IL-6 Regulates MMP-10 Expression *via* JAK2/STAT3 Signaling Pathway in a Human Lung Adenocarcinoma Cell Line

XIAOYING ZHANG[1], PEIJI YIN[1], DONGMEI DI[1], GUANGHUA LUO[1], LU ZHENG[1], JIANG WEI[1], JUN ZHANG[1], YUANPING SHI[1], JICHEN ZHANG[1] and NING XU[2]

[1]*Comprehensive Laboratory, The Third Affiliated Hospital, Suzhou University, Changzhou 213003, P.R. China;*
[2]*Section of Clinical Chemistry and Pharmacology, Institute of Laboratory Medicine, Lund University, S-221 85 Lund, Sweden*

Abstract. *We previously reported that matrix metalloproteinase (MMP)-10 mRNA levels were significantly lower in tumor tissues than in adjacent normal tissues in human non-small cell lung cancer (NSCLC), whereas protein levels of MMP-10 were higher in the tumor tissues than the adjacent tissues. The mechanism of this divergence is still unknown. In the present study the role of Janus kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) on interleukin (IL)-6 mediated regulation of MMP-10 expression was investigated in a human lung adenocarcinoma cell line (A549 cells) and the molecular regulatory mechanism of MMP-10 expression was explored. A549 cells were stimulated by different concentrations of IL-6 with or without AG490, a specific JAK2 inhibitor. It was demonstrated that IL-6 moderately reduced the MMP-10 mRNA levels, whereas it significantly enhanced the MMP-10 protein mass in the A549 cells. This phenomenon mimicked the divergence of mRNA level and protein mass of MMP-10 in human NSCLC. Moreover, the present study indicated that IL-6 regulation of MMP-10 expression was via the JAK2/STAT3 pathway. STAT3 mRNA levels were significantly increased when the cells were treated with IL-6, whereas when AG490 (50 μM) was added to the cell cultures, IL-6-induced increase of STAT3 mRNA levels was abolished. Meanwhile, AG490 blocked the IL-6-induced inhibition of MMP-10 mRNA as well as blocking the IL-6-induced increase of MMP-10 protein mass in the A549 cells. Neither IL-6 nor AG490 influenced JAK2 mRNA levels in the A549 cell cultures. It is concluded that the JAK2/STAT3 pathway is involved in the IL-6-mediated regulation of MMP-10, and IL-6 can moderately reduce MMP-10 mRNA levels and strongly increase MMP-10 protein mass in human lung adenocarcinoma A549 cells. Contrasting effects of IL-6 on MMP-10 mRNA level and protein concentration in A549 cells may partially explain the divergence of MMP-10 mRNA level and protein mass in human NSCLC.*

Correspondence to: Xiaoying Zhang, Comprehensive Laboratory, The Third Affiliated Hospital of Suzhou University, Jiangsu Changzhou 213003, P.R. China. Tel: +86 51986181278, Fax: +86 51986621235, e-mail: Xiaoyingzhang6689996@msn.com / Ning Xu, MD, PhD., Section of Clinical Chemistry and Pharmacology, Institute of Laboratory Medicine, Lund University, S-221 85 Lund, Sweden. Tel: +46 46173462, Fax: +46 46130064, e-mail: ning.xu@med.lu.se

Key Words: MMP-10, IL-6, JAK2/STAT3, lung cancer.

