Signal Transducer and Activator of Transcription 3 Is Required for the Oncogenic Effects of Non–Small-Cell Lung Cancer–Associated Mutations of the Epidermal Growth Factor Receptor

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Abstract

Somatic mutations in the epidermal growth factor receptor (EGFR) occur frequently in lung cancer and confer sensitivity to EGFR kinase inhibitors gefitinib and erlotinib. These mutations, which occur in the kinase domain of the protein, also render EGFR constitutively active and transforming. Signal transducers and activators of transcription 3 (STAT3) transduces signals from a number of oncogenic tyrosine kinases and contributes to a wide spectrum of human malignancies. Here, we show that STAT3 is activated by mutant EGFRs and is necessary for its downstream phenotypic effects. Inhibiting STAT3 function in fibroblasts abrogates transformation by mutant EGFR. In non–small-cell lung cancer cells, STAT3 activity is regulated by EGFR through modulation of STAT3 serine phosphorylation. Inhibiting STAT3 function increases apoptosis of these cells, suggesting that STAT3 is necessary for their survival. Finally, a group of genes constituting a STAT3 signature is enriched in lung tumors with EGFR mutations. Thus, STAT3 is a critical mediator of the oncogenic effects of somatic EGFR mutations and targeting STAT3 may be an effective strategy for treating tumors characterized by these mutations. (Cancer Res 2006; 66(6): 3162-8)

Introduction

Although lung cancer remains the leading cause of death from cancer in the United States, significant progress has been made toward elucidating the molecular underpinnings of this disease. The epidermal growth factor receptor (EGFR) tyrosine kinase has been implicated in the pathogenesis of lung cancer, and this has prompted the development of small-molecule inhibitors of EGFR kinase activity (1). Gefitinib (ZD1839, Iressa; AstraZeneca, Wilmington, DE) and erlotinib (OSI-774, Tarceva; Genentech, San Francisco, CA) both inhibit EGFR kinase activity with nanomolar potency, and several clinical trials using these agents alone or in combination with conventional chemotherapy have been done (2). These trials indicated that both EGFR inhibitors led to clinical response in only a minority of patients. The molecular determinant of this response was found to be somatic mutations in the gene encoding the EGFR (3–5). Tumors from patients responding to gefitinib and erlotinib harbored EGFRs with mutations that cluster in the kinase domain of the protein (6, 7). These mutations render the kinase constitutively active, and it has recently been shown that expression of these mutant proteins is sufficient to transform rodent fibroblasts and human lung epithelial cells (8, 9).

Following activation by either ligand binding or mutation, EGFR initiates a cascade of signal transduction pathways that alter the biology of the cell through transcriptional and post-translational mechanisms. The signaling pathways that mediate these changes include the Ras–Raf–mitogen-activated protein (MAP) kinase (MAPK), phosphoinositide 3-kinase–AKT, and signal transducers and activators of transcription (STAT) 3 and STAT5 signal transduction pathways (10). The STAT family of transcription factors are activated by phosphorylation on a conserved tyrosine residue, leading to dimerization, nuclear translocation, and DNA binding (11). STAT1, STAT3, and STAT5 are also phosphorylated on a serine residue in their COOH terminus; this phosphorylation is dispensable for dimerization, nuclear translocation, and DNA binding, but is required for maximal transcriptional activity of some genes (12). STAT3 is persistently activated in a wide variety of hematologic and epithelial malignancies and is required for the survival of many tumor cell lines (13, 14). It is activated by diverse receptor and non–receptor tyrosine kinases, including EGFR, platelet-derived growth factor receptor, Janus-activated kinases (JAK), and src. STAT3 is an important mediator of the oncogenic effects of EGF and transforming growth factor–α in squamous cell carcinoma of the head and neck (15), and is activated by EGF in a mouse model of skin carcinogenesis (16). Furthermore, several non–small-cell lung cancer cell lines contain constitutively active STAT3 (17). Finally, nontransformed epithelial cells engineered to express various non–small-cell lung cancer (NSCLC)-associated EGFR mutants contain elevated levels of activated STAT5 and STAT3 (8), and we recently showed that STAT3 is activated by several of these EGFR mutants in a genetically defined system (9).

