Different maspin functions in the lung adenocarcinoma A549 and SPC-A1 cell lines

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Abstract. Mammary serine protease inhibitor (maspin) is a tumor suppressor gene that is silenced in the majority of cancer cells during metastatic progression by transcriptional and epigenetic mechanisms. The function of maspin in non-small cell lung cancer cells (NSCLC) has not been clearly defined. In the present study, the expression of maspin in NSCLC cell lines, in particular, the adenocarcinoma cell lines, was heterogeneous. While the expression levels of maspin in PC-9 and H460 cell lines were intact, the expression of maspin in the A549 and SPC-A1 cells was hardly detected. Ectopic expression of maspin in A549 cells carrying the *K-ras* gene point mutation significantly inhibited cell migration and invasion abilities, which was associated with downregulated expression of matrix metalloproteinase-2 and integrin β1. Ectopic expression of maspin in SPC-A1 cells harboring the wild-type *K-ras* gene predominantly affected cell growth via targeting the AKT signaling molecules. Maspin functions differently in lung adenocarcinoma cells, possibly due to the varied molecular characteristics.

Introduction

Lung cancer is a leading cause of cancer mortality worldwide. Lung cancer is generally divided into non-small cell lung cancer cells (NSCLC) and SCLC, in which the NSCLC constitutes 80-85% of lung cancers. The three major NSCLC subtypes are adenocarcinoma, squamous cell carcinoma and large cell carcinoma. NSCLC, which is characterized by slow tumor cell growth and dissemination, is refractory to chemotherapy and chest radiotherapy. Several driven genes have been identified in NSCLC, such as *EGFR*, *c-MET* and the *ALK-EML4* fusion gene (1). Treatment for lung cancer is mainly based on tumor stage, tumor pathology and molecular pathology. Understanding thoroughly the molecular mechanism underlying the aberrant cellular events driving lung cancer progressions is crucial for developing novel treatments.

Mammary serine protease inhibitor (maspin), also known as Serpin B5, was first identified in 1994 (2). Maspin belongs to the serine protease inhibitor superfamily, and is predominantly localized in the cytoplasm, but is also localized in the nucleus and membrane, and is secreted. The exact roles of maspin in cancer are far more complicated than initially thought due to the conflicting experimental and clinical data that have been reported. In prostate cancer, the expression of maspin is frequently absent (3). Overexpression of maspin is considered an independent factor in predicting a favorable prognosis in breast cancer and lung squamous cell cancer (4-6). However, it has been also reported that the increased nuclear maspin expression predicts a favorable prognosis, whereas the enhanced cytoplasmic expression is associated with early-relapsing and unfavorable prognosis in breast cancer (7). Enhanced expression of maspin is correlated with unfavorable prognosis in pancreatic, ovarian and colorectal cancer (8-10). High maspin expression correlates with an unfavorable outcome in colorectal cancer in stage III and IV, suggesting that maspin may have a stage-specific function possibly associated with cancer cell dissemination and/or metastatic outgrowth (11). The maspin expression in the cytoplasm has recently been reported as an independent unfavorable prognostic indicator of patients with lung adenocarcinoma with tumor size <3 cm (12). The complexity in the clinical significance of maspin expression indicates that maspin expression is possibly influenced by a variety of factors including cell type, genetic background and endogenous expression. The downregulated maspin expression may be due to aberrant cytosine methylation and chromatin condensation of the maspin promoter in cancer cells (13). A recent bioinformatic study suggests that maspin is not commonly underexpressed in cancer, and the perturbation of genes near maspin, such as *PHLPP1*, may explain the poor survival in patients with low maspin expression (14).