It is well known that malignant tumor cells have the ability to degrade both extracelluar matrix (ECM) and basement membranes, promote an angiogenic response, evade immune surveillance and avoid elimination during their growth, spread and metastasis (1). Many proteolytic enzymes have been demonstrated to be involved in the aforementioned processes, of which the most important are matrix metalloproteinases (MMPs) (2-4). The MMP family contains at least 24 zinc-dependent endopeptidases and has a central role in tumor invasion, metastasis, angiogenesis and malignant cell proliferation (2, 5). According to their substrate specificities and structures, MMPs can be classified into five different subgroups, *i.e.* collagenases, gelatinases, stromelysins, the membrane-type MMPs and other MMPs. MMP-10, also known as stromelysin-2, is one of the well-characterized members of the MMP family, and has relatively broad substrate specificity compared to other MMPs. It has been demonstrated that MMP-10 was overexpressed in several human tumors of epithelial origin, including gastric cancer (6), bladder transitional cell carcinoma (7), esophageal carcinoma (8), skin carcinoma (9) and non-small cell lung cancer (NSCLC) (10), which suggested that MMP-10 may play an important role in the development and progression of malignant tumors.

Interleukin-6 (IL-6), a pleiotropic cytokine with a wide range of biological activities including immune regulation, hematopoiesis, inflammation and oncogenesis can be produced by various types of human normal and transformed

Table I. *Sequences of primers and probes of GAPDH, JAK2, STAT3 and MMP-10.*

Genes	Sequence (5'→3')
GAPDH Sense primer	GGAAGGTGAAGGTCGGAGTC
GAPDH Anti-sense primer	CGTTCTCAGCCTTGACGGT
GAPDH Probe	FAM-TTTGGTCGTATTGGGCGCCTG-TAMRA
JAK2 Sense primer	GCCTTCTTTCAGAGCCATCAT
JAK2 Anti-sense primer	GTGTAGGATCCCGGTCTTCAA
JAK2 Probe	FAM-AGGATAGGTGCCCTGGGGTTTTCTG-TAMRA
STAT3 Sense primer	CCTGCAAGAGTCGAATGTTCTC
STAT3 Anti-sense primer	TATCAGCACAATCTACGAAGAATCAAGCAGT
STAT3 Probe	FAM-GCCTTCTTTCAGAGCCATCAT-TAMRA
MMP-10 Sense primer	TGTACCCACTCTACAACTCATTCACA
MMP-10 Anti-sense primer	TGAATGCCATTCACATCATCTTG
MMP-10 Probe	FAM-AGCTCGCCCAGTTCCGCCTTTC-TAMRA

tumor cells. It has been demonstrated that IL-6 may be involved in the regulation of the immune response, acute phase reaction and cell differentiation as well as cell proliferation (11, 12). The binding of IL-6 to the IL-6 receptor activates Janus kinases (JAKs) and then signal transducers and activators of transcription (STATs) after tyrosine phosphorylation (13). Subsequently, activated STATs form dimers and translocate to the nucleus where they bind to specific promoter elements of target genes and further regulate their transcription. An increasing number of studies have indicated that STATs activation, particularly STAT3, may be involved in transformation and tumor progression (14, 15). Constitutive activation of STAT3 has been detected in many cancer tissues (14-17). Oncostatin M-induced MMP gene expression in chondrocytes requires the JAK/STAT signaling pathway (18). STAT3 activation plays a decisive role in *MMP-1* and *MMP-10* induction by epidermal growth factor in T24 bladder cancer cells (16).

Our previous studies demonstrated that the *MMP-10* mRNA level in tumor tissues was lower than in adjacent normal lung tissues in NSCLC, however MMP-10 protein levels in the tumor tissues were obviously higher than the adjacent normal lung tissues (10). The detailed mechanism concerning this divergence of mRNA level and protein level is still unknown. The aim of the present study was to analyze the role of the JAK2/STAT3 signaling pathway in IL-6-mediated MMP-10 expression in a human lung adenocarcinoma cell line and to explore the mechanism of MMP-10 expression.