It is not known which of the signal transduction pathways downstream of mutant EGFR are required to mediate its oncogenic properties. Given the role of STAT3 in a wide range of human malignancies, and the fact that it is activated by EGF in various cell types, we sought to determine whether STAT3 is necessary for the oncogenic effects of somatic mutant EGFRs. We find that STAT3 is activated in fibroblasts expressing mutant EGFRs, as well as in two NSCLC lines with naturally occurring EGFR mutations, and that this activation is required for the transformation and survival, respectively, of these cells.
Materials and Methods

Plasmids. STAT3 Y705F (STAT3F; ref. 18) was inserted into the mammalian expression vector pBCEMV. pIRES-GFP and pLNCX2 were from Clontech (Mountain View, CA). m67 pTATA TK-luc was kindly provided by J. Bromberg (Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY). The Renilla luciferase reporter plgRL tk-luc was from Promega (Madison, WI).

Cell culture, inhibitors, and growth factors. NIH3T3 cells expressing mutant EGFR have been described previously (9). H3255 and HCC-827 lung adenocarcinoma cells were kindly provided by Pasi Janne (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA) and were cultured as described (9, 19). Gefitinib was kindly provided by AstraZeneca and was used at 1 μmol/L unless otherwise noted. The MAP/ extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) inhibitor PD98059 (Cell Signaling Technology, Danvers, MA) was used at 20 μmol/L, the MEK2 inhibitor U0126 (Cell Signaling Technology) was used at 10 μmol/L, the src inhibitor PP2 (Calbiochem, San Diego, CA) was used at 10 μmol/L, and the JAK inhibitor AG490 (Calbiochem) was used at 50 μmol/L. EGF (R&D Systems, Minneapolis, MN) was used at 50 ng/mL. For Western blot analysis, following treatment with inhibitors or stimulation with EGF, cells were plated on coverslips or in 6 cm dishes overnight in 0.5% serum and then treated with inhibitors for 2 hours or stimulated with EGF for 30 minutes.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40 (a nonionic detergent), 0.5% sodium deoxycholate, and 0.1% SDS] containing 1 mmol/L phenylmethanesulfonyl fluoride, 1 μg/mL pepstatin, and 1 mmol/L sodium vanadate on ice for 15 minutes. Fifty micrograms of protein were resolved on 8% or 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Antibodies were as follows: STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated STAT3 (pSTAT3; Cell Signaling Technology), serine pSTAT3 (20), phospho-ERK1/2 (Cell Signaling Technology).

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were done essentially as described (21). Briefly, 1 × 10⁶ cells were starved overnight in 0.5% serum and then treated with inhibitors for 2 hours. Nuclear extracts were prepared and incubated with 32P-radio-labeled probe from the sis-inducible element (hSIE; 5′-TGGAGGCTGACATTTCCCCCTAATTGTGCTGA-3′) and its complement; refs. 22, 23) in binding buffer for 15 minutes at room temperature. For antibody blocking experiments, extracts were incubated with an anti-STAT3 antibody (Santa Cruz Biotechnology) for 20 minutes before the binding reaction. Proteins-DNA complexes were resolved by nonnaturating gel electrophoresis and were detected by autoradiography.

Luciferase assays. Cells on a 6 cm dish were transfected with 7.2 μg m67-luciferase and 0.8 μg pHRL plasmids using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Sixteen hours later, cells were plated at 50,000 per well onto a 24-well plate. After adhering, they were treated with inhibitors for 24 hours. Cells were then lysed and relative luciferase was measured using the dual-luciferase reagents from Promega using a Luminoskan Ascent luminometer (ThermoLab Systems, Helsinki, Finland). STAT3-dependent luciferase production was normalized to control Renilla values.