However, it is generally believed that maspin functions as a tumor suppressor. Maspin suppresses multiple malignant behaviors of cancer cells, including cell proliferation,

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apoptosis, migration, invasion and angiogenesis. Maspin inhibits breast cancer progression by increased apoptosis. Increased maspin expression sensitizes the apoptotic response of tumor cells to various chemical reagents (15,16). The effect of maspin on apoptosis originates from the cytoplasmic fraction and is mediated by Bax (17). Overexpression of Bax also enhances apoptosis through the mitochondrial permeability transition. A mitochondrial death signaling pathway is induced that involves the localization of maspin to the mitochondria in breast cancer cells (18). Overexpression of maspin sensitizes prostate cancer cells to apoptosis by inhibiting hypoxia-induced AKT activation (19). Maspin inhibits tumor metastasis by suppressing the invasion and motility abilities in breast and prostate cancer cells (20). Maspin could inhibit cancer-induced bone matrix remodeling and induce prostate cancer cell re-differentiation *in vivo* (21). Maspin regulates the cell surface-bound uPA/uPAR-dependent cell detachment in prostate cancer cells (20,22). Maspin can directly bind to integrin β1, leading to the inactivation of integrin β1 and inhibition of the migration ability in cancer cells (23).

Maspin expression is significantly higher in NSCLC, such as squamous cell carcinoma and adenocarcinoma, compared to in SCLC (24). In NSCLC patients, the expression of maspin is also an independent prognostic factor. A statistically significant longer overall survival has been found in patients with a higher expression of nuclear maspin, and unfavorable prognosis is present in patients with a higher intensity of cytoplasmic maspin expression (25). It has also been reported that nuclear maspin, but not the maspin expression in both the nucleus and cytoplasm, correlates with improved survival of lung adenocarcinoma, and maspin nuclear localization inversely correlates with vascular endothelial growth factor-A (26). Maspin is a molecular target of p63, which is a critical factor for cell invasion and progression in the absence of wild-type p53 or in the presence of mutant p53 in NSCLC cells (27). High frequency of co-expression of maspin and p63, as well as maspin and p53, has been detected in squamous cell carcinoma (28). Restoration of maspin expression suppresses cell invasion in NCSLC cells (29).

The aim of the present study was to understand the function of maspin in different NSCLC cell lines. The etopic expression of maspin was established successfully in the A549 and SPC-A1 cell lines. Multiple cellular functions were investigated, including cell growth, migration and invasion. The expression of maspin in NSCLC, in particular, the adenocarcinoma cell lines, was heterogeneous. While the expression of maspin was almost absent in the A549 and SPC-A1 cells, the expression of maspin in the PC-9 and H460 cell lines was intact. Ectopic expression of maspin in A549 cells significantly inhibited cell migration and invasion abilities; however, ectopic expression of maspin in SPC-A1 cells affected cell growth via targeting the AKT signaling molecules.

Materials and methods

Tissue culture. Cell lines PC-9, A549, SPC-A1 and H460 were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. All the cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Cell transfection. The maspin overexpression vector was constructed on the backbone of the MSCV vector. Full-length coding sequences (CDS) of maspin was amplified by polymerase chain reaction (PCR) from human normal breast tissue and cloned into the MSCV vector (MSCV-hMaspin). Phoenix A packaging cells were transfected with MSCV-hMaspin or MSCV by FuGENE HD (Roche, Beijing, China). Virus supernatants were collected and target cells were infected with the virus supernatants. For obtaining stable maspin-expressing cells, the cells were selected for two weeks in the presence of puromycin (5 μg/ml).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol reagent (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. RNA yield and purity were determined by NanoDrop 1000 (Thermo Fisher Scientific, Suzhou, China). Equivalent amounts of RNA (2 μg) were reverse-transcribed with SuperScript M-MLV (Promega, Shanghai, China). Triplicates were performed for all RT-qPCR reactions with a LightCycler 480 System (Roche). Primers for RT-qPCR were designed using Primer-BLAST (PubMed). Primers were synthesized from Invitrogen (Beijing, China). The reference (β-actin) and target genes were run together. The reaction with was set up with 2X LC480 SYBR-Green I Master mix (Roche), according to the manufacturer's instructions. For data analysis, a target gene transcript was quantified in comparison to the reference gene (β-actin).