Materials and Methods

Cell line and reagents. The human lung adenocarcinoma cell line, A549, was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (SIBS, CAS, Shanghai, China). Bovine serum albumin (BSA), fetal calf serum (FCS) and RPMI-1640 culture medium were purchased from the Gibco Corporation (Carlsbad, CA, USA). IL-6 was obtained from PeproTech Inc (Rocky Hill, New jersey, USA) and AG490, a specific JAK2 inhibitor from the Alexis Corporation (Plymouth Meeting, PA, USA). The total RNA purification kit was purchased from the Shenergy Biocolor BioScience and Technology Company, Shanghai, China. The first strand cDNA synthesis kit was obtained from Fermantas, Vilnius, Lithuania. The primers and TaqMan probes of glyceraldehyde-3 3-phosphate dehydrogenase (GAPDH), JAK2, STAT3 and MMP-10 were synthesized by the Sangon Biological Engineer Technology and Services Limited Corporation, Shanghai, China. The whole-cell extraction kit and mouse anti human MMP-10 monoclonal antibody was obtained from Chemicon International Inc, (Billerica, MA, USA). The ECL chemiluminescence kit, mouse anti-human GAPDH and peroxidase conjugated goat anti-mouse IgG were purchased from the MultiSciences Biotech Co, Ltd, Hangzhou, China. The LightCycler real-time RT-PCR System was from Roche Applied Science, Mannheim, Germany. Electroblotting apparatus, gel imaging system and Quantity One software v4.6.2 were obtained from Bio-Rad, Hercules, CA, USA. Polyvinylidene difluoride (PVDF) membranes were obtained from the Millipore Company, Billerica, MA, USA.

Cell cultures. The A549 cells were cultured in RPMI-1640 with 10% FCS in the presence of benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) under standard culture conditions (5% CO_2, 37˚C). The cells were seeded into 6-well cell culture clusters and allowed to grow to 50-70% confluence. Prior to the experiments, the cells were washed twice with phosphate-buffered saline (PBS) and once with serum-free RPMI-1640. The experimental medium RPMI-1640 with 10% FCS and different concentrations of IL-6 (0, 12.5, 25 or 50 ng/ml) with or without AG490 (50 μM) was added. The cells were cultured at 37˚C for 24 h.

Total RNA extraction and RT-PCR. The total RNA was extracted using the total RNA purification kit according to the manufacturer's instructions. The purity and concentration of the total RNA were determined by spectrophotometer. Only samples with optical density (OD) 260/280 ratios from 1.80 to 2.00 were chosen. Six μl total RNA were reverse transcribed into cDNA by using the First strand cDNA synthetic kit according to the manufacturer's instructions. The synthetic cDNA were preserved at –30˚C. The primers and TaqMan probes of *GAPDH, JAK2, STAT3* and *MMP-10* were designed

according to the National Center for Biotechnology Information (NCBI) database (NM_002046, NM_004972, NM_003150 and NM_002425, respectively) by using Primer Primier 5.0 software (Palo Alto, CA, USA) (Table I). All the PCRs were performed on a LightCycler in a final volume of 25 μl. Optimum reaction conditions were obtained with 2.5 μl of 10×PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM 4×dNTPs, 0.25 μl of 5 U/μl common Taq DNA polymerase, 0.1 μl of 100 μM specific sense primer(s), 0.1 μl of 100 μM specific antisense primer(s), 0.1 μl of 100 μM specific probe(s) and 2 μl template cDNA. Finally, 17.95 μl ddH_2O was added to the reaction mixture. The cycling conditions for *GAPDH, JAK2, STAT3* and *MMP-10* were as follows: initial denaturation at 95˚C for 3 min, followed by 40 cycles at 95˚C for 15 s, and 60˚C for 45 s, collecting the fluorescence signal at 60˚C. From each amplification plot, a threshold cycle (Ct) value was calculated, then according to the 2(-Delta Delta Ct) method, the relative mRNA levels of *JAK2, STAT3* and *MMP-10* were calculated (19).

Western blotting analysis. After being plated at the appropriate seeding density, the cells were incubated with IL-6 (25 ng/ml) in the presence or absence of AG490 (50 μM) for 24 h, the total intracellular proteins were extracted and the total protein concentrations were determined according to the manufacturer's instruction for the whole cell extraction. For immunoblotting, cell lysates containing 30 μg of total protein were applied and separated on the SDS-PAGE. The proteins were transferred to PVDF membranes using an electroblotting apparatus. The membranes were blocked in blocking solution BSA/tris buffered saline (TBS)-T (TBS containing 4% Tween 20 and 3% BSA) overnight at 4˚C. The blots were incubated with primary antibody (mouse anti-human MMP-10) for 2 h at room temperature and washed three times with TBS-T. The blots were then incubated with secondary antibody (peroxidase-conjugated goat anti-mouse IgG) for 2 h at room temperature, washed three times with TBS-T, and then developed using a ECL chemiluminescence kit and exposed to the BioMax Film (Kodak, Rochester, NY, USA). The same blot was stripped and reprobed for GAPDH.

Statistical analyses. Statistical analysis was performed with Graphpad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). The results are expressed as means±SE. Two comparisons were analyzed by Student's *t*-test. Multiple comparisons were analyzed by the one-way ANOVA/Tukey. Significance was established at a *p*-value less than 0.05.

Results

Influence of IL-6 on the regulation of JAK2, STAT3 and MMP-10. Basal mRNA levels of *JAK2, STAT3* and *MMP-10* were determined in cells incubated without IL-6. With increased concentrations of IL-6, the *JAK2* mRNA levels remained almost unvarying (Figure 1), whereas *STAT3* mRNA levels significantly increased with 25 ng/ml IL-6 (by about 44%, $p<0.05$) (Figure 1). Moreover, *MMP-10* mRNA levels moderately decreased when the cells were incubated with IL-6 (Figure 1), however MMP-10 protein mass significantly increased with the peak point at 25 ng/ml IL-6 ($p<0.01$) (Figure 2 A and B).

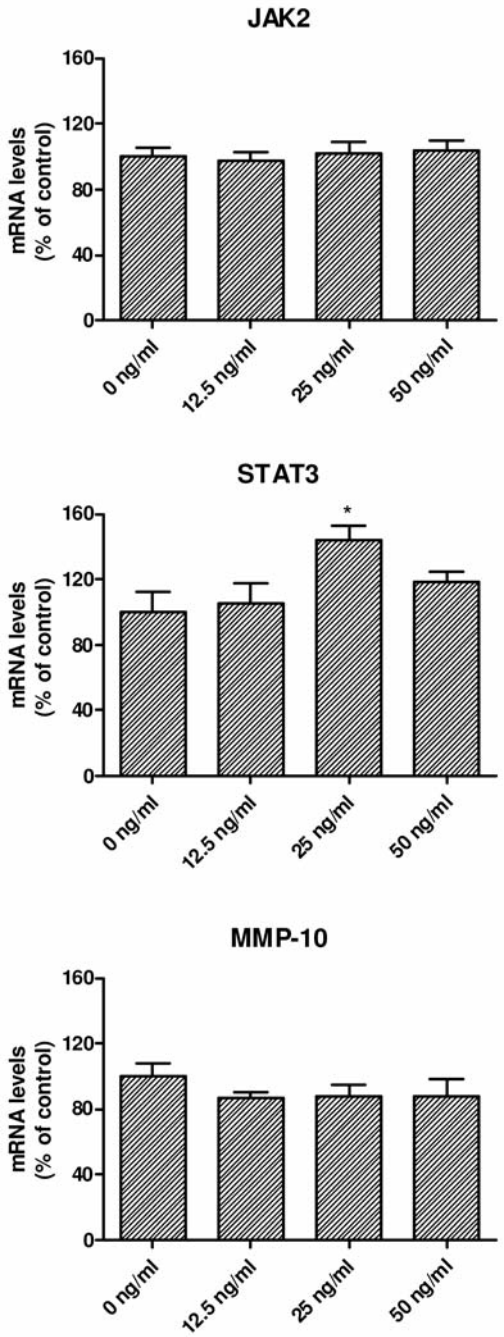

Figure 1. *Effects of IL-6 on the mRNA levels of JAK2, STAT3 and MMP-10. The mRNA levels of JAK2, STAT3 and MMP-10 were determined by real-time RT-PCR analysis after 24 h culture with or without IL-6. Mean±SE (n=6). Control group (0 ng/ml IL-6) is represented as 100%. *p<0.05 vs. 0 ng/ml group.*

