

EML4-ALK-mediated Activation of the JAK2-STAT Pathway is Critical for Non-small Cell Lung Cancer Transformation

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Abstract

Background: The echinoderm microtubule-associated protein-like-4 anaplastic lymphoma kinase (EML4-ALK) fusion gene was identified in a small subset of non-small cell lung cancer (NSCLC) patients, who responded positively to ALK inhibitors. The aim of this study was to characterize the mechanism of NSCLC transformation by EML4-ALK.

Methods: HEK293 and NIH3T3 cells were transfected with EML4-ALK-V3 or pcDNA3.1-NC, and H2228 cells were transfected with siRNA-EML4-ALK or siRNA-NC. Cell viability and proliferation were measured by CCK-8 and EDU methods, respectively. Apoptosis was detected by flow cytometry. Gene expression profiles were generated from a signal pathway screen of EML4-ALK-regulated lung cancer cell transformation and verified by qPCR and western blotting. The interaction and colocalization of JAK2-STAT pathway components and EML4-ALK were determined by co-immunoprecipitation (Co-IP) and immunohistochemistry/ immunofluorescence, respectively.

Results: Several genes involved in the JAK2-STAT pathway were identified by Microarray. JAK2 and STAT6 were constitutively phosphorylated in H2228 cells, which was downregulated by EML4-ALK silencing. Ectopic expression of EML4-ALK in HEK293 and NIH3T3 cells resulted in activating of JAK2 and STAT1, STAT3, STAT5, and STAT6. In EML4-ALK-transfected HEK293 and EML4-ALK-positive H2228 cells, activated STAT6 and JAK2 colocalized with ALK. STAT3 and STAT6 were found to be phosphorylated and translocated to the nucleus of H2228 cells following IL4 or IL6 treatment. Apoptosis increased and cell proliferation and DNA replication decreased in H2228 cells following EML4-ALK knockdown. In contrast, HEK293 cell viability increased following EML4-ALK over-expression, while H2228 cells viability significantly decreased after treatment with ALK or JAK-STAT pathway inhibitors.

Conclusions: Taken together, our data suggest that aberrant expression of EML4-ALK leads to JAK2-STAT signaling pathway activation, an essential part of the development of non-small cell lung cancer.

Background

Non-small cell lung cancer (NSCLC) is the most malignant tumor in the world[1]. Prognosis of patients with NSCLC has improved largely owing to targeted therapy over recent years. In contrast to traditional chemotherapy and radiotherapy, targeted cancer therapy has garnered increasing attention for its more tumor specific in efficacy and fewer toxicities [2]. Patients with non-small-cell lung cancer harboring epidermal growth factor receptor (EGFR)-mutations may benefit from EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib.[3,4]. Therefore, targeted therapies are essential for prolonging survival in NSCLC patients with specific genotype.

The fusion gene Echinoderm Microtubule-associated protein-Like 4 (EML4)- Anaplastic Lymphoma Kinase (ALK) occur in approximately 5% of patients with NSCLC as a result of a recurrent chromosome inversion[5]. The EML4-ALK fusion appears to be more common in female patients and non-smoking patients with adenocarcinoma [6]. In general, the EML4-ALK fusion gene occurs in NSCLC without

mutation in epidermal growth factor receptor (EGFR). Patients who harbor EML4-ALK fusions may not be sensitive to EGFR TKIs or traditional chemotherapy, but sensitive to ALK inhibitors [6]. Various break and fusion points within the EML4 locus in NSCLC cells give rise to different isoforms of EML4-ALK [5-11]. The most common EML4-ALK variants are 1 and 3, which account for about 60% of EML4-ALK-positive lung cancer cases.

Although mouse NIH3T3 cells forced to express human EML4-ALK fusion transformed foci in culture and subcutaneous tumours in nude mice, which indicated that the potentially tumorigenic EML4-ALK may be a key driving factor in the development of lung cancer [12]. Inhibition of ALK by ALK inhibitors could suppress the growth and induce apoptosis in lung cancer cells [13-15], suggesting that ALK inhibition is a potential strategy for the treatment of EML4-ALK-positive NSCLC. However, the molecular mechanisms for how EML4-ALK regulate the cell proliferation or survival of lung cancer cells harboring EML4-ALK are still unknown.

In this study, we found that EML4-ALK activated the JAK2-STAT signaling pathway. We also identified changes in the expression of multiple target genes that are regulated by the STATs in high-throughput microarray analysis. We hypothesized that the JAK2-STAT pathway plays an important role in the development of lung cancer driven by EML4-ALK. EML4-ALK fusion gene phosphorylates JAK2, and constitutively active the STAT-1,3,5,6, P-STATs resulted in the cell viability increasing of the EML4-ALK-positive cells.

Methods

Cell culture, cytokines, and inhibitors

Human embryo kidney 293 cells (HEK293) and the human lung adenocarcinoma cell line H2228, which harbors the EML4-ALK variant-3 fusion gene, were obtained from the American Type Culture Collection. The NIH3T3 mouse embryonic fibroblasts were obtained from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Gibco™ Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂. All cytokines were purchased from PeproTech (USA), diluted as indicated by the manufacturer, and frozen in aliquots at -80°C. IL4 and IL6 were diluted in sterile ultrapure water. All inhibitors were purchased from Selleck Chemicals (USA) with dilution and storage according to the manufacturer.

Patients and samples

EML4-ALK-positive samples were obtained from seven NSCLC patients, as previously described [10]. The control group was patients with EML4-ALK-negative. Written informed consent was obtained from each patient and the work was approved by the Ethics Committee of Tianjin Medical University General Hospital. The demographic and clinical characteristics of the EML4-ALK-positive patients used in study was showed in Table 1

Microarray gene expression analysis

Total RNA from H2228 cells was used to prepare biotinylated target cRNA using the Affymetrix One-cycle cDNA synthesis kit following the manufacturer's instructions (Affymetrix, Santa Clara, CA). Then the biotinylated cRNA was fragmented and hybridized to the GeneChip Human Genome U133 plus 2.0 array (Affymetrix, Inc.), which contains more than 54,000 transcripts and expressed sequence tags. Raw Data were analyzed using Affymetrix GeneChip Operating Software (GCOS) 1.4 and filtered via 2-fold expression levels. P value cutoff of 0.05. Annotations were analysed using a combination of interactive query at NetAffx (www.affymetrix.com) and R suite. Clustering analysis made using MultiExperiment Viewer (The Institute for Genomic Research, <http://www.tigr.org/tdb/microarray/>).

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed as previously described [10]. After being washed in PBS and blocked with 5% bovine serum albumin for 15 min at room temperature. Then sections were incubated the primary antibody against ALK (1:50, DAKO North America, USA), p-STAT3, p-STAT6, and p-JAK2 (1:50, Cell Signaling Technology, Inc., USA) at 4°C overnight. Negative controls were set up by replacement of antibody with PBS. Cytoplasmic staining was considered positive for ALK.

Immunofluorescence labeling was performed as previously described [10]. Anti-ALK antibodies (DAKO North America, USA) were used at a dilution of 1:50, and p-JAK2, p-STAT3, and p-STAT6 antibodies (Cell Signaling Technology, Inc., USA) were used at a dilution of 1:100 for 2 h at 37°C. AlexaFluor 488-conjugated goat anti-rabbit (Invitrogen) and Alexa Fluor 594-conjugated goat anti-mouse were used as secondary antibody. Images were taken by an inverted fluorescence microscope (NIKON, Tokyo, Japan).

SiRNA and Plasmid construction

The EML4-ALK siRNA oligonucleotides (target sequence: CCTGTCAGCTCTTGAGTCA, positive-sense strand 5'-CCUGUCAGCUCUUGAGUCA-dTdT-3', antisense strand 5'-dTdT-GGACAGUCGAGAACUCAGU-3') were synthesized at RiboBio (Guangzhou, China). A scrambled siRNA duplex was used as a negative control. The EML4-ALK variant-3 cDNA expression construct was engineered by cloning the EML4-ALK PCR product generated from H2228 into the pcDNA3.1 vector (Invitrogen) using the EcoRI and XbaI sites (F: 5'-CGGAATT CACTCTGTCGGTCCGCTGAATGAA-3'. R: 5'-GCTCTAGACCAC GGTCTTAGGGATCCCAAGGAAGAGAA-3').

Protein extraction, immunoprecipitation, and western blotting

For the preparation of total cell lysates, cells were washed with PBS and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X100) containing a protease inhibitor cocktail (Roche, Mannheim, Germany), 50 mM NaF, and 1 mM Na₃VO₄. The samples were cleared at 12000 rpm for 15 min at 4°C, and the proteins quantified using the BCA protein assay (Thermo Scientific, MA, USA) using bovine serum albumin (BSA) as a standard. Equal amounts of proteins (10 to

40 µg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Amersham Biosciences, NJ). After washing, membranes were incubated with primary antibody overnight at 4°C under gentle rocking. Primary antibodies included anti-ALK, JAK2, STAT1, STAT3, STAT5, and STAT6, and anti-phospho-ALK, JAK2, STAT1, STAT3, STAT5, and STAT6 antibodies (all 1:1000 dilution), which were purchased from Cell Signaling Technology (Danvers, MA, USA). And then exposed to HRP-conjugated secondary antibody (1:1000 dilution, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. Bands were visualized using Pierce ECL Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunoprecipitation was performed on 800 µg of total protein by adding anti-JAK2 antibodies overnight at 4°C. The immune complexes were precipitated with Protein A-Sepharose 4B (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at 4°C. Immunoprecipitated proteins were washed, recovered by boiling in 5×SDS sample loading buffer, and then separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes at 100 V for 60 min in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) and probed with primary antibodies against ALK, JAK2 and phospho-STAT6. The following steps were done as above.