Western blot analysis. Whole-cell extracts were prepared with radioimmunoprecipitation assay buffer according to the standard instructions. Extracts (5 μg) were separated on an SDS-PAGE gel and transferred to nitrocellulose membranes. Following blocking, membranes were probed with individual antibodies (Abs). Membranes were washed and further probed with an appropriate secondary Ab. Proteins were detected and scanned with an Odyssey system (LI-COR Biosciences, Lincoln, NE, USA). Band density was normalized to the tubulin or β-actin reference. Abs against uPA (sc-14019) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Human maspin Abs (554292) was purchased from BD Pharmingen (San Diego, CA, USA). Abs against ERK (#4695), AKT (#4691), p-AKT (ser473, #4060), p-PDK1 (#3438) and intergrin-β1 (#9699) were obtained from Cell Signaling Technology (Beverly, MA, USA). β-tubulin (SAM1002) were purchased from Sunshine Bio Science and Technology Co., Ltd. (Guangzhou, China). β-actin (AT0001) was purchased from CMCTAG (Milwaukee, WI, USA).

Cell growth assay. The cell growth assay was performed with the xCELLigence RTCA instrument (Roche). In the assay, impedance for indicated times was continuously monitored by the system, and the value was indicated as 'cell index', which was determined by the number of cells seeded, the overall size and morphology of the cells, and the degree to which the cells interact with the sensor surface. The assay was set up according to the manufacturer's instructions. Following running the background blank with 100 μl RPMI-1640 supplemented with 10% FBS in each well of the E-Plate, cells were seeded in wells (8,000 cells/well for A549 cells and 25,000 cells/well for

SPC-A1 cells) and the program was run. The cell index was continuously monitored by the system, and data was collected and analyzed by RTCA software 1.2.

Cell migration and invasion assay. The cell migration assay was also performed with the xCELLigence RTCA instrument. In this assay, a CIM-plate assembled with an upper and lower chamber was used. RPMI-1640 (180 μl) supplemented with 10% FBS was added to each well on the lower chamber. Cells were suspended in the serum-free media and added into the upper chamber. Following attachment, cell migration towards the lower chamber containing RPMI-1640 supplemented with 10% FBS was continuously monitored, and data was collected and analyzed by RTCA software 1.2. For the cell invasion assay, wells of the upper chamber were pre-coated with Matrigel (cat. no. 356234; BD Biosciences, Shanghai, China) for ≥4 h.

Gel electrophoresis and zymography. Cells were plated in 100-mm dishes until a 70-80% confluence was reached. Cells were rinsed twice with phosphate-buffered saline and fed with serum-free medium. Conditioned medium was collected after 24 h and centrifuged to remove the cell debris. The conditioned medium was subsequently mixed with non-reducing sample buffer (without β-mercaptoethanol), and loaded on an 8% SDS-PAGE gel containing 0.1% gelatin. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were incubated at 37˚C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 8.0), and stained with 0.5% Coomassie blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel to produce clear bands against an intensely stained background.

Cell cycle analysis. In order to estimate cell cycle, cells were detected using flow cytometry. After 48-h continuous culture, cells were harvested and fixed with 70% ethanol for 24 h at 4˚C. Subsequently, the single cell suspensions were prepared to stain DNA using propidium iodide staining, based on the manufacturer's instructions. Cell cycle was measured by FACSCalibur (BD Biosciences) with at least three independent experiments performed.

Statistical analysis. All the experiments were repeated at least three times. The data are expressed as the mean ± standard deviation from experiments in replicate. All the statistical analysis was performed by GraphPad software (GraphPad Software, La Jolla, CA, USA). The differences between groups were evaluated using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of maspin expression in individual lung cancer cell lines. The maspin mRNA expression level was measured in the NSCLC PC-9, A549, SPC-A1 and H460 cells by RT-qPCR. As shown in Fig. 1, the maspin expression was detected in the H460 and PC-9 cells, while it was barely detected in the A549 and SPC-A1 cells. The protein expression of maspin in the H460 and PC-9 cells was detectable, while the maspin expression in the A549 and SPC-A1 cells could not be detected. The expression of maspin was comparable in the H460 and PC-9 cells.