Soft agar assays. NIH3T3 cells expressing the EGFR ins mutant were transfected with STAT3F-IRES-GFP or the empty IRES-GFP vector. After 48 hours, green fluorescent protein (GFP)-positive cells were counted to normalize for transfection efficiency. Cells (1 × 10⁶) were plated in 0.5% soft agar containing 500 μg/mL of the antibiotic G418 sulfate (to select for neomycin-resistant cells) on a layer of 0.5% base agar. After 3 weeks, resistant cells were starved overnight in 0.5% base agar. Cells on a 6 cm dish were transfected with 7.2 μg m67-luciferase and 0.8 μg pHRL plasmids using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Sixteen hours later, cells were plated at 50,000 per well onto a 24-well plate. After adhering, they were treated with inhibitors for 24 hours. Cells were then lysed and relative luciferase was measured using the dual-luciferase reagents from Promega using a Luminoskan Ascent luminometer (ThermoLab Systems, Helsinki, Finland). STAT3-dependent luciferase production was normalized to control Renilla values.

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RNA interference and viability assays. Retrovirus-expressing short hairpin RNA targeting STAT3 was produced as described previously (24). HCC-827 cells were infected with 1 mL viral supernatant supplemented with 2 mL medium with 4 μg/mL polybren. After 24 hours, puromycin was added at 1 μg/mL and cells were selected for 2 days. Resistant cells were immediately analyzed by Western blot for STAT3 expression and plated at 6,000 per well on a 96-well plate. After adhering overnight, cells were left untreated or treated with varying concentrations of gefitinib. Cell viability was measured after 24, 48, and 72 hours using the CellTiter-Glo luminescence-based ATP assay (Promega).

Retroviral infection and Annexin V apoptosis assays. Retrovirus-expressing STAT3F or STAT3A was produced as described previously (24). HCC-827 cells were infected with 1 mL viral supernatant supplemented with 2 mL medium with 4 μg/mL polybren. Cell culture medium was changed after 12 hours, and cells were harvested 72 hours postinfection. To measure the extent of apoptosis, cells were analyzed for Annexin V-FITC and propidium iodide staining (Beckman Coulter, Miami, FL) to detect the levels of phosphatidylserine and the extent of membrane permeability, respectively. The percentage of Annexin V–positive cells was measured by flow cytometry.

Gene set enrichment analysis. Genes associated with pSTAT3 in breast tumors were determined as previously described (24). Briefly, genes differentially expressed in tumors with high nuclear pSTAT3 staining versus those with no nuclear pSTAT3 were ranked based on signal-to-noise scores. Sets comprising the top 20, 50, 100, 200, or 300 genes were then analyzed for differential expression in lung tumors with mutant versus wild-type EGFR using gene set enrichment analysis, as described previously (24–26).

Results

STAT3 activation in fibroblasts transformed by mutant EGFRs. To determine if STAT3 is activated by mutant EGFRs, we used genetically defined mouse fibroblasts engineered to express wild-type EGFR or various mutant forms found in lung tumors. We previously showed that NIH3T3 cells expressing EGFR with an L858R (3T3/EGFR L858R) or an exon 20 insertion (3T3/EGFR ins) mutation, both of which occur in NSCLC, displayed elevated STAT3 tyrosine phosphorylation compared with NIH3T3 cells expressing wild-type EGFR (9). To confirm and extend these results, we focused on the ins mutant, which was the most transforming mutant identified and also led to the most prominent STAT3 activation. NIH3T3 cells expressing the exon 20 insertion mutant EGFR contain increased STAT3 tyrosine phosphorylation compared with cells with empty vector (Fig. 1A). As shown previously, expression of wild-type EGFR had no effect on downstream signaling pathways, including STAT3 phosphorylation (ref. 9 and data not shown). This phosphorylation observed in cells expressing the ins mutant was dependent on EGFR kinase activity, as it could be inhibited by treatment with gefitinib (Fig. 1B). Under these conditions, gefitinib led to almost complete suppression of EGFR autophosphorylation (Fig. 1A, asterisk). To determine if this elevated tyrosine phosphorylation translated to increases in DNA binding, an EMBA was done. Nuclear extracts from cells expressing the ins mutant had elevated levels of STAT DNA-binding activity, and this could be inhibited with an antibody against STAT3, confirming that STAT3 DNA binding was increased in these cells (Fig. 1B). To address the functional consequences of this elevated DNA binding, a luciferase reporter assay was done. Expression of the ins and L858R mutants led to increased transcription from a STAT-dependent promoter (Fig. 1C; data not shown). STAT3 DNA-binding and transcriptional activity could be inhibited by gefitinib, indicating that both are dependent on EGFR kinase activity, STAT3 can also become phosphorylated on a serine residue (Ser727) in its COOH-terminal transactivation domain. Expression of both the ins and L858R mutant EGFR also led to serine phosphorylation of STAT3, and this required EGFR kinase activity (Fig. 1D).

