IL-6 induces neuroendocrine dedifferentiation and cell proliferation in non-small cell lung cancer cells

Kuo-Ting Chang, Chun-Ming Tsai, Yih-Chy Chiou, Chao-Hua Chiu, King-Song Jeng, and Chi-Ying F. Huang

1Graduate Institute of Life Sciences, National Defense Medical Center, Taipei; 2Division of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli County; 3Section of Thoracic Oncology, Chest Department, Taipei Veterans General Hospital, and Department of Medicine, School of Medicine, National Yang-Ming University, Taipei; 4Institute of Molecular Biology, Academia Sinica, Nankang, Taipei; 5Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei; and 6Department of Computer Science and Information Engineering, National Taiwan University, Taipei, Taiwan

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IL-6 induces neuroendocrine dedifferentiation and cell proliferation in non-small cell lung cancer cells. Am J Physiol Lung Cell Mol Physiol 289: L446–L453, 2005. First published May 13, 2005; doi:10.1152/ajplung.00089.2005.—Interleukin-6 (IL-6) has been identified as an important growth regulator of lung cancer cells. Elevation of serum levels of IL-6 has been found in a subpopulation of lung cancer patients, but rarely in patients with benign lung diseases. Approximately 15% of non-small cell lung cancer (NSCLC) tumors exhibit neuroendocrine (NE) properties (NSCLC-NE) and have been suggested to have the biological characteristics similar to small cell lung cancer (SCLC) with early metastasis and initial responsiveness to chemotherapy. We recently showed that IL-6 promotes cell proliferation and downregulates the expression of neuron-specific enolase (NSE), one of the major NE markers) in NSCLC-NE cells. In this study, we show that IL-6 stimulates a transient increase of tyrosine phosphorylation of STAT3 in a dose-dependent fashion. Inhibition of STAT3 signaling pathway by either AG-490 (JAK2-specific inhibitor) or overexpression of STAT3Y705F (a dominant-negative STAT3) reverses NSE expression in IL-6-treated NSCLC-NE cells. In addition, IL-6 induces phosphorylation and activation of p38 MAPK. SB-203580, a p38 MAPK-specific inhibitor, inhibits IL-6-induced p38 MAPK phosphorylating activity and suppresses IL-6-stimulated cell proliferation. Together, our results indicate that STAT3 signaling pathway is involved in IL-6-induced NE differentiation and that p38 MAPK is associated with IL-6-stimulated growth regulation in NSCLC-NE cells. These data suggest that both kinase pathways play critical roles in the pathogenesis of NSCLC-NE malignancies, providing new molecular targets for future therapeutic approaches.

Address for reprint requests and other correspondence: C.-Y. F. Huang, Natl. Health Research Inst., Div. of Molecular & Genomic Medicine, 35, Keyan Rd., Zhunan Town, Miaoli County 350, Taiwan, R. O. C. (e-mail: chiying@nhri.org.tw).

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NSCLC (38%), and, most importantly, it is probably a prognostic marker of clinical relevance (17).

The IL-6/IL-6 receptor reaction activates several signal transduction pathways that have been separately implicated in regulating cancer cell growth and differentiation. Currently, there are at least three signal transduction pathways associated with IL-6 receptor signaling. The first one is the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway. STAT proteins are a family of latent cytoplasmic transcription factors that are activated by cytokines and growth factors. STATs are activated after tyrosine phosphorylation by JAKs associated with cytokine receptors. Subsequently, activated STATs form dimers and translocate to the nucleus where they bind specific promoter elements of target genes and regulate their transcription (12, 13, 20, 21). Some members of the STAT family play roles in cellular differentiation, survival, and proliferation. Among STAT family members, STAT3 is the most frequently associated with deregulated cell growth and neoplasia (19). At present, the effect of IL-6 on tumors is controversial. Most notably, IL-6 has been shown to mediate growth arrest and NSE upregulation in LNCaP (a human prostate cancer cell line) and murine M1 leukemia cells by STAT3 (34, 42, 43). On the other hand, other investigators have suggested that ectopic expression of IL-6 stimulates prostate cancer cell growth accompanied by activation of the STAT3 signaling pathway (29) or by activating extracellular signal-regulated kinase (ERK) through cross talk with the growth factor receptor kinase erbB2 (39) in LNCaP cells. It is not clear what causes such discrepancies even for the same cell line.

