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Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells

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Overexpression of receptor tyrosine kinases including the epidermal growth factor receptor (EGF-R) as well as nonreceptor tyrosine kinases, such as Src, have been implicated in the formation of human lung cancers. In addition, cytokines like interleukin-6 (IL-6) have been demonstrated to modulate lung cancer cell growth and elevated levels of IL-6 have been shown to be an adverse prognostic factor for patients with lung cancer. Despite a large body of evidence pointing to their potential importance, few direct studies into the role of signal transducers and activators of transcription (STAT) pathways in human lung cancer have been undertaken. Here we demonstrate that multiple nonsmall cell lung cancer cell lines demonstrate constitutive Stat3 DNA-binding activity. Stat3 DNA-binding activity is specifically upregulated by the addition of epidermal growth factor (EGF), IL-6, and hepatocyte-derived growth factor (HGF). Furthermore, the stimulation of Stat3 DNA-binding activity by EGF requires the activity of EGF-R tyrosine kinase as well as Src-kinase, while the upregulation of Stat3 activity by IL-6 or HGF requires only Src-kinase activity. Treatment of A549 lung cancer cells with PD180970 or SU6656, both pharmacological inhibitors of Src-kinase, resulted in reduced Src and Stat3 activity, cell cycle arrest in G2, and reduced viability of cells accompanied by induction of apoptosis. Treatment of Stat3-positive A549 and H358 cells with antisense Stat3 oligonucleotides results in complete loss of Stat3 DNA-binding activity and apoptosis, while Stat3-positive H1299 cells remained healthy. Finally, an adenoviral vector expressing a dominant-negative Stat3 isoform results in loss of Stat3 DNA-binding activity, apoptosis, and reduced cellular viability. These results demonstrate a role of Stat3 in transducing survival signals downstream of tyrosine kinases such as Src, EGF-R, and c-Met, as well as cytokines such as IL-6, in human nonsmall cell lung cancers.

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Introduction

Each year approximately 170 000 people in the United States will be diagnosed with cancer of the lung, with nearly 160 000 of patients succumbing to the illness (Greenlee *et al*., 2000). The majority of patients present with their illness in an advanced stage that precludes a curative surgical resection. Others present with surgically resectable disease, undergo a curative procedure and yet ultimately succumb to the subsequent development of distant metastatic disease. Despite advances in the treatment of advanced disease, progress has been slow. While chemotherapy has clearly shown a clinically significant benefit, the effect is modest and patients with metastatic disease ultimately die of their disease (Non-small cell lung cancer collaborative group, 1995).

One promising family of targets for cancer therapy are the tyrosine kinase growth factor receptors located on the cell surface. These growth factor receptors transmit signals through diverse pathways, which ultimately lead to alterations in gene expression important in the regulation of cell growth and survival. A number of nonreceptor tyrosine kinase signaling molecules are also important in the regulation of cell growth and survival. Perhaps the best studied is the c-Src proto-oncogene, which was discovered after identification of the viral Src oncogene from the Rous sarcoma virus (Jove and Hanafusa, 1987; Irby and Yeatman, 2000).

v-Src leads to activation of a number of different signaling cascades and can lead to transformation of a number of different cell types. The activation of receptor tyrosine kinases and Src also leads to activation of the signal transducers and activators of transcription (STAT) pathway. Accumulation of evidence from many laboratories has demonstrated the critical role of STATs in the molecular pathology of cancer (Bowman *et al*., 2000; Bowman and Jove, 1999). Originally identified as key components linking cytokine signals to transcriptional events in cells, STATs more recently have been demonstrated to play a major role in tumor formation.

STATs are a family of latent cytoplasmic transcription factors which form dimers when activated by tyrosine kinase signals and translocate to the nucleus to regulate expression of genes by binding to elements within promoters (Darnell, 1997). In addition to cytokine receptors, STATs are also activated by tyrosine kinase growth factor receptors such as the epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R), as well as nonreceptor tyrosine kinases such as Src and BCR-Abl, and cytokines such as IL-6 (Garcia *et al*., 1997; Bowman *et al*., 2000; Garcia and Jove, 1998). Recent work has demonstrated that v-Src-induced cellular transformation is dependent on Stat3-mediated transcription of cellular genes (Bowman *et al*., 2000; Bromberg and Darnell, 2000). In addition, a constitutively activated mutant of Stat3 can enhance cellular proliferation and survival as well as lead to cellular transformation, providing genetic evidence for the intrinsic oncogenic potential of Stat3 (Bromberg *et al*., 1999).

Recent work has demonstrated the activation of specific STATs in human cancers, including breast cancer, multiple myeloma and leukemia, lymphomas, and head and neck cancers (Bowman *et al*., 2000). While few direct studies into the role of STATs in human lung cancer have been undertaken, a large body of evidence points to their potential importance. First, tyrosine kinase growth factor receptors are overexpressed in a large number of human lung cancers, with non-small cell lung cancers demonstrating overexpression of EGF-R and its ligands EGF, amphiregulin, and TGF-alpha, while some small cell lung cancers demonstrate c-kit overexpression (Antoniades *et al*., 1992; Rusch *et al*., 1993, 1997; Takanami *et al*., 1995; Kawai *et al*., 1997; Krystal *et al*., 2000; Brabender *et al*., 2001). Lung cancer cell lines can produce soluble EGF or TGF-alpha and targeting the EGF-R with either monoclonal antibodies or small molecule inhibitors can lead to inhibition of tumor growth and in some cases tumor regression (Lee *et al*., 1992; Rabiasz *et al*., 1992; Lei *et al*., 1998, 1999; Fang and Chen, 1999; Ciardiello *et al*., 2000; Sirotnak *et al*., 2000; Wang *et al*., 2000a; Norman, 2001). Second, Src, a major upstream regulator of STAT activity, has also been suggested to be activated in human lung cancers (Mazurenko *et al*., 1992; Budde *et al*., 1994). Third, one study suggested a role for Stat3 activation in a lung cancer cell line transformed by c-*erb*B2 (Fernandes *et al*., 1999). Fourth, cytokines can activate STAT activity in human lung fibroblasts (Doucet *et al*., 2000). Finally, IL-6, a known upstream regulator of STAT signal transduction acting through the gp130 receptor, has been found to be elevated in nearly 40% of lung cancer patients (Yanagawa *et al*., 1995). Cancer cell lines of nonsmall cell origin have been found to express IL-6 mRNA and protein, and antisense oligonucleotides targeting IL-6 results in reduced IL-6 synthesis and cellular proliferation (Bihl *et al*., 1998). These results suggested a possible autocrine role of IL-6 in the growth regulation of lung cancer cells. Therefore, lung carcinoma cells and lung stromal cells have a number of potential upstream regulators of the STAT family.

Growing evidence points to a number of important STAT target genes that have been implicated in the formation of tumors. First, STATs have been demonstrated to affect genes important in cell cycle control such as cyclin D1, the cyclin-dependent kinase inhibitor p21, and c-Myc (Turkson *et al*., 1998; Kiuchi *et al*., 1999; Odajima *et al*., 2000). In addition, STATs are also thought to regulate the process of programmed cell death or apoptosis, most notably through the upregulation of the antiapoptotic proteins Bcl-xL and Mcl-1. In a myeloma tumor model, IL-6-dependent Stat3 signaling upregulates the Bcl-x promoter and contributes to cell survival (Catlett-Falcone *et al*., 1999). A recent study demonstrated that a constitutively activated Stat3 protein protects fibroblasts from apoptosis induced by UV light and serum withdrawal (Shen *et al*., 2001). Significantly, these cell cycle and apoptosis genes are also found to be deregulated in human lung cancers. While amplification of the cyclin D1 or c-Myc gene is a rare event in nonsmall cell lung cancers, enhanced protein levels are frequently found in these tumors (Carbone, 1997). Therefore, one could envision a model where by constitutive STAT activation leads to increased transcription of cyclin D1 and Myc genes and higher levels of cyclin D1 and Myc protein.

We sought to delineate the potential role of Stat3 in human nonsmall cell lung carcinomas by studying cell lines of nonsmall cell origin. Our emphasis has been on Stat3, which appears to be the more frequent family member implicated in tumor formation and survival signaling in epithelial cells (Bowman *et al*., 2000). Our results demonstrate that non-small cell lung carcinoma cells have constitutive Stat3 DNA-binding activity which can be upregulated by EGF, IL-6, and the hepatocyte growth factor (HGF). Inhibition of Stat3 through pharmacological inhibition of upstream molecules such as Src and JAK leads to reduced cell viability and increased apoptosis. Direct inhibition of Stat3 by antisense oligonucleotides or dominant-negative Stat3 results in apoptosis in two of three lung cancer cells that have constitutive Stat3 activity but not lung carcinoma cells which lack constitutive Stat3 activity.

Results

Nonsmall cell lung cancer cell lines exhibit constitutive Stat3 DNA binding

To characterize the potential role of STATs in nonsmall cell lung cancer, we first sought to determine the incidence and pattern of STAT DNA binding in a collection of cell lines of nonsmall cell origin. All cell lines were grown in RPMI media and 5% fetal bovine serum (FBS). Subconfluent dishes of these tumor cells were harvested and nuclear extracts prepared for assays specific for DNA-binding activity of STATs. STAT activity is measured by electrophoretic mobility shift assay (EMSA) using the ^{32}P-labeled c-*fos* sis-inducible element (hSIE) probe that binds activated Stat1 and Stat3 proteins with high affinity. This radiolabeled