Effect of JAK2 inhibitor, AG490, on the IL-6 mediated regulation of STAT3 and MMP-10. The cells were cultured with IL-6 (25 ng/ml) in the presence or absence of AG490 (50 μM) for 24 h, and cells cultured without IL-6 or AG490

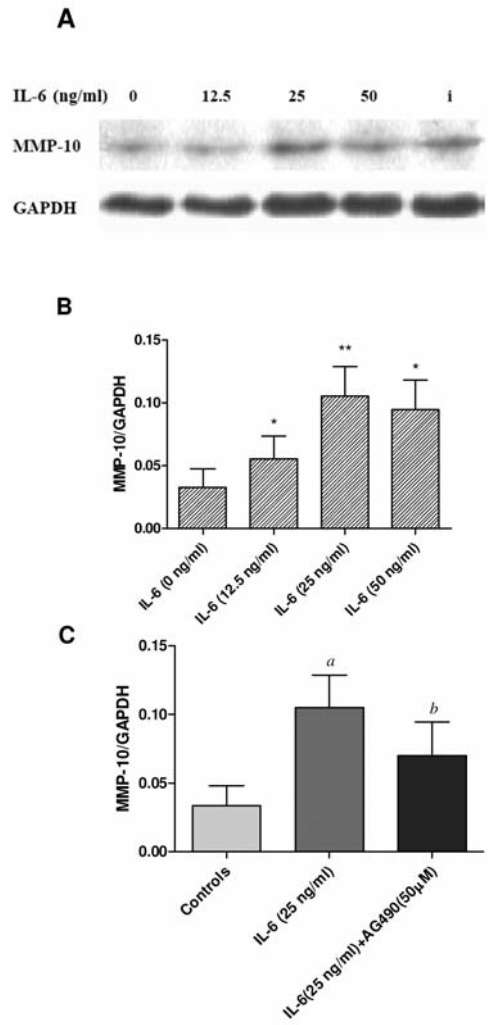

Figure 2. *Effect of IL-6 and AG490 on MMP-10 protein levels. MMP-10 protein mass determined by Western blotting after 24 h culture. Panel A: representative Western blot. i: Cells cultured with 25 ng/ml IL-6 and 50 μM AG490. Panel B: *p<0.05 and **p<0.01 vs. 0 ng/ml group. Panel C: control, no IL-6 or AG490; [a]p<0.01 vs. controls; [b]p<0.05 vs. IL-6 (25 ng/ml). Mean±SE (n=6).*

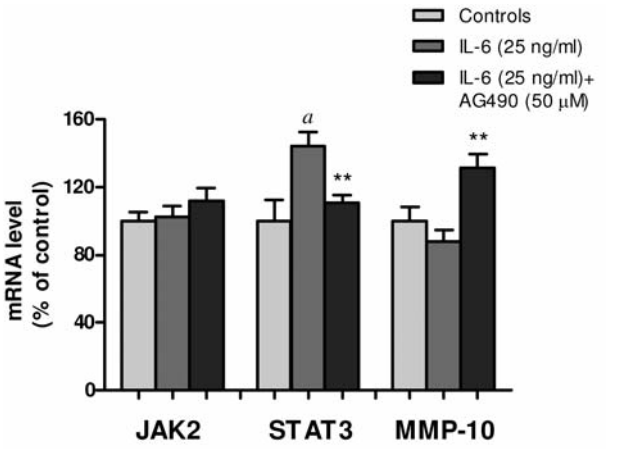

Figure 3. *Effects of JAK2 inhibitor, AG490, on mRNA levels of JAK2, STAT3 and MMP-10. The mRNA levels of JAK2, STAT3 and MMP-10 were determined after 24 h culture by real-time RT-PCR analysis. Mean±SE (n=6). Control group, no IL-6 or AG490, is represented as 100%. [a]p<0.05 vs. controls; **p<0.01 vs. 25 ng/ml IL-6 group.*

were used as controls. As shown in Figure 3, *JAK2* mRNA levels were unchanged, whereas AG490 blocked the IL-6 induced increase of *STAT3* mRNA levels ($p<0.01$) (Figure 3). Moreover, the *MMP-10* mRNA levels in the A549 cells treated with 50 μM AG490 together with 25 ng/ml IL-6 were significantly higher than in the cells cultured with 25 ng/ml IL-6 alone ($p<0.01$) (Figure 3), which indicated that AG490 inhibited IL-6-mediated down-regulation of MMP-10 mRNA. Moreover as shown in Figure 2C, AG490 significantly inhibited the IL-6-mediated increase of MMP-10 protein mass in the A549 cell cultures.

Discussion

Recently, it has been reported that the JAK/STAT signaling pathway participates in cell proliferation, differentiation, apoptosis and plays an integral role in intracellular signaling *via* cytokine receptors (20, 21). In the present study, IL-6 moderately reduced the *MMP-10* mRNA levels and significantly increased the MMP-10 protein levels in the human lung cancer A549 cells, which mimicked the divergence of mRNA levels and protein mass of MMP-10 in human NSCLC. Moreover this IL-6-mediated inhibition of *MMP-10* mRNA levels and increase of MMP-10 protein concentration was significantly blocked by the addition of a specific JAK2 inhibitor, AG490, which may suggest that IL-6-mediated regulation of MMP-10 occurs *via* a JAK2/STAT3 signaling pathway, mainly *via* STAT3 because when the A549 cells were stimulated by IL-6 for 24 h, the *JAK2* mRNA levels remained unchanged, but the *STAT3* mRNA levels were significantly increased.

The present study further confirmed our previous finding that the divergence of *MMP-10* mRNA levels and protein mass occurred in human NSCLC tissues. Contrasting effects of IL-6 on *MMP-10* mRNA level and protein concentration in A549 cells may partially explain this divergence. First, STAT3 may function as an activator of gene transcription, it may also inhibit the transcription of some other genes. It has been demonstrated that activated STAT3 inhibited *p53* transcription (22). In the present study, IL-6 up-regulated the *STAT3* mRNA levels, which could potentially provide more substrates for activated JAK. As a result, more STAT3 could be activated, translocated to the nucleus and bound to DNA

functional elements, inhibiting the transcription of *MMP-10* genes. Meanwhile activated STAT3 may increase the expression of genes associated with translation and therefore promote translation of *MMP-10* mRNA. Second, the half-life of mRNA is usually much shorter than protein. The *MMP-10* mRNA may be degraded by RNAase quickly.

These studies demonstrated that IL-6 regulated MMP-10 protein mass in a dose-dependent manner, whereas there was no such phenomenon observed on IL-6-mediated inhibition of *MMP-10* mRNA levels.

References

1 Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*: 161-174, 2002.

2 Gill JH, Kirwan IG, Seargent JM, Martin SW, Tijani S, Anikin VA, Mearns AJ, Bibby MC, Anthoney A and Loadman PM: MMP-10 is overexpressed, proteolytically active, and a potential target for therapeutic intervention in human lung carcinomas. Neoplasia *6*: 777-785, 2004.

3 Duffy MJ and McCarthy K: Matrix metalloproteinases in cancer: prognostic markers and targets for therapy (review). Int J Oncol *12*: 1343-1348, 1998.

4 Puente XS, Sanchez LM, Overall CM and Lopez-Otin C: Human and mouse proteases: a comparative genomic approach. Nat Rev Genet *4*: 544-558, 2003.

