis buffer to a concentration of 1 μ g/ μ L and precleared with protein G-Sepharose. Samples were then incubated for 2 h or overnight at 4°C with anti-EphA2 antibody (Millipore) and precipitated with protein G-Sepharose. Density quantitation was done using NIH ImageJ (31).

Migration assay

Untreated cells were grown to confluence in six-well dishes. Alternatively, cells were plated and transfected with siRNA, as described above, then allowed to reach confluence (~48–60 h after transfection) before beginning the assay. Once the cells reached confluence, sterile pipette tips were used to make a scrape in the monolayer, the cells were gently washed with PBS, and medium containing the indicated treatment was added. Serial photographs of the same area were taken from 0 to 12 h. Cell migration was measured by comparing the size of the wound over time with the original wound size. ImageJ was used to measure the size of the wound at each time point, and all wound sizes were normalized to the original wound size.

Rho GTPase activity assay

The RhoA G-LISA assay from Cytoskeleton was used to assess RhoA activity after Rho activation per manufacturer's instructions. Briefly, subconfluent cells were serum starved and then treated with EA1-Fc or control Fc. Cells were lysed, clarified, and snap-frozen in liquid nitrogen. Protein concentration was calculated, equalized between all samples with lysis buffer, mixed with binding buffer, and applied to the G-Lisa plate. After incubation to bind active GTPase, the plate was incubated with primary antibody, then incubated with horseradish peroxidase-conjugated secondary antibody, and detected with horseradish peroxidase; absorbance was measured at 490 nm.

Statistical analysis

The log-rank test was used to test differences between groups. The cutoff for high versus low EphA2 expression was defined on the basis of a mean score of 110. Kaplan-Meier curves for time to recurrence and time to metastasis were produced. Multivariable Cox proportional hazard regression models were done to determine the effect of EphA2 expression status on recurrence distributions after adjusting for the potential risk factors (age, gender, race, smoking status, tumor histology, and tumor stage). The final multivariable model was obtained using a backward selection approach. All tests were two-sided, and $P < 0.05$ was considered statistically significant. SAS 9.1.3 and S-PLUS 7.0 were used for the analyses.

Results

Downregulation of EphA2 decreases proliferation of NSCLC cell lines

To examine the role of EphA2 in NSCLC progression and to assess its possible use as a therapeutic target, we used two methods of downregulating EphA2 in this study: One method involved EA1-Fc, the dimerized ligand for EphA2, and the other used EphA2-specific siRNA (17, 32). EA1-Fc produces a transient increase in phosphorylation of EphA2, leading to decreased total EphA2 through rapid internalization and degradation of the receptor (19, 29).

To determine the effect of EphA2 downregulation on the proliferation of NSCLC cells, we used a clonogenic growth assay in which we seeded cells at a low density, treated them with EA1-Fc or control-Fc for 14 days, and then assessed them by measuring colony size and number. Baseline EphA2 expression in our seven NSCLC cell lines is shown in Fig. 1A; ligand treatment of five of these cell lines resulted in decreased colony size in four (Fig. 1B and C). We observed no difference in the colony numbers of treated and untreated cells. To further characterize this effect, we chose two cell lines that experienced a decrease in colony size (i.e., sensitive lines, H322 and H441), incubated them with EA1-Fc or control-Fc for 10 days, and counted the cells. As early as 6 days after EphA2 knockdown, we observed a decrease in the number of cells when cells were incubated with EA1-Fc (Fig. 1D and E).

The significantly smaller colony size in EA1-Fc-treated cell lines, without differences in colony number, suggests either cell cycle arrest or apoptosis in the treated cells. We next examined the effects of EphA2 downregulation on cell cycle progression and apoptosis in H441, H1299, and H322 NSCLC cell lines. Our analysis revealed no changes in cell cycle progression either by EA1-Fc- or EphA2-specific siRNA-mediated downregulation of EphA2 in H1299 cells, consistent with the colony formation assay (Fig. 1F and data not shown). In contrast, H322 and H441 cells underwent cell cycle arrest (Fig. 1F). Low levels of baseline apoptosis, measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, remained unchanged in all three cell lines regardless of treatment. Likewise, we found no evidence that EA1-Fc induced autophagy or senescence (data not shown).