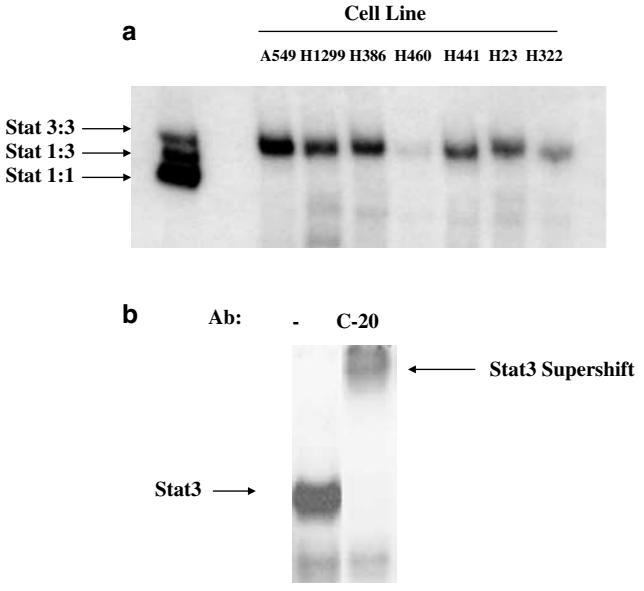


Figure 1 Constitutive activation of Stat3 in human nonsmall cell lung carcinoma cell lines. **(a)** EMSA analysis of STAT DNA-binding activity in nuclear extracts from human nonsmall cell lung cancer cell lines grown in 5% FBS performed using radiolabeled hSIE probe, which binds activated Stat1 and Stat3 with high affinity. Lane 1 consists of control extracts demonstrating positions of Stat3 : 3, Stat 1 : 3, and Stat 1 : 1 complexes and are indicated by arrows. **(b)** Incorporation of an antibody (C-20) which specifically recognizes Stat3 into the EMSA demonstrates a supershift of Stat3

probe c-*fos* sis-inducible element corresponds to a high-affinity variant of the hSIE which binds Stat1 and Stat3 homodimers as well as Stat1 : Stat3 heterodimers (Yu *et al*., 1995). As shown in Figure 1a, constitutive Stat3 DNA-binding activity is demonstrated in all but one cell line studied, while no constitutive Stat1 DNA-binding activity nor Stat1 : Stat3 heterodimers were observed in these exponentially growing carcinoma cells. Other notable findings include a low but detectable level of Stat3 activation in the H460 lung cancer cell line and differing extents of Stat3 binding in the different cell lines tested. To confirm that the band indicated by an arrow represents Stat3, we performed a Stat3 supershift using a specific antibody that recognizes Stat3 but not Stat1 or Stat5. The supershift performed with A549 cells shown in Figure 1b demonstrates reduced mobility of this complex, indicating that the band identified with an arrow is indeed Stat3. These findings suggest that constitutive activation of Stat3 in human lung cancers may play a role in the malignant phenotype of these cells.

Stat3 DNA binding is stimulated by both EGF and IL-6

Given the existence of constitutive Stat3 activity found in multiple nonsmall cell lung cancer cell lines, we next sought to identify tyrosine kinases that can activate Stat3. Previous studies using a breast cancer cell line model found that EGF was able to activate Stat3, while in a myeloma model, Stat3 activity was upregulated by IL-6 (Catlett-Falcone *et al*., 1999; Garcia *et al*., 2001). Since the EGF receptor and IL-6 cytokine have been implicated in the pathogenesis of nonsmall cell lung

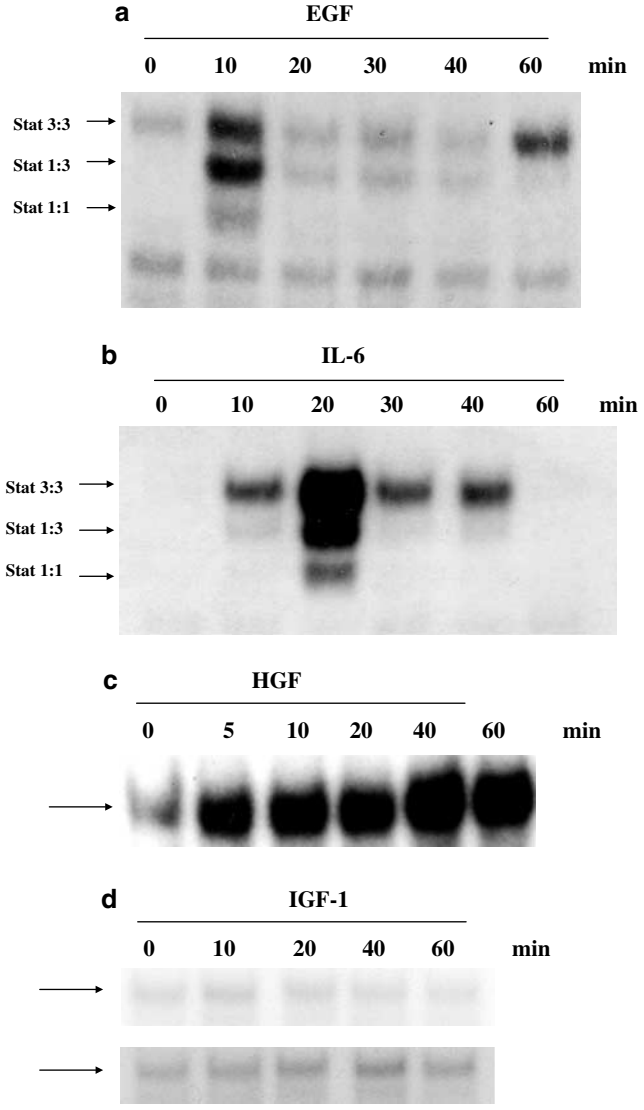


Figure 2 EGF, IL-6, and HGF stimulate Stat3 DNA binding in a time-dependent manner. A549 cells were serum deprived for 24 h in RPMI media alone and subsequently stimulated with addition of exogenous EGF, IL-6, and HGF as described. At the time points indicated, cells were harvested and nuclear extracts prepared for EMSA as previously described. **(a)** Addition of exogenous EGF stimulates Stat3 : 3, Stat 1 : 3, and Stat 1 : 1 complexes in a time-dependent manner with peak activation at 10 min followed by rapid inactivation. **(b)** Addition of exogenous IL-6 stimulates Stat3 : 3, Stat 1 : 3, and Stat 1 : 1 complexes in a time-dependent manner with peak activation at 20 min followed by rapid inactivation. **(c)** Addition of exogenous HGF stimulates only Stat3 : 3 activity in a time-dependent manner with peak activation at 40 min. Stat3 is indicated by arrow. **(d)** Two representative experiments are shown demonstrating minimal to absent stimulation of Stat3 activity by addition of exogenous IGF-1 to A549 cells. Stat3 is indicated by arrow

cancers, we determined whether these ligands could lead to activation of Stat3. A549 cells, which express the receptors for EGF and IL-6, were grown in serum-free media for 24 and then stimulated with exogenous EGF or IL-6. At various times after stimulation by EGF or IL-6, cells were harvested and nuclear extracts were prepared. As shown in Figure 2a, the addition of EGF

leads to the rapid activation of Stat3:3, Stat 1 : 3, and Stat 1 : 1 DNA-binding activity with a peak activity at 10 min followed by return to baseline levels 1 h after stimulation. In a similar pattern, Figure 2b demonstrates that the addition of IL-6 leads to activation of Stat3:3, Stat 1 : 3, and Stat 1 : 1 DNA-binding activity with peak activity at 20 min but to a greater extent to that seen with addition of EGF. IL-6 is a very potent stimulator of STAT activity and in order to have clear bands by EMSA, this film is exposed for shorter amounts of time compared with Figure 1 or 2a. With equal exposure times, the band at time 0 is very apparent. These results indicate that both EGF and IL-6 can activate Stat3 in a human lung cancer cell line. We also tested for the ability of HGF to stimulate the DNA-binding activity of Stat3. We were unable to demonstrate the activation of Stat3 or Stat 1 DNA-binding activity in A549 lung carcinoma cells (which have been reported to have c-Met expression) stimulated with HGF, but could demonstrate the activation of Stat3 in H358 lung carcinoma cells that express c-Met. H358 cells were serum deprived for 24 h, stimulated with exogenous HGF, and nuclear extracts prepared for EMSA. As demonstrated in Figure 2c, HGF leads to a rapid enhancement of Stat3 DNA binding in a time-dependent manner. Therefore, growth factors and cytokines implicated in the biology of lung cancer can activate Stat3 signaling.

We also attempted to determine if insulin-like growth factor 1 (IGF-1) and its receptor could upregulate Stat3 activity. A549 cells that express the receptor for IGF-1 were serum deprived as above and subsequently stimulated by addition of 30 ng/ml of IGF-1. Nuclear extracts were prepared from cells treated for different lengths of time and analysed for Stat3 DNA-binding activity by EMSA. Despite multiple attempts, we were unable to demonstrate any significant enhancement of Stat3 activity by addition of IGF-1 to A549 lung carcinoma cells. Figure 2d shows two independent experiments that demonstrate no increase in Stat3 DNA-binding activity. Therefore, at least in these lung carcinoma cells, IGF-1 does not appear to stimulate the activity of Stat3.

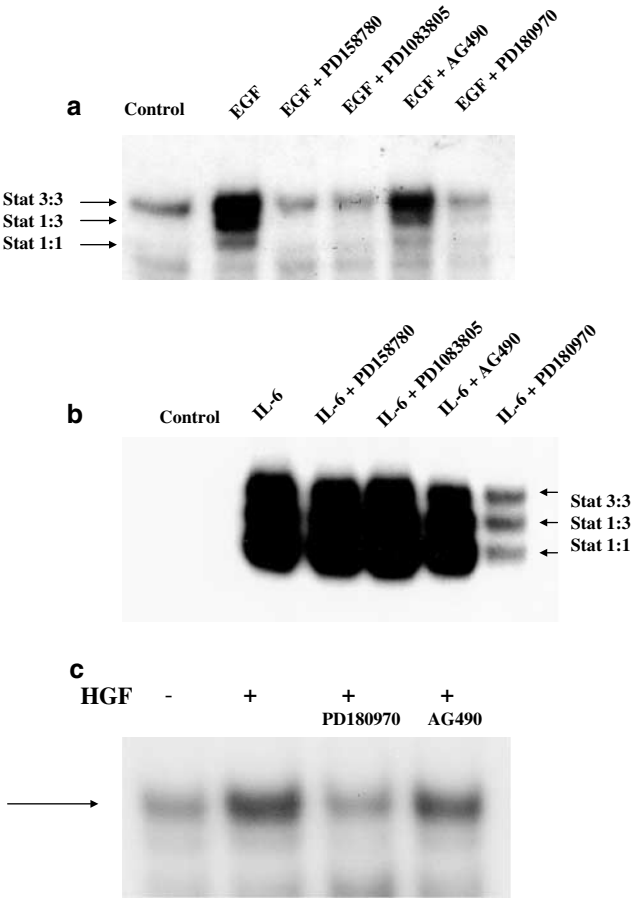


Figure 3 Stimulation of Stat3 DNA-binding activity by EGF, IL-6, and HGF requires Src-kinase activity. A549 or H358 cells were serum deprived for 24 h as before and pretreated with the listed inhibitors for 1.5 h before addition of mitogens. Concentrations used include 500 nM of PD158780, 500 nM of PD0183805, 500 nM of PD180970, and 50 μM of AG490. The cells were stimulated with mitogen for 10 min (EGF) or 20 min for IL-6 and HGF and were subsequently harvested and nuclear extracts prepared for EMSA. (**a**) EGF-dependent STAT activation requires EGF-R kinase activity and Src-kinase activity, since pretreatment of A549 cells with PD158780, PD0183805, or PD180970 prevents activation of Stat3 : 3, Stat 1 : 3, and Stat 1 : 1. **(b)** IL-6-dependent STAT activation requires Src-kinase activity, since pretreatment of A549 cells with PD180970 prevents complete activation of Stat3 : 3, Stat 1 : 3, and Stat 1 : 1. **(c)** HGF-dependent Stat3 activation requires Src-kinase activity, since pretreatment of H358 cells with PD180970 prevents activation of Stat3 : 3 DNA-binding activity