To further elucidate the kinases involved in STAT3 activation downstream of mutant EGFRs, we used specific pharmacologic inhibitors. STAT3 tyrosine phosphorylation was dependent on EGFR and src kinase activities, as it could be inhibited by gefitinib and the
src inhibitor PP2, but was independent of JAK2 kinase activity (Fig. 1D; data not shown). This is consistent with previous results regarding EGF-induced STAT3 phosphorylation by wild-type EGFR (27, 28). STAT3 serine phosphorylation has previously been shown to be mediated by ERK1/2 (29). Consistent with this, STAT3 serine phosphorylation was sensitive to the MEK1 inhibitor PD98059, suggesting that it is dependent on MEK1/2 activity. Indeed, ERK1/2 are constitutively phosphorylated in cells expressing both mutants, and this phosphorylation is EGFR dependent (Fig. 1D). Together, these results suggest that the constitutive STAT3 serine phosphorylation downstream of mutant EGFRs may be mediated by ERK1/2. This is consistent with previous studies showing ERK1/2-induced STAT3 Ser727 phosphorylation both in vivo and in vitro (29).

**STAT3 activation in human lung cancer cells expressing mutant EGFRs.** To determine if the results in mouse fibroblasts were reflective of human lung cancer cells, we determined whether STAT3 was activated in two NSCLC cell lines with naturally occurring mutant EGFRs. H3255 cells contain the L858R EGFR mutation, and HCC-827 cells contain an E746_A750 EGFR deletion. STAT3 was tyrosine phosphorylated under basal conditions in both cell lines, and this phosphorylation could be increased with EGF stimulation. However, this phosphorylation was not affected by treatment with gefitinib, suggesting that it is not dependent on EGFR kinase activity (Fig. 2A). The antibody to tyrosine-phosphorylated STAT3 cross-reacts with an epitope on the EGFR that arises from receptor autophosphorylation, as has been found with other pSTAT-specific antibodies (30). Gefitinib also inhibited this autophosphorylation, confirming that the inhibitor works in these cell types at this concentration (Fig. 2A, asterisk). To address whether STAT3 DNA-binding activity was increased in these cells, EMSA was done on nuclear extracts from each cell type. A STAT3-DNA complex was observed in both cell types, confirming STAT3 activation (Fig. 2B). However, these complexes were not inhibited by gefitinib treatment, demonstrating that STAT3 DNA binding is independent of EGFR kinase activity in these two cell lines. JAK2 and src frequently phosphorylate STAT3 in tumor cells. To address