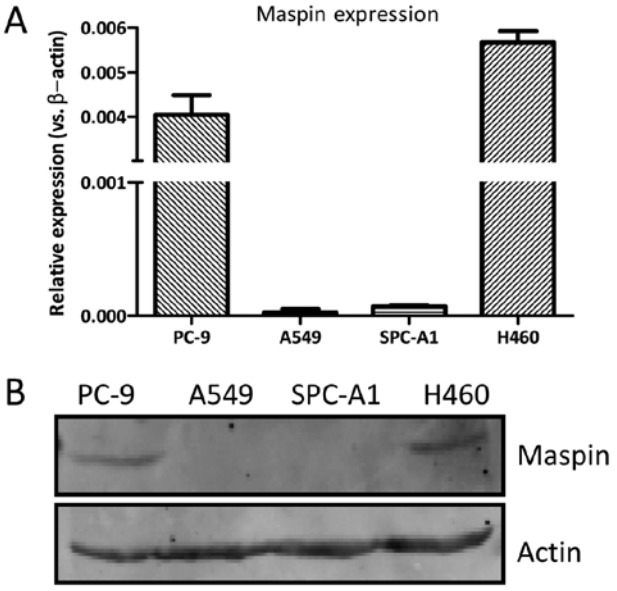


Figure 1. Maspin expression in the different NSCLC cell lines. (A) Maspin mRNA expression between the four NSCLC cell lines. β-actin normalized gene expression, measured in triplicates is exhibited. Data were analyzed with the Pfaffl method that provides a means for quantification of a target gene transcript in comparison to the β-actin gene. (B) Protein levels of maspin expression in the four NSCLC cell lines were determined by western blotting. The level of each protein was normalized against actin.

Establishing maspin-overexpressing cell lines. In order to generate a stable maspin-overexpressing cell line, a maspin expression vector was constructed on the backbone of the MSCV vector. Full-length CDS of the maspin gene was amplified by PCR and cloned into the MSCV vector (MSCV-hMaspin). Phoenix A packaging cells were transfected with MSCV-hMaspin or the control vector (MSCV), and followed by infecting target cells A549 (A549-hMaspin and A549-ctrl) and SPC-A1 (SPC-A1-hMaspin and SPC-A1-ctrl) with the virus supernatants, respectively. The stably transfected cell lines were established by puromycin selection for two weeks. As shown in Fig. 2A, the purities of the established cells were measured by the frequencies of green fluorescent protein expression by flow cytometry analysis, and were 97.7 and 96.2% for A549-hMaspin and A549-ctrl cells, and 98.5 and 97.4% for SPC-A1-hMaspin and SPC-A1-ctrl cells, respectively.

The selected monoclones were further expanded and examined for maspin expression by RT-qPCR. The maspin mRNA expression level was increased ~20-fold in A549-hMaspin cells, compared to that of the A549-ctrl cells (Fig. 2B). The maspin expression in the whole-cell extracts of the A549-hMaspin cells became detectable (Fig. 2C). Similarly, the maspin mRNA expression level was increased ~100-fold in SPC-A1-hMaspin cells, compared to that of the SPC-A1-ctrl cells (Fig. 2D). The maspin expression in the whole-cell extracts of the SPC-A1-hMaspin cells could also be detected (Fig. 2E). Taken together, these results indicate that