Activation of Stat3 DNA binding by EGF, IL-6, and HGF requires Src-kinase activity

In addition to receptor tyrosine kinases (such as EGF-R) and cytokine receptors, constitutive activation of Stat3 can also result from other oncoproteins with tyrosine kinase activity, such a v-Src, v-Abl, v-Fps, v-Eyk, v-Ros, and Bcr-abl (Zhong *et al*., 1994; Garcia *et al*., 1997; Garcia and Jove, 1998). Other studies have demonstrated the necessary role of Stat3 in transformation mediated by v-Src (Bromberg *et al*., 1998; Turkson *et al*., 1998). Recent studies have demonstrated a role of both Src and JAK in the constitutive activation of Stat3 in human breast carcinoma cell lines (Garcia *et al*., 2001). Since our results above implicated both EGF and IL-6 in the activation of Stat3 and other reports suggested a role of Src in the pathogenesis of human nonsmall cell lung cancers, we determined the tyrosine kinase requirements for ligand-induced activation of Stat3 by EGF, IL-6, and HGF. A549 cells were serum deprived for 24 h and pretreated for 1.5 h with DMSO (control), PD180970 (a specific inhibitor of Src-kinase), PD158780 (a specific inhibitor of EGF-R tyrosine kinase activity), PD0183805 (an irreversible pan-HER kinase inhibitor), and AG490 (a specific inhibitor of JAK) (Fry, 1999; Levitzki, 1999; Kraker *et al*., 2000). Cells were subsequently stimulated with either EGF or IL-6 as before and harvested 10 to 20 min after stimulation and nuclear extracts were prepared for EMSA. As shown in Figure 3a, addition of exogenous EGF leads to enhanced Stat3 DNA-binding activity,

which is prevented by pretreatment with inhibitors of the EGF receptor tyrosine kinase (PD158780 and PD1083805). In addition, cells treated with PD180970 did not display activation of Stat3 by EGF, indicating a requirement for Src-kinase in the activation of Stat3 by EGF. Pretreatment of cells with AG490 had no effect on the ability of EGF to activate Stat3 DNA-binding activity.

The requirement of EGF-R, Src, and JAK kinase activity for activation of Stat3 by IL-6 was also tested in a similar manner using pharmacological inhibitors. In Figure 3b, cells were treated as above with tyrosine kinase inhibitors and stimulated with IL-6 for 20 min. As demonstrated before, IL-6 led to potent induction of Stat3 activity. Neither inhibitors of the EGF receptor tyrosine kinase nor JAK kinase had any measurable affect on the ability of IL-6 to stimulate Stat3 DNA-binding activity. This lack of effect with EGF-R or JAK inhibitors was seen even with longer pretreatment for up to 12 h (data not shown). By contrast, pretreatment of A549 cells with PD180970 prevents the full activation of Stat3 by IL-6, implicating the activity of Src-kinase as being necessary for full IL-6-mediated stimulation of Stat3. However, we do detect some activation of STATs in the presence of PD180970, possibly implicating another kinase other than Src in the activation of STATs by IL-6. We conclude that EGF stimulation of Stat3 activity requires the activity of the EGF receptor and Src-kinase activity, while full IL-6-mediated Stat3 activity requires Src-kinase activity.

We also studied the ability of HGF to stimulate Stat3 activity. The human nonsmall cell lung cancer cell line H358, which expresses c-Met, the receptor for HGF, was serum deprived and pretreated for 1.5 h with either PD180970 or AG490. Exogenous HGF was added to cells and nuclear extracts were prepared 20 min after stimulation. As demonstrated in Figure 3c, addition of HGF leads to enhancement of Stat3 DNA binding which is blocked by pretreatment with PD180970 but not AG490. These results suggest a critical role of Src-kinase activity in the activation of Stat3 by HGF. Taken together, we conclude that Src-kinase activity is required for full activation of Stat3 by EGF, HGF, and IL-6 in these lung cancer cells.

Inhibition of Src-kinase leads to downregulation of Stat3, G2 cell cycle arrest, and apoptosis in lung cancer cells

Since Stat3 is phosphorylated and its DNA-binding activity is upregulated by multiple proteins with tyrosine kinase activity, inhibition of upstream regulators of Stat3, such as Src, could have an effect on lung cancer cells growing in culture. To better define a role of Stat3 in growth and survival of lung cancer cells, as well as identify tyrosine kinases which result in constitutive Stat3 activation, we treated cells with pharmacological inhibitors of tyrosine kinases for up to 3 days and assessed their affect on cell viability. A549 and H358 human lung cancer cells growing in 5% FBS were plated at low density and treated with DMSO as control, PD180970 (Src-kinase inhibitor), PD158780 (EGF-R kinase inhibitor), PD0183805 (pan-HER kinase inhibitor), or AG490 (JAK kinase inhibitor) (Fry, 1999; Levitzki, 1999; Kraker *et al*., 2000; Garcia *et al*., 2001). Fresh media with fresh inhibitors were exchanged every 24 h and viable cells were counted every 24 h after drug treatment for 72 h. As demonstrated in Figure 4a, inhibition of both Src and JAK by the pharmacological inhibitors leads to reduced cell viability in both A549 and H358 lung carcinoma cells. Inhibition of EGF-R kinase with PD158780 or pan-HER kinase with PD0183805 had a slight growth inhibitory effect in A549 cells mainly manifested by reduced cell number 72 h after drug treatment. The inhibition of cell viability by PD180970 or AG490 appears to be independent of the status of p53, since H358 cells have a mutation in p53 that negates its function (Ling *et al*., 2000).

To correlate the inhibition of lung cancer cell viability by PD180970 and AG490 with Stat3 DNA-binding activity, we treated A549 cells with the same inhibitors for 24 h and assessed Stat3 activity by EMSA. Figure 4b demonstrates that treatment with PD180970 or AG490 leads to a reduction in Stat3 DNA-binding that correlates with the effect of the inhibitors seen in the cell growth assay. Treatment with PD158780 or PD0183805 had no affect on Stat3 activity that paralleled the minimal reduction in cell growth in the above cell viability assay.

Since these preliminary findings suggested that Src-kinase may be responsible for Stat3 activation in these cells, we assayed for Src levels and activation using antibodies which recognized total c-Src and phosphorylated Src activity. Tyr416 antibody detects endogenous levels of Src, Lyn, Fyn, Lck, Yes, and Hck when phosphorylated at tyrosine 416 in the activation loop. Total cell extracts were analysed from A549, H358, and H1299 lung cancer cells. As shown in Figure 4c, total c-Src protein was found in all lung cancer cells tested, with H1299 cells expressing the highest level, while levels of phosphorylated Src demonstrated higher levels in A549 and H358 cells compared to H1299 cells. Therefore, c-Src protein is present and activated Src kinase exists in these lung cancer cells, albeit to different levels.

To further study the effect of Src on Stat3 activity and control of lung cancer cell growth, we performed both dose- and time-dependent studies using PD180970 on A549 cells. Previous work had identified this compound selective against c-Src and Lck, while having minimal affect on autophosphorylation of receptors for bFGF, PDGF, and EGF (Kraker *et al*., 2000). Cells were treated with increasing concentrations of PD180970 and cells assayed for phosphorylated and total Src protein after 24 h. PD180970 results in a dose-dependent reduction in phosphorylated Src with major reductions seen at 500 nM and above (Figure 5a). Total Src protein was unchanged as was actin levels. We next treated A549 cells with 500 nM of PD180970 and assayed for changes in phosphorylated Src, total Src, phosphorylated Stat3, and total Stat3 after various times of treatment. PD180970 lead to a time-dependent reduction in phosphorylated Src levels with major reductions seen after 12 h of treatment and no presence of phosphorylated Src 24 h or later (Figure 5b). We also