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**Figure 1.** STAT3 is activated in fibroblasts expressing mutant EGFR. A, cells expressing wild-type (wt) EGFR or the ins mutant were starved in 0.5% serum overnight and then treated with 1 μmol/L gefitinib for 2 hours. STAT3 tyrosine phosphorylation was determined by Western blotting. *, phosphorylated EGFR detected by the pSTAT3 antibody. B, cells were treated as in (A), and STAT3 DNA-binding activity was measured by EMSA using a hSIE probe. The STAT3-DNA complex could be disrupted with an antibody against STAT3. Arrow, STAT3-DNA-binding complex. C, cells were transfected with a STAT3-dependent luciferase reporter (m67-luc) and treated with 1 μmol/L gefitinib for 24 hours. Levels of STAT3-dependent luciferase production were measured and normalized to Renilla luciferase (phRL-luc). D, cells were starved in 0.5% serum overnight and then treated with 1 μmol/L gefitinib or 20 μmol/L PD98059 for 2 hours. STAT3 serine phosphorylation and ERK1/2 phosphorylation were determined by Western blotting.

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**Figure 2.** STAT3 is activated in lung cancer cells with mutant EGFR. A, cells were starved in 0.5% serum overnight and left unstimulated or stimulated with 50 ng/mL EGF or treated with 1 μmol/L gefitinib for 2 or 24 hours. STAT3 tyrosine phosphorylation was determined by Western blotting. *, phosphorylated EGFR. B, cells were treated as in (A), and STAT3 DNA-binding activity was measured by EMSA using a hSIE probe. The STAT3-DNA complex could be disrupted with an antibody against STAT3. Arrow, STAT3-DNA-binding complex in H3255 cells; arrowhead, STAT3-DNA complex in HCC-827 cells consistent with a STAT3:STAT1 heterodimer. C, cells were starved in 0.5% serum overnight and then treated with 1 μmol/L gefitinib or 20 μmol/L PD98059 for 2 hours, and STAT3 serine phosphorylation and ERK1/2 phosphorylation were determined by Western blotting. D, cells were transfected with a STAT3-dependent luciferase reporter (m67-luc) and treated with 20 μmol/L PD98059 or 10 μmol/L U0126 for 24 hours. Levels of STAT3-dependent luciferase production were measured and normalized to Renilla luciferase (phRL-luc).
whether these kinases are responsible for the constitutive STAT3 phosphorylation observed in H3255 and HCC-827 cells, cells were treated with pharmacologic inhibitors of each kinase. Neither PP2 nor AG490 inhibited STAT3 tyrosine phosphorylation, indicating that in these cell lines neither JAK2 nor src is responsible for STAT3 phosphorylation (data not shown).

Given the prominent phosphorylation of STAT3 on Ser727 in fibroblasts expressing mutant EGFR, we considered the possibility that this phosphorylation might be elevated in these NSCLC cell lines. STAT3 was constitutively phosphorylated on Ser727 in both cell lines, and, in contrast to tyrosine phosphorylation, this phosphorylation was inhibited by gefitinib treatment (Fig. 2C). Again, this correlated with an inhibition of constitutive ERK1/2 phosphorylation. Direct inhibition of MEK1 with PD98059 decreased ERK1/2 phosphorylation and STAT3 serine phosphorylation in parallel, suggesting that ERK1/2 is responsible for this phosphorylation event in these cells (Fig. 2C).

Because phosphorylation of STAT3 on Ser727 has been shown to be required for maximal transcriptional activation in some contexts, we wished to determine whether inhibition of EGFR kinase activity led to a decrease in STAT3-dependent transcription through inhibition of serine phosphorylation. We measured STAT3 transcriptional activity using a STAT3-dependent luciferase reporter gene in H3255 and HCC-827 cells. Treatment of both cell types with pharmacologic inhibitors of MEK1 or MEK1/2 led to a marked decrease in STAT3-dependent transcription (Fig. 2D). This suggests that mutant EGFRs regulate STAT3 activity via a MEK-dependent mechanism.

STAT3 activity contributes to EGFR-induced transformation of fibroblasts. NIH3T3 cells expressing mutant EGFRs are transformed, shown by their loss of contact inhibition, their anchorage-independent growth, and their ability to form tumors in nude mice (9). To determine if STAT3 is required for this transformation, 3T3/EGFR ins cells were transfected with a vector expressing GFP (pIRES-GFP) or STAT3Y705F (pSTAT3F-IRES-GFP), which functions as a dominant negative protein. Transfected cells were selected in soft agar containing G418, and colonies were counted 3 weeks later. GFP-positive cells were counted at 2 days posttransfection and used to normalize for transfection efficiency. Cells expressing STAT3F formed fewer colonies in soft agar than cells expressing empty vector (Fig. 3A and B). Stable pools were also created by infecting cells with retrovirus expressing STAT3F or

![Figure 3](image-url)

Figure 3. Requirement of STAT3 for EGFR-induced transformation of fibroblasts. A, cells were transfected with empty vector (pIRES-GFP) or dominant negative STAT3 (STAT3F-IRES-GFP) and selected in soft agar containing G418. After 21 days, cells were photographed by phase microscopy (×100; left) and following staining with crystal violet (right). B, number of macroscopically visible colonies (>0.2 mm diameter) were counted in triplicate and normalized to GFP-positive cells (counted 48 hours posttransfection) to control for transfection efficiency.

![Figure 4](image-url)

Figure 4. Requirement of STAT3 for growth and survival of lung cancer cells with mutant EGFR. A, HCC-827 cells were infected with retrovirus expressing short hairpin RNA against STAT3 (pRS/STAT3) and selected in puromycin for 48 hours. Levels of total STAT3 were analyzed by Western blotting. B, immediately following selection in puromycin, cells were plated in 96-well plates and their viability was measured at 0, 24, and 48 hours using an ATP-based viability assay. C, immediately following selection in puromycin, cells were plated in 96-well plates, allowed to adhere overnight, and treated with 1 nmol/L gefitinib. Their viability after 48 hours was measured using an ATP-based viability assay, and the decrease in viability is expressed relative to untreated cells. Four replicates were done per condition for all viability assays. D, cells were infected with retrovirus expressing empty vector, STAT3F, or STAT3A, and the extent of apoptosis was measured after 72 hours by quantifying Annexin V–positive cells on a flow cytometer.
empty vector and selecting in G418. Resistant cells were plated in soft agar, and colonies were counted 3 weeks later. Again, cells expressing STAT3F formed fewer colonies than cells containing empty vector (data not shown). Thus, STAT3 activity contributes to EGFR-induced transformation of NIH3T3 cells.

**STAT3 contributes to the survival of lung cancer cells expressing mutant EGFRs.** We next determined whether STAT3 plays a role in the growth and survival of lung cancer cells with mutant EGFRs. HCC-827 cells were infected with a retrovirus expressing short hairpin RNA targeting STAT3, or a vector control, and selected in puromycin for 2 days (Fig. 4A). HCC-827 cells in which STAT3 has been knocked down proliferated more slowly than control cells (Fig. 4B). These cells also displayed increased sensitivity to low concentrations of gefitinib (Fig. 4C). To determine if inhibiting STAT3 activity directly induces apoptosis in these cells, cells were infected with retrovirus expressing one of two inhibitory forms of STAT3, and the amount of apoptosis was measured 72 hours later. STAT3F contains the critical tyrosine residue (Tyr705) mutated to phenylalanine, and likely serves as a dominant negative protein by inhibiting receptor binding and phosphorylation of wild-type STAT3. It is possible that STAT3F would inhibit activation of other STAT family members as well. STAT3A contains a serine-to-alanine mutation at residue 727 and should compete with wild-type STAT3 at the promoters of target genes. Both forms function to inhibit endogenous STAT3 (31). Expression of each protein led to an ~2-fold increase in the amount of apoptosis in HCC-827 cells (Fig. 4D). Additionally, cells expressing each protein proliferated more slowly than control cells and also displayed increased sensitivity to gefitinib (data not shown). These results suggest that both tyrosine and serine phosphorylation of STAT3 contribute to the survival of these cells and suggest that inhibition of STAT3 activity may be one mechanism by which gefitinib kills these tumor cells.

**A STAT3 signature is present in lung tumors with EGFR mutations.** If STAT3 mediates the oncogenic effects of mutant EGFRs, then STAT3 target genes should be expressed in lung tumors harboring mutant receptors. A data set for this analysis was obtained by sequencing EGFR in 127 lung adenocarcinomas for which oligonucleotide microarray gene expression data was previously generated (32). Sequencing of EGFR in these samples revealed kinase domain mutations in 13 of the 127 tumors.5 We compared the gene expression profiles of tumors with EGFR mutations to those without and determined whether a group of genes previously shown to correlate with activated STAT3 in breast tumors (ref. 24; Supplementary Table S1) was enriched in the former class. This group of genes was significantly enriched in lung tumors containing EGFR mutations as measured by gene set enrichment analysis (Fig. 5A and B; refs. 25, 26). Thus, an expression signature for STAT3 activation is present in these lung tumors harboring mutant EGFR, suggesting that STAT3 activation may play an important mechanistic role in the development of these tumors.

**Discussion**

Targeted therapies against oncogenic tyrosine kinases have proven effective in treating several types of cancer. Small-molecule inhibitors of EGFR lead to clinical response in some patients with NSCLC, and this response correlates with activating mutations in the kinase domain of EGFR. These mutant proteins are sufficient to transform mouse fibroblasts and human epithelial cells and are required for the survival of NSCLC cell lines. Understanding the biological changes induced by mutant EGFR and its contribution to oncogenesis requires a thorough understanding of the downstream signal transduction pathways it activates. Here, we show that STAT3 is activated by various forms of mutant EGFR—including the common L858R and E746_A750 deletion mutants as well as the potently transforming exon 20 insertion mutant—and may contribute to the oncogenic effects of these mutations in fibroblasts and human lung cancer cells.

In fibroblasts expressing mutant EGFR, STAT3 is constitutively phosphorylated on both Tyr705 and Ser727. Phosphorylation on Tyr705, which is critical for STAT3 dimerization, nuclear translocation, and DNA-binding, requires EGFR and src kinase activity, but is independent of JAK2. The serine phosphorylation requires EGFR and MEK kinase activity, suggesting that it occurs via EGFR-initiated activation of the Ras-Raf-MAPK pathway, culminating in ERK1/2 activation and phosphorylation of STAT3 Ser727. These two phosphorylation events render STAT3 maximally transcriptionally active, and inhibiting these phosphorylations with the EGFR inhibitor gefitinib leads to a significant decrease in STAT3-dependent transcription.

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5 K. Naoki and M. Meyerson, manuscript in preparation.
In lung cancer cells expressing both types of EGFR mutations, STAT3 is also phosphorylated on both Tyr705 and Ser277. However, the tyrosine phosphorylation does not require EGFR kinase activity as it is unaffected by gefitinib treatment. Furthermore, this tyrosine phosphorylation is independent of src and JAK2 kinase activities. Thus, an as yet unidentified kinase is responsible for the constitutive STAT3 phosphorylation observed in these cell lines. Interestingly, a previous report indicated that inhibiting JAK2 led to decreased viability of different lung cancer cells expressing mutant receptors (8). Our data would suggest that this effect is not due to inhibition of STAT3, as JAK2 inhibition does not affect STAT3 tyrosine phosphorylation in the cell lines we examined. The constitutive serine phosphorylation in these cells does require EGFR and MEK kinase activity, suggesting that ERK1/2 kinases are responsible for STAT3 Ser277 phosphorylation in these cell lines as well. Although the MEK1 and MEK1/2 pharmacologic inhibitors are relatively specific, it remains formally possible that another kinase is inhibited by these compounds and that this inhibition is responsible for the observed STAT3 phosphorylation (33, 34). Nonetheless, inhibiting this serine phosphorylation leads to a decrease in STAT3-dependent transcription, indicating that serine phosphorylation is required for maximal transcriptional activity of STAT3.

The finding that pathogenic mutations of the EGFR lead to constitutive serine phosphorylation of STAT3 has a precedent in other tumors. Constitutive phosphorylation of STAT3 on Ser277 is also seen in the malignant cells of chronic lymphocytic leukemia (CLL), but not their normal counterparts (20). The role that this serine phosphorylation of STAT3 plays in the pathogenesis of CLL is unclear, but it may serve to amplify signals for survival and proliferation induced by cytokines. For example, in myeloid cells, stimuli that cause STAT3 tyrosine phosphorylation and serine phosphorylation synergize with one another at the levels of gene expression and biological output (35). Thus, by promoting serine phosphorylation of STAT3 in lung epithelial cells and enhancing STAT3-dependent gene expression, activating EGFR mutations may have profound effects on cellular function.

STAT3 was shown to be constitutively activated in a number of lung cancer cell lines (17). However, this study did not address EGFR mutation status or STAT3 serine phosphorylation, which we have shown is required for maximal activity. Examining the prevalence of STAT3 tyrosine and serine phosphorylation and the association of each phosphorylation with EGFR mutations in primary lung cancer samples will be of paramount importance.

Tumors become dependent on the oncogenes that initiated transformation, a phenomenon known as oncogene addiction (36). Inhibiting mutant EGFR in NSCLC cells leads to induction of apoptosis (19). Our results suggest that one mechanism by which this occurs is through inhibition of STAT3-driven transcription. Inhibiting STAT3 serine phosphorylation through expression of STAT3A mimicked the effect of EGFR inhibition on STAT3 function and led to significant apoptosis. This suggests that STAT3 serine phosphorylation is an important component of the prosurvival signals that emanate from EGFR.

We previously identified a group of genes based on their high expression in breast tumors with activated STAT3. We find that this group of genes is enriched in lung tumors with mutant EGFR. This suggests that STAT3 promotes oncogenesis by regulating a common set of genes in distinct tumor types, consistent with previous findings (24). Furthermore, this suggests that STAT3 is likely mediating the oncogenic role of these mutant proteins in vivo through altering the transcriptional profile of the tumor cell. Indeed, STAT3 target genes have been implicated in promoting the survival and proliferation of tumor cells, as well as the induction of angiogenesis by tumors (37). Identifying the specific STAT3 targets that contribute to malignancy in NSCLC remains an important goal.

Elucidating the molecular pathways downstream of EGFR is important for several aspects of NSCLC pathology. First, a number of tumors that respond to EGFR inhibitors do not contain mutations in EGFR, and understanding the mechanism of sensitivity of these tumors is of importance. Second, tumors that initially respond to EGFR inhibitors eventually progress and become refractory to EGFR inhibition. Although in some of these cases, an additional mutation in EGFR was identified that conferred resistance to the inhibitor, in the other cases no second mutation was identified, suggesting that other mechanisms of resistance exist (38, 39). Understanding how signaling pathways downstream of mutant EGFR, including STAT3, contribute to the response and resistance of lung tumors to EGFR inhibitors should lead to the improved treatment of these tumors. Furthermore, given the critical role STAT3 plays in mediating the oncogenic effects downstream of mutant EGFR, pharmacologic inhibitors of STAT3 may prove useful in the treatment of NSCLC. Indeed, the ins mutant, which leads to robust STAT3 activation, seems to be relatively resistant to gefitinib, and tumors with these mutations may respond to treatment less well than tumors with other mutations (9). Thus, targeting STAT3 in these tumors, either alone or in combination with EGFR inhibitors, may have a beneficial effect. Finally, a number of other solid tumors contain activated EGFR, and our findings suggest that STAT3 may play a critical role in the pathogenesis of these tumors as well.

Acknowledgments

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References

7. Janne PA, Engelman JA, Johnson BE. Epidermal growth factor receptor mutations in non-small-cell lung cancer:
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