5 Miyata Y, Iwata T, Maruta S, Kanda S, Nishikido M, Koga S and Kanetake H: Expression of matrix metalloproteinase-10 in renal cell carcinoma and its prognostic role. Eur Urol *52*: 791-797, 2007.

6 Aung PP, Oue N, Mitani Y, Mitani Y, Nakayama H, Yoshida K, Koguchi T, Bosserhoff AK and Yasui W: Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene *25*: 2546-2557, 2006.

7 Seargent JM, Loadman PM, Martin SW, Naylor B, Bibby MC and Gill JH: Expression of matrix metalloproteinase-10 in human bladder transitional cell carcinoma. Urology *65*: 815-820, 2005.

8 Mathew R, Khanna R, Kumar R, Mathur M, Shukla NK and Ralhan R: Stromelysin-2 overexpression in human esophageal squamous cell carcinoma: potential clinical implications. Cancer Detect Prev *26*: 222-228, 2002.

9 Kerkela E, Ala-aho R, Lohi J, Grenman R, M. Kahari V and Saarialho-Kere U: Differential patterns of stromelysin-2 (MMP-10) and MT1-MMP (MMP-14) expression in epithelial skin cancers. Br J Cancer *84*: 659-669, 2001.

10 Zhang X, Zhu S, Luo G, Zheng L, Wei J, Zhu J, Mu Q and Xu N: Expression of MMP-10 in lung cancer. Anticancer Res *27*: 2791-2795, 2007.

11 Chang KT, Tsai CM, Chiou YC, Chiu CH, Jeng KS and Huang CY: IL-6 induces neuroendocrine dedifferentiation and cell proliferation in non-small cell lung cancer cells. Am J Physiol Lung Cell Mol Physiol *289*: L446-453, 2005.

12 Schafer ZT and Brugge JS: IL-6 involvement in epithelial cancers. J Clin Invest *117*: 3660-3663, 2007.

13 Heinrich PC, Behrmann I, Muller-Newen G, Schaper F and Graeve L: Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J *334(Pt 2)*: 297-314, 1998.

14 Leong PL, Andrews GA, Johnson DE, Dyer KF, Xi S, Mai JC, Robbin PD, Gadiparthi S, Burke NA, Watkins SF and Grandis JR: Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci USA. *100*: 4138-4143, 2003.

15 Mejias-Luque R, Peiro S, Vincent A, Van Seuningen I and de Bolos C: IL-6 induces MUC4 expression through gp130/STAT3 pathway in gastric cancer cell lines. Biochim Biophys Acta *1783*: 1728-1736, 2008.

16 Itoh M, Murata T, Suzuki T, Shindoh M, Nakajima K, Imai K and Yoshida K: Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. Oncogene *25*: 1195-1204, 2006.

17 Gong A, He M, Krishna Vanaja D, Yin P, Karnes RJ and Young CY: Phenethyl isothiocyanate inhibits STAT3 activation in prostate cancer cells. Mol Nutr Food Res *53*: 878-886, 2009.

18 Li WQ, Dehnade F and Zafarullah M: Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway. J Immunol *166*: 3491-3498, 2001.

19 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods *25*: 402-408, 2001.

20 Won HH, Park I, Lee E, Kim JW and Lee D: Comparative analysis of the JAK/STAT signaling through erythropoietin receptor and thrombopoietin receptor using a systems approach. BMC Bioinformatics *10(Suppl 1)*: S53, 2009.

21 Zhang X and Darnell JE Jr: Functional importance of Stat3 tetramerization in activation of the alpha 2-macroglobulin gene. J Biol Chem *276*: 33576-33581, 2001.

22 Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R, Briggs J, Karras J, Cress WD, Pardoll D, Jove R, Chen J and Yu H: Role of Stat3 in regulating p53 expression and function. Mol Cell Biol *25*: 7432-7440, 2005.

Received July 1, 2009
Revised September 29, 2009
Accepted October 6, 2009