# Two serine residues of non-metastasis protein 23-H1 are critical in inhibiting signal transducer and activator of transcription 3 activity in human lung cancer cells

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**Abstract.** Constitutive activation of signal transducer and activator of transcription 3 (STAT3) in numerous cancers, including lung cancer, is one of the major mechanisms of tumor progression and metastasis. The authors previously reported that the metastasis suppressor non-metastasis protein 23-H1 (Nm23-H1) negatively regulates STAT3 activity by inhibiting its phosphorylation on Tyr705. Nm23-H1 is a multifunction protein that has three different kinase activities. By transfecting the five mutants that inactivated three different kinase activities respectively into Nm23-H1 deficient lung cancer cell lines, it was identified that Nm23-H1$^{S44A}$ (Ser44 to Ala) and Nm23-H1$^{S120G}$ (Ser120 to Gly) mutant forms were unable to suppress STAT3 phosphorylation on Tyr705, resulting in increased expression of fibronectin and matrix metalloproteinase-9. Notably, protein inhibitor of activated STAT3 was also involved in Nm23-H1$^{S44A}$- and Nm23-H1$^{S120G}$-mediated suppression of STAT3 phosphorylation. The present results indicated that Ser44 and Ser120 sites of Nm23-H1 may be responsible for its biological suppressive effects of STAT3 and tumor metastasis, which may contribute to illuminate the metastasis suppression function of Nm23-H1 in lung cancer.

## Introduction

Lung cancer is the leading cause of cancer-associated mortality throughout the world (1). The most common causes for mortality of patients with lung cancer are treatment failure and metastasis (1). Therefore, understanding the molecular mechanisms that contribute to tumor metastasis, and developing strategies for targeting tumor invasion and metastasis may have important clinical and social significance.

Aberrant signal transducer and activator of transcription-3 (STAT3) signaling promotes initiation and progression of human cancer. A previous study demonstrated that constitutive activation of STAT3 in numerous cancers, including lung cancer, is one of the major mechanisms of tumor progression and metastasis (2). STAT3 binds to the promoter of the matrix metalloproteinase (MMP)-9 and MMP-2 genes, leading to their transcriptional activation and expression, thus resulting in the degradation and remodeling of the extracellular matrix (3,4). In human breast cancer, activation of STAT3 by interleukin-6 (IL-6) was also revealed to induce Twist expression (5), which may lead to epithelial-mesenchymal transition (EMT). Constitutively activated STAT3 in tumors is sufficient to increase vascular endothelial growth factor (VEGF) expression and induce angiogenesis *in vivo* (6). VEGF, hypoxia-inducible factor-1α and hepatocyte growth factor are prominent transcriptional targets for STAT3 (7-9). STAT3 may be activated by cytokines, growth factors and oncogenes (10,11). Phosphorylation of Tyr705 at the C-terminal domain of STAT3 activates STAT3. In normal cells, STAT3 is actsivated transiently, as it is tightly controlled by several negative protein modulators, including the family of suppressor of cytokine signaling proteins 1-7,

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the protein inhibitors of activated STATS (PIAS) and several protein tyrosine phosphatases (12-14). Therefore, the constitutive STAT3 activity in metastatic tumors may be attributed to a loss-of-function or reduction of expression of inhibitory protein during cancer progression, and tumor metastasis suppressors may also serve a role in regulating STAT3 activity.

A previous study was focused on the role of the tumor metastasis suppressor Nm23-H1 in the regulation of STAT3 activity (15). Nm23-H1 was the first metastasis suppressor identified in a mouse tumor model (16). Reduction or loss of Nm23-H1 expression is associated with tumor progression and metastasis (17). Nm23-H1 is a multifunction protein, with three enzyme activities *in vitro*: Nucleoside diphosphate kinase (NDPK); histidine kinase; and 3'-5' exonuclease activity. A previous study has suggested that histidine kinase activity and Ser44 phosphorylation are associated with the suppression of tumor metastasis by Nm23-H1 (18). Based on the aforementioned studies, the present study successfully generated five different recombinant plasmids of Nm23-H1 that inactivate different kinase activities, using the short-hairpin RNA (shRNA)-resistant Nm23-H1 expression vector as a template (19). The present study investigated the role of critical Ser residues of Nm23-H1 in suppression of STAT3 activity.

## Materials and methods

*Cell culture and transfection.* The human lung adenocarcinoma A549 cell line was obtained from American Type Culture Collection (Manassas, VA, USA), cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). A549/Nm23-H1-shRNA cells, stably expressing Nm23-H1-specific shRNA, were obtained and characterized in our laboratory (The Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Center and Institute, Tianjin Medical University General Hospital, Tianjin, China) by transducing A549 cells with the lentiviral construct containing the Nm23-H1 shRNA sequence. The cells were grown in 6-well plates and transfected with PolyJet™ *in vitro* DNA transfection reagent (SignaGen Laboratories, Rockville, MD, USA) for 15 min at room temperature in 1 ml of medium, according to the manufacturer's protocol.