The second one is the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. The PI3-K pathway regulates various cellular processes, such as proliferation, growth, apoptosis, and cytoskeletal rearrangement. Akt, a serine-threonine kinase, is activated by recruitment to the plasma membrane through direct contact of its pleckstrin-homology domain with phosphatidylinositol-3,4,5-triphosphate, and phosphorylation at threonine 308 and serine 473 (47). In LNCaP cells, IL-6 may induce a remarkable NE-like differentiation phenotype, with neurite extension and enhanced expression of NSE by the activation of PI3K/Etk (a member of Btk tyrosine kinase family) (40). Recent growing evidence suggests the involvement of PI3K/Akt in IL-6-dependent survival and proliferative responses in several types of cells. In IL-6-dependent 7TD1 B-cell hybridoma cells, IL-6 induced PI3-K/Akt activation is essential for the optimal growth through upregulation of antiapoptosis protein XIAP (52).

The third one is the mitogen-activated protein kinase (MAPK) pathway. The superfamily of MAPKs (38) include three major groups: the ERKs, the Jun NH2-terminal kinases (JNKs), and the p38 MAPK (37). The MAPK signaling cascade is activated by a wide variety of different cellular stimuli. It has been suggested that JAK and STAT proteins are activated by IL-6 in multiple myeloma (MM) cells independently of cell proliferation, whereas ERK was activated only in cells showing a proliferative response to IL-6 (35). However, the signal cascades mediating IL-6-induced cell growth are not fully defined. Accumulating evidence indicates that MAPK pathways can mediate signals that either promote or suppress the growth of malignant cells. For example, members of the p38 MAPK are primarily activated by stress stimuli but are also activated during engagement of various cytokine receptors by their ligands. The functions of p38 MAPK include the regulation of apoptosis, induction of cell differentiation, cell growth, and inflammation (10). Moreover, p38 MAPK has been reported to be associated with acute-phase protein secretion from HepG2 hepatoma cells and proliferation of B9 hybridoma cells in the presence of IL-6 (50). These findings support the idea that p38 MAPK may play a role in the process of cell proliferation.

The aim of this study is to identify the possible IL-6-mediated signaling pathway(s) involved in controlling NE differentiation and cell proliferation in NCI-H820. In the present study, we demonstrate that STAT3 phosphorylation is involved in NSE downregulation and p38 MAPK activation is associated with cell proliferation upon IL-6 stimulation. These results suggest that two major signaling pathways, STAT3 and p38 MAPK, are involved in the biological functions of regulating NE dedifferentiation and cell growth in NSCLC-NE cells, respectively.

![Fig. 1. STAT3 activation is induced by IL-6 in NCI-H820. A: IL-6 activated STAT3 by tyrosine 705 phosphorylation but did not activate other STATs involved in the JAK-STAT pathways. NCI-H820 cells (5 × 10^5) were seeded overnight, and ACL-4 culture medium was changed to RPMI basal medium for starvation. After 16 h, cells were either left untreated or treated with recombinant human (rh) IL-6 (50–200 ng/ml) for 10 min. Cells were harvested and lysed. Equal amounts of protein were analyzed by Western blotting using antibodies specific to the phosphorylated (p) or nonphosphorylated antibodies as indicated. Positive controls (+) were HeLa and NIH/3T3 cell lysates provided by Cell Signaling Technology. B: neither the serine (473) nor threonine (308) of Akt was phosphorylated after rhIL-6 stimulation. Positive control (+) indicates that NCI-H820 cells were treated with 1.5 nM heregulin β-1 for 15 min. The results shown are representative of 3 independent experiments.](http://ajplung.physiology.org/Downloadedfrom)
MATERIALS AND METHODS

Cell line and reagents. The NCI-H820 human adenocarcinoma cell line, which has NE properties, was obtained from the National Cancer Institute (NCI)-Navy Medical Oncology Branch (Division of Cancer Treatment, NCI, Bethesda, MD) from tumor specimens obtained from previously untreated patients (27). Recombinant human IL-6 (rhIL-6) and heregulin β-1 were purchased from R & D Systems (Minneapolis, MN). Antibodies against STAT1, STAT3, Akt, ERK, and p38 (including phosphorylated forms) were obtained from Cell Signaling Technology (Beverly, MA). Anti-STAT5 (including phosphorylated forms) antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-NSE antibody was obtained from Neomarkers (Fremont, CA). Anti-β-actin antibody and AG-490 were obtained from Calbiochem (La Jolla, CA). SB-203580 was from Promega (Madison, WI). All other reagents were of analytical grade.

Cell culture, treatment of rhIL-6, and transfection. NCI-H820 cells were cultured and maintained in serum-free ACL-4 medium (Invitrogen, Grand Island, NY) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). In 6 days of treatments, ACL-4 medium was renewed with the indicated concentration of IL-6 every 2 days. For short-term stimulation experiments, cells were starved in RPMI-1640 for 16 h followed by stimulation with IL-6 (10–200 ng) in RPMI-1640. When inhibitors were used, AG-490 was added to the medium and incubated for 16 h before rhIL-6 treatment (26, 31). For SB-203580 treatment (28), cells were pretreated with SB-203580 for 90 min before rhIL-6 stimulation. The rhIL-6 constitutive expression construct pCMV-IL-6 was transfected into NCI-H820 cells with the TransFast transfection reagent (Promega) according to the manufacturer’s instructions. Stable transfectants were isolated and cultured in the selective culture medium with 200 μg/ml of the antibiotic Geneticin (G418, Invitrogen) for 3–4 wk. G418-resistant clones were isolated and expanded. Expression of soluble rhIL-6 was confirmed by ELISA analysis (Diaclone Research, Besançon, France) of supernatant, cell lysates, and expanded. Expression of soluble rhIL-6 was confirmed by ELISA analysis (Diaclone Research, Besançon, France) of supernatants from transfectant, control vector, and parental cell populations.

Cell growth. NCI-H820 cells were seeded in a T-25 culture flask at a density of 1 × 10⁵ cells per 4 ml of ACL-4 medium with or without IL-6 (20 ng/ml). The medium was replaced every 2 days. Cells were disaggregated with a trypsin-EDTA solution, neutralized, and resuspended in culture medium. Viable cell counts were determined by trypsin blue exclusion with a hemocytometer. The mean of the hemocytometer counts of cells from triplicate flasks was determined every other day for 6 days for growth-curve and doubling-time determinations. The reported results were the means of three independent experiments. Paired t-tests were performed to evaluate whether significant differences in cell proliferation occurred between the tested conditions. All tests were two sided, and significance was assumed if P < 0.05.

Construction of the pStat3Y705F Adeno-X expression vector and infection. The dominant form of STAT3, Stat3Y705F, was generated by PCR-based mutagenesis (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) employing STAT3 as the template. Stat3Y705F was subcloned into pIRE52/EGFP shuttle vector, which was flanked by PI-Sce I and I-Ceu I. The resulting construct was then subcloned into Adeno-X Viral DNA vector, which was predestiged with PI-Sce I and I-Ceu I, according to the manufacturer’s instructions (BD Biosciences Clontech, Palo Alto, CA) to produce recombinant adenovirus. The adenovirus construct of Stat3Y705F, designated Ad-DNSTAT3, was transfected into a low-passage HEK-293 cell line with PacI-digested recombinant Adeno-X DNA, and recombinant adenovirus was harvested 10–14 days later. Virus titers were determined by with the Adeno-X Rapid Titer Kit (BD Biosciences Clontech). NCI-H820 cells were infected with 50–100 multiplicities of infection (MOI) of either Ad-DNSTAT3 or vector control (Ad-CTL) for 48 h. Viral infection was confirmed by visual observation of EGFP expression in infected cells.

Preparation of cell lysates and Western blot analysis. To investigate the cell signaling pathway, NCI-H820 cells were first cultured in ACL-4 medium for 16 h. Cells then either were left untreated or treated with rhIL-6 (50, 100, and 200 ng/ml) or Neu differentiating factor (heregulin; 1.5 nM, as the positive control) for 15 min. The harvested cells were lysed in radioimmunoprecipitation assay buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40], and cell lysates were prepared and analyzed by Western blotting with NSE or β-actin antibodies. Similar results were obtained in 3 independent experiments. The numbers in parentheses are the relative expression fold compared with the first lane (1.00).
P-40, and 0.42% NaF) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Total cell lysates (50 μg) were resuspended in SDS sample buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (NEB Life Science, Boston, MA) and detected with various antibodies. The PVDF membrane was then blocked with 3% BSA/PBST (PBS plus 0.1% Tween 20) for 1 h at room temperature. Antibodies were incubated with the membrane for 1 h at room temperature. The membranes were washed with PBST at room temperature for 30 min, and the wash was repeated three times. The resulting IgGs were detected by incubation with secondary antibodies conjugated to horseradish peroxidase and developed using an enhanced chemiluminescence system (LumiGLO; KPL, Gaithersburg, MD) and exposed to BioMax film (Kodak, Rochester, NY). All experiments were performed in triplicate.

p38 MAPK kinase activity assay. Cell lysates (600 μg) were incubated overnight with 20 μl of immobilized phospho-p38 MAPK (threonine 180/tyrosine 182) monoclonal antibody beads (50% slurry) (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instructions. After extensive washing, the kinase reaction was performed in the presence of 100 μM cold ATP and 2 μg of activating transcription factor (ATF-2) fusion protein at 30°C for 30 min. Phosphorylation of ATF-2 at threonine 71 was measured by Western blotting using phospho-ATF-2 (threonine 71) antibody. Phosphorylation of ATF-2 at threonine 71 was measured by Western blotting using phospho-ATF-2 (threonine 71) antibody.

RESULTS

Activation of STAT3 in lung adenocarcinoma cells is mediated by IL-6. In this study, we investigated whether the IL-6-elicited NE differentiation in lung adenocarcinoma cells might use signaling pathways similar to those in prostate cancer cells. A panel of phosphorylated antibodies, which represent the activation state of the signaling pathways, was used to rapidly screen the possible involvement of the above-mentioned pathways. Of the 16 lung cancer cell lines surveyed, NCI-H820 was selected for further analysis because it contains a high gene expression of IL-6 receptor and exhibits NE markers, such as NSE (9). NCI-H820 treated with the indicated concentrations of rhIL-6 resulted in a dramatic elevation of STAT3 tyrosine phosphorylation. NCI-H820 treated with 50 ng/ml of rhIL-6 was downregulated for 4 days to ~0.35-fold and for 6 days to ~0.32-fold (Fig. 2B). To evaluate the effect of AG-490 on NE differentiation, NCI-H820 was preincubated with 50 μM AG-490 and then treated with rhIL-6 (50 ng/ml) for 4 and 6 days. As shown in Fig. 2B, AG-490 could reverse the downregulation of NSE expression in the rhIL-6-treated NCI-H820.

Although AG-490 has been broadly used for JAK/STAT signaling pathway identification, it should be noted that this tyrphostin inhibitor specifically blocks JAK2 activation. Therefore, the second approach was to employ the dominant-negative form of STAT3 (STAT3Y705F), in which tyrosine 705 has been replaced by phenylalanine, to ascertain the involvement of STAT3 in rhIL-6-induced NSE downregulation. Be-

![Graphical representation of the results showing the effect of AG-490 on NE differentiation in NCI-H820 cells.](http://ajplung.physiology.org/)
cause of the low transfection efficiency of NCI-H820, we prepared an adenovirus delivery system to increase the sensitivity of our assay. The adenovirus constructs [i.e., dominant-negative STAT3 and control vector (vehicle)] were designated Ad-DNSTAT3 and Ad-CTL, respectively. NCI-H820 was infected with 100 MOI of Ad-DNSTAT3 and Ad-CTL, respectively. After 48 h, infected cells were treated with increasing concentrations of rhIL-6 (5 and 20 ng/ml) for 10 min. The results showed that overexpression of Ad-DNSTAT3 could completely block IL-6-induced tyrosine phosphorylation of STAT3 but did not affect the phosphotyrosine status of JAK2 (Fig. 3A). For long-term (6 days) treatment, NCI-H820 was infected with 50 MOI of Ad-DNSTAT3 and Ad-CTL, respectively, and then assayed for NSE expression. Figure 3B shows that Ad-DNSTAT3, but not Ad-CTL, could reverse the down-regulation of NSE expression when cells were stimulated with rhIL-6. Together, these findings raise the possibility that activation of STAT3 is an important mediator for IL-6-mediated NE differentiation in NCI-H820.

IL-6 activates p38 MAPK in NCI-H820 cells. We next investigated whether the three well-known MAPK pathways were involved in the modulation of IL-6 in NCI-H820. Again,
three phosphorylated antibodies, which represent the activation state of the p38 MAPK, ERK, and JNK signaling pathways, were used to rapidly screen the possible involvement of IL-6-elicted signaling pathways. NCI-H820 was treated with rhIL-6 (20 ng/ml) for 10 min. Cell lysates were examined by Western blotting with antibodies to the phosphorylated forms of p38 MAPK, ERK, and JNK. As shown in Fig. 4A, p38 phosphorylation was elevated in IL-6-treated cells. In contrast, ERK and JNK were not phosphorylated in response to rhIL-6 stimulation (results not shown). To provide additional evidence for the participation of p38 MAPK in rhIL-6 stimulation, we performed an immunoprecipitation/kinase assay using antibody against phosphorylated p38 MAPK. ATF-2 served as an in vitro substrate for p38 MAPK in the kinase assay. The results showed that p38 MAPK activity was enhanced 8.35-fold upon induction with rhIL-6 (Fig. 4B).

A pyridinyl imidazole compound, SB-203580, which has been shown to inhibit the p38 MAPK signaling pathway by binding to the ATP pocket of p38 MAPK (49), was used to examine the activity of p38 MAPK. In this assay NCI-H820 cells were exposed to 10 μM SB-203580 for 90 min before stimulation with rhIL-6 (20 ng/ml). p38 MAPK phosphorylation was slightly inhibited by SB-203580 (Fig. 4B), whereas 10 μM SB-203580 was sufficient to reduce p38 MAPK activity to the basal level (Fig. 4B). A similar effect was seen in another NSCLC-NE cell, NCI-H1355 (data not shown). These data support the idea that IL-6 activates p38 MAPK in NCI-H820 cells.

IL-6-elicted cell proliferation is mediated by p38 MAPK in NCI-H820 cells. In agreement with previous observation, treatment with rhIL-6 promoted cell growth as determined by direct cell counting (Fig. 5A). The finding prompted us to investigate whether the JAK/STAT and/or p38 MAPK signaling pathways were involved in IL-6-elicted cell proliferation. Treatment with AG-490 or overexpression of Ad-DNSTAT3 failed to demonstrate a dependency of cell growth on STAT3 activation in NCI-H820 (data not shown). In contrast, pretreatment of NCI-H820 with SB-203580 (5 μM) effectively blocked rhIL-6 (20 ng/ml)-induced cell proliferation (P < 0.05, n = 3, compared with the IL-6-stimulated cell growth) (Fig. 5A). To further corroborate these findings and elucidate the biological role of IL-6 in the NCI-H820 cells, an rhIL-6 constitutive expression construct was transfected into NCI-H820 and generated a stable rhIL-6-overexpressing subclone, which was designated H820.ILSC. Subsequently, we examined the H820.ILSC cell growth in the presence or absence of SB-203580. As shown in Fig. 5B, SB-203580 significantly suppressed H820.ILSC cell growth ~2.24-fold at concentrations of 5 μM (P < 0.05, n = 3) and 4.46-fold at 10 μM (P < 0.001, n = 3). Together, these data support the idea that IL-6-elicted cell proliferation is mediated through p38 MAPK.

DISCUSSION

IL-6, a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis, is produced by various types of human normal and transformed tumor cells, involved in regulation of the immune response, acute phase reaction, cell differentiation, and cell proliferation (18, 33). This pleiotropism implies a complex signaling cascade that enables the development of different pathways and distinct biological functions. It is well established that the binding of IL-6 to IL-6 receptor activates the JAK/STAT signal transduction pathway, where STAT3 plays a central role in transmitting the signals from the membrane to the nucleus (Fig. 6). The biological readout of STAT3 can be proliferation, survival, apoptosis, and/or differentiation, depending on the target tissues. STAT3 was first described as the acute-phase response factor, a transcription factor activated by IL-6/IL-6 receptor in human hepatoma cells (50). Recent studies have demonstrated the activation of STAT3 in human cancers, e.g., multiple myeloma, leukemia, and lymphoma (6). In addition, STAT3 has been suggested to be a key player in the pathogenesis of diverse human cancers, making this molecule a prime target for novel therapies.
The importance of STAT3 activation via IL-6 in prostate cancer development has been suggested. In LNCaP cells, IL-6 treatment could increase the growth of the cells concomitant with activation of STAT3 (19, 29). In contrast, other reports have observed that treatment of LNCaP cells with IL-6 results in terminal differentiation (NSE upregulation) and growth inhibition associated with STAT3 activation (42, 43). However, the molecular basis for the apparent contradiction remains unknown. On the other hand, IL-6-mediated proliferation of BAF3 cells is not dependent on activation of STAT3 (44). Although few direct studies into the role of STAT3 in human lung cancer have been undertaken, one recent study has demonstrated the role of STAT3 activation 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 NSCLCs (41). In the present study, the aim is to delineate the potential role of STAT3 in human non-small lung carcinomas by studying cell lines of non-small cell origin. We show that IL-6 elicited NSE downregulation in NCI-H820 is mediated by STAT3 activation using treatment with the JAK2-selective inhibitor (AG-490) (Fig. 2) and ectopic expression of a dominant-negative form of STAT3 (Fig. 3). Moreover, our results suggest that in IL-6-dependent NSCLC-NE cells, the JAK/STAT pathway appears to be more important in the regulation of NE differentiation than in cell growth promotion. To the best of our knowledge, this is the first report to directly demonstrate a role for STAT3 of the STAT family in NE differentiation in the established human NSCLC-NE cell line NCI-H820.

In addition to the classic JAK/STAT pathway, other signaling cascades may play important roles in signal transduction via the IL-6 receptor. It has been reported that ERK activation is essential for IL-6-dependent MM cell proliferation, whereas the phosphorylation of STAT1 and STAT3 is unrelated to melanoma cell growth (35). In contrast, the p38 MAPK pathway has been shown to be involved in cell proliferation in IL-6 signaling in B9 hybridoma cells (50). Therefore, different intracellular signaling pathways may contribute to the regulation of cell proliferation. In the present study, we show that activation of the p38 MAPK pathway, but not ERK or JNK pathway, is necessary for the induction of IL-6 (Fig. 4). Moreover, inhibition of p38 MAPK activity by SB-203580 resulted in suppression of the growth of cells induced by IL-6 and growth of an IL-6-expressing transfectant (Fig. 5), suggesting that p38 MAPK activation may play a critical role in IL-6-induced cell growth of NSCLC-NE cells. Together, the proposed signaling modules (Fig. 6), which depict IL-6-dependent NE dedifferentiation and cell proliferation in NSCLC-NE cells, exert their effect through the STAT3 and p38 MAPK signaling pathways.

Various new pharmacological agents are currently under clinical development for treatment of malignant tumors, including agents that either block growth-promoting signaling cascades or trigger apoptotic signals in neoplastic cells. The realization of signaling cascades that play important roles in the regulation of apoptosis and growth of cancer cells has led to extensive studies. Growing evidence points to a number of important STAT3 target genes that have been implicated in the formation of tumors (7, 12, 19). Inhibition of STAT3 through pharmacological blockage of upstream molecules such as Src and JAK may reduce the tumor formation. Our findings strongly suggest that the STAT3 and p38 MAPK signaling systems may play important roles in the development of lung cancers by either autocrine or paracrine mechanisms. This raises the possibility that, in addition to targeting STAT3, targeting the p38 MAPK pathway may have therapeutic implications in the treatment of NSCLC-NE tumors. Our results demonstrate that a thorough understanding of how specific signaling pathways regulate downstream gene expression may provide rationale for using targeted therapy to enhance cell killing.

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