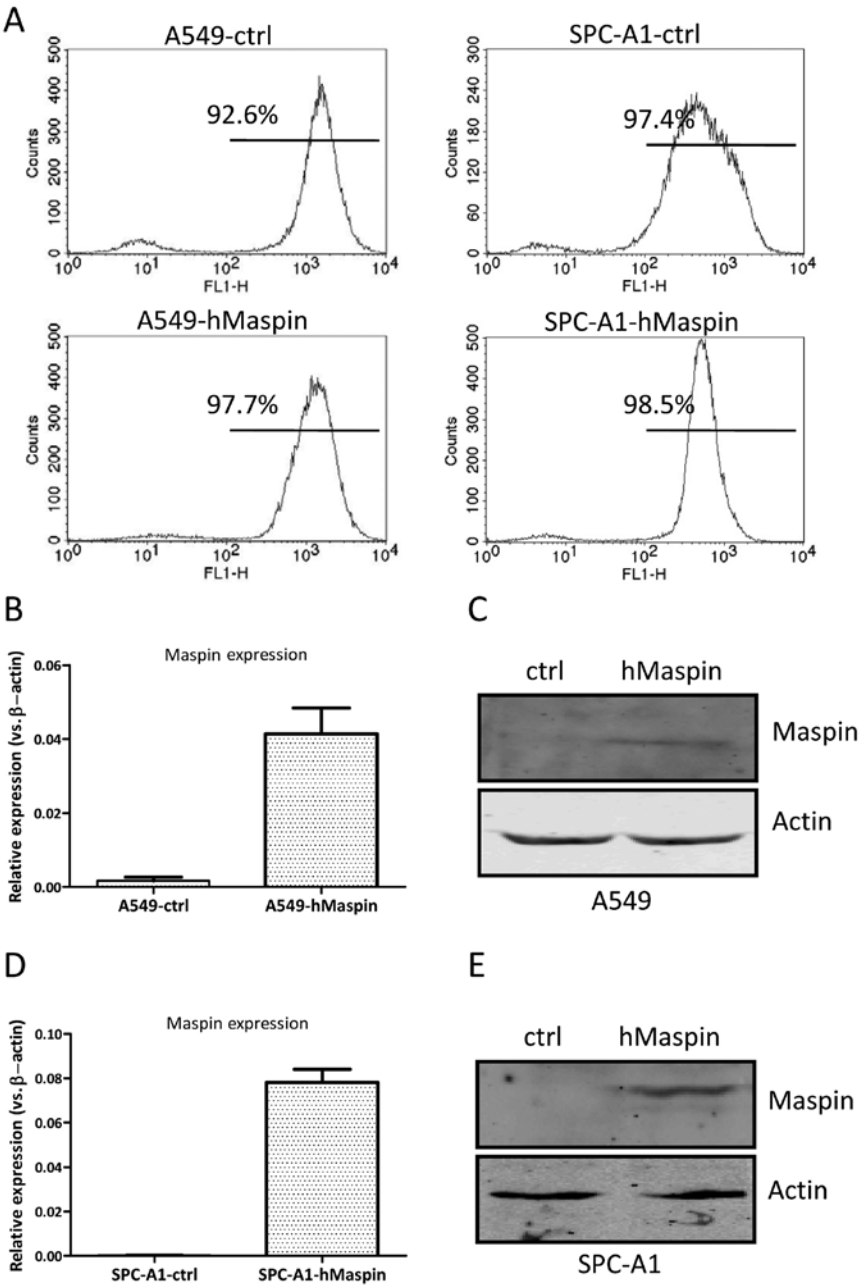


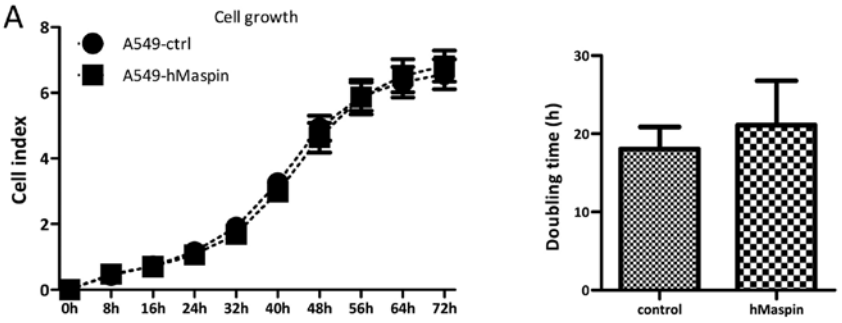
Figure 2. Establishing maspin-overexpressing cell lines. (A) The green fluorescent protein signals of the control cell lines and maspin-overexpressing cell lines were examined by flow cytometry, respectively. (B and D) Maspin expression at mRNA levels were detected in the established cell lines. (C and E) Protein levels of maspin expression were detected in the established cell lines by western blotting.

the stable maspin overexpressing cell lines, A549-hMaspin and SPC-A1-hMaspin, were established successfully.