CCK-8 assay

The Cell Counting Kit-8 (Beyotime, Shanghai, China) was used according to the manufacturer's instructions. Approximately, 3×10^3 NIH3T3 or HEK293 cells in the exponential growth phase were seeded into 96-well plates and transiently transfected with pcDNA3.1-EML4-ALK-V3, EML4-ALK siRNA, control vector, or control siRNA for 24, 48, or 72 h. H2228 cells (1×10^4 cells/well) were cultured in 96-well plates for 24 h and then treated with TAE (0, 0.001, 0.01, 0.1, 1, 10, 100 µM) alone or in combination with SH-4-54 (0, 8, 12, 18, 27, 40, 0 µM) or ruxolitinib (0, 4, 16, 64, 256, 1024, 4096 µM) for an additional 24 h before performing the CCK8 assay. Then, 10 µL of CCK8 solution was added into each well and incubated for 1 h. The absorbance of each well was quantified at 450 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA). All data were calculated from triplicate wells.

5-Ethynyl-2'-deoxyuridine (EdU) staining

Cells were stained with the Cell-Light™ EdU stain kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, cells were cultured with 50 µM EdU for 2 h followed by two washes with PBS and fixation with 4% paraformaldehyde. After permeabilization with 0.5% Triton X-100 and washing with PBS, the cells were dyed with Apollo (Red) and Hoechst 33342 (Blue) in dark for 30 min and analyzed by fluorescence microscopy.

Flow cytometry analysis of apoptotic cells

H2228 cells (2×10^5 cells/well) were seeded into 6-well plates and cultured for 24 h. The cells were transfected with siRNA-NC or siRNA-EML4-ALK and cultured for another 24 h. The transfected cells were

stained using the Annexin V-FITC Apoptosis Analysis Kit (BD Biosciences, CA, USA) and analyzed by flow cytometry using a FACSAria™ flow cytometer (BD Biosciences, CA, USA).

Statistical analysis

All data were analyzed using the Statistical Package for Social Sciences (version 16.0., SPSS Inc., Chicago, Illinois, USA). The Student's t-test was used to identify statistical significance between two experimental groups. Multivariate analysis of variance (MANOVA) was used to identify statistical significance for H2228 cells viability for the three inhibitor groups. $P < 0.05$ indicated statistical significance.

Results

Oncogenic EML4-ALK tyrosine kinase activates JAK-STAT6 signaling pathway

To explore the mechanism of EML4-ALK-regulated lung cancer cell transformation, we used microarray analysis to generate gene expression profiles for H2228 cells transfected with EML4-ALK or scrambled control siRNA. This analysis identified 800 upregulated and downregulated genes that were changed at least 1.5-fold by EML4-ALK siRNA knockdown compared to the scrambled control. Further analysis revealed that a number of the altered genes were involved in the JAK2-STAT pathway (Figure 1A). Real-time PCR was used to validate the microarray data for the upregulated and downregulated genes that were previously reported to be regulated by STAT6. This analysis confirmed the microarray results (Figure 1B).

We also generated differential gene expression profiles for HEK293 cells transfected with EML4-ALK variant 3 or empty vector control. The microarray analysis identified 241 upregulated and 21 downregulated genes (Figure 1A). Consistent with the EML4-ALK knockdown experiment, expression of genes involved in the JAK2-STAT pathway, including IL4R, LIF, IL24, IL11, IL15RA, IL6R, SOCS3, and OSMR, was clearly upregulated, which was confirmed by real-time PCR (Figure 1B). Taken together, these data indicate that the JAK2-STAT signal pathway may be associated with the function of EML4-ALK.

STAT6 is constitutively active in EML4-ALK-positive lung cancer cells and tissues

To investigate the activation status of the STAT proteins in EML4-ALK-positive lung cancer cells, the levels of the phosphorylated STAT proteins (P-STATs) in H2228 cells were analyzed by immunoblotting. As demonstrated in Figure 2A, the H2228 cells were positive for p-STAT6. JAK2 was also constitutively active. Knockdown of EML4-ALK by siRNA in H2228 cells downregulated the phosphorylation levels of JAK2 and STAT6.

The cellular localization of p-STAT6, p-JAK2, and ALK in the EML4-ALK-positive lung cancer tissues was analyzed by immunohistochemistry. As shown in Figure 2B, p-JAK2 and p-STAT6 were detected in the cytoplasm of lung cancer cells in EML4-ALK-positive lung cancer tissues. As previously reported [10], ALK-positive staining was not seen in each primary tumor cell, indicating the intratumor heterogeneity of

ALK rearrangements in primary tumors. Similarly, the p-STAT6 and p-JAK2 staining was not observed in every primary tumor cell. By comparing the positive staining area of ALK and p-STAT6 in serial sections, it was apparent that both ALK- and p-STAT6-positive staining areas overlapped (Figure 2B).

As shown in Table 1 for seven EML4-ALK-positive lung cancer patients, p-STAT6 were only positively staining in 5 of 7 ALK-positive patients with primary tumors, and p-JAK2 was found in all these seven primary tumors. In As previously reported [10], there were only three cases (Cases 2, 5, and 6) with ALK-positive staining in the metastatic tumors. Consistent with the ALK staining, p-JAK2 was only present in these three cases. However, p-STAT6 staining was not observed in any of the six cases that had metastatic tumors.

The JAK2-STAT pathway is activated upon EML4-ALK stimulation

Because HEK293 cells lack functional endogenous STAT6 but express other components of the IL4 signaling pathway, we transfected the EML4-ALK variant-3 cDNA plasmid into HEK293 cells. EML4-ALK transfection resulted in intense activation of JAK2, STAT1, STAT3, STAT5, and STAT6 as demonstrated by their phosphorylation. Similar results were obtained by EML4-ALK transfection into NIH3T3 cells (Figure 3). Furthermore, the gene expression profiles of HEK293 cells versus EML4-ALK-transfected HEK293 cells indicated that the expression levels of the STAT6 pathway genes (e.g., IL4R, MAF, SOCS3, IL4 IL15RA, and IL6R) were significantly upregulated. These data demonstrated that key members of the JAK-STAT signal pathway could form a molecular network with EML4-ALK and be involved in the tumorigenicity mediated by EML4-ALK.

EML4-ALK interacts with JAK2 and activates the JAK2-STAT pathway by phosphorylation

To determine the interactions between the JAK2-STAT pathway components and EML4-ALK, we first examined the protein expression in H2228 and EML4-ALK-transfected HEK293 cells and discovered that activated STAT6 colocalized with ALK-positive cells (Figure 4A). We also examined whether EML4-ALK could directly interact with JAK2, resulting in the activation of the JAK2-STAT pathway. We performed Co-IP experiments using an anti-JAK2 antibody in lysates from EML4-ALK-transfected HEK293 cells. Both ALK and p-STAT6 co-immunoprecipitated with JAK2 (Figure 4B), confirming a protein interaction between EML4-ALK and JAK2-STAT6.

IL4 and IL6 activated JAK2-STAT pathway of EML4-ALK-positive cells

Because EML4-ALK interacted with the JAK2-STAT pathway resulting in the phosphorylation of STAT proteins in EML4-ALK-positive lung cancer cells, we investigated whether IL4 or IL6 could activated the JAK2-STAT pathway in these cells. We examined the activation states of STAT3 and STAT6 in H2228 cells following IL4 /IL6 treatment and found that both STAT3 and STAT6 were phosphorylated and translocated into the nucleus (Figure 5A). JAK-STAT pathway was activated in existing endogenous EML4-ALK after IL4 or IL6 treatment. These data demonstrate that EML4-ALK may be involved in the IL4/IL6/JAK/STAT signaling pathway in EML4-ALK-positive lung cancer cells.

We further employed microarray analysis to generate the gene expression profile of H2228 cells stimulated with IL4 compared to unstimulated cells. This analysis identified 280 genes that were upregulated at least 1.5-fold following IL4 stimulation. Further analysis revealed that a number of the identified genes were involved in the JAK2-STAT pathway, regulation of the actin cytoskeleton, the cell cycle, cell adhesion, and positive regulation of cell proliferation (Figure 5B). These data suggested that IL4 could activate the JAK2-STAT pathway in EML4-ALK-positive cells.

The effect of oncogenic EML4-ALK tyrosine kinase on the biological behaviors of lung cancer cells

To study the effect of oncogenic EML4-ALK on the biological behaviors of lung cancer cells, we measured cell apoptosis following either EML4-ALK knockdown in H2228 cells or expression of EML4-ALK variant 3 in HEK293 cells (Figure 6A). The percentage of H2228 apoptotic cells increased from $5.36 \pm 0.33\%$ to $10.86 \pm 3.06\%$ following EML4-ALK knockdown ($P = 0.013$, Figure 6B). This result demonstrated that EML4-ALK could function as an anti-apoptosis factor.

HEK293 cell viability increased following EML4-ALK expression ($P < 0.05$, Figure 6C). While exploring EML4-ALK tumorigenicity signaling pathways, we found that cell viability and DNA replication ability of HCC827 cell decreased following knockdown of EML4-ALK or STAT6 expression with siRNA (Figure 6D, 6E). These results indicated that modulation of EML4-ALK or STAT6 levels in EML4-ALK-positive cells could cause the similar effect on biological characteristics of the cells.

Lastly, we analyzed H2228 cell viability after ALK or JAK-STAT pathway inhibitor treatment (Figure 6F). Compared to ALK inhibitor (TAE684) alone ($74.08 \pm 12.26\%$), the viability of EML4-ALK-positive H2228 cells significantly decreased further when TAE684 was combined with the JAK2 inhibitor ruxolitinib ($54.51 \pm 13.64\%$, $P = 0.023$) or STAT inhibitor SH-4-54 ($39.98 \pm 15.02\%$, $P = 0.021$). These results showed that JAK/STAT signaling as the downstream of EML4-ALK fusion gene involves in regulating cell proliferation and cell survival in NSCLC cells.

Discussion

The JAK family of non-receptor tyrosine kinases includes four members (JAK1, JAK2, JAK3, and TYK2) [16]. The JAK-STAT signal pathway is normally activated in response to ligand engagement of cytokine receptors that triggers auto-cross phosphorylation of the receptor-associated JAK kinases and subsequent tyrosine phosphorylation of STAT factors. The STATs then dimerize, translocate to the nucleus, and activate the transcription of target genes. JAK2 activation plays an important role in the signal transduction induced by cytokines (e.g., IL4, IL6, INF- α , INF- β , INF- γ) and growth factors (e.g., growth hormone, prolactin, leptin, erythropoietin, thrombopoietin) [17].

The STAT signaling pathway is an essential signaling pathway for many cytokines, hormones, and growth factors that regulate cell proliferation, differentiation, development, and survival [18]. There are six main STAT family members, STAT1-6. STAT3, STAT5, and STAT6 are activated in a high proportion of breast tumors and act as mammary oncogenes [19]. Constitutive activation of STAT proteins occurs in a

variety of blood cancers (e.g., leukemia, lymphoma, and multiple myeloma)[20,21] and solid tumors (e.g., brain, head and neck, breast, lung, pancreatic, and prostate cancer) [22,23]. STAT6 activation can promote tumor cell proliferation. High STAT6 expression is an important survival factor for prostate cancer cells and can control the progress of this disease[24]. Knockdown of STAT3 using siRNA inhibits the growth of tumor cells and induces apoptosis[25]. STAT5 is a key protein in prostate cancer survival; in CWR22RV and LNCaP prostate cancer cells, STAT5 gene silencing can induce cell death[26]. In addition, constitutive activation of STAT proteins is closely related to the malignant transformation of cancer and associated with the occurrence and development of tumors[27].

Abnormal activation of the JAK-STAT pathway is a feature of many types of cancer [28]. This abnormal activation is associated with tumorigenesis, likely because the activated target genes for this pathway encode anti-apoptotic proteins (e.g., Bcl-2 and Bcl-X). By increasing the levels of anti-apoptotic proteins (Bcl-2, Bcl-xl, Mcl-1), proliferation-related proteins (e.g., cyclin-D1 and Myc), and angiogenesis factor VEGF, and decreasing p21 and p27 to promote the transition from G1 to S phase of the cell cycle, the STATs inhibit tumor cell apoptosis and promote cell proliferation. In addition, the coordinated repression of STAT3 and c-Jun can restrain Fas-mediated cell apoptosis[29], and STAT6-mediated downregulation of p27kip1 and Gif-1 expression can control cell proliferation[30].

In normal cells, STAT activation is usually short-term and accurately regulated, such that STATs are transported back into the cytoplasm within hours, and the activation signal is attenuated [18]. JAK-STAT pathway proteins are degraded or deactivated by negative regulation factors (e.g., SOCS and PIAS) in cells [31]. In contrast, STATs can be constitutively active in tumor cells [28]. Potential mechanisms of continuous STAT activation include[31]: (1) Tumor-secreted or paracrine cytokines or growth factors, such as IL4 or IL6, can generate signaling through the corresponding receptor-activated tyrosine kinase on the cell membrane (e.g., JAK2) to constitutively activate the STAT proteins; (2) Inactivation of negative regulatory factors (e.g., proteases, suppressors of cytokine signaling (SOCS, including SOCS-1), phosphatases, PTP1B, PIAS1, and PIAS3) can lead to STAT activation; (3) Some oncogenes and tyrosine kinases (e.g., v-SRC, v-Abl, v-Ros, Etk/BMX, and Lck) can cause sustained activation of STAT3 and STAT6 and (4) Many JAK point mutations have been found in human leukemia cells (e.g., JAK2V617F), which cause constitutive activation of STAT proteins by JAK2 kinase and abnormal activation of the JAK2-STAT signal pathway. In patients with primary mediastinal B cell lymphoma, a point mutation in the STAT6 DNA binding domain caused sustained activation of STAT6[31].

In our study, EML4-ALK siRNA in H2228 cells or EML4-ALK expression in HEK293 cells resulted in changes in the expression of JAK-STAT signaling pathway-related genes, including multiple STAT target genes. Indeed, the presence of the EML4-ALK enhanced the survival of tumor cells and promoted cell proliferation. Through western blot experiments, JAK2 and STAT6 sustained activation was found in EML4-ALK positive H2228 cells, after siRNA silence, JAK2, and STAT6 phosphorylation levels decreased. HEK293 cells of EML4-ALK transfection phosphorylated JAK2, STAT1, 3, 5, and 6 also have resulted in obvious phosphorylation. By immunohistochemistry observation of EML4-ALK positive lung cancer tissue and immunofluorescence observations of H2228 cells and transfection EML4-ALK 293 cells,

STAT6 were phosphorylated, and its expression parts were consistent with ALK in EML4-ALK expression positive cells. In subsequent co-immunoprecipitation experiments, we used the JAK2 antibodies to precipitate out of the ALK and STAT6 protein, and the results confirmed that with JAK2 with EML4-ALK and STAT6 on the same protein complexes, each played a role in relation to the other. After IL4 and IL6 stimulating H2228 cells, we found that the P-STAT3 and P-STAT6 transport into the nucleus.

Takeover, the results of the current study suggest that the JAK2-STAT signaling pathway plays an important role in the EML4-ALK-mediated occurrence and development of lung cancer. Sustained activation of EML4-ALK or ALK causes JAK2 phosphorylation and activation of STAT1, STAT3, STAT5, and STAT6. This study provides the preliminary basis for an interaction mechanism between EML4-ALK and the JAK-STAT pathway and the construction of a molecular network. It provides new concepts for the potential role of the JAK-STAT signaling pathway in EML4-ALK-mediated tumorigenesis and biological activity, and in deepening our understanding of targeted lung cancer therapy.

Conclusions

In conclusion, our data suggest that aberrant expression of EML4-ALK leads to JAK2-STAT signaling pathway activation, an essential part of the development of non-small cell lung cancer.

Abbreviations

EML4: echinoderm microtubule-associated protein-like-4; ALK: anaplastic lymphoma kinase; NSCLC: non-small cell lung cancer; JAK2: Janus Kinase 2; STAT: signal transducer and activator of transcription; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitors; EdU: 5-Ethynyl-2'-deoxyuridine; SOCS: Suppressor of cytokine signaling; IL4: Interleukin 4

Declarations

Ethics approval and consent to participate: This study was approved by the Ethical Review Committee of Tianjin Medical University General Hospital. All biological samples and images were obtained with patients' written informed consent.

Consent for publication: Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions

JC and HYL designed and supervised of the study. YL, YWL, HYL and JC wrote the manuscript. YL, YWL and HBZ performed the experiments. RFS and ZHZ assisted with the performance of some experiments. All authors analyzed the data together, discussed the manuscript and approved the final manuscript.

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Tables

Table 1. EML4-ALK-positive NSCLC patient characteristics and associated JAK2 and STAT6 protein expression

Case number	Smoking status	Histology [H&E]	pTNM	ALK/P-JAK2/P-STAT6 IHC at primary sites	ALK/P-JAK2/P-STAT6 -IHC at metastatic sites
1-147	Non	AD	T2aN2M0	+/+/+	-/-/-
2-159	Non	Mixed-AD	T1N2M0	+/+/-	+/+/-
3-161	Non	Mixed -AD	T1N2M0	+/+/+	-/-/-
4-170	Non	Ad + SCC	T2bN2M0	+/+/-	-/-/-
5-177	Non	Mixed -AD	T2aN1M0	+/+/+	+/+/-
6-184	Yes	Mixed -AD	T2aN1M1	+/+/+	+/+/-
7-98	Non	Mucinous BAC	T2aN0M0	+/+/+	/

AD:adenocarcinoma;SCC:squamous carcinoma;BAC:bronchioloalveolar carcinoma;+:postive;-:negative.

Figures

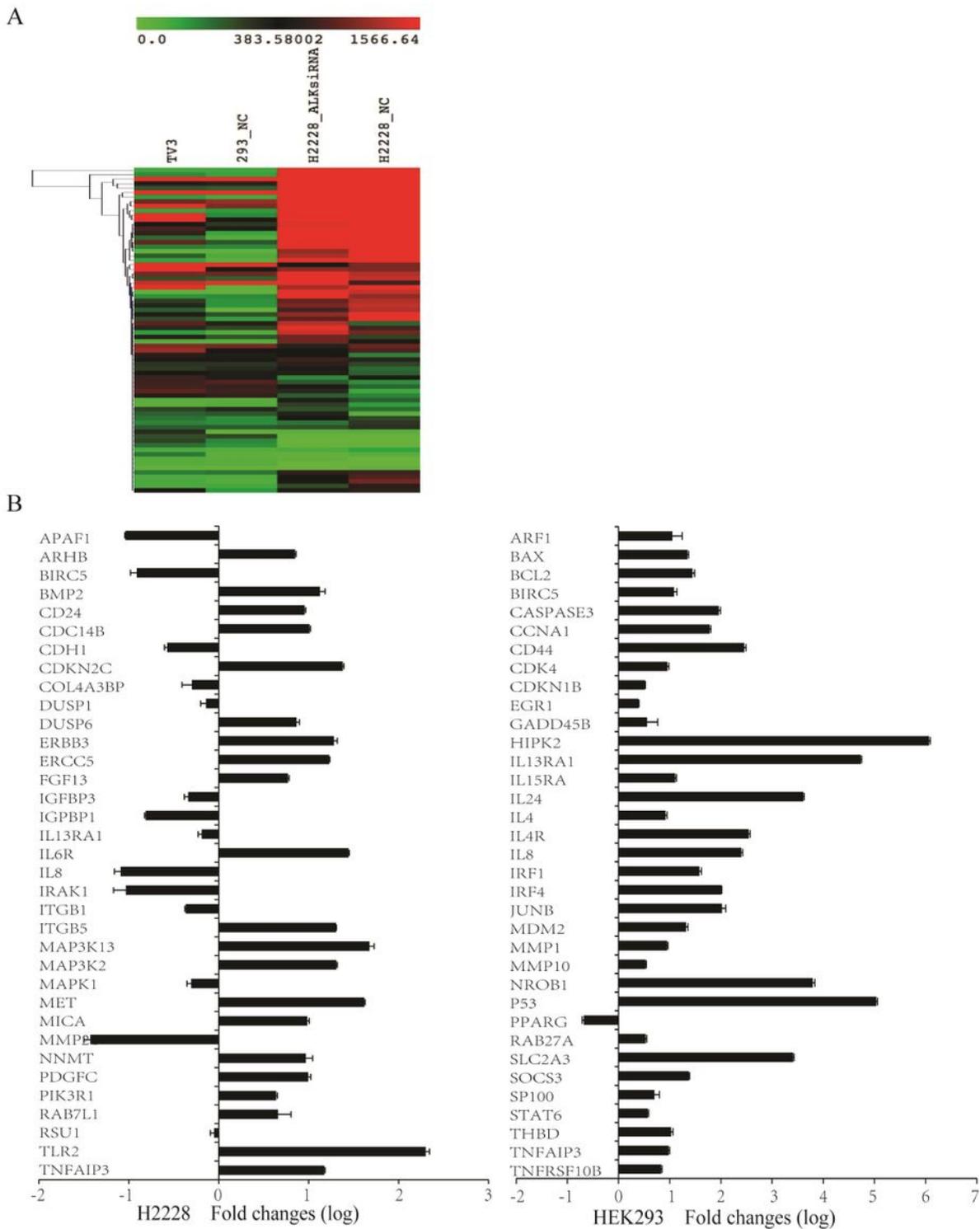


Figure 1

Microarray analysis of H2228 cells without or with EML4-ALK knockdown and HEK293 cells with or without EML4-ALK variant 3 expression. (A): The heat map associated with a number of JAK2-STAT pathway genes. (B): Expression of JAK2-STAT pathway-related genes measured by real-time PCR to verify the microarray results.

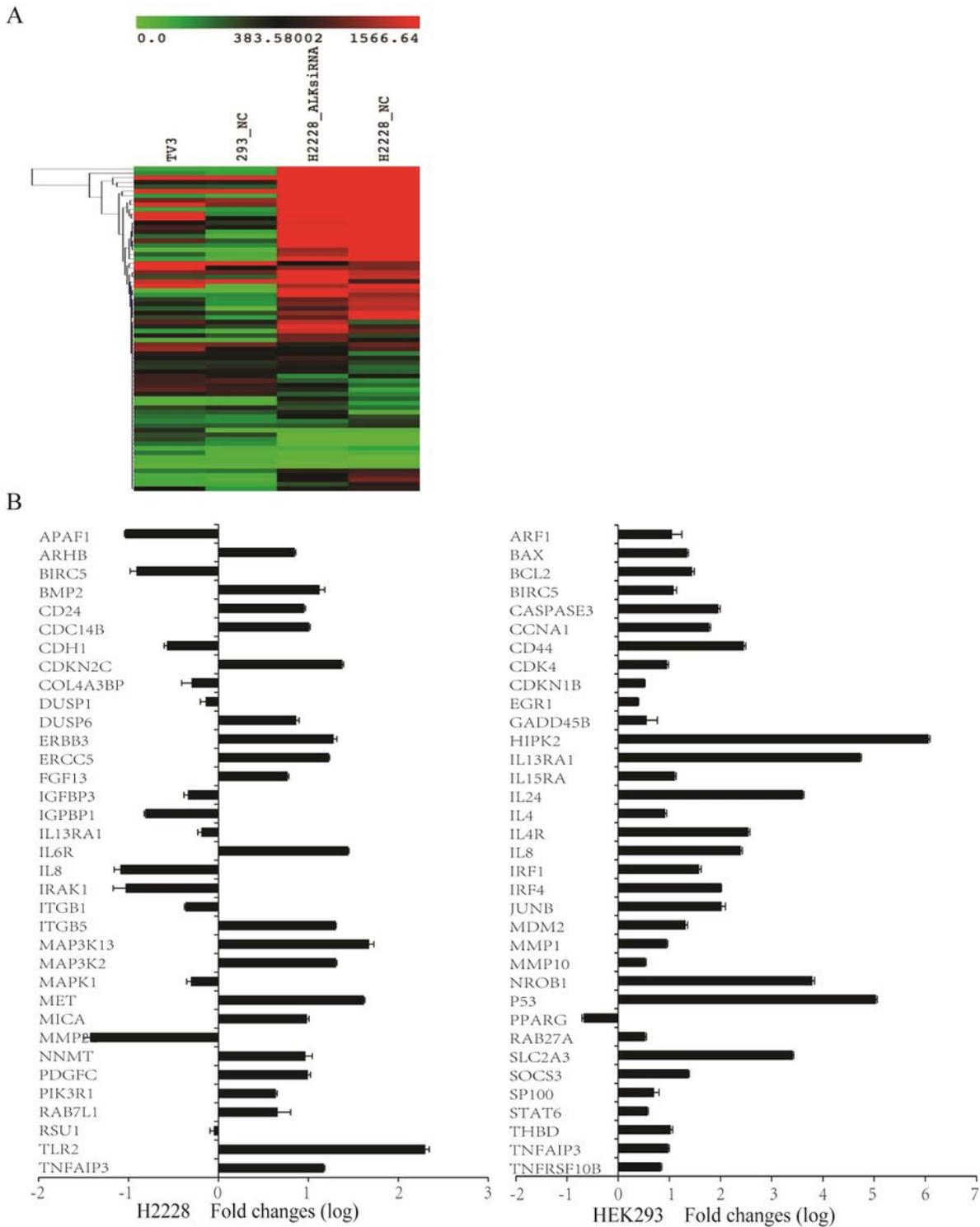


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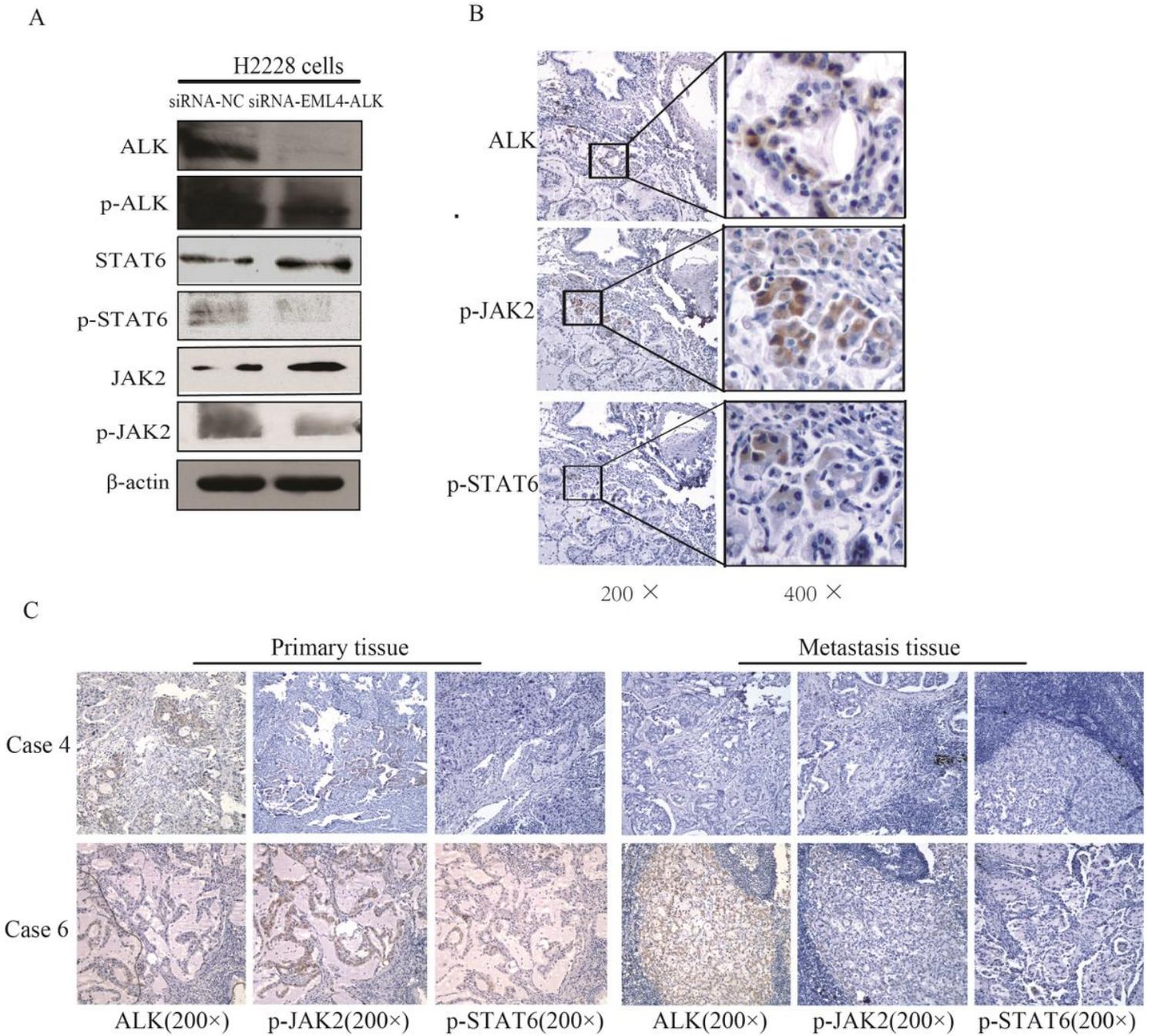


Figure 2

The activation status of STAT proteins in EML4-ALK-positive lung cancer. (A): STAT6 and JAK2 were constitutively phosphorylated in H2228 cells. Phosphorylation of JAK2 and STAT6 was downregulated following EML4-ALK knockdown in H2228 cells. (B): ALK-, p-STAT6-, and p-JAK2-positive staining coincide in primary tumor tissue.

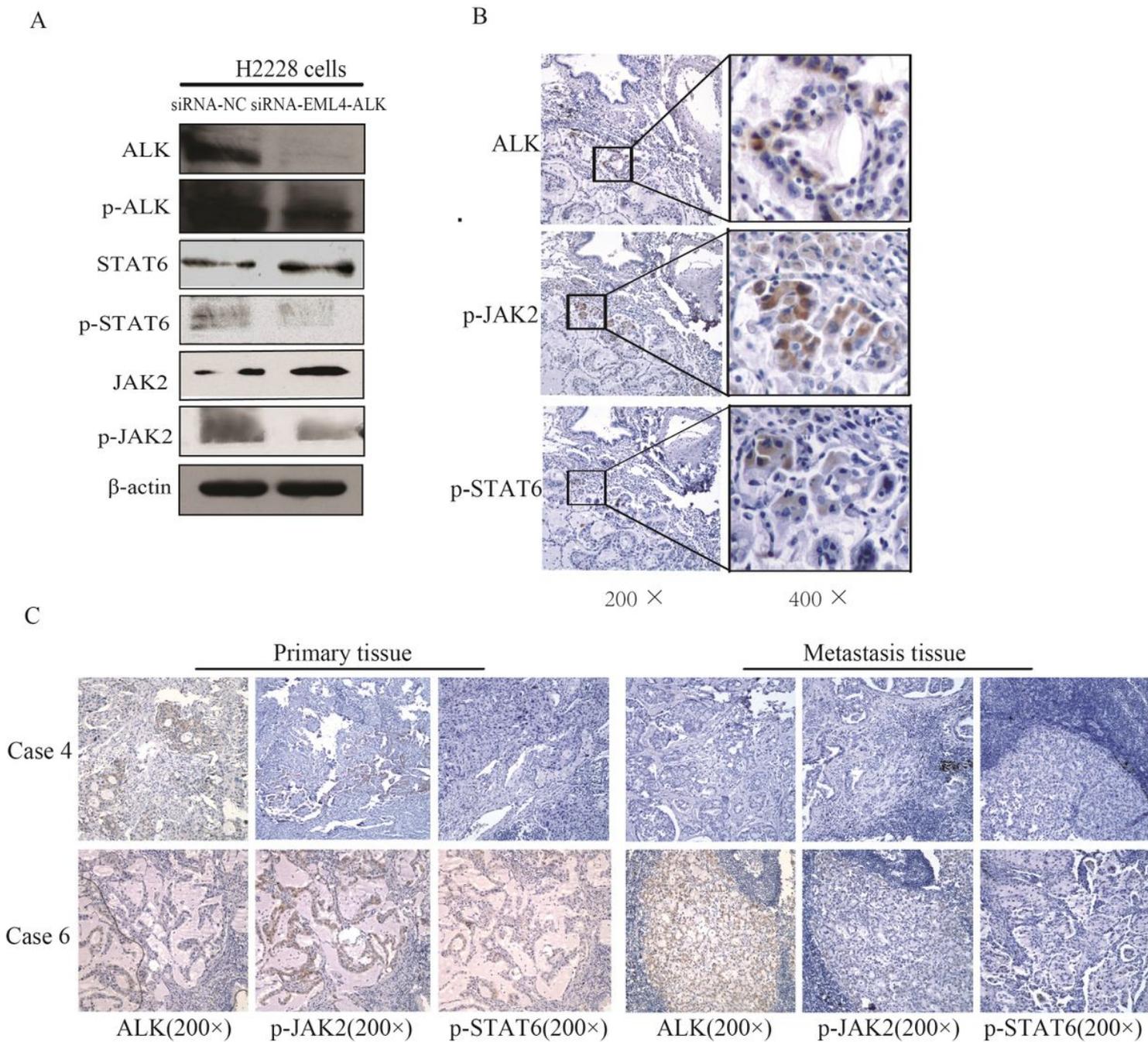


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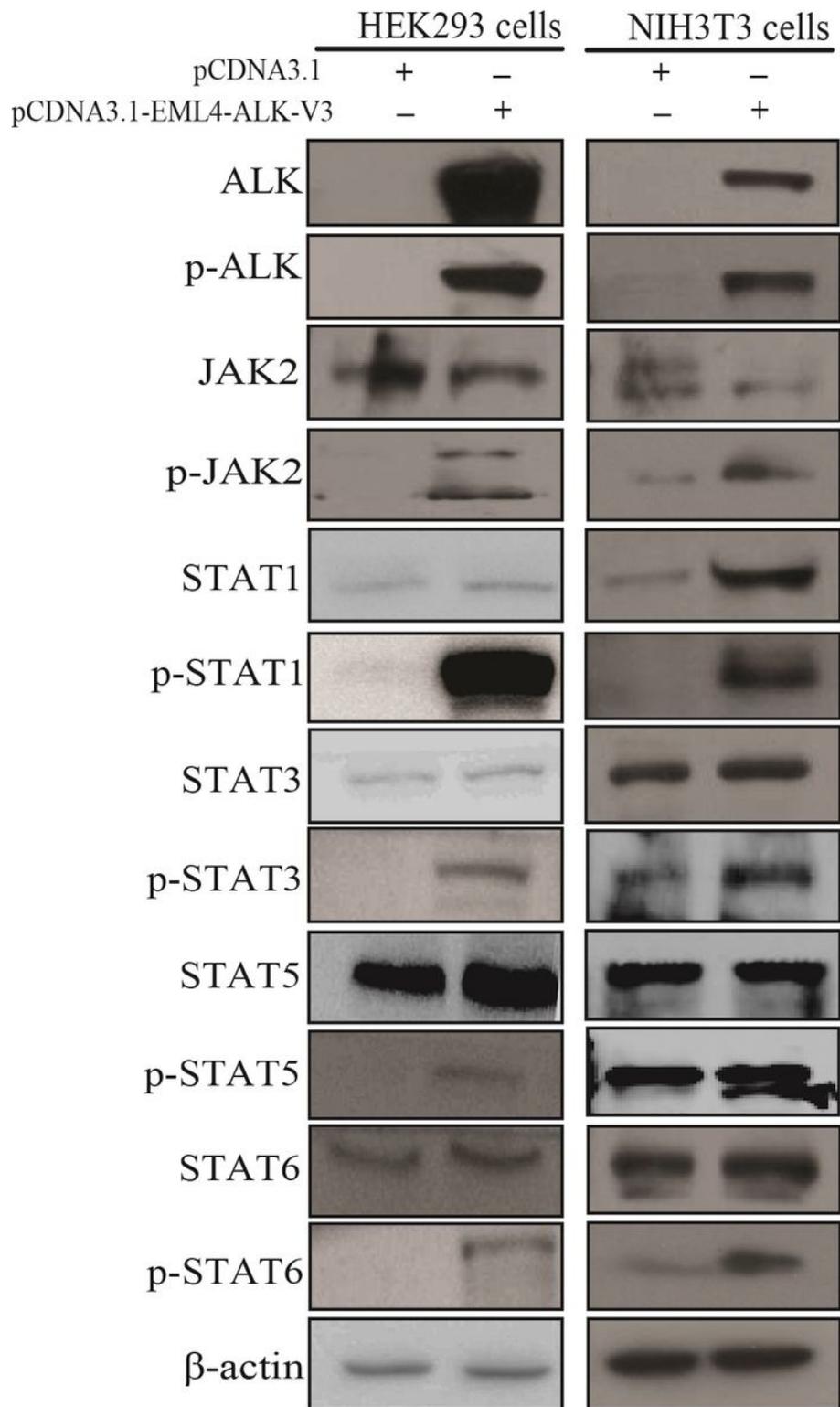


Figure 3

EML4-ALK transfection resulted in activation of JAK2, STAT1, STAT3, STAT5, and STAT6 in both HEK293 and NIH3T3 cells.

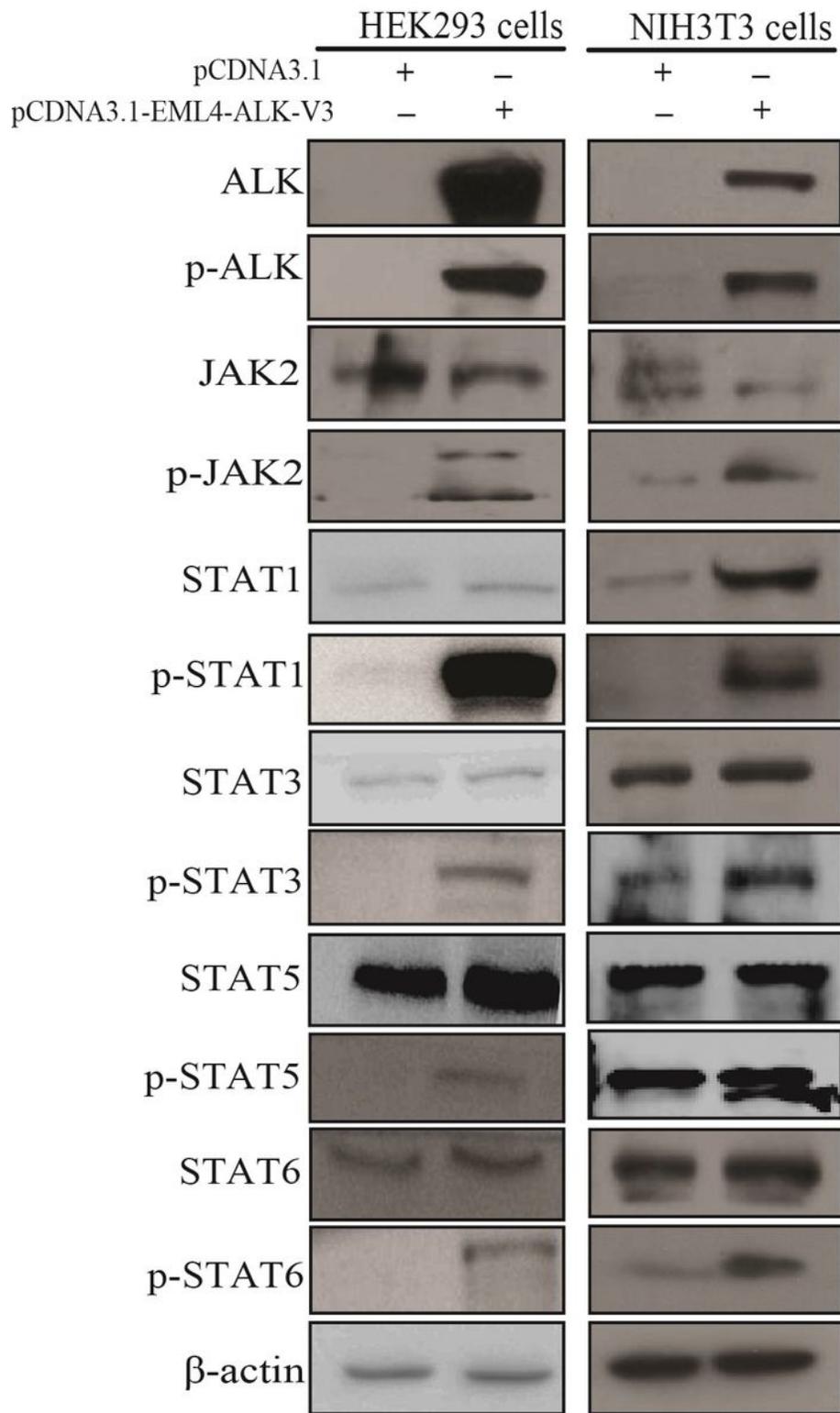


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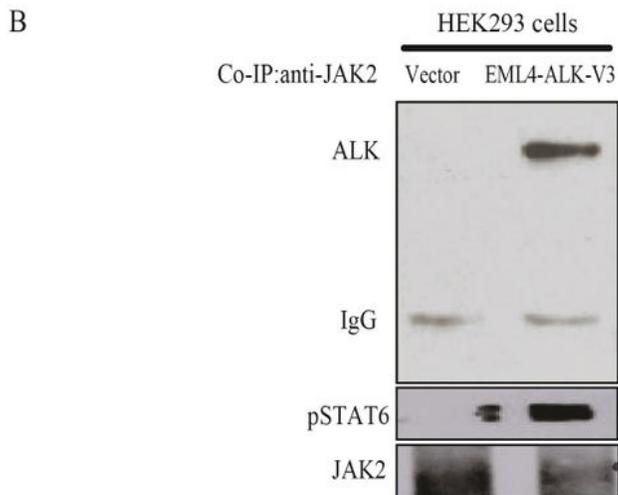
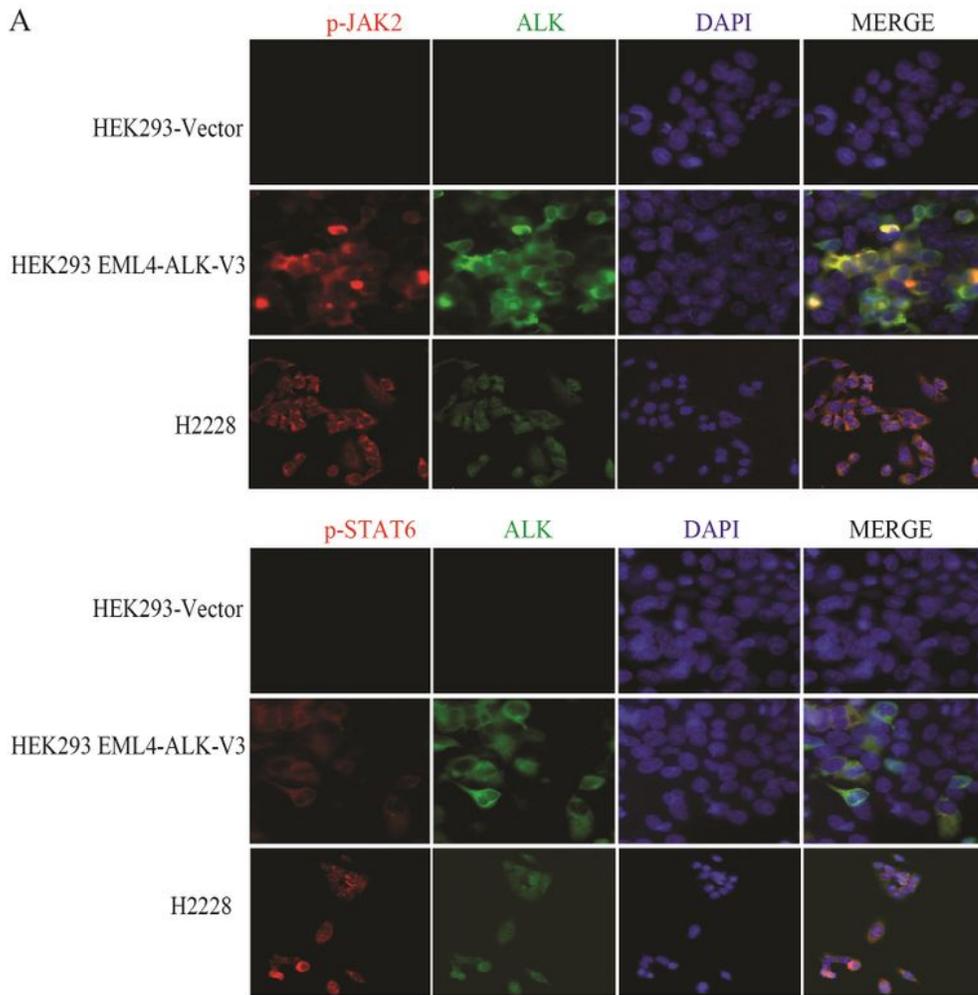


Figure 4

The interactions between JAK2-STAT pathway components and EML4-ALK. (A): In EML4-ALK-transfected HEK293 cells and H2228 cells, Active STAT6 and JAK2 colocalized with ALK-positive cells. (B): Co-IP using anti-JAK2 antibody in lysates from EML4-ALK-transfected HEK293 cells. ALK and phosphorylated STAT6 protein were co-immunoprecipitated with JAK2.

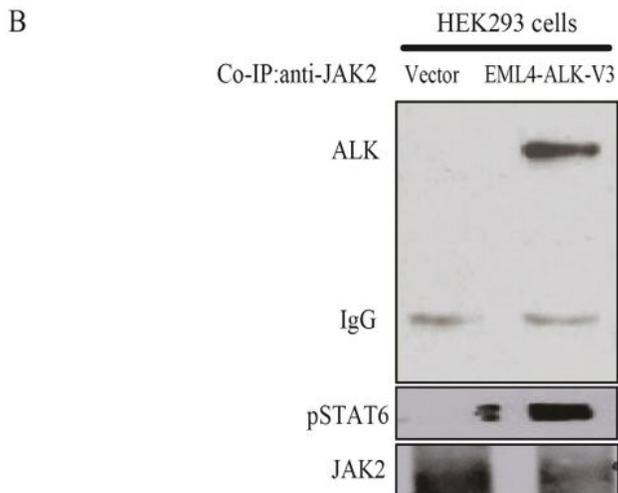
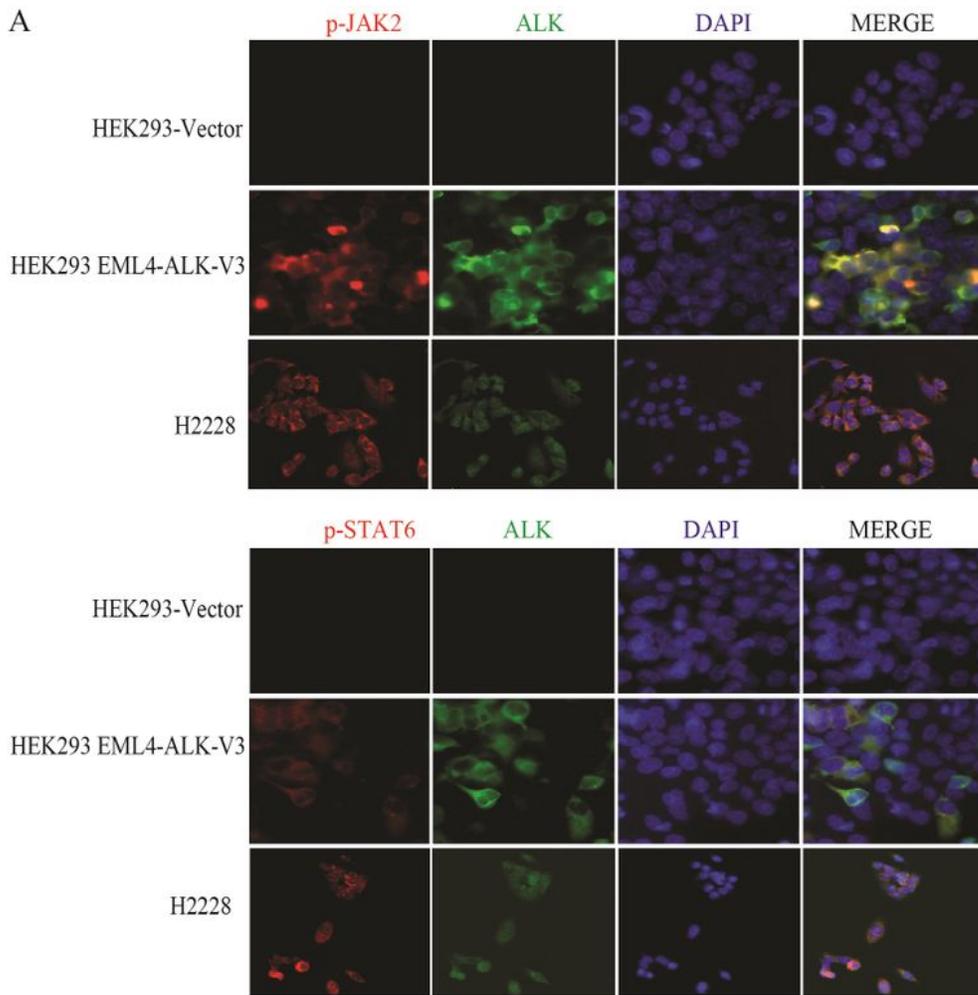


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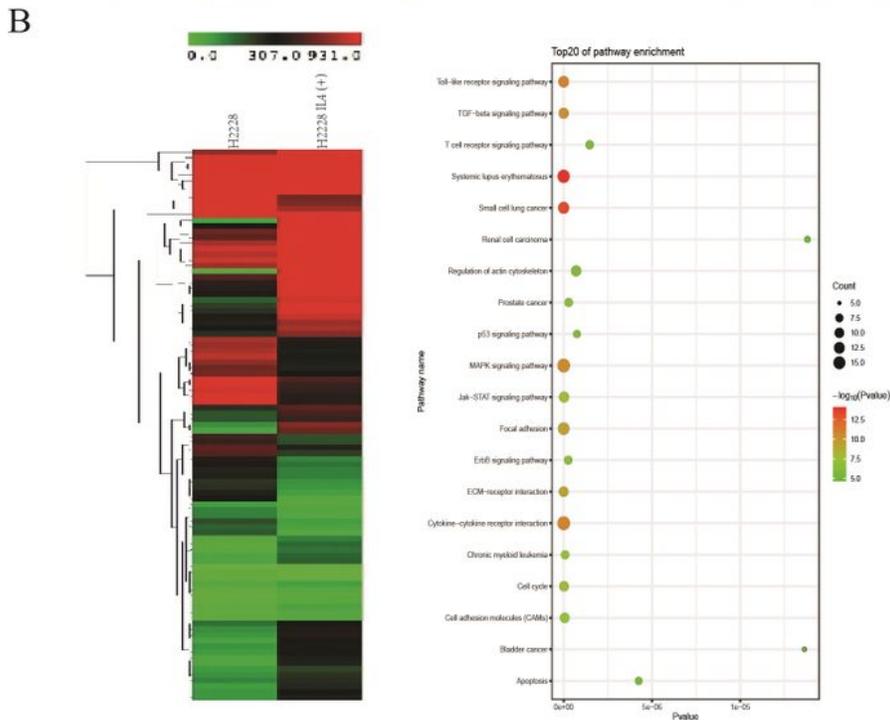
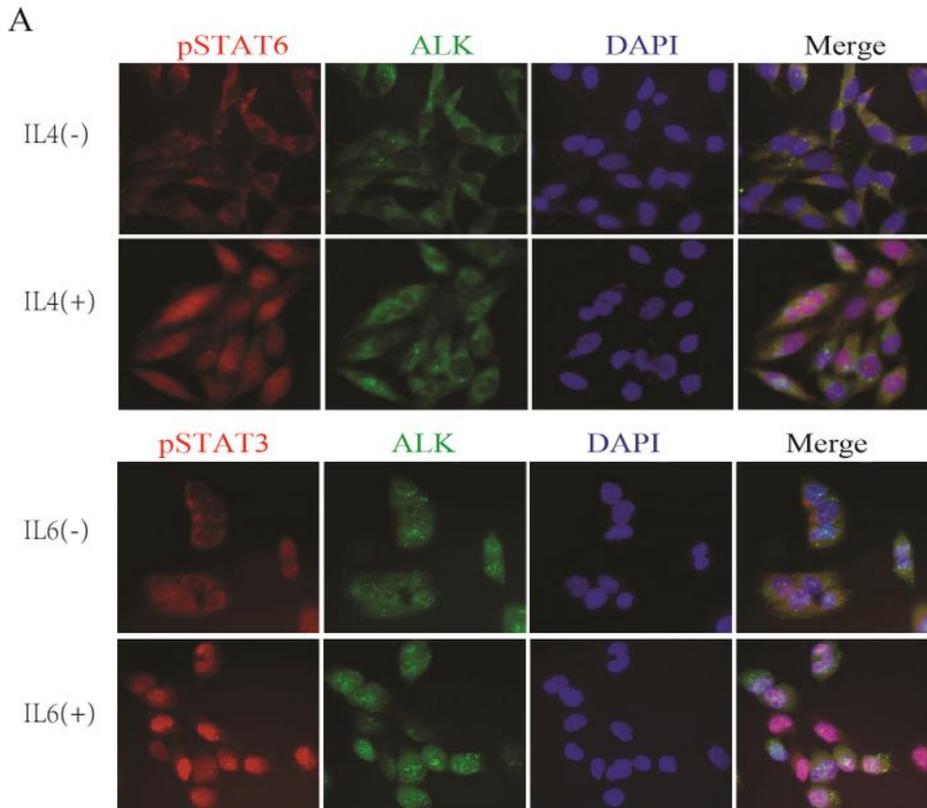


Figure 5

IL4 and IL6 affected the JAK2-STAT pathway of EML4-ALK-positive cells. (A): STAT3 and STAT6 were phosphorylated and translocated to the nuclei of H2228 cells following treatment with IL4 or IL6. (B): Microarray analysis for H2228 cells without or with IL4 stimulation. The heat map was associated with JAK2-STAT pathway, regulation of actin cytoskeleton, the cell cycle, cell adhesion, and positive regulation of cell proliferation.

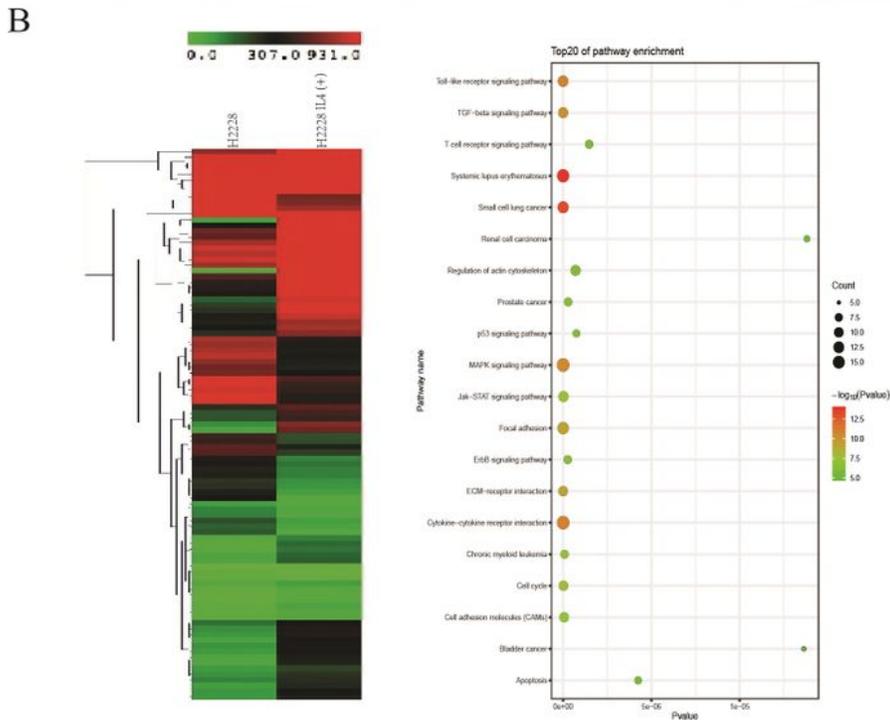
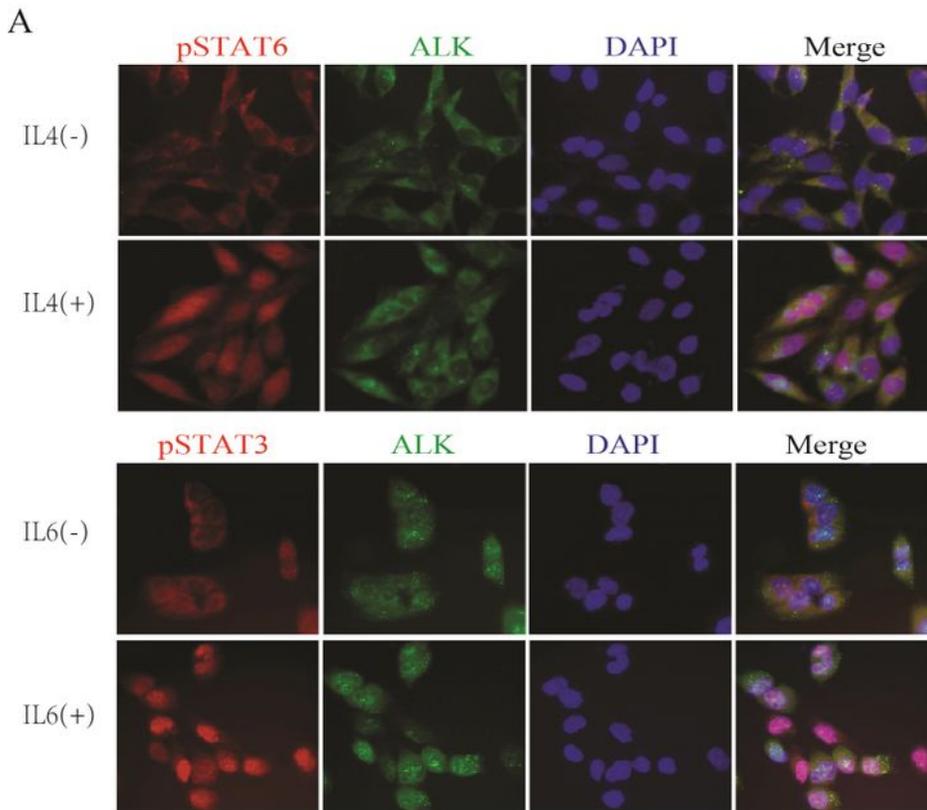