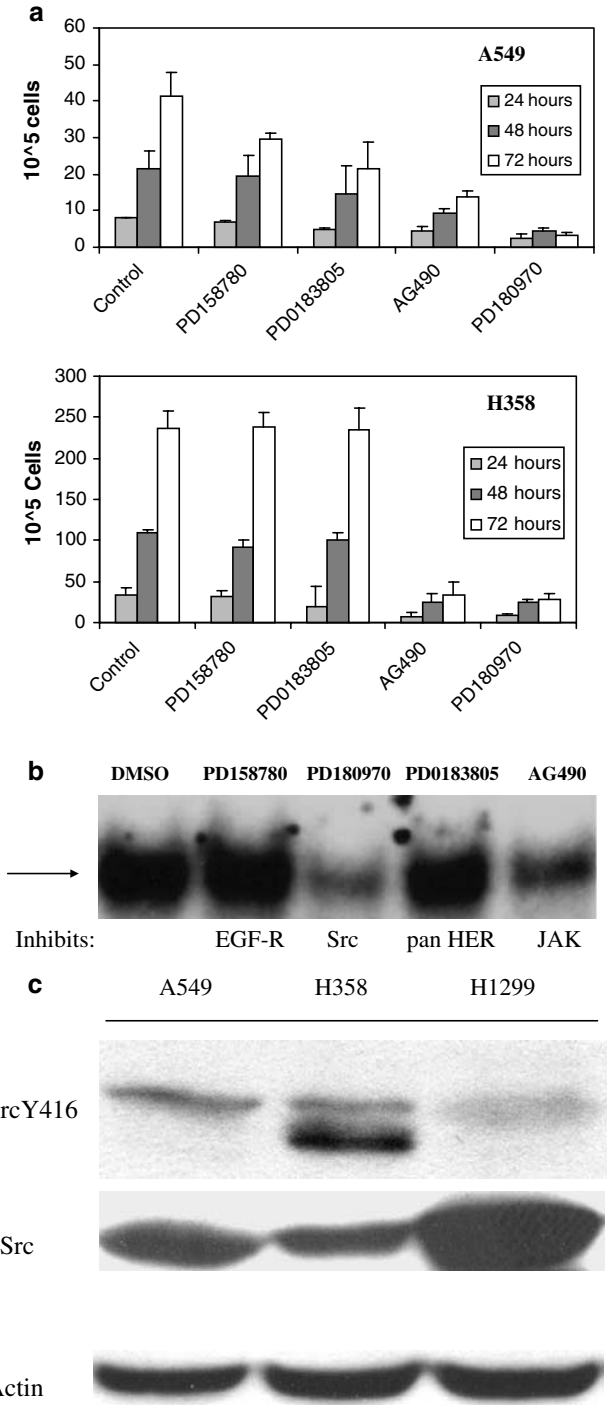
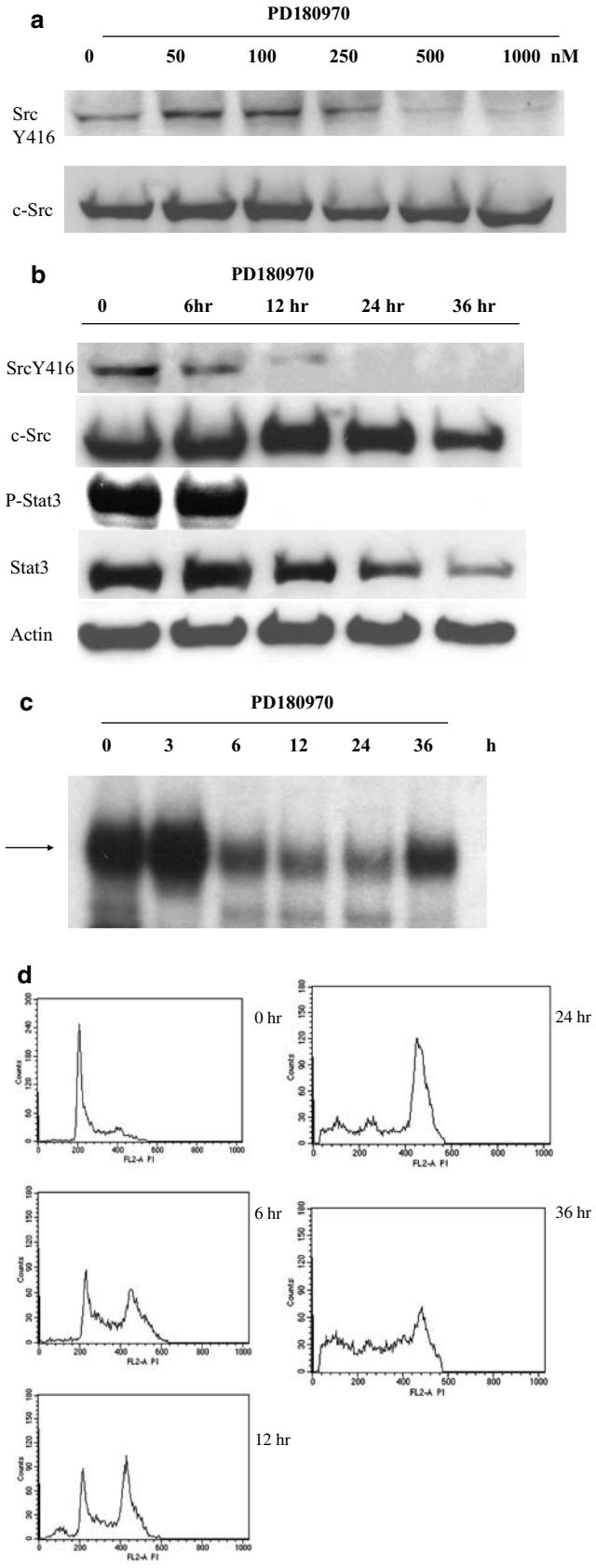


Figure 4 Effects of tyrosine kinase inhibition on lung cancer cell growth and correlation with Stat3 activity. **(a)** A549 and H358 human lung cancer cell lines were treated with 500 nM PD158780, 500 nM of PD0183805, 500 nM PD180970, or 50 μM AG490 for 72 h and viable cell numbers were estimated by cell counting on hemocytometer using trypan blue dye exclusion. Three separate experiments were performed and the results averaged. Error bars represent one standard deviation calculated from the three experiments. **(b)** A549 cells were grown in RPMI media and 5% FBS and treated with the tyrosine kinase inhibitors listed for 24 h. Cells were harvested and nuclear extracts prepared for EMSA. **(c)** A549, H358, and H1299 cells were harvested for total protein and probed with antibodies specific for SrcY416 and total c-Src levels, as well as actin to control for protein loading

noticed a slight reduction in total Src levels although not to the extent seen with phosphorylated Src. Levels of phosphorylated Stat3 were reduced in a similar temporal pattern seen with phosphorylated Src, with absent levels seen after 12 h of treatment. The total levels of Stat3 were also reduced with prolonged exposure to PD180970 consistent with an autoregulatory role of Stat3 in control of its own promoter (Ichiba *et al*., 1998). Equal protein loading was confirmed by probing the membranes with an antibody for actin. In addition, we harvested parallel treated cells and assayed for Stat3 DNA-binding activity by EMSA. Figure 5c demonstrates that Stat3 DNA binding activity is reduced by treatment with PD180970 within 6 h of exposure and becomes maximally reduced after 12 h. To understand how inhibition of phosphorylated Src and Stat3 by PD180970 leads to reduced cell viability, we treated a parallel group of A549 cells with PD180970 and assayed for effects on cell cycle using propidium iodide (PI) staining. A549 cells were treated with 500 nM of PD180970 and harvested at various times for cell cycle analysis. As demonstrated in Figure 5d, treatment of A549 cells with PD180970 led to early growth arrest manifested by accumulation of cells with G2 DNA content, followed by the appearance of cells with sub-G1 DNA content indicative of apoptosis starting approximately 12 h after treatment.

Since pharmacological inhibitors can have other targets besides Src, we wished to confirm our results with PD180970 using a second pharmacological inhibitor of Src-kinase. SU6656 was previously found to be a selective Src-kinase inhibitor compared with concentrations required to inhibit other tyrosine kinases and could inhibit PDGF- and Src-driven mitogenesis. In addition, it could inhibit PDGF-stimulated Myc induction but was not a good inhibitor of PDGF autophosphorylation (Blake *et al*., 2000). We treated A549 cells with escalating doses of SU6656 and performed phosphorylated and total c-Src Western blots. SU6656 reduced phosphorylated Src levels using 20 μM while lower concentrations had no significant effect on levels of phosphorylated Src (Figure 6a). Similar to the findings with PD180970, exposure of A549 cells to 20 μM of SU6656 resulted in a time-dependent reduction in phosphorylated Src and phosphorylated Stat3 levels (Figure 6b). In addition, Stat3 levels were again reduced consistent with the autoregulatory role of Stat3. Cell cycle analysis similarly demonstrated growth arrest in G2 at 24 h with a suggestion of DNA fragmentation with 36 h of exposure (Figure 6c). Therefore, use of pharmacological inhibitors of Src-kinase with PD180970 and SU6656 both resulted in reductions in phosphorylated Src and Stat3, cell cycle arrest in G2, and evidence of apoptosis.

Morphological examination of the cells also suggested that cell death, not just cell cycle arrest, was caused by treatment with PD180970 and SU6656. Treated cells had altered cell morphology, including alterations in cell size and shape, increasing amounts of cellular debris, and the appearance of vacuolated cells. To perform another confirmatory assay for induction of apoptosis



by Src-kinase inhibition with PD180970 and SU6656, cells were collected 48 h after treatment and apoptosis scored by detection of DNA fragmentation using Apo-BrdU incorporation. As shown in Figure 6d, cells treated with PD180970 or SU6656 underwent apoptotic cell death indicated by the large fraction of cells incorporating Apo-BrdU above that of control. These results suggest that inhibition of Src-kinase and the accompanying reduction in Stat3 activity are associated with reduced lung cancer cell viability and increased apoptosis.

Antisense Stat3 oligonucleotides induce apoptosis in lung carcinoma cells

Our above results demonstrate that the enhancement of Stat3 activity by EGF, IL-6, and HGF requires the activity of Src-kinase. Furthermore, prolonged inhibition of Src-kinase by PD180970 or SU6656 is associated with lung cancer cell apoptosis and reduced Stat3 DNA-binding. Since Src likely controls multiple pathways in addition to Stat3, we next sought to demonstrate directly the effect of inhibition of Stat3 on A549 lung cancer cells. We used antisense oligonucleotides directed against Stat3 to specifically inhibit the expression of Stat3 and a control mismatch oligonucleotide that should have no effect on Stat3 expression. A549 cells were transfected with no oligonucleotides, antisense, or mismatch oligonucleotides and 24 h later cells were collected and total protein and nuclear extracts were prepared. To rule out nonspecific transfection-related effects, we transfected cells either with liposomes (Lipofectin) or electroporation and observed identical effects. Figure 7a demonstrates reduction in the total Stat3 protein content of cells by antisense Stat3 oligonucleotide while no reduction was seen in cells treated with mismatch oligonucleotide compared with control. The reduction in Stat3 levels was accompanied by the activation of apoptosis as evidenced by cleavage of poly-(ADP-ribose) polymerase (PARP) as early as 24 h after transfection, while no cleavage of PARP was seen with mismatch oligonuclotides. As shown in Figure 7b, antisense Stat3 transfection resulted in nearly complete inhibition of Stat3 DNA-binding activity as assayed by EMSA, while mismatch oligonucleotide treatment had no affect on Stat3 activity compared to control cells. At 48 h after treatment, cells treated with antisense Stat3 demonstrated reduced numbers and appearance of altered cell size and shape with accompanying increase in cellular debris and vacuolated cells (see Figure 7c). By contrast, cells treated with mismatch

Figure 5 Effects of Src-kinase inhibition by PD180970 on Src-Stat3 signaling and cell cycle progression in A549 lung cancer cells. (**a**) A549 cells were treated with increasing doses of PD180970 and total protein collected after 24 h of exposure and probed for SrcY416 and total c-Src levels. (**b**) A549 cells were exposed for varying times to 500 nM of PD180970 and total proteins probed for activated and total Src and Stat3. (**c**) Parallel treated cells were prepared for nuclear extracts and STAT EMSA performed as before. (**d**) Parallel treated cells were collected for cell cycle analysis using PI staining

oligonucleotide appeared to have no change in cellular number, size, shape, nor had increased cellular debris suggesting cell death. To provide additional evidence to support the role of Stat3 in controlling apoptosis in A549 cells, the cells were collected and assayed for DNA fragmentation by incorporation of Apo-BrdU. Results from three independent experiments were pooled and percent apoptosis determined by the percentage of cells incorporating Apo-BrdU. Cells treated with antisense Stat3 oligonuclotides had increased Apo-BrdU incorporation, indicative of apoptosis, while mismatch oligonuclotide-treated cells had minimal evidence of nicked DNA above that of control cells (Figure 7d). Therefore, antisense inhibition of Stat3 induced apoptosis, indicated by PARP cleavage and DNA fragmentation, in A549 lung carcinoma cells that have constitutive Stat3 activation.