EphA2 knockdown transiently inhibits ERK1/2 activity

To determine the mechanism underlying EphA2-mediated proliferation, we next examined the effects of EphA2 downregulation on downstream signaling in NSCLC in cells that had been sensitive (H441 and H322) and resistant (H1299) to the effects of EphA2 knockdown on colony size. After treating cells with EA1-Fc, we observed a transient decrease in activated ERK1/2 in H441 and H322 cells but not in H1299 cells (Fig. 2). In NSCLC cells, EphA2 knockdown had no consistent effect on other pathways known to affect proliferation (i.e., AKT or signal transducer and activator of transcription 3), showing a specific effect of EphA2 on ERK1/2.

To determine if this effect on ERK1/2 was sufficient to account for the biological effects that we observed, we transiently inhibited ERK1/2 in H441 cells using the mitogen-activated protein/ERK kinase (MEK) inhibitor PD98059. Incubation of cells with PD98059 led to a rapid and profound inhibition of ERK1/2, which recovered to near baseline levels once PD98059

was removed (Fig. 3A), mirroring the effect of EA1-Fc on ERK1/2 (Fig. 2A and B). Transient inhibition of ERK1/2 resulted in reduced colony size without affecting the colony number in a clonogenic assay, similar to the results seen after EA1-Fc treatment (Fig. 3B).

Knockdown of EphA2 inhibits tumor cell migration

Expression of EphA2 has been implicated in migration and metastasis in a number of cancer models. To determine whether EphA2 affects NSCLC migration, we examined the effects of EphA2 modulation on NSCLC cells in a wound healing assay. Cells were grown to confluence on cell culture dishes and wounded, and the ability of the cells to migrate into the wound was compared between control and EA1-Fc-treated cells. In five of six cell lines tested, EA1-Fc treatment resulted in decreased migration of NSCLC cells (Fig. 4A and B). To ascertain whether the effects on migration were dependent on EphA2 activation or loss of expression, we transfected H441 and H1299 cells with EphA2-specific siRNA and measured migration. In both cases, cells treated with EphA2-specific siRNA migrated more slowly than cells transfected with nontargeting (scrambled) siRNA (Fig. 4C and D), showing that the

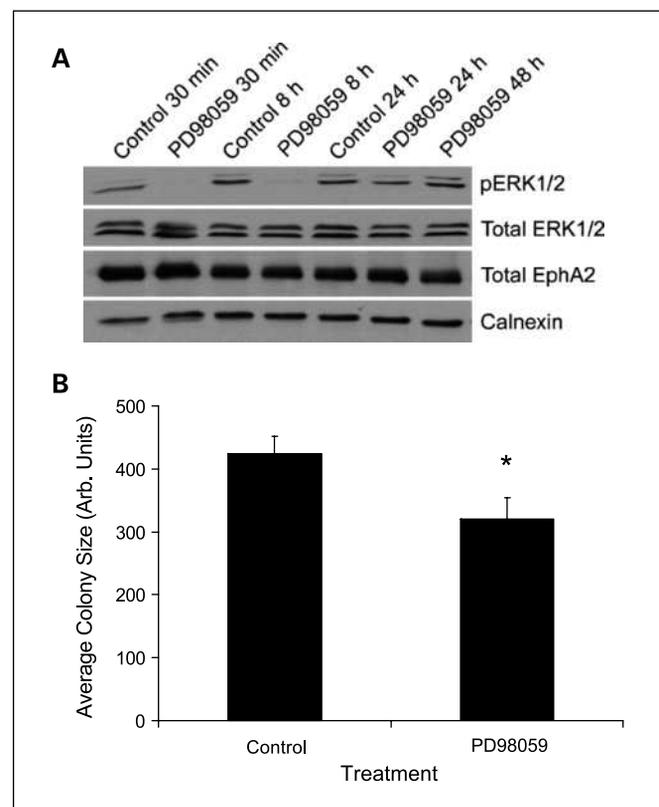


Fig. 3. Transient inhibition of ERK1/2 results in decreased proliferation. *A*, H441 cells were incubated with the MEK inhibitor PD98059 or vehicle control for from 30 min to 8 h. In the 24-h and 48-h samples, the medium was changed after 8 h of exposure to PD98059 or vehicle control to remove the drug or control. Cells were assayed by Western blot with the indicated antibodies. PD98059 inhibits ERK1/2 activity (pERK1/2) but has no effect on total ERK1/2 or EphA2 protein levels. *B*, H441 cells were seeded sparsely, allowed to adhere overnight, and then treated with PD98059 or vehicle control for 8 h every 3 d to mimic the effect of EA1-Fc treatment on ERK1/2 activity in the clonogenic assay. After 14 d, cells were fixed, stained with crystal violet, digitized, and analyzed for colony size using ImageJ. Bars, SD. *, $P \leq 0.05$.

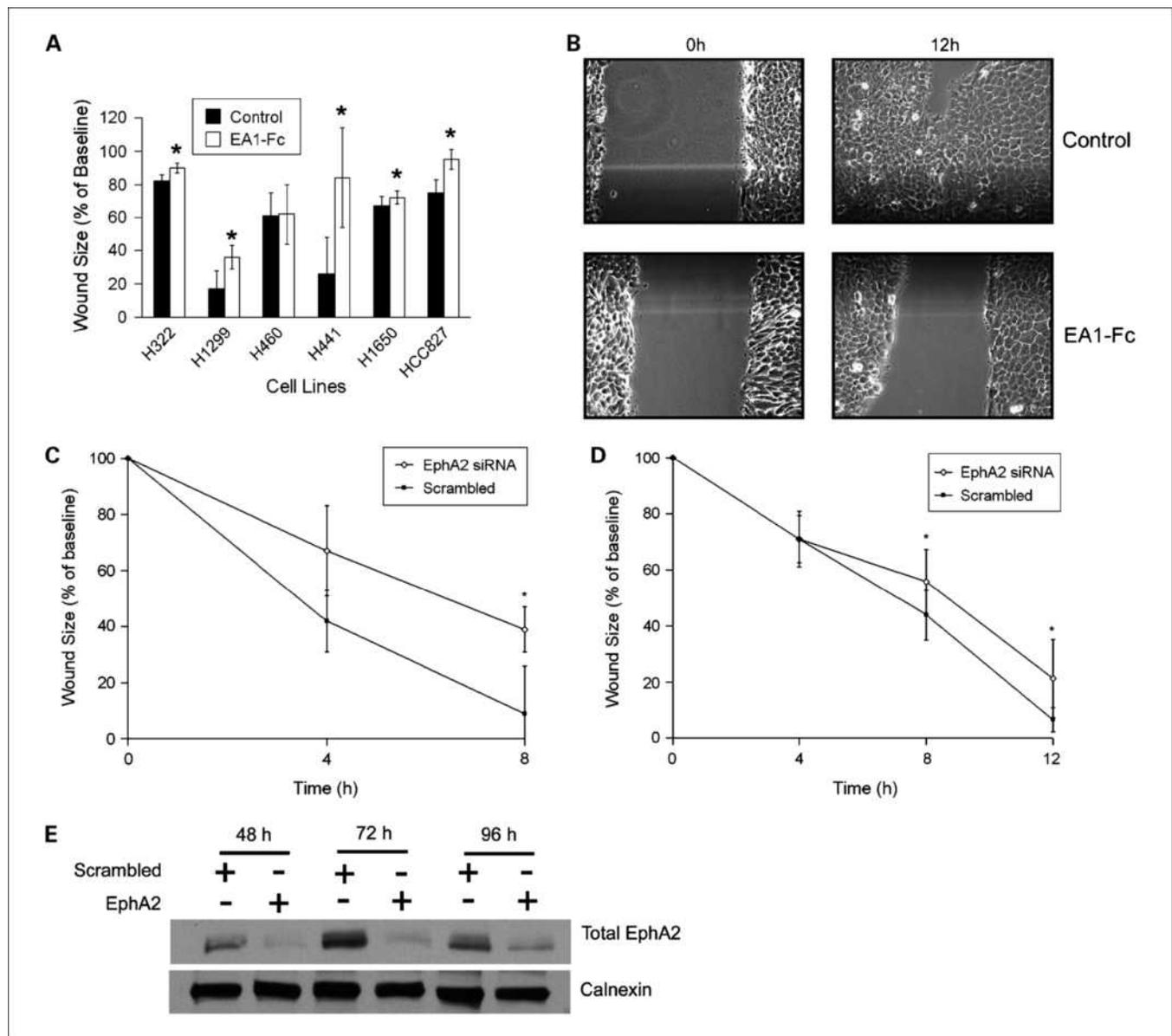


Fig. 4. EphA2 protein downregulation inhibits migration. NSCLC cell lines were incubated with EA1-Fc (A and B) or EphA2-specific siRNA (C and D) to deplete EphA2 protein levels. Cells were grown to confluence, and then a wound was made to the monolayer. The wound area was measured in control and treated cells at the indicated time points. H441 cells (C) and H1299 cells (D) were transfected with nonspecific siRNA (scrambled) or EphA2-specific siRNA. Bars, SD. *, $P \leq 0.05$. E, Western blotting of H441 cells transfected with EphA2 siRNA.

effect on tumor cell migration stems from a specific decrease in EphA2. In H1299 cells, EphA2-specific siRNA resulted in a more than 90% reduction in total EphA2 protein. In H441 cells, EphA2-targeting siRNA resulted in a 75% to 95% reduction in total EphA2 protein (Fig. 4E).