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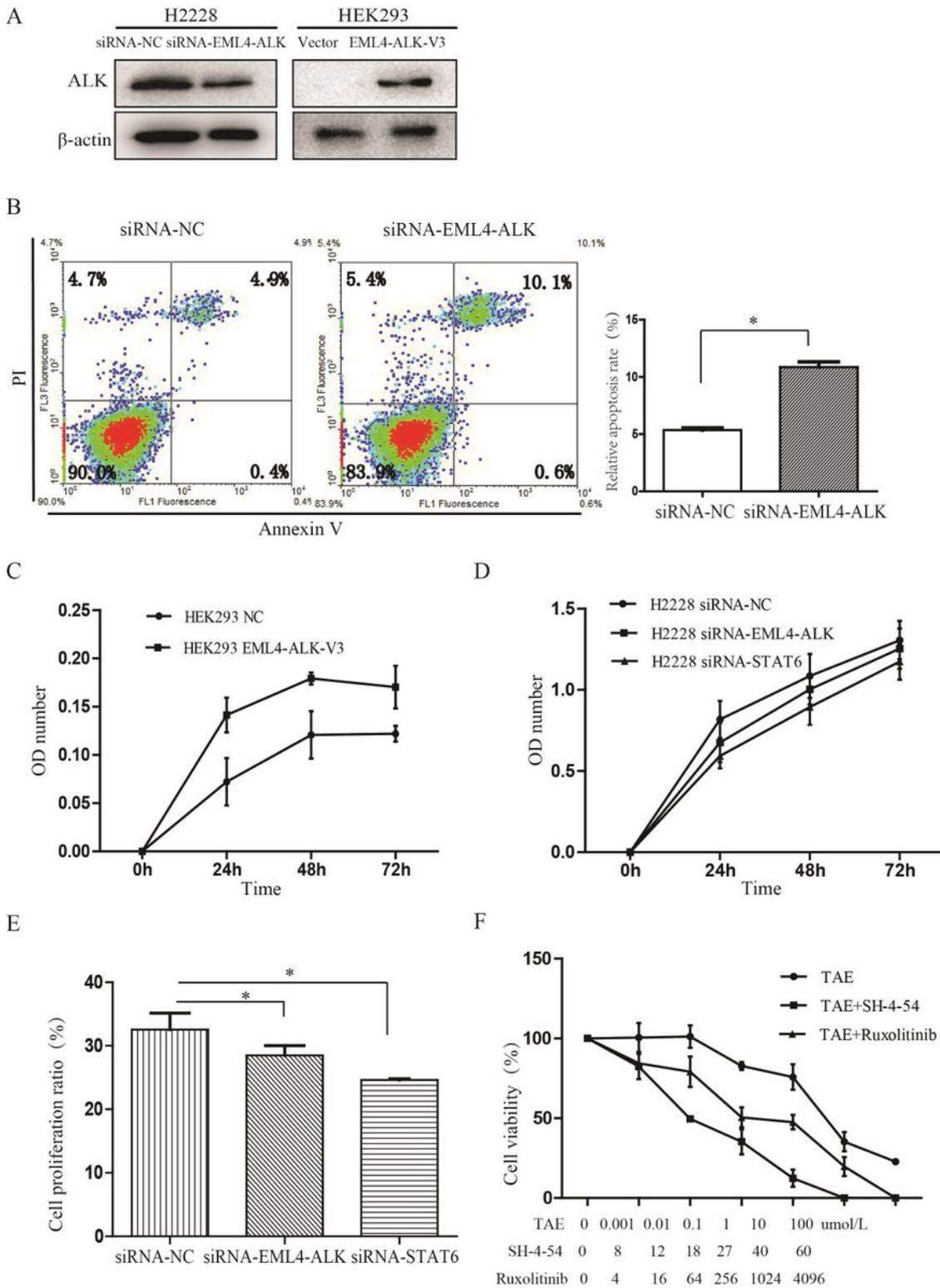


Figure 6

Effect of oncogenic EML4-ALK tyrosine kinase on the biological behaviors of lung cancer cells. (A): EML4-ALK levels in transfected H2228 and HEK293 cells, (B): Increased apoptosis in H2228 cells following EML4-ALK knockdown measured by flow cytometry. (C): Cell viability was increased by EML4-ALK expression in HEK293 cells measured by the CCK8 assay. (D-E): H2228 cells viability and cell

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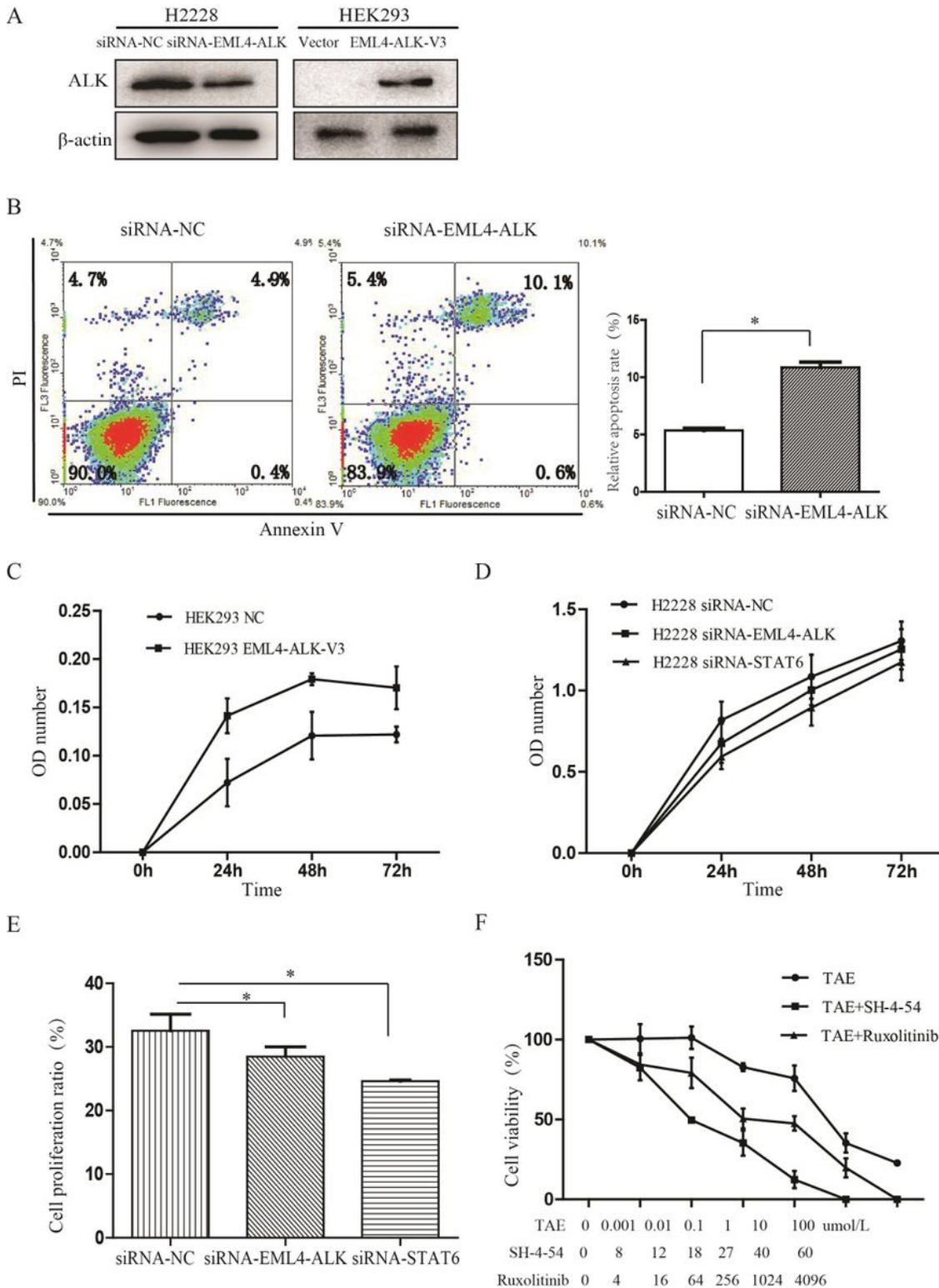


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