*Plasmid construction.* Site-directed mutagenesis of the Nm23-H1 gene was performed by the overlap extension polymerase chain reaction (PCR) method (Primers sequences in Tables I and II; BGI, Schenzhen, China). All PCR reactions all contained 3 components; PCR1 contained pcDNA3.1 (+)-resistant-shRNA-nm23-H1 as template, forward and reverse primers for amplification of mutant DNA and the upstream DNA PCR product named P1; PCR2, forward and reverse primers for amplification of mutant DNA and the downstream DNA PCR product named P2; PCR3, use P1 and P2 as template, forward and reverse primers for the 3rd PCR and the final products were obtained from joining P1 and P2. Thermocycling conditions: 94˚C for 2 min for pre-degeneration; 94˚C for 30 sec for degeneration; 60˚C 30 sec for annealing; 72˚C 45 sec for extending for a total

Table I. Primers targeting with side of the mutation region.

| Primer name | Primer sequence, 5'-3' |
|---|---|
| Forward | (GC)<u>GGATCC</u>ATGGCCAACTGTGAGCGAA |
| Reverse | (CG)<u>TCTAGA</u>TCATTCATAGATCCAGTTCTGA |

The bases in brackets are protective bases and the bases that are underlined present the enzyme loci.

Table II. Primer for the insertion of the intended mutations.

| Mutation, primer name | Primer sequence (5'-3') |
|---|---|
| Ser44 | |
| Fm44 | GAAATTCATGCAAGCT<u>G</u>(T)CCGAAGATCTTCTCAAGG |
| Rm44 | CCTTGAGAAGATCTTCGG<u>C</u>(A)AGCTTGCATGAATTTC |
| Pro96 | |
| Fm96 | CGGGGAGACCAAC<u>T</u>(C)CTGCAGACTCCAAGC |
| Rm96 | GCTTGGAGTCTGCAG<u>A</u>(G)GTTGGTCTCCCCG |
| His118 | |
| Fm118 | CAAGTTGGCAGGAACATTATAT<u>T</u>(CA)TGGCAGTGATTCTGTGGAGA |
| Rm118 | TCTCCACAGAATCACTGCCA<u>AA</u>(TG)TATAATGTTCCTGCCAACTTG |
| Ser120 | |
| Fm120 | GGAACATTATACATGGC<u>G</u>(A)GTGATTCTGTGGAGAGT |
| Rm120 | ACTCTCCACAGAATCAC<u>C</u>(T)GCCATGTATAATGTTCC |

The bases in brackets are the unchanged bases next to the mutated bases and bases that are underlined are the mutated bases. Fm, forward mutation primer; Rm, reverse mutation primer.

of 30 cycles. After the last cycle, a 72˚C for 8 min step was used for extension, and 4˚C for termination). Pure plasmid containing Nm23-H1 gene (shRNA-resistant) was prepared. The desired five mutations were constructed and cloned into the eukaryotic pcDNA3.1Hygro(+) vector, consisting of Nm23-H1$^{S44A}$ (Ser44 TCC mutates to Ala GCC), Nm23-H1$^{P96S}$ (Pro96 CCT mutates to Ser TCT), Nm23-H1$^{H118F}$ (His118 CAT mutates to Phe TTT), Nm23-H1$^{S120G}$ (Ser120 AGT mutates to Gly GGT) and Nm23-H1$^{P96S-S120G}$ (P96S combination mutation with S120G). These five recombinant plasmids maintain the integrity of Nm23-H1 protein, but change the activity of kinases. The results of DNA sequencing confirmed that the base sequences of the genes were completely concordant with the experimental design. A549/nm23-H1-shRNA cells were

transfected with these five mutants, and the expression of the mutant proteins was determined by western blot analysis, as previously described (19).

*Small interfering RNA (siRNA)*. Nm23-H1-specific siRNA (sense, 5'-GGAACACUACGUUGACCUGtt-3' and antisense, 5'-CAGGUCAACGUAGUUCCtt-3') was used to knockdown the expression of Nm23-H1. Scrambled siRNA was used for control experiments. All siRNAs were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were transfected with 10 nM of specific or control siRNA using 1 $\mu$l GenMute siRNA & DNA transfection reagent (SignaGen Laboratories). After 24 h, cells were treated with 1 or 10 ng/ml IL-6 (Roche Diagnostics, Indianapolis, IN, USA).

*Western blot analysis*. Western blot analysis was performed as previously described (20). Specific antibodies against Nm23-H1 (#sc-514515; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p-STAT3Tyr705 (#9145), STAT3 (#9139), MMP-9 (#13667), Twist1 (#46702) (Cell Signaling Technology, Inc., Danvers, MA, USA), E-cadherin (#33-4000; Invitrogen; Thermo Fisher Scientific, Inc.), fibronectin (#F7387) and $\beta$-actin (#A5441; Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) were used for western blot analysis, all diluted to 1:1,000.

*Wound healing assay*. The cells were seeded at a density of $4 \times 10^4$ onto 6-well plates and cultured to 40-60% confluence. Cells were transfected with Nm23-H1 siRNA, and after 24 h, the confluent cell monolayer was scratched with a pipette tip to produce a straight line. The detached cells were washed with PBS two times. The cells were then cultured in serum-free RPMI-1640 medium and treated with IL-6 (1,10 ng/ml) for an additional 48 h at 37°C. The open gap was then inspected and images were captured at a magnification of x200, as shown in Fig. 1 (Nikon TE 2000-E; Nikon Corporation, Tokyo, Japan). Activity of cell migration was calculated as the number of cells entering into the rectangle. Experiments were repeated three times.

*In vitro cell invasion assay*. To evaluate the invasive activity of tumor cells, a cell invasion assay kit (Millicell Standing Cell Culture 24-well PCF 8.0 $\mu$m, #PI8P01250; EMD Millipore) was used according to the manufacturer's protocol. Briefly, $0.5 \times 10^6$ cells were transfected with Nm23-H1 siRNA or control siRNA (5 pmol) for 24 h using GenMute siRNA and DNA transfection reagent (SignaGen Laboratories) as aforementioned, and were harvested with 0.25% Trypsin/EDTA and suspended with serum-free medium to a final concentration of $0.5 \times 10^6$ cells/ml. A total of 300 ml of suspension was added to the lower chamber interior of the inserts, which contained 500 ml complete culture medium with 10% FBS. IL-6 was added to the upper chamber with the concentration of 5 $\mu$g/ml. After 48 h, the invading cells that attached to the bottom of the membrane were counted by capturing images under a microscope (magnification, x4; Nikon TE 2000-E; Nikon Corporation), subsequent to the removal of non-invasive cells and stained with crystal violet. In total 6 fields of view were evaluated. Experiments were repeated a minimum of three times.

## Results

*Nm23-H1 suppresses cell migration and invasion by inhibiting STAT3 phosphorylation on Tyr705 in lung cancer cells*. The authors previously determined that Nm23-H1 negatively regulates STAT3 phosphorylation at Tyr705, but how this Nm23-H1 function affects tumor metastasis remains unclear. To investigate whether Nm23-H1 executes its suppression of tumor metastasis by negative regulation of STAT3, an *in vitro* model of IL-6-induced STAT3 and siRNA targeting of Nm23-H1 to knock down the expression of Nm23-H1 was established. Western blot analysis was performed to detect the expression of metastasis-associated proteins in A549 cells transfected with control siRNA or Nm23-H1 siRNA (Fig. 1A). The results demonstrated that IL-6-induced STAT3$^{Tyr705}$ phosphorylation was increased in Nm23-H1-deficient cells. Notably, the expression of the epithelial cell marker E-cadherin was suppressed by IL-6, which was inhibited in Nm23-H1-deficient cells. By contrast, the expression of fibronectin and MMP-9 was induced by IL-6, and enhanced by Nm23-H1 siRNA transfection.

Wound healing and cell invasion assay in A549 cells were performed to determine cell migration and invasion ability following Nm23-H1 gene knockdown, with or without treatment with IL-6, which is a good activator of STAT3 phosphorylation. The data revealed that IL-6 increased cell migration and invasion (Fig. 1B and C), which were enhanced following Nm23-H1 siRNA transfection. Nm23-H1 expression was then rescued by the transfection and expression of Nm23-H1 cDNA in A549/Nm23-H1-shRNA cells that stably expressed Nm23-H1-specific-shRNA (19). Scratch test data revealed that Nm23-H1 cDNA inhibited wound healing induced by IL-6 (Fig. 1D). These results indicated that promotion of lung cancer cell migration and invasion via IL-6 induced p-STAT3$^{Tyr705}$ may be suppressed by Nm23-H1.

*Nm23-H1 Ser residues are required for the negative regulation of p-STAT$^{Tyr705}$ to suppress tumor metastasis*. Nm23-H1 is a multifunction protein with three enzyme activities, consisting of NDPK, histidine kinase and 3'-5' exonuclease activity. Histidine kinase activity was identified to be associated with the suppression of tumor metastasis (18). Ser44 phosphorylation was reported to be associated with Nm23-H1 suppression of tumor metastasis; Pro96 to Ser and Ser120 to Gly retain the nucleoside-diphosphate kinase (NDPK) activity and lose the histidine-dependent protein phosphotransferase activity; His118 to Phe loses NDPK activity. The authors previously identified (18) that Nm23-H1 suppresses cell migration and invasion by inhibiting STAT3 phosphorylation and activity in lung cancer cells; however, the kinase activity of Nm23-H1 that was involved remains unclear. Based on a previous study (19), five site-directed mutants were successfully constructed, including Nm23-H1$^{S44A}$, Nm23-H1$^{P96S}$, Nm23-H1$^{H118F}$, Nm23-H1$^{S120G}$ and combined Nm23-H1$^{P96S-S120G}$. These mutants inactivated different kinase activities, but maintained the integrity of the Nm23-H1 protein using the shRNA-resistant Nm23-H1 expression vector as a template. As is shown in Fig. 2A, the expression of Nm23-H1 was rescued by transfection of site-directed mutagenesis of Nm23-H1 cDNA into A549/nm23-H1-shRNA cells. To identify which type of mutant was unable to reverse
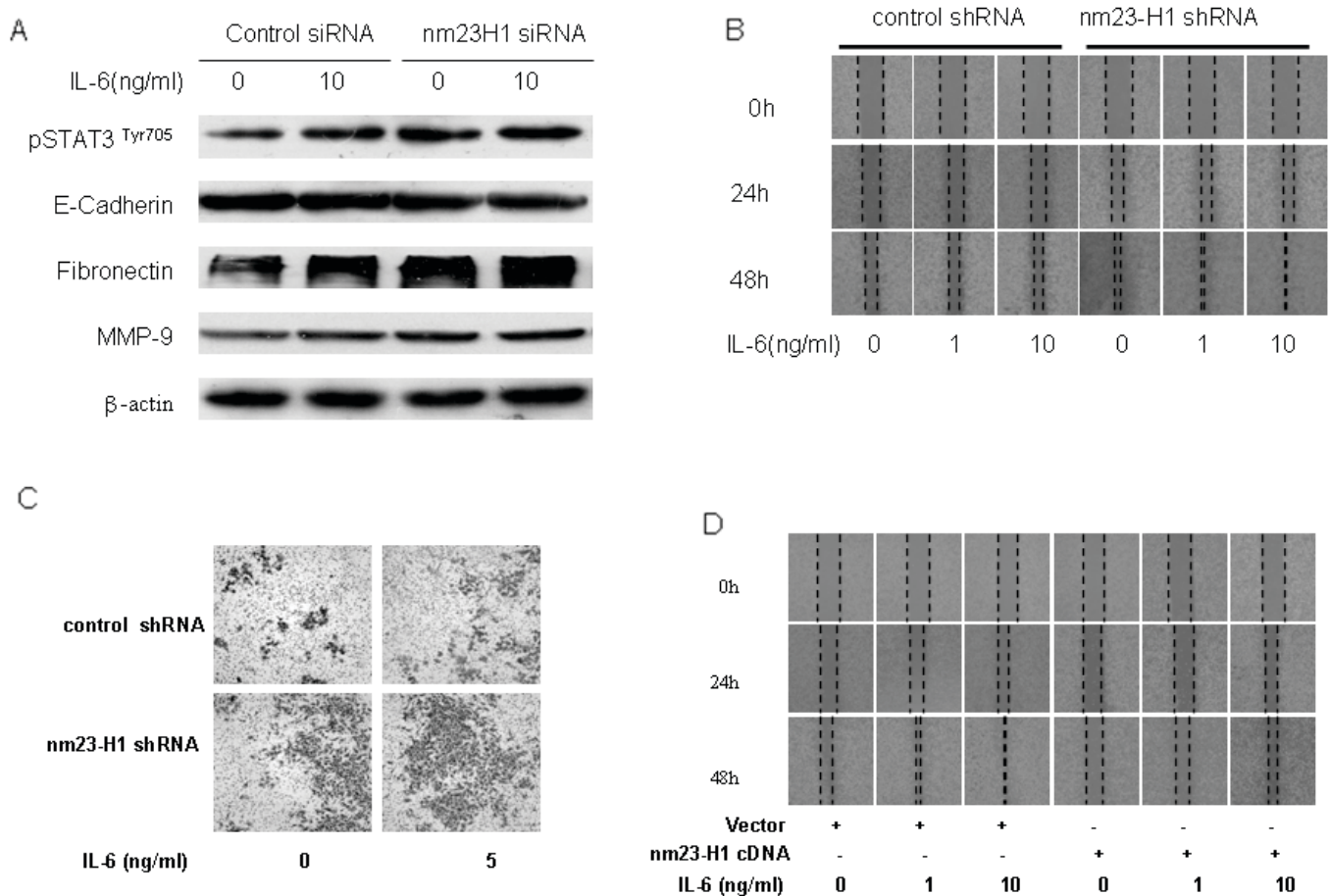
Figure 1. Nm23-H1 suppresses cell migration and invasion by inhibiting STAT3 phosphorylation and activity in lung cancer. (A) A549 cells were seeded onto 6-well plates and transfected with Nm23-H1 specific siRNA, with non-specific as control. After 48 h post-transfection, cells were untreated or treated with IL-6 for an additional 1 h. Western blot analysis for the expression of p-STAT3$^{Tyr705}$, total STAT3, E-cadherin, fibronectin, Twist, MMP-9 and Nm23-H1. β-actin control was included to verify equal protein loading in the lanes. Compared with control siRNA-treated cells, cells with Nm23-H1 deficiency showed significantly increased levels of p-STAT$^{Tyr705}$ and fibronectin induced by IL-6, while E-cadherin levels decreased. (B) Wounds were scratched 24 h after A549 cells were transfected with Nm23-H1 siRNA, and cells were untreated or treated with different concentration of IL-6 for an additional 48 h. The dotted line shows the migratory changes of Nm23-H1 silencing. (C) Images of the invading cells in Boyden chamber were captured under microscopy (magnification, x4) 48 h after the addition of IL-6 to A549 cells transfected with Nm23-H1siRNA. (D) The A549/Nm23-H1-shRNA cells were transfected with Nm23-H1 cDNA to rescue Nm23-H1 expression levels to evaluate the migration ability using a wound healing assay. STAT3, signal transducer and activator of transcription 3; p-STAT3, phospho-STAT3; MMP-9, matrix metalloproteinase-9; IL-6, interleukin-6; siRNA, small interfering RNA.

the inhibition of STAT3 activity induced by Nm23-H1 deficiency, the p-STAT3$^{Tyr705}$ protein levels were examined in A549/nm23-H1-shRNA cells transfected with these site-directed mutant forms of Nm23-H1 cDNA. Western blot analysis data revealed that in Nm23-H1$^{P96S}$, Nm23-H1$^{H118F}$ and combined Nm23-H1$^{P96S-S120G}$-transfected A549/nm23-H1-shRNA cells, the phosphorylation of STAT3$^{Tyr705}$ was suppressed compared with the control group. By contrast, in Nm23-H1$^{S44A}$ and Nm23-H1$^{S120G}$-transfected cells, the phosphorylation of STAT3$^{Tyr705}$ was almost equal to the control, suggesting that these sites (Ser44 and Ser120) are critical to suppress increased STAT3 activity induced by Nm23-H1 deficiency (Fig. 2B).

A549/nm23-H1-shRNA cells with or without IL-6 treatment were then transfected with site-directed mutagenesis of Nm23-H1$^{S44A}$ and Nm23-H1$^{S120G}$, and changes in tumor metastasis ability were determined using a wound healing assay. Western blot analysis for the EMT protein markers E-cadherin and fibronectin, and other metastasis-associated proteins, including MMP-9, showed that Nm23-H1$^{S44A}$ and Nm23-H1$^{S120G}$

were able to reverse the expression of these proteins (Fig. 2C). The wound healing assay results indicated that mutant S44A-transfected cells migrated faster than Nm23-H1 cDNA and mutant S120G-transfected cells (Fig. 2D). These data indicated that Nm23-H1 Ser residues were required for negative regulation of p-STAT3$^{Tyr705}$ to suppress tumor metastasis.

*PIAS3 mediates the negative regulation of p-STAT$^{Tyr705}$ by Nm23-H1.* Suppressor of cytokine signaling 3 (SOCS3) and PIAS3 are known as the important negative regulators in the STAT3 pathway, and their loss of function or reduction may contribute to constitutive STAT3 activity in metastatic tumors. Therefore, SOCS3 siRNA and PIAS3 siRNA were respectively co-transfected with Nm23-H1 cDNA into A549/nm23-H1-shRNA cells. As shown in western blot analysis results, Nm23-H1 cDNA inhibited IL-6 induced p-STAT3$^{Tyr705}$ (Fig. 3A and B). However, cells that were co-transfected with SOCS3 siRNA did not change the phosphorylation levels of STAT3$^{Tyr705}$ suppressed by Nm23-H1 (Fig. 3A). In
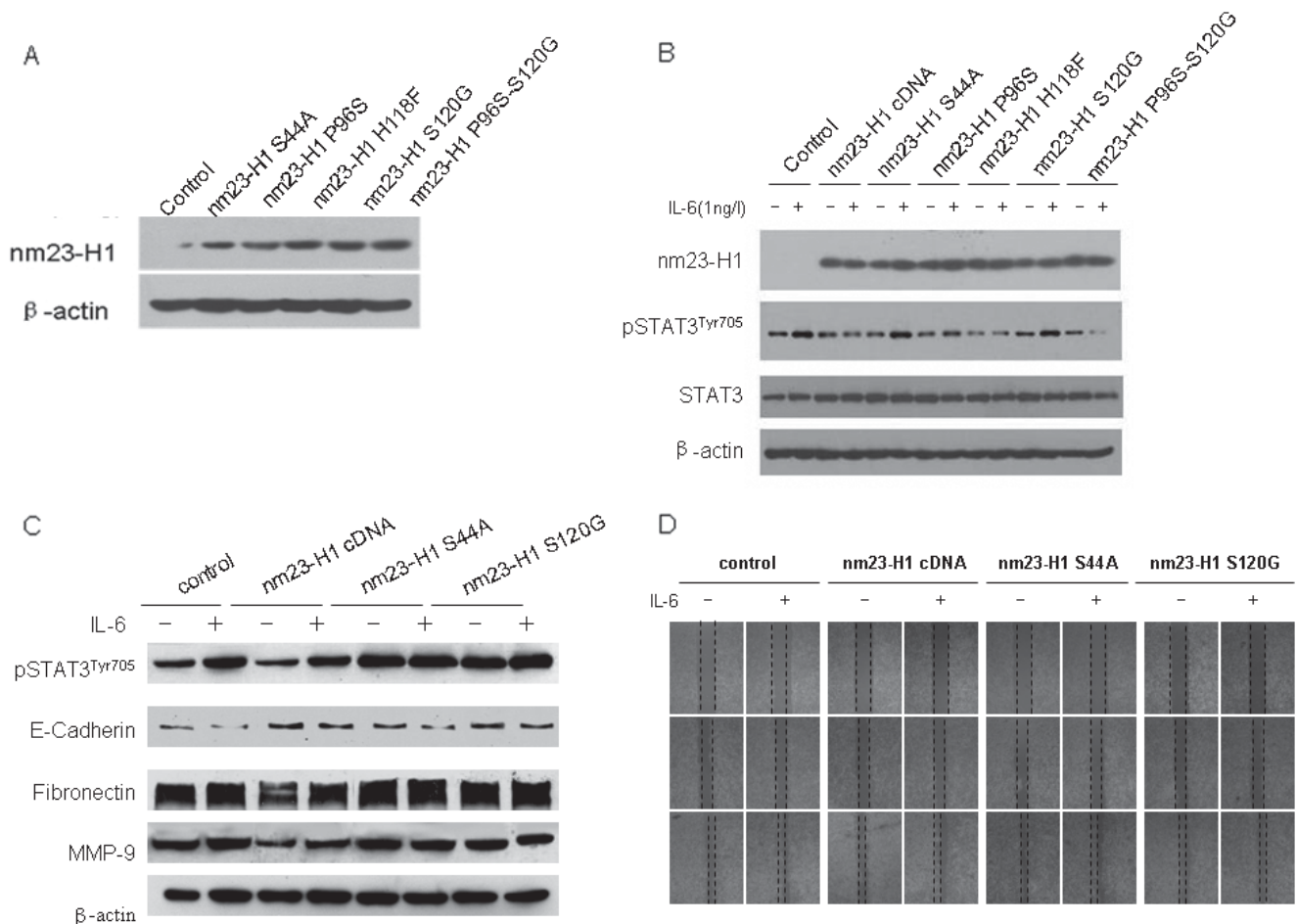
Figure 2. Site-directed mutagenesis of Nm23-H1 cDNA, S44A and S120G were unable to reverse the negative regulation of p-STAT$^{Tyr705}$ by Nm23-H1 cDNA. (A) Nm23-H1 expression was rescued by transfection with a shRNA-resistant Nm23-H1 expression vector generated by site-directed mutagenesis of Nm23-H1 cDNA into A549/Nm23-H1-shRNA cells and Nm23-H1 protein levels were determined using western blot analysis. (B) Western blot analysis of p-STAT3$^{Tyr705}$ levels in cells transfected with site-directed mutagenesis of Nm23-H1 cDNA. In Nm23-H1cDNA P96S, H118F and P96S-S120G-transfected A549/Nm23-H1-shRNA cells, the phosphorylation of STAT3$^{Tyr705}$ was suppressed compared with the control group; by contrast, in Nm23-H1 cDNA, S44A and S120G-transfected cells, the phosphorylation of STAT3$^{Tyr705}$ was almost equal to the control. (C) Western blot analysis for STAT3$^{Tyr705}$ and epithelial-mesenchymal transition protein markers E-cadherin and fibronectin levels in A549/Nm23-H1-shRNA cells transfected with site-directed mutagenesis of Nm23-H1 cDNA, S44A and S120G. (D) Wounds were scratched 24 h after A549/Nm23-H1-shRNA cells were transfected with Nm23-H1 cDNA or Nm23-H1$^{S44A}$ and Nm23-H1$^{S120G}$, and cells were untreated or treated with IL-6 for an additional 48 h. The dotted line shows the migratory changes of Nm23-H1 silencing. STAT3, signal transducer and activator of transcription 3; p-STAT3, phospho-STAT3; IL-6, interleukin-6.

PIAS3 siRNA and Nm23-H1 cDNA co-transfected cells, p-STAT3$^{Tyr705}$ levels increased compared with Nm23H1 cDNA alone (Fig. 3B). These data indicated that PIAS3 was involved in the negative regulation of p-STAT3$^{Tyr705}$ by Nm23-H1.

To identify which type of mutant was involved in this regulation, Nm23-H1$^{S44A}$ and Nm23-H1$^{S120G}$ were co-transfected with PIAS3 siRNA into A549/nm23-H1-shRNA cells. Western blot analysis for phosphorylation levels of STAT3$^{Tyr705}$ and the tumor metastasis-associated proteins showed that in the Nm23-H1$^{S44A}$ and PIAS3 siRNA co-transfected group, the expressions of STAT3$^{Tyr705}$, fibronectin and MMP-9 increased, whilst the expression of E-cadherin was decreased, compared with protein levels in Nm23-H1$^{S120G}$ and PIAS3 siRNA co-transfected group (Fig. 3C and D). Cell invasion analysis also indicated that PIAS3 siRNA alone was able to promote cell invasion ability, which was enhanced by the combined transfection of Nm23-H1$^{S44A}$ with PIAS siRNA (Fig. 3E). Thus, PIAS3 was involved in the inhibition of STAT3 activity by Nm23-H1, which may contribute to the suppression of tumor metastasis.

## Discussion

A previous study in an animal model of human brain metastasis revealed higher levels of STAT3 activity in human brain metastasis tissues compared with primary melanoma tissues, indicating constitutive activation of STAT3 occurs not only in oncogenesis, but also in melanoma brain metastasis (21). A previous study demonstrated that Nm23-H1, which was the first identified metastasis suppressor gene with an inverse association between its expression and metastasis progression (22), serves a key role in regulating STAT3 activity (15). It was identified that IL-6-dependent induction of tyrosine phosphorylation and activity of STAT3 was significantly decreased by Nm23-H1 (15). In the present study, the role of Nm23-H1 kinase activity and critical serine residues in metastasis was investigated by its inhibition of IL-6 inducing STAT3$^{Tyr705}$ phosphorylation and activity. The present study demonstrated that Ser44 and Ser120 residues of Nm23-H1 are responsible for this negative regulation of STAT3 activity to suppress cell
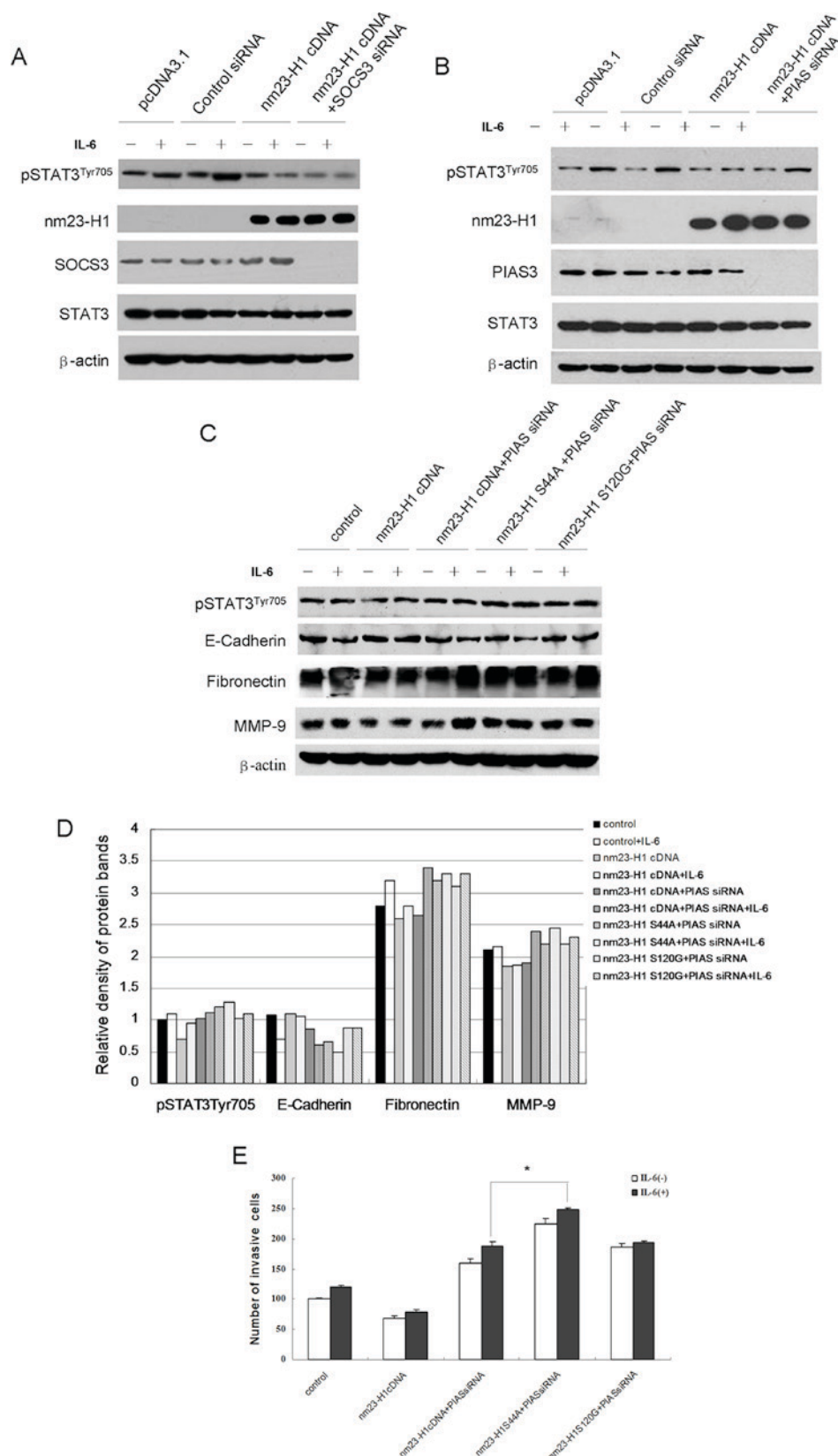
Figure 3. PIAS3 mediates the suppression of cell migration mediated through the inhibition of STAT3 phosphorylation by Nm23-H1. (A) SOCS siRNA and (B) PIAS3 siRNA were individually co-transfected with shRNA-resistant Nm23-H1 cDNA into A549/Nm23-H1-shRNA cells; following 72 h transfection, IL-6 was added for an additional an hour, and then western blot analysis was used to examine the phosphorylation of STAT3$^{Tyr705}$. In SOCS siRNA and Nm23-H1 cDNA co-transfected cells, phosphorylation of STAT3$^{Tyr705}$ was extremely similar to that in Nm23H1 cDNA cells; while in PIAS siRNA and Nm23-H1 cDNA co-transfected cells, phosphorylation of STAT3$^{Tyr705}$ increased compared with Nm23H1 cDNA cells. (C) Western blot analysis was used to examine the phosphorylation of STAT3$^{Tyr705}$ and the levels of the metastasis-associated proteins E-cadherin, fibronectin and MMP-9 in A549/Nm23-H1-shRNA cells transfected with Nm23-H1 cDNA alone or in combination with PIAS3 siRNA. (D) The relative density of protein lands of phosphorylation of STAT3$^{Tyr705}$, E-cadherin, fibronectin and MMP-9 as indicated in (C). (E) The number of the invading cells in Boyden chamber of each treatment were analyzed using t-test ($^*$P<0.05). STAT3, signal transducer and activator of transcription 3; PIAS3, protein inhibitor of activated STAT3; p-STAT3, phosphor-STAT3; IL-6, interleukin-6; MMP-9, matrix metalloproteinase-9; siRNA, small interfering RNA; shRNA, short-hairpin RNA; SOCS, Suppressor of cytokine signaling 3.

migration and invasion, and PIAS3 may contribute to the effect of Nm23-H1-associated inhibition of STAT3 phosphorylation and activity.

Nm23-H1 is a multifunctional protein with at least three enzyme activities *in vitro*: Nucleoside diphosphate kinase; histidine kinase; and 3'-5' exonuclease. Only the histidine kinase activity has been revealed to be associated with metastasis suppression *in vitro* (18). A study using melanoma showed that the Ser44 phosphorylation level of the Nm23-H1 protein is associated with the suppression of tumor metastasis (22), which is in accordance with the present study in lung cancer cells. In the present study, Nm23-H1 Ser120 was also involved in the inhibition of STAT3 phosphorylation on Tyr705 and the suppression of cell migration and invasion. Nm23-H1 can interact with T-cell lymphoma invasion and metastasis-1, a specific activator of Ras-related C3 botulinum toxin substrate 1, and inhibits its activation (23). However, whether this combination is required for the negative regulation of STAT3 activity by Nm23-H1 requires additional study.

STAT3 constitutive activation in cancers via its Src homology 2 (SH2) domain. Src kinase or the kinase activity of the receptor is able to phosphorylate STAT3 on Tyr705. Phosphorylated STAT3 on Tyr705 translocates to the nucleus and has been reported to be involved in the expression of genes that promote angiogenesis, metastasis, growth and survival (24). A previous study indicated a phosphatase-stable phosphopeptide mimics target to the SH2 domain of STAT3 may inhibit the phosphorylation of STAT3$^{Tyr705}$ in cultured tumor cells (25). Drugs that control the activation of STAT3 signaling pathway, particularly in their phosphorylated state, are required for gene expression (26). In the present study, Nm23-H1, a metastasis suppressor gene, which inhibits the phosphorylation of STAT3 on Tyr705 via its Ser44 phosphorylation, provides a novel antitumor metastasis strategy in lung cancer A549 cells.

It was also observed that PIAS3 siRNA and Nm23-H1 cDNA co-transfection increased the phosphorylation level of STAT3 on Tyr705 and the expression of the mesenchymal marker fibronectin. PIAS3 is an endogenous inhibitor of STAT3 that negatively regulates the transcriptional activity of STAT3 and cell growth, and it is demonstrated to limit the majority of human lung squamous cell carcinomas. PIAS3 has been reported to inhibit cell growth in non-small cell lung cancer cell lines by inducing apoptosis (27). The expression level of PIAS3 in squamous cell lung cancer is low and may predict overall survival (28). However, there is little evidence for the role of PIAS3 in lung cancer metastasis. In the present study, PIAS3 was shown to be involved in negative regulation of p-STAT3$^{Tyr705}$ via Nm23-H1 in the suppression of metastasis, which may provide indirect evidence for the requirement of PIAS3 in tumor metastasis. However, the effect of PIAS3 on Nm23-H1 requires additional study.

In conclusion, the present study firstly demonstrated that the Nm23-H1 residues Ser44 and Ser120 are responsible for the negative regulation of STAT3 phosphorylation on Tyr705, in which PIAS3 is involved, and thus lead to the suppression of lung cancer cell metastasis *in vitro*. The present study may contribute to the understanding of Nm23-H1-associated suppression of metastasis in lung cancer and provide a novel view for the treatment of metastatic cancers.

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