Maspin overexpression does not affect A549 cell growth. In order to test whether the overexpression of maspin may affect cell growth, a real-time xCELLigence system using E-plates was carried out on the A549-ctrl and A549-hMaspin cells. As shown in Fig. 3A, there was no significant difference between the cell index of A549-hMaspin and A549-ctrl cells during the 72-h continuous monitoring. The doubling times of A549-hMaspin and A549-ctrl were 21.12±5.66 and 18.5±2.82 h, respectively. The cell cycle analysis and cellular DNA content measurement were examined by flow cytometry, and the distributions of G_0-G_1, S and G_2-M phases in the A549-ctrl and A549-hMaspin cells were comparable (Fig. 3B).

Maspin overexpression attenuates the migration and invasion abilities of the A549 cells. The migration ability affected by the overexpression of maspin was examined by the real-time xCELLigence system using CIM-plates. After 8-h culturing, the A549 cells overexpressing maspin migrated significantly slower compared to the A549-ctrl cells, and there was a significantly statistical difference in the migration assay between the two established cell lines (Fig. 4A).

The invasion ability affected by the overexpression of maspin was also measured by the xCELLigence system using matrigel (dilution at 1:30)-coated CIM-plates. After



B

	G_0-G_1 (%)	S (%)	G_2-M (%)
A549-ctrl	72.14±0.27	13.62±0.29	13.52±0.57
A549-hMaspin	69.97±4.74	11.13±2.85	17.69±7.92

Figure 3. Maspin overexpression does not affects the cell growth of A549 cells. (A) The cell growth curves between the A549-ctrl and A549-hMaspin cell lines were detected by xCELLigence system using the E-plate. Doubling time was calculated by RTCA software according to the manufacturer's instructions. (B) The cell cycle analysis between the two established cell lines was examined by flow cytometry. The table presents the data of three phases (G_0-G_1, S and G_2-M).