To extend our findings with A549 cells, we also performed similar experiments with Stat3-positive H358 and H1299 lung carcinoma cells. As shown in Figure 8a, antisense Stat3 transfection results in loss of Stat3 in both H358 and H1299 cells compared with mismatch oligonucleotides. Activation of apoptotic pathways was demonstrated in H358 cells, evidenced by PARP cleavage in cells treated with antisense Stat3, while no evidence of apoptosis was seen in H1299 cells despite absent Stat3 protein. We also assayed the effect of Stat3 antisense oligonucleotides on H460 lung carcinoma cells that have minimal Stat3 DNA binding as demonstrated in Figure 1a. H460 cells were transfected with up to 10 μM of mismatch or antisense Stat3 oligonucleotides by electroporation. Stat3 activity as measured by EMSA showed minimal DNA binding with prolonged exposure of the EMSA which was reduced by Stat3 antisense but not mismatch oligonucleotides (Figure 8b). We found no effect on cellular morphology or cell number in H460 cells treated with antisense Stat3 (Figure 8b) and no increased incorporation of Apo-BrdU was observed. These data support the conclusion that antisense Stat3 is specific and does not induce cell death in H460 cells, which have minimal Stat3 activity. Figure 8c summarizes our results with antisense Stat3 and four lung carcinoma cells tested, including three Stat3-positive cell lines (A549, H358, and H1299) and one Stat3-negative cell line (H460). Antisense Stat3 resulted in apoptosis in A549 and H358 cells, while no observation of apoptosis was seen in H1299 cells. These findings demonstrate that direct inhibition of Stat3 leads to apoptotic cell death and suggests that Stat3 controls apoptotic pathways in certain human lung cancer cells.

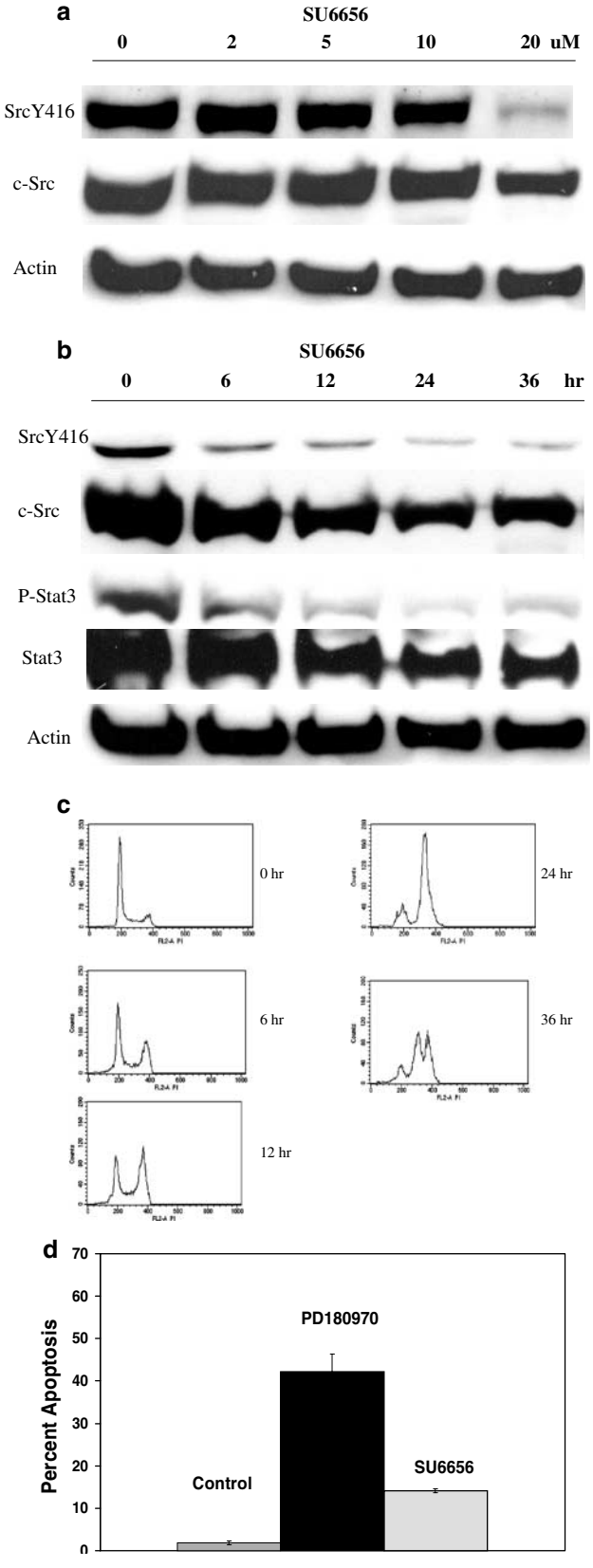


Figure 6 Effects of Src-kinase inhibition by SU6656 on Src-Stat3 signaling and cell cycle progression in A549 lung cancer cells. (**a**) A549 cells were treated with increasing doses of SU6656 and total protein collected after 24 h of exposure and probed for SrcY416 and total c-Src levels. (**b**) A549 cells were exposed for varying times to 20 μM SU6656 and proteins total proteins probed for activated and total Src and Stat3. (**c**) Parallel treated cells were collected for cell cycle analysis using PI staining. **(d)** A549 cells were treated with DMSO, 500 nM PD180970, or 20 μM SU6656 for 48 h. Cells were collected and prepared for Apo-BrdU staining as described in Methods. Percent of cells undergoing apoptosis represent percentage of cells staining positive for BrdU. The results represent pooled results from two independent experiments and error bars represent one standard deviation of the two experiments

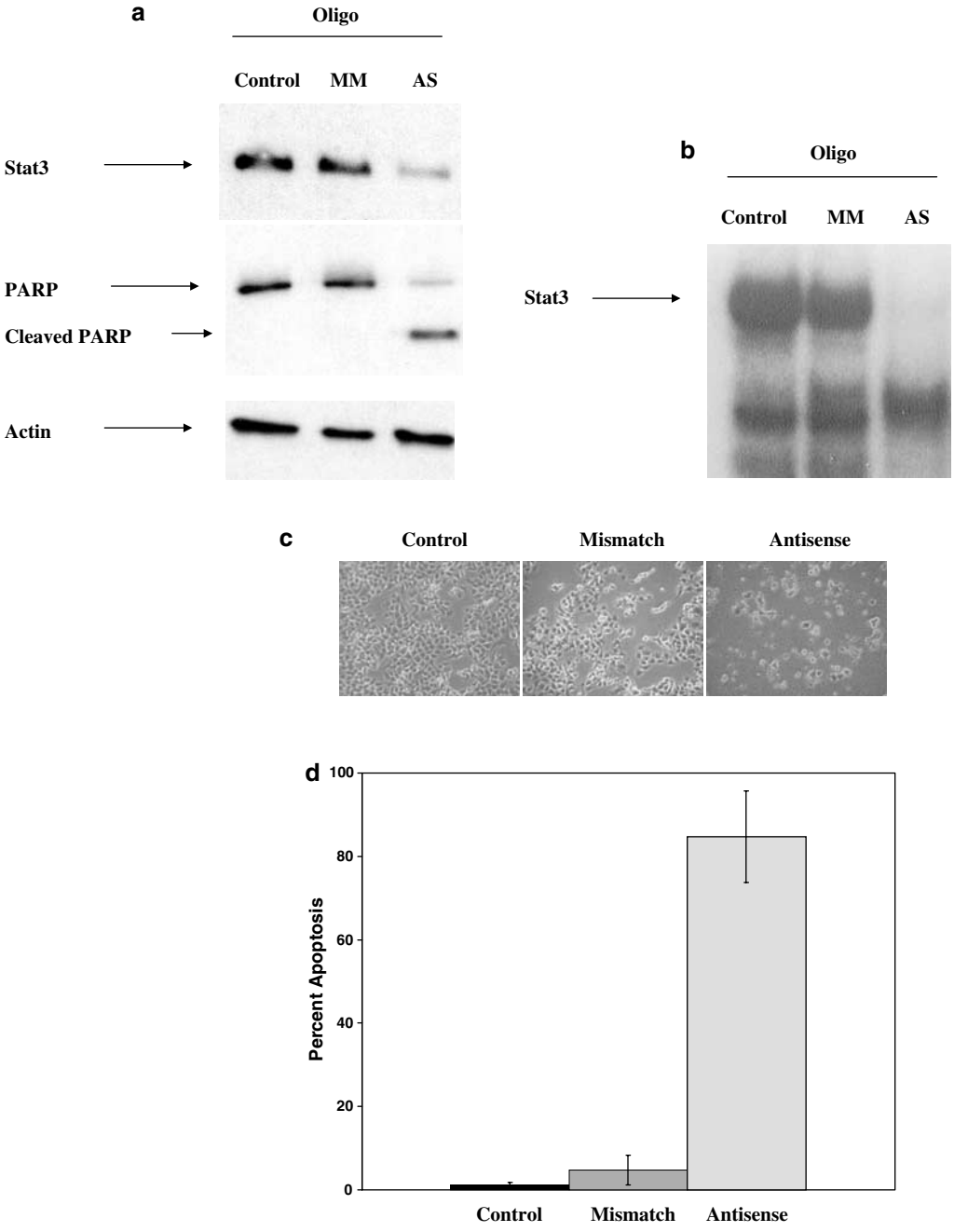


Figure 7 Effects of antisense Stat3 oligonucleotide on A549 lung cancer cell growth and induction of apoptosis. (**a**) A549 cells were transfected with either 500 nM antisense Stat3 oligonucleotide or 500 nM mismatch oligonucleotide and harvested 24 h later for preparation of total protein or nuclear extract. Control cells were mock-transfected and received no oligonucleotides. **(b)** Nuclear extracts from A549 cells were prepared for EMSA analysis after oligonucleotide treatment for 24 h demonstrating that Stat3 DNA-binding activity is abrogated by antisense oligonucleotides but not mismatch oligonucleotides. **(c)** Photomicrographs of A549 cells 48 h after transfection with antisense Stat3, mismatch Stat3, or mock transfected demonstrating cell death with treatment of cells with antisense Stat3. **(d)** A549 cells were collected 48 h after transfection and prepared for Apo-BrdU staining as described in Methods. The results represent pooled results from three independent experiments and error bars represent one standard deviation of the three experiments

Adenoviral delivery of dominant negative Stat3 induces apoptosis in A549 cells

To confirm our results with Stat3 antisense oligonucleotides, we created an adenoviral vector that expresses a dominant-negative form of Stat3 called Stat3-EVA. This construct has two mutations in the DNA binding site of Stat3 which prevents binding to DNA but has no effect on tyrosine phosphorylation or dimerization (Horvath *et al*., 1995). A549 cells were grown as before and infected with two multiplicities of infection (MOI) and controls consisted of cells infected with adenovirus

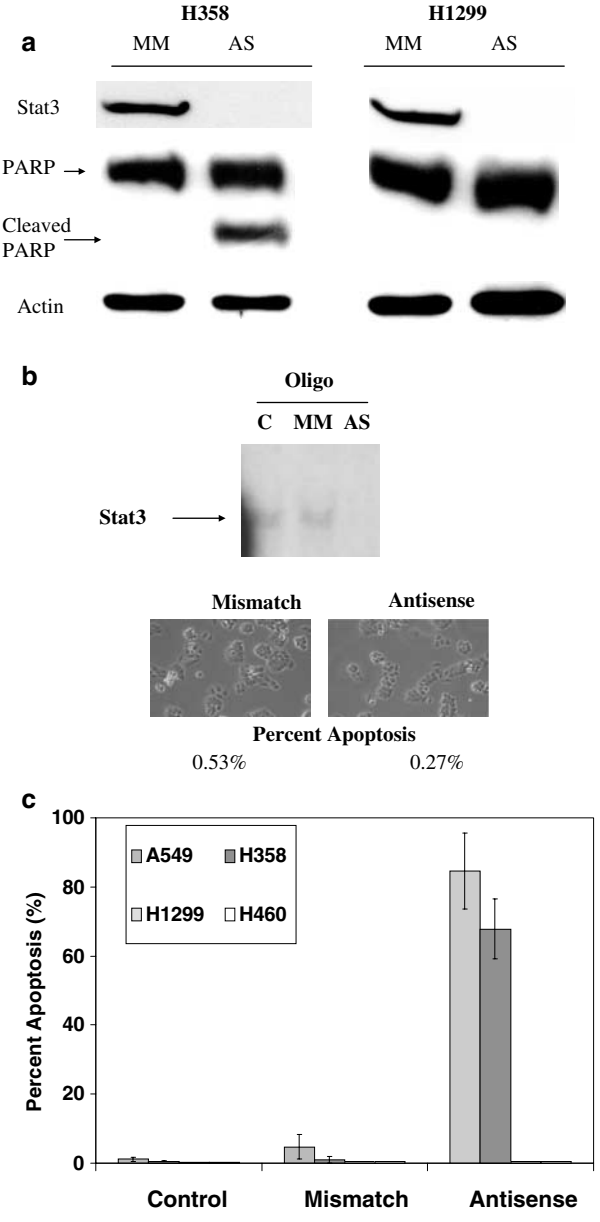


Figure 8 Effects of antisense Stat3 oligonucleotide on Stat3-positive H358 and H1299 cells and Stat3-negative H460 lung cancer. (**a**) H358 and H1299 cells were transfected with either 500 nM of mismatch or antisense Stat3 oligonucleotides for 24 h and total protein analysed for Stat3 levels and PARP cleavage. (**b**) H460 cells were transfected with either 10 μM antisense Stat3 oligonucleotide or 10 μM mismatch oligonucleotide as described and harvested 24 h later and nuclear extracts were prepared for EMSA. Control cells were mock-transfected and received no oligonucleotides. With prolonged film exposure, slight amounts of Stat3 DNA-binding activity are seen in control and mismatch-treated cells but abrogated with antisense Stat3. Photomicrographs of H460 cells 48 h after transfection with antisense Stat3, mismatch Stat3, or mock transfected demonstrate and no difference in cellular morphology no increase in apoptosis measured by Apo-BrdU. (**c**) Summary of experiments with antisense Stat3 in Stat3-positive A549, H358, and H1299 lung carcinoma cells and Stat3-negative H460 cells. Cells were collected 48 h after transfection and prepared for Apo-BrdU staining as described in Methods. The results represent pooled results from three independent experiments and error bars represent one standard deviation of the three experiments

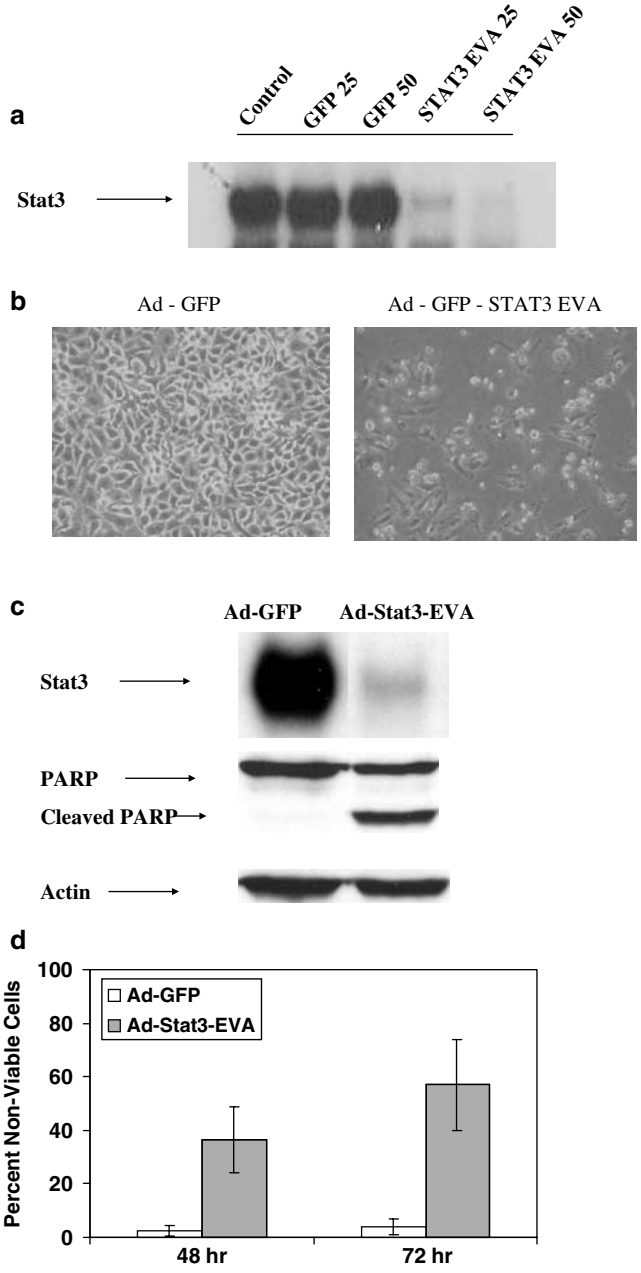


Figure 9 Effects of adenoviral vector expressing Stat3-EVA, a dominant-negative form of Stat3. (**a**) A549 cells were infected with either Ad-GFP or Ad-GFP-Stat3-EVA as described and harvested 24 h later and nuclear extracts were prepared for EMSA. Control cells were mock-infected and received no adenovirus. Numbers after virus infection refer to multiplicity of infection (MOI). (**b**) Photomicrographs of A549 cells 96 hours after infection with Ad-GFP or Ad-GFP-Stat3-EVA. **(c)** A549 cells were collected 24 h after infection and prepared for EMSA analysis for Stat3 in addition to total protein for PARP analysis. (**d**) A549 cells were assayed for viability by trypan blue exclusion after infection with either Ad-Stat3-EVA or Ad-GFP at the times indicated. The results represent pooled results from three independent experiments and error bars represent one standard deviation of the three experiments

expressing green fluorescent protein (GFP). At 24 h after infection, the cells were harvested and nuclear extracts prepared for EMSA. Figure 9a demonstrates loss of

Stat3 DNA binding when cells were infected with 25 or 50 MOI of Ad-Stat3-EVA but no inhibition of DNA binding was seen with cells infected with Ad-GFP at equivalent viral doses. Infection of cells with Ad-Stat3-EVA also resulted in a dose-dependent reduction in Stat3 DNA-binding activity (data not shown). Cell were infected at MOI of 5, 10, 25, and 50 and EMSA was performed after 36 h of infection. Infection with a MOI of 5 resulted in nearly 90% reduction in Stat3 activity, while higher doses were associated with near complete loss of Stat3 activity assayed by EMSA.

We treated A549 cells with Ad-Stat3-EVA or Ad-GFP at an MOI of 10 and observed the cells in culture. After approximately 48 h of infection, cells infected with Ad-Stat3-EVA became rounded, refractile, and began to float and at 96 h after infection widespread cell death was apparent in the cells infected with Ad-Stat3-EVA but minimal effects were seen with cells infected with Ad-GFP (Figure 9b). We were unable to assay for apoptosis using apo-BrdU incorporation, since the FITC-labeled antibody and GFP expressed by the adenoviral vector overlap in fluorescence. However, cells infected with Ad-Stat3-EVA demonstrated PARP cleavage indicative of apoptosis, while cells infected with Ad-GFP did not demonstrate PARP cleavage (Figure 9c). Finally, cells infected with Ad-Stat3-EVA demonstrated reduced viability assayed by trypan blue staining while cells infected with Ad-GFP had no significant affect on cellular viability (Figure 9c). Therefore, two distinct approaches to inhibit Stat3 function, one by antisense oligonucleotides and one using dominant-negative Stat3 adenovirus, similarly demonstrate that loss of Stat3 function in A549 lung carcinoma cells results in cell death through apoptosis.

Discussion

Our results suggest that activation of Stat3 may participate in the malignant phenotype of nonsmall cell lung carcinomas through the regulation of apoptotic pathways. Our findings with cancer cell lines of nonsmall cell origin demonstrate that the majority of cell lines tested have constitutive Stat3 DNA-binding activity. Similar to observations in other tumor types, the activation of Stat3 is the consequence of upstream regulation by various receptor and nonreceptor tyrosine kinases. We identified EGF, HGF, and IL-6 as important regulators of Stat3 DNA-binding activity. These ligands were chosen based on their role in the biology of lung cancer. Similar to other investigations, we found that Src-kinase activity is required for the full activation of Stat3 DNA-binding activity by EGF, HGF, and IL-6 in lung cancer cells (Chaturvedi *et al*., 1998; Reddy *et al*., 2000). Furthermore, inhibition of Src-kinase activity by two pharmacological inhibitors resulted in reduced Stat3 activity and cell cycle arrest in G2 followed by the appearance of apoptotic cells. Finally, to directly demonstrate a role for Stat3 in controlling cell survival in lung carcinoma cells, we showed that antisense Stat3 oligonucleotides or dominant-negative Stat3 results in the induction of apoptosis.

EGF serves as a potent and rapid inducer of Stat3 activity in A549 lung cancer cells. Significantly, this activation appears to require the action of Src-kinase, since pretreatment with PD180970 ablates the activation by exogenous EGF. Our results are consistent with a previous report demonstrating a requirement for Src-kinase in EGF and heregulin-induced activation of Stat3 in human A431 epidermoid cells (Olayioye *et al*., 1999). Similarly, others have demonstrated that c-Src is required for phosphorylation of EGF-R and critical for DNA synthesis induced by exogenous EGF (Tice *et al*., 1999). Our data are consistent with a model where inactive Stat3 preassembles with EGF receptors which are activated by ligand binding in a manner dependent on Src-kinase activity (Wang *et al*., 2000b). Our result contrasts to another study in human breast carcinoma cell lines which found no requirement of Src-kinase in ligand-dependent activation of Stat3 by EGF (Garcia *et al*., 2001). In addition, a recent report demonstrated that neuregulin-1, a ligand for HER family of receptors, can activate JAK-STAT signaling in lung cancer cells (Liu and Kern, 2002). It is likely that cell type factors determine how STATs are regulated by various tyrosine kinases. Our results are also consistent with work on squamous cell cancers of the head and neck (SCCHN), another tobacco-related epidermal cancer, where TGF-alpha/EGF-R autocrine loops play an important role in the development of the disease. Importantly, studies on SCCHN clearly demonstrate that targeting Stat3 leads to growth inhibition and apoptosis through the down-regulation of Bcl-xl (Grandis *et al*., 1998, 2000a, b; Song and Grandis, 2000).

IL-6 and its receptor have also been implicated in the molecular progression of nonsmall cell lung carcinoma (Yanagawa *et al*., 1995; Martin *et al*., 1999). Our results presented above demonstrate that IL-6 can lead to enhanced Stat1 and Stat3 activity and this stimulation is dependent on Src-kinase activity since reduced stimulation results when cells are pretreated with PD180970. This result is consistent with the work of others who demonstrated that signal transduction through IL-6 activates Src family kinases, most notably Hck kinases (Hallek *et al*., 1997; Schaeffer *et al*., 2001). A more recent report has demonstrated that IL-6 activation of Stat3 did not require JAK activity but instead was inhibited by inhibitors of MAPK pathway (Kopantzev *et al*., 2002). In addition, in myeloid cells, IL-3 induces STATs through Src-kinases rather than JAKs (Chaturvedi *et al*., 1998). Therefore, it is likely that cell type differences determine how cytokines signal to STATs. Our results are also in agreement with others who have found Stat3 activity upregulated by IL-6 in a human myeloma tumor model (Catlett-Falcone *et al*., 1999). In a myeloma tumor model, enhanced Stat3 activity by IL-6 stimulation conferred resistance of myeloma tumor cells to pro-apoptotic stimuli at least in part through direct Stat3 regulation of bcl-x (Catlett-Falcone *et al*., 1999). It remains unclear what the exact role of IL-6 plays in lung cancer growth, but our results suggest that

Stat1 and Stat3 signaling may be important determinants of a lung cancer cell's response to IL-6.

In addition to EGF and IL-6, our results also demonstrate that HGF can act to stimulate Stat3 activity in a manner requiring the function of the Src family of kinases. In addition to the largely well-known role of TGF-alpha and the EGF receptor in lung cancer biology, a role for another receptor tyrosine kinase, c-Met, and its ligand HGF have also been implicated in lung cancer oncogenesis (Olivero *et al*., 1996; Tsao *et al*., 1998). Others studying mammary carcinoma cells similarly found c-Src-kinase activity to be required for HGF-induced motility and anchorage-independent growth of mammary carcinoma cells and suggested a mechanism for autocrine regulation of tumor cell growth through a HGF-Src-Stat3-HGF loop (Rahimi *et al*., 1998; Hung and Elliott, 2001). In addition, a recent study found that dominant negative Stat3 prevented HGF/SF-Met-mediated tumor growth in athymic mice (Zhang *et al*., 2002). Our data suggest a similar HGF-Src-Stat3 signaling cascade could be occurring in human lung cancers and may explain the negative prognostic implication of HGF/c-Met overexpression in human lung cancers (Ichimura *et al*., 1996; Siegfried *et al*., 1997, 1998).

We also studied the effect of IGF-1 on STAT activity in lung carcinoma cells given previous findings demonstrating the ability of IGF-1 to stimulate the growth of serum-deprived lung cancer cells and targeting the IGF-1 receptor can lead to tumor suppression (Ankrapp and Bevan, 1993; Favoni *et al*., 1994; Jiang *et al*., 1999). Despite the reported presence of IGF-1 receptors on the surface of A549 lung carcinoma cells, we were unable to convincingly demonstrate that addition of IGF-1 to these cells stimulated the DNA-binding activity of Stat3. Other investigators, however, have demonstrated the ability of IGF-1 and its receptor to activate Stat3 in a manner dependent on JAKs, suggesting that Stat3 may be an important signaling mechanism downstream of IGF-1 for certain cell types (Takahashi *et al*., 1999; Zong *et al*., 2000).

When lung cancer cells were treated for prolonged periods with the inhibitors of Src-kinase, we found a reduction in cell number and viability that was associated with a parallel reduction in Stat3 activity. We found no reduction of Stat3 activity with inhibitors of EGF-R or pan-HER kinase that correlated with minimal effects seen on cell growth. Treatment of A549 cells with the Src inhibitors PD180970 and SU6656 leads to early cell cycle arrest, predominant in G2, as well as a large induction of apoptosis that was independent of p53 status, since H358 cells with mutated p53 similarly underwent apoptosis. Our work is consistent with the reports of other groups demonstrating a role of Src family protein tyrosine kinases in the transition from G2 phase to mitosis (Fumagalli *et al*., 1994; Taylor and Shalloway, 1994; Roche *et al*., 1995). A previous report also demonstrated the ability of PD173955, a selective pharmacological inhibitor of Src-kinase, to reduce *in vitro* activity of Yes and Src, induce mitotic arrest in breast carcinoma cells, and induce apoptosis (Moasser *et al*., 1999). One question that remains is which members of the family of Src are important in nonsmall cell lung carcinoma cells, since these inhibitors can inhibit the activity of multiple family members (Blake *et al*., 2000; Kraker *et al*., 2000). This has been more extensively studied in small cell lung cancers, where Lck and Yes have been implicated in the ability of stem cell factor/Kit mediation proliferation (Krystal *et al*., 1998).

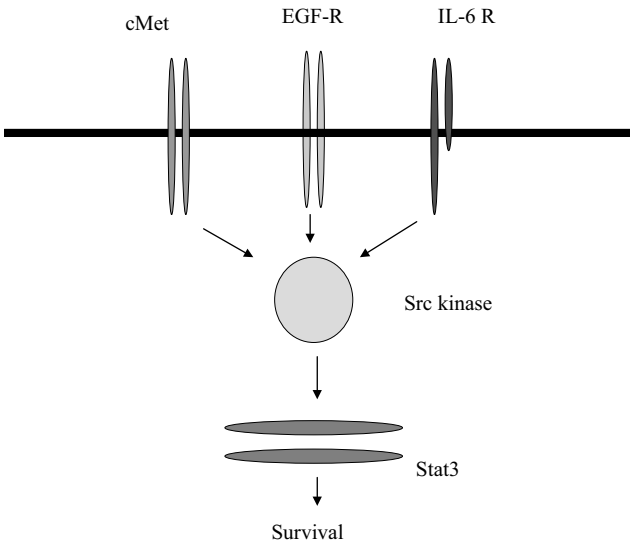


Figure 10 Receptor tyrosine kinases such as EGF-R and c-Met as well as cytokine receptors for IL-6 lead to activation of Stat3 which regulates the survival of lung cancer cells. A model of how receptor tyrosine kinases (such as EGF-R and c-Met), receptors for cytokines (such as IL-6), and nonreceptor tyrosine kinases (such as Src) may protect lung cancer cells from death signals through the upregulation of Stat3 activity

To demonstrate directly the role of Stat3 in lung cancer survival, we used two approaches to disrupt Stat3 function. First, treatment of A549 and H358 cells with antisense oligonucleotides directed against Stat3 lead to reduction in Stat3 protein levels and Stat3 DNA binding followed by the induction of apoptosis indicated by PARP cleavage and DNA fragmentation. Furthermore, H460 cells that have low levels of Stat3 DNA binding were unaffected by Stat3 antisense, suggesting that survival of these cells is not dependent on Stat3-mediated signaling. Second, an adenoviral vector expressing a dominant-negative form of Stat3 reduced Stat3 DNA binding and resulted in apoptosis. These results indicate that activation of Stat3 by receptor tyrosine kinases such as EGF-R and c-Met, cytokine receptors such as for IL-6, and nonreceptor kinases such as Src are important in the regulation of survival pathways in certain lung cancer cells that depend on Stat3 for survival (Figure 10). Importantly, the mere presence of activated Stat3 is not sufficient to predict that apoptosis will result from targeting Stat3, since Stat3-positive H1299 cells were resistant to apoptosis induced by antisense Stat3. Receptor tyrosine kinases activate multiple signaling pathways in addition to Stat3 that can lead to enhanced tumor growth and survival. A

recent report demonstrated a role of constitutive PI3K/Akt in lung cancer cell survival and enhanced resistance of lung cancer cells to chemotherapy (Brognard *et al*., 2001). Interestingly, we found no constitutive Stat3 activity in the H460 lung carcinoma cell line that does have constitutive Akt activity, and H1299 cells, which also have constitutive Akt activity, were insensitive to apoptosis induced by Stat3 inhibition (Brognard *et al*., 2001). Therefore, Stat3 signaling may be dispensable for tumors that have upregulation of other survival signals such as Akt. We hypothesize that other survival signals can prevent apoptosis resulting from loss of Stat3 activity and it will therefore be important to understand how Stat3 signaling cooperates with other survival-signaling mechanisms in cancer cells.

Consistent with its role as a transcription factor that directly affects gene expression, Stat3 likely regulates a group of genes that control apoptotic pathways. These results are consistent with previous studies demonstrating constitutive Stat3 activation in a number of human cancer models, including breast, multiple myeloma, head and neck squamous cell carcinomas, and various hematological malignancies (Garcia *et al*., 1997; Catlett-Falcone *et al*., 1999; Bowman *et al*., 2000; Epling-Burnette *et al*., 2001a; Garcia *et al*., 2001). Ongoing studies are attempting to delineate the genes and pathways regulated by Stat3 in lung carcinoma cells. In a myeloma tumor model, IL-6-dependent STAT3 signaling upregulates the Bcl-x promoter and contributes to cell survival, while in large granular T cells, cytokine-dependent Stat3 signaling was necessary for transcriptional upregulation of Mcl-1 (Catlett-Falcone *et al*., 1999; Epling-Burnette *et al*., 2001a, b). Therefore, it is likely that Stat3 regulates a set of genes in the bcl-2 family and inhibition of Stat3 changes the balance between pro- and antiapoptotic family members.

Materials and methods

Cell lines and cell culture

Human nonsmall cell lung cancer cell lines were purchased from ATCC or were a gift provided by Dr Gerold Bepler. Cells were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco) supplemented with 5% FBS (Hyclone) and 1% penicillin/streptomycin (Gibco). Subconfluent cells were prepared for nuclear extracts as detailed below. Cell viability was determined by counting with a hemocytometer using trypan blue dye exclusion.

Nuclear extract preparation and EMSA

STAT DNA-binding assays were performed as described previously (Yu *et al*., 1995; Garcia *et al*., 1997). Briefly, nuclear extracts were prepared from cultured cells in hypertonic buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 0.5 mM PMSF, 0.1 mM aprotinin, 1 mM leupeptin, and 1 mM antipain). Normalized extracts containing 3–8 μg of total protein were incubated with a double-stranded ^{32}P-radiolabeled hSIE oligonucleotide probe as described previously (Yu *et al*., 1995; Garcia *et al*., 1997). Protein–DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography.

Anti-Stat3 (C-20) polyclonal antibodies used to identify specific Stat3 complexes by EMSA were obtained from Santa Cruz Biotechnologies. For use in supershift assays, 1ug of the C-20 antibody was incubated with nuclear extracts for 20 min at room temperature prior to addition of radiolabeled probe(s) and electrophoresis. Protein-DNA complexes were detected as described above.

Western blot analysis

Cell lysates were normalized for total protein content (50 μg) and subjected to SDS–PAGE. Primary antibodies used in these studies consisted of Stat3 (Transduction Laboratories, #S21320), phosphorylated Stat3 Y705 (Cell Signaling, #9131) PARP (Cell Signaling, #9542), phosphorylated Src family Y416 (Cell Signaling, #2101), c-Src (Cell Signaling, #2107), and beta-actin (Sigma, A5441). Detection of proteins was accomplished using horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) purchased through Amersham.

Treatment of human nonsmall cell lung cancer cell lines with EGF, IL-6, and HGF and tyrosine kinase selective inhibitors

For experiments involving treatment with EGF, cells were placed in serum-free RPMI media for 24 h and then stimulated with EGF (Gibco) at a dose of 50 ng/ml. For experiments involving treatment with IL-6, cells were placed in serum-free RPMI media for 24 h and then stimulated with IL-6 (Pharmingen) at a dose of 30 ng/ml. For experiments involving treatment with HGF, cells were placed in serum-free RPMI media for 24 h and then stimulated with HGF (Calbiochem) at a dose of 30 ng/ml. For experiments involving treatment with IGF-1, cells were placed in serum-free RPMI media for 24 h and then stimulated with IGF-1 (Gibco) at a dose of 30 ng/ml. Dishes were incubated at 37°C for times indicated prior to preparation of the nuclear extracts. For treatment with PD158780 (Fry, 1999), PD180970 (Kraker *et al*., 2000), and AG490 (Levitzki, 1999), stock solutions of the compounds in 100% DMSO were diluted directly into the media to indicated concentrations followed by incubation at 37°C. For cell growth and viability assays, media plus inhibitors were changed every 24 h.

Cell cycle analysis

Percentages of cells of G1, S, and G2/M were determined by PI staining. Following treatment with tyrosine kinase inhibitors, cells were trypsinized and washed with cold PBS followed by fixation with ethanol at 4°C. The next day cells were incubated in the dark for 1 h in buffer containing 10 mg/ml PI and 500 mg/ml RNase A. A total of 10 000 cells per experimental condition were used for processing and analysis of fluorescence on a Becton–Dickinson FACScan using Cell Quest software.

Apoptosis assays

Apoptosis was assayed using PARP antibody (Cell Signaling) to detect full-length PARP (116 kDa) as well as the cleaved large fragment (89 kDa). Protein collection and immunoblots were prepared as above. Apoptosis was also assayed using Pharmingen Apo-BrdU Kit without modification. Following treatment with either tyrosine kinase inhibitors or transfection with either antisense or mismatch oligonucleotides, cells were trypsinized and resuspended in PBS. Cells were counted and

$1–2 \times 10^6$ cells were fixed in 1% paraformaldehyde in PBS on ice, pelleted, washed twice in PBS, and fixed with ice-cold 70% ethanol overnight. The next day cells were pelleted, resuspended, and washed with wash buffer. Pelleted cells were resuspended with reaction buffer, TdT enzyme, and br-dUTP for 1 h at 37°C. Cells were subsequently rinsed with 1.0 ml of rinse buffer and resuspended with fluorescein labeled Anti-BrdU in the dark for 30 min at room temperature. PI and RNAse are added and incubated for 30 min. Data from 10 000 cells per experimental condition were acquired and analysed for fluorescence on a Becton–Dickinson FACScan using Cell Quest software.

Antisense oligonucleotides and transfections

Stat3 antisense and mismatch oligonucleotides were kindly provided by ISIS Pharmaceuticals. The oligonucleotides used for these studies were synthesized as methoxyethylribose-modified phosphorothioates on an automated DNA synthesizer, as described previously (Monia *et al*., 1993). The sequences were as follows: Human Stat3 antisense: 5′-GCT CCA GCA TCT GCT GCT TC-3′; 5 base mismatch: 5′-GCT CCA ATA CCC GTT GCT TC-3′. These chimeric oligonucleotides contain 2′-*O*-methoxyethyl-modified residues that provide enhanced resistance to exonuclease degradation flanking a 2′-deoxynucleotide/phosphorothioate region (gap) that supports RNase H activation. Oligonucleotides were analysed by capillary gel electrophoresis and judged to be at least 85% full-length material.

Oligos were transfected into A549 or H460 human lung cancer cells using either encapsulated liposomes or electroporation. A549 cells (100 000–200 000 cells in a 60 mm dish) were transfected with 500 nM of oligonucleotides with 15 μg of Lipofectin (Gibco) in 1.5 ml of OptiMEM media (Gibco). Cells were first washed twice with nonserum-containing RPMI media followed by addition of the preincubated mixture of oligonucleotides, lipofectin, and OptiMEM media. Cells were incubated with the oligonucleotides for 4 h, the mixture was removed and the cells were washed once with RPMI plus 5% FBS and then incubated for the indicated times in RPMI plus 5% FBS. For immunoblots, cells were harvested 24 h after transfection. For Apo-BrdU assays for apoptosis, cells were harvested at 48 h post-transfection, washed and analysed by flow cytometry as described.

For electroporation, cells were grown to approximately 20–30×10^6 in number, tyrpsinized, and incubated with oligonucleotides at a final concentration of 10 μM. Cells were electroporated in a Biorad electroporator at 1000 μF and 230 V in OPTI-MEM, Eagle's media in a total volume of 400 μl. Cells were subsequently replated in 10 cm dishes in RPMI with 5% FBS and 1% penicillin/streptomycin and placed in a 5% CO_2 and 37°C incubator overnight. The next morning the cells were washed with PBS and new media with serum and antibiotics was added. For EMSA assays, cells were harvested 24 h after electroporation as described. For Apo-BrdU assays for apoptosis, cells were harvested at 48 h post-transfection, washed and analysed by flow cytometry as described.

Adenoviral vectors

We developed adenoviral vectors that express a dominant-negative form of Stat3 termed Stat3-EVA. This mutant form of Stat3 fails to bind DNA and therefore acts as a dominant-negative form of Stat3. Using the mouse Stat3 cDNA sequence, amino acids 434 and 435 were mutated from glutamic acid to alanine, and amino acids 461–463 were mutated from valine to alanine using PCR-based mutagenesis (Horvath *et al*., 1995). Ad-Stat3 EVA was constructed by digesting the plasmid-contained Stat3-EVA with *Sal*I and *Xba*I and ligating this fragment into the AdTrack-CMV plasmid used to construct recombinant adenoviruses as described by He *et al*. (1998). Viral stocks were created and purified as described previously and virus titers were determined by both an indirect immunofluorescent assay specific for the 72K E2 gene product and a flow cytometric method that titers adenovirus containing GFP (DeGregori *et al*., 1995; Nevins *et al*., 1997; Hitt *et al*., 2000). Concentrations of adenovirus detailed in each experiment were placed directly into the medium of cells and incubated for the desired times. Viral infection was confirmed by visually observing GFP expression in infected cells.

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