Depletion of EphA2 transiently inhibits RhoA activity but does not affect c-Src or FAK activation

Eph receptors have been shown to affect migration through interactions with c-Src and Rho GTPase family members. We examined the effect of EA1-Fc on the activation of Rho GTPases RhoA, Rac1, and CDC42 (Fig. 5A). Treatment of cells with EA1-Fc led to a transient decrease in RhoA and CDC42 activity at early time points in NSCLC cells. EA1-Fc did not

affect the levels of total RhoA (data not shown). A pan-Rho inhibitor, which inhibits RhoA, RhoB, and RhoC, but not Rac1 and CDC42, did not affect migration (Fig. 5B). Inhibiting Rho in the presence of EA1-Fc or anti-EphA2 siRNA did not affect EphA2 downregulation-dependent inhibition of migration, suggesting that EphA2-dependent migration does not require Rho activation in NSCLC cells.

Given our data suggesting a role for EphA2 in NSCLC migration and the literature associating EphA2 with c-Src and FAK, we looked for an association between EphA2 and c-Src activity at Tyr416 (pSrc) in resected tissue from NSCLC patients but found none (Spearman correlation coefficient = 0.096; $P = 0.119$). We next examined the effects of EphA2 modulation on Src and FAK activation in NSCLC cell lines. EA1-Fc

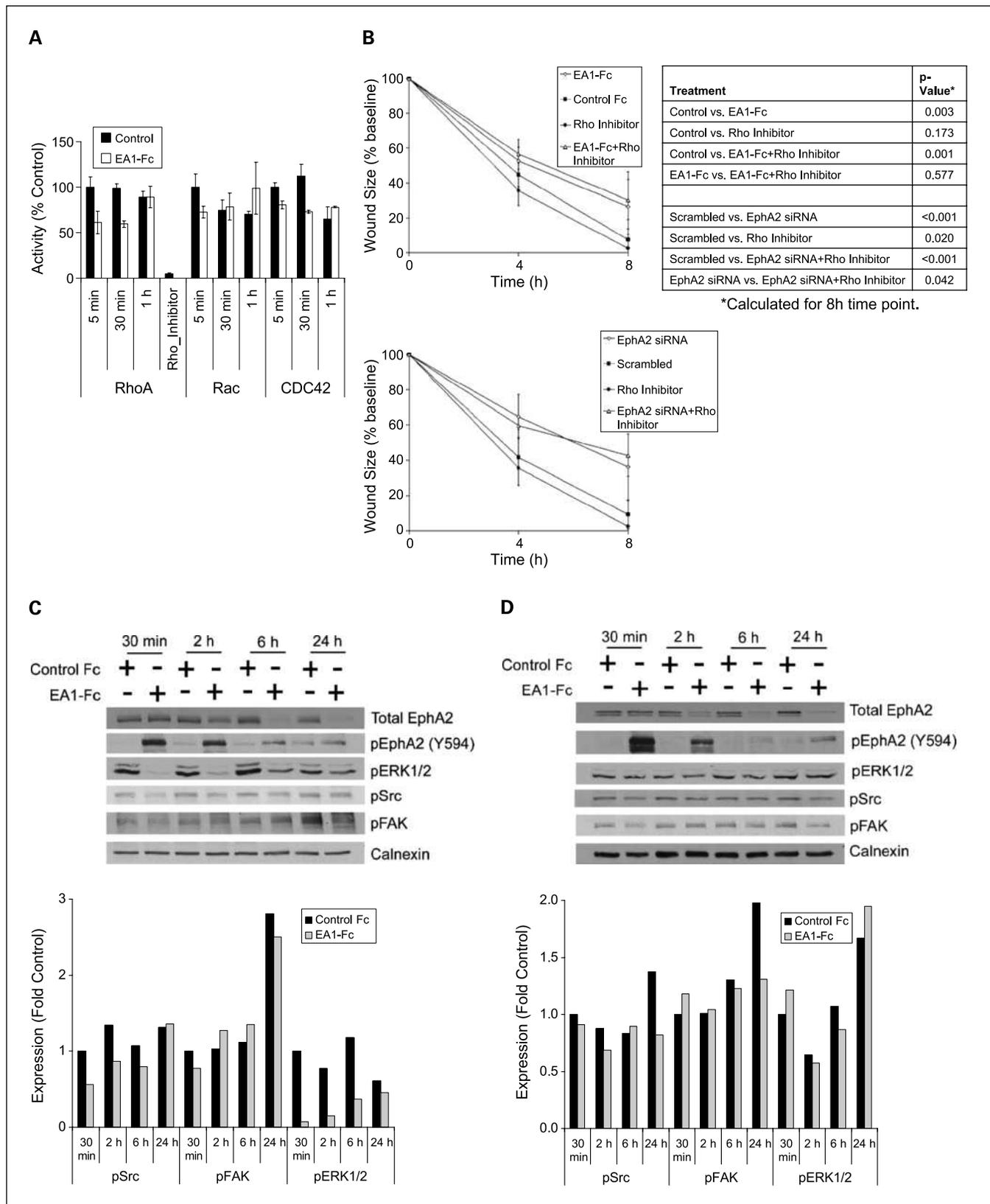


Fig. 5. Downregulation of EphA2 transiently inhibits RhoA activity but does not consistently affect the activation status of c-Src or FAK. *A*, H441 cells were incubated with EA1-Fc or control Fc, and RhoA, Rac, and CDC42 activities were measured by G-LISA at the indicated time points. *B*, H441 cells were treated with a Rho inhibitor, either alone or combined with EA1-Fc (top) or anti-EphA2 siRNA (bottom), and tumor cell migration was measured using a wound assay. H441 (*C*) and H1299 (*D*) cells were incubated with EA1-Fc or control Fc for various lengths of time and protein expressions were measured by Western blotting at the indicated time points. The blots were quantified using ImageJ, and expression was corrected for the loading control (calnexin).

did not consistently affect Src or FAK activity (Fig. 5C and D), showing that EphA2 does not affect migration via modulation of c-Src or FAK activity in NSCLC tumors.

EphA2 expression correlates with decreased time to recurrence and metastasis

To extend our analysis *in vivo* and evaluate the role of EphA2 in regulating migration and proliferation in patients with NSCLC, we measured the levels of EphA2 expression in tumors and compared the levels to the rates of recurrence and metastasis. For this analysis, we divided the results into two groups based on low and high EphA2 expression as previously described (7). High EphA2 expression was associated with decreased time to recurrence and metastasis (Fig. 6). In a multivariate analysis, high EphA2 expression was an independent prognostic factor for predicting time to recurrence (hazard ratio, 1.54; 95% confidence interval, 1.03-2.31; $P = 0.038$) and time to metastases (hazard ratio, 1.58; 95% confidence interval, 1.05-2.39; $P = 0.029$) after adjusting for tumor stage.

EphA2 expression in AAH lesions and the bronchioloalveolar component of adenocarcinomas

EphA2 was expressed in the cytoplasm of 19 of the 22 (86%) AAHs and in 15 of the 21 (71%) adenocarcinomas we assessed

(Fig. 7). The score of expression was slightly higher in AAH lesions (mean score, 104.1; SD, 65.6) than in tumors (mean score, 83.3; SD, 68.7). We examined AAHs and corresponding tumors in three patients, and the level of expression was similar in both tissue types: a mean score of 126.0 (SD 64.6) in AAH and 133.3 (SD 5.8) in tumors. EphA2 was expressed in 16 of the 18 (89%) cases of bronchioloalveolar (noninvasive) component, and the mean score of the expression (72.2; SD, 64.8) was similar to that in corresponding invasive tumors (70.0; SD, 67.6).

Discussion

The results of our present study advance our understanding of the role of EphA2 in the proliferation and migration of NSCLC cells and metastasis of NSCLC tumors, all factors related to progression, and in the pathogenesis of NSCLC. We showed that knockdown of EphA2 results in decreased NSCLC cell proliferation and migration *in vitro*. Likewise, increased EphA2 expression in NSCLC tumors correlated with time to recurrence and metastasis. EphA2 expression also was common in premalignant tissues. These findings support our hypothesis that EphA2 promotes NSCLC progression and is a potential target for therapy and prevention.

EA1-Fc treatment significantly reduced the sizes of colonies in four of five cell lines but did not reduce colony number. This result suggests that the same proportion of cells that were seeded (placed in medium) in each group was able to form colonies, but that once the colonies were formed, proliferation was slower in EA1-Fc-treated than in untreated cells. Growth curve analysis indicated a slowing growth rate and arrest at G₁ in EA1-Fc-treated NSCLC cell lines. Apoptosis did not increase in the treated cells, and thus we attribute the smaller colony size to a decreased rate of proliferation. This finding is consistent with previous data (19, 33) showing that knocking down Eph receptor expression reduced cell proliferation and colony-forming efficiency in various non-lung models including breast carcinoma.

One mechanism by which EphA2 may affect cancer cell progression is in interacting with the Ras/ERK pathway (19, 20, 34). Conflicting data have shown that the signaling effects of EphA2 on ERK were highly context or cell type dependent. Stimulation of EphA2 in prostate epithelial cells, fibroblasts, keratinocytes, and endothelial cells caused a rapid (within 5-10 minutes) and sustained (1-2 hours) inhibition of ERK1/2 (19). In contrast, phospho-EphA2 led to the rapid activation (within 2 minutes) of ERK1/2 in breast and prostate cancer cells by binding the adaptor protein SHC (21). The interactions between EphA2 and ERK1/2 have never been investigated previously in NSCLC. We examined the effects of EA1-Fc treatment on ERK1/2 activity in NSCLC cell lines. We observed that EA1-Fc treatment transiently decreased ERK1/2 activity in two of three NSCLC cell lines (the nonresponsive line was H1299 and is discussed in the next paragraph). Our previous work and those of others established a relationship between EphA2 and *K-Ras* mutation in patient tumors and cell lines (7, 20, 34). Of note, although ligand activation of EphA2 was found to have opposite effects on ERK activation in studies by Miao et al. (19) and Nasreen et al. (22), both groups observed decreased cell proliferation in response to EphA2 activation in epithelial cells. There is evidence that Eph receptors have

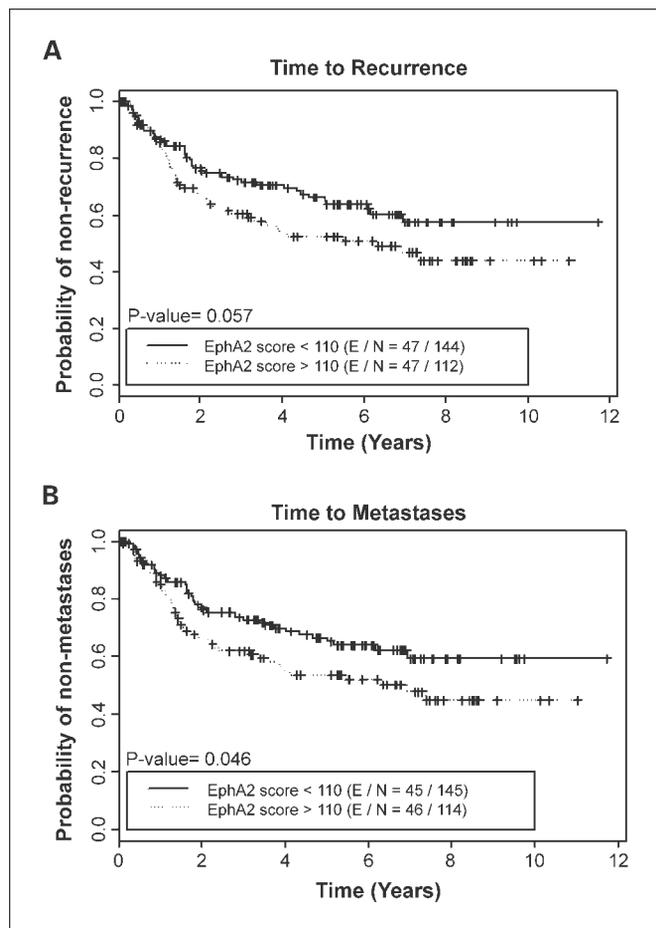


Fig. 6. High EphA2 expression correlates with decreased time to recurrence and metastases. Kaplan-Meier curves for time to recurrence (A) and time to metastases (B) in low versus high EphA2-expressing tissues of NSCLC patients.

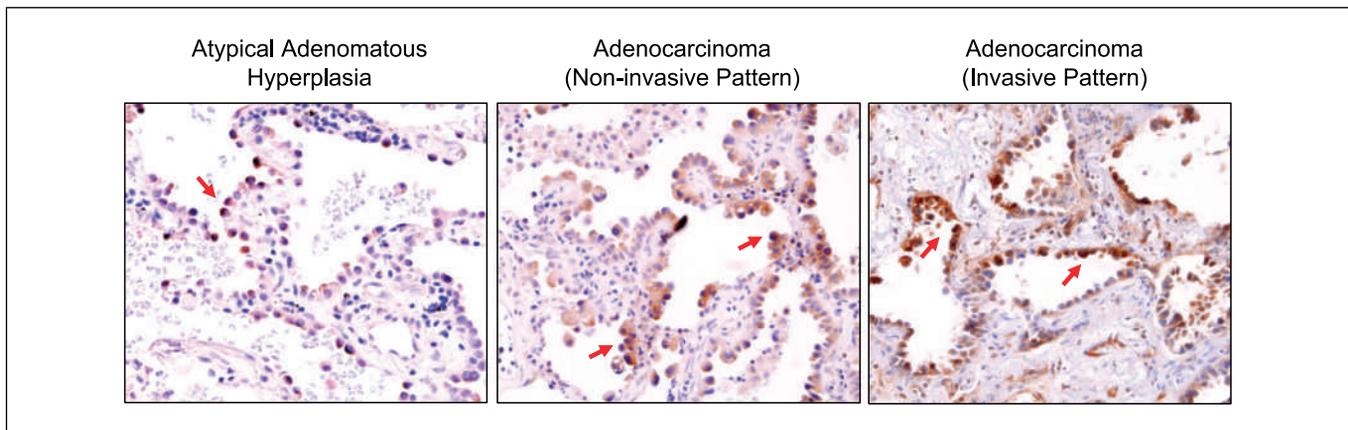


Fig. 7. Microphotographs ($\times 200$ magnification) of representative examples of EphA2 immunohistochemical staining in premalignant tissue (AAH) and malignant tissue (adenocarcinoma). Arrows, brown immunostaining for expression of EphA2 in the cytoplasm of AAH cells and adenocarcinoma cells (both of the noninvasive and invasive components).

cell-type-specific effects. Furthermore, a recent study described the requirement for EphA2 in ErbB2-driven mammary tumors but not in polyoma virus middle T (PyV-mT)-driven tumors in mice (35). These data support a model in which the oncogene context of EphA2 expression may determine the biological effects of EphA2 activation.

EA1-Fc treatment did not decrease proliferation or ERK1/2 activity in H1299 cells, suggesting that decreased proliferation in NSCLC cell lines after EA1-Fc treatment depends on inhibition of ERK1/2 activity. This distinct result (versus positive effects in other lines) may be related to differential phosphorylation status of EphA2 in H1299 cells. As expected, EA1-Fc treatment downregulated total EphA2 protein expression, as detected within 2 hours and sustained for 72 hours, and increased EphA2 phosphorylation immediately (within 5 minutes) and robustly. Although total EphA2 phosphorylation was transient in H322 (Fig. 2A) and H441 (Fig. 2B) cells, total receptor phosphorylation was sustained for up to 72 hours in H1299 cells (Fig. 2C). In contrast, phosphorylation at Y594 in H1299 cells was transient (Figs. 2C and 5C). Mutations of *EphA2* in the juxtamembrane region (Y594) were previously shown not to affect the association of EphA2 with the proliferation-associated p85 subunit of phosphatidylinositol 3-kinase but to affect its association with migration-associated Vav2, suggesting that Y594 is important for migration but not for proliferation (36), which is consistent with our observation that EA1-Fc inhibited migration but not proliferation in H1299 cells.

Because Eph receptors play a role in cell migration during development, it is not surprising that upregulation of Eph receptors in cancer may contribute to cell motility. Using a wound healing assay, we observed that the downregulation of EphA2, either by ligand or siRNA, inhibited migration in all cell lines but H460. The lack of an effect in this line may be explained by its oncogene context. Three cell lines in our panel, H460, H441, and H1299, harbor *Ras* mutations. However, H441 cells also exhibit increased EGFR activity due to gene amplification, which may affect signaling to and from EphA2. H1299 cells carry a mutation in *N-Ras* rather than *K-Ras*. *N-Ras* and *K-Ras* mutations have been noted to exert differential effects on cells; *K-Ras* mutations contributed to cancer progres-

sion in a mouse model, whereas *N-Ras* mutations did not (37). Therefore, H460 cells may represent the only "true" *Ras* mutant among our panel of cell lines, suggesting a role for the *Ras*/ERK pathway in EphA2-mediated migration within NSCLC cell lines. Further examination of EphA2-mediated migration in the presence and absence of *Ras* mutations could address the role of *K-Ras* mutations in EphA2-mediated tumor cell migration.

Eph receptors have been reported to interact with several proteins frequently implicated in tumor cell migration and invasion. The best described of these interactions to date are EphA2 with Src, FAK, and the Rho GTPases. The EphA2 ligand EA1 activated Src and its downstream effector FAK, which led to Rho-dependent contractility, in prostate carcinoma cells (24). Conversely, EA1 activation of EphA2 suppressed integrin function and decreased FAK phosphorylation in other systems (29, 38, 39). AKT-dependent phosphorylation of EphA2 promoted cell migration, which was inhibited by EphA2 ligand binding (18). Growing evidence points toward regulation of the Rho GTPases by Eph receptors (24, 40, 41). In light of the implication of both increased and lost expression of Rho family members in cancer (42), the role of Rho GTPases in cancer is still controversial. A recent study found that EA1 activation of EphA2 led to increased RhoA activity in a Src/FAK-dependent manner in prostate and breast carcinoma cells (24). Rho activity has been shown to both impede and promote cell migration (reviewed in ref. 43) and to be inhibited in cells expressing a signaling-defective mutant of EphA2 (44). Our examination of Rho GTPase activity in H441 cells showed a transient decrease in RhoA and CDC42 activity at early time points. We did not observe, however, any effect of a Rho inhibitor, either alone or in combination with EphA2 downregulation, on NSCLC migration, suggesting that Rho GTPases do not modulate EphA2-mediated migration in NSCLC. The Rho inhibitor did inhibit RhoA, RhoB, and RhoC and thus may mask the differential effects of Rho family members, for example, those of RhoB, which behaves very differently from other Rho family members and may suppress tumorigenesis (42).

Aberrant c-Src activation has been observed in NSCLC and a number of other cancers (45). c-Src and FAK activation

together mediate integrin signaling and control cell motility. EphA2 is constitutively associated with FAK in PC3 cells, and ligand activation of EphA2 results in the dissociation and dephosphorylation of FAK, thereby inhibiting cell migration (38, 39). Increased FAK activation, however, also has been observed in response to ligand activation of EphA2 (24, 46). Combined with the association of EphA2 expression with more aggressive tumors, these data strongly suggest a role for EphA2 in Src/FAK- and Rho-dependent tumor cell migration, although the effects of EphA2 activation seem to be cell type dependent rather than universal. We did not detect any association between c-Src activity and EphA2 expression in NSCLC patient tumors, and we found no changes in c-Src or FAK activity levels in response to ligand stimulation of EphA2 in NSCLC cell lines. Furthermore, c-Src inhibition reduces migration in these cell lines (47). These data further support the hypothesis that the role of EphA2 in tumor cell migration may be exquisitely context dependent.

In light of the evidence suggesting a role for EphA2 in metastatic progression (7–14), we examined the relationship between EphA2 expression and clinical outcome of NSCLC patients. We showed that high EphA2 expression is an independent prognostic factor for decreased time to recurrence and metastasis. These results agree with those from a previous NSCLC study, in which EphA2 expression predicted a poor prognosis and brain metastases (8). Although we found no correlation of EphA2 with brain metastasis in our current study, our sample contained few patients with brain metastases.⁵ Combined with our previous work correlating high

EphA2 expression with decreased survival, these data suggest that EphA2 may be a useful prognostic marker in NSCLC.

To our knowledge, this is the first study to examine the expression of EphA2 in premalignant lesions of any type. Whereas the sequential preneoplastic changes have been defined for squamous carcinomas that arise in the central region of the lungs, they have been poorly documented for adenocarcinomas (48). Adenocarcinomas may be preceded by morphologic changes, including AAH in peripheral airways (48). Similar to lung adenocarcinomas, AAHs had a high frequency of EphA2 expression, suggesting that this abnormality is a frequent and early phenomenon in the pathogenesis of lung adenocarcinoma. This finding is supported by our observation that the noninvasive bronchioloalveolar component was similar in frequency and levels of EphA2 expression to the corresponding invasive component of adenocarcinomas.

We report here that downregulation of EphA2 inhibits the proliferation and migration of NSCLC cell lines and the first evidence that EphA2 is expressed in premalignant lesions of lung adenocarcinoma. Furthermore, we have shown that the ligand-mediated inhibition of proliferation is dependent on inhibition of ERK1/2 activity. We also have shown that activation of EphA2 can transiently inhibit the activity of Rho family members RhoA and CDC42 but has no effect on Src or FAK activity in NSCLC cell lines. These data and the observation that EphA2 expression correlates with metastasis in NSCLC patients suggest a causative function for EphA2 in aggressive NSCLC. Therefore, EphA2 may be a promising target for the prevention and therapy of NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

⁵ Unpublished data.

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Jennifer M. Brannan, Banibrata Sen, Babita Saigal, et al.

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