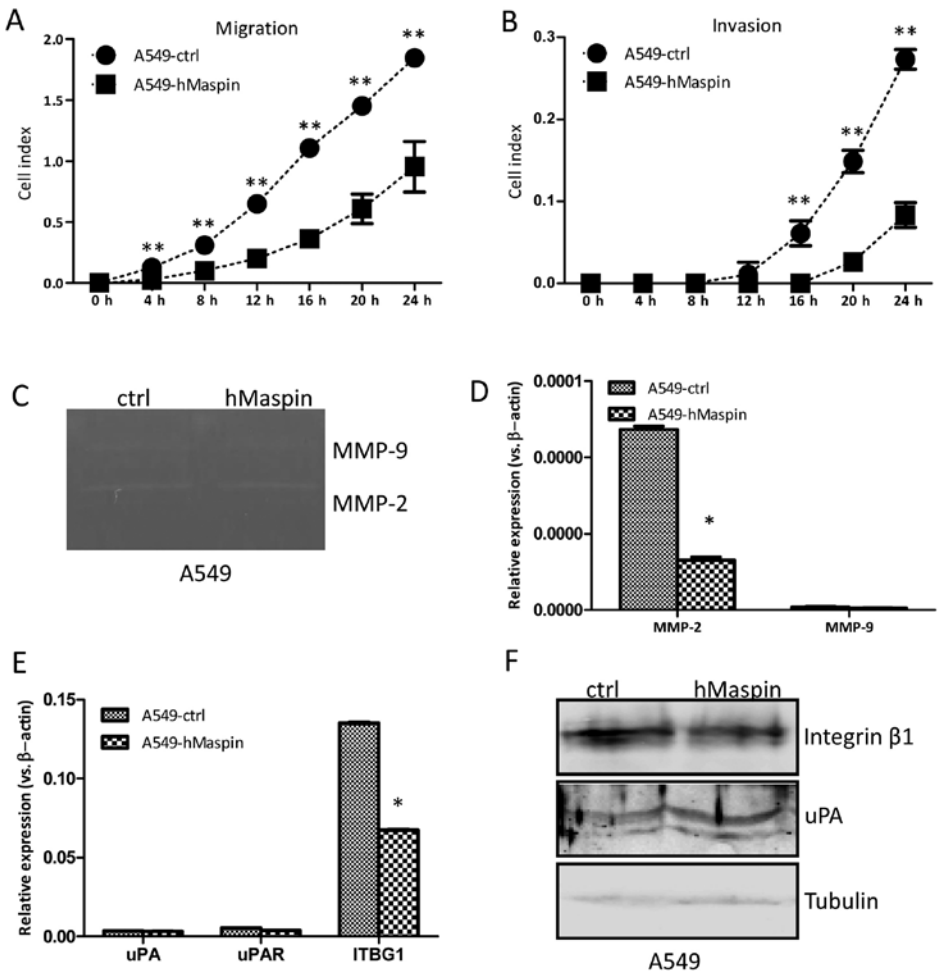


Figure 4. Maspin overexpression inhibits the migration and invasion abilities of A549 cells. (A) The migration ability of A549-ctrl and A549-hMaspin cell lines were detected by the real-time xCELLigence system using CIM-plates. (B) The invasion ability of A549-ctrl and A549-hMaspin cell lines were detected by the real-time xCELLigence system using Matrigel (1:30 dilution)-coated CIM-plates. (C) MMP-2 and MMP-9 activities were detected by the gelatin zymography experiment. (D) The mRNA expression of *MMP-2* and *MMP-9* genes was examined by RT-qPCR. (E) The mRNA expression levels of uPA, uPAR and *ITGB1* genes were examined by RT-qPCR. (F) Integrin β1 and uPA expression at the protein level were analyzed by western blotting. The level of each protein was normalized against tubulin. *P<0.05, **P<0.01. MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

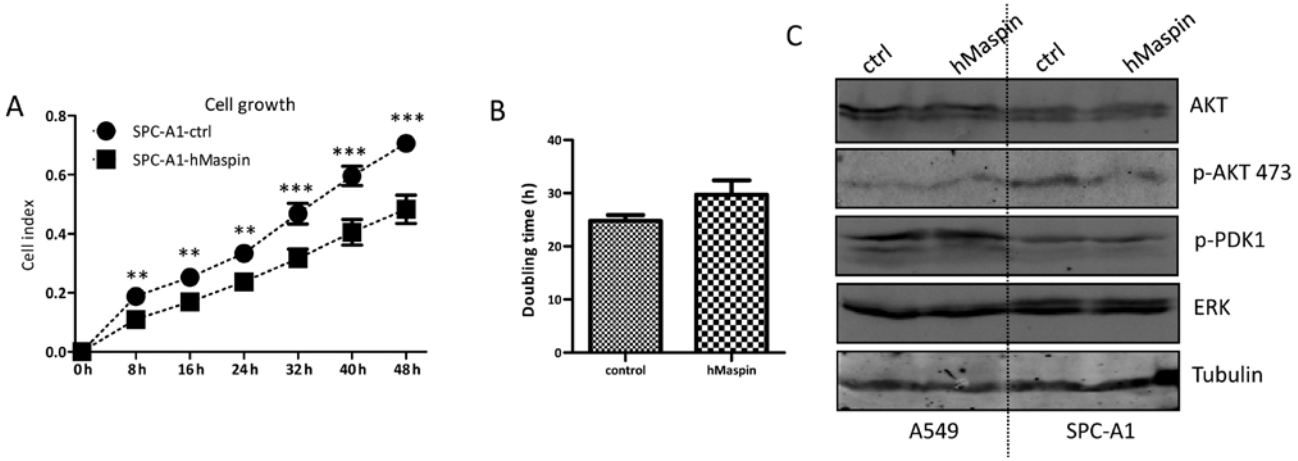


Figure 5. Maspin overexpression inhibits the cell growth of SPC-A1 cells. (A) The cell growth curves between the SPC-A1-ctrl and SPC-A1-hMaspin cell lines were detected by xCELLigence system using E-plate. (B) Doubling time was calculated by RTCA software according to the manufacturer's instructions. (C) The protein expression level of AKT, pAKT (Ser-473), pPDK1 and ERK was analyzed by western blotting. The level of each protein was normalized against tubulin. **P<0.01, ***P<0.001.

16 h culturing, the A549 cells overexpressing maspin invaded through the Matrigel slower compared to the A549-ctrl cells, and there were statistically significant differences in the invasion ability between the two established cell lines (Fig. 4B). The gelatin zymography assay was further performed to explore the relative amounts of active matrix metalloproteinases (MMPs). As shown in Fig. 4C, the MMP-2 activities were decreased in the A549-hMaspin cells compared to that of the A549-ctrl cells. Furthermore, the expression of *MMP-2* in the A549-hMaspin cells at the mRNA level was markedly decreased, while the expression of *MMP-9* remained unchanged (Fig. 4D). The expression of uPA and uPAR, which are involved in the migration and invasion of cancer cells, was not changed in the A549-hMaspin cells (Fig. 4E). The integrin β1 expression at the protein level was significantly decreased in the A549-hMaspin cells compared to that of the A549-ctrl cells (Fig. 4F). Thus, the results here indicated that overexpression of maspin suppressed the migration and invasion abilities of the A549 cells, which was associated with the reduced activities of MMP-2, and the downregulated integrin β1 expression.

Maspin overexpression inhibits SPC-A1 cell growth. Similarly, the cell growth of SPC-A1 cells affected by the maspin overexpression was detected. The SPC-A1 cells overexpressing maspin grew much slower than that of the SPC-A1-ctrl cells, and there was a statistically significant difference between the two established cell lines during the 72-h continuous monitoring (Fig. 5A). The doubling times of SPC-A1-hMaspin and SPC-A1-ctrl were 24.77±1.11 and 29.73±2.08 h, respectively (Fig. 5B).

The PI3K/AKT signaling pathway has been implicated in the tumor cell proliferation and apoptosis resistance. In order to understand whether the ATK signaling is involved in the decreased cell growth of the SPC-A1 cells overexpressing maspin, the key molecules in the AKT signaling were examined by western blotting. The expression of AKT was comparable in the SPC-A1-ctrl and SPC-A1-hMaspin cells. However, the expression of phosphorylated AKT was clearly decreased in SPC-A1-hMaspin cells. The expression of phosphorylated PDK1 and ERK was not affected by the overexpression of maspin in the SPC-A1 cells (Fig. 5C). These key molecules in the AKT signaling were also examined in the A549-ctrl and A549-hMaspin cells, and there was no significant difference between the two cell lines.

Maspin overexpression does not affect the migration and invasion abilities of SPC-A1 cells. The migration and invasion abilities were also investigated in the SPC-A1-ctrl and SPC-A1-hMaspin cells using the real-time xCELLigence system. As opposed to the A549 cells, the overexpression of maspin did not affect either the migration or the invasion ability of the SPC-A1 cells. There was no significant difference between the two established cell lines during the 24-h continuous monitoring for the migration and the invasion assay (Fig. 6A and B). Furthermore, the mRNA expression of the *MMP-2*, *MMP-9*, uPA, uPAR and *ITGB1* genes, which are involved in the migration and invasion of cancer cells, was comparable between the SPC-A1-ctrl and SPC-A1-hMaspin cells (Fig. 6C).

Discussion

Maspin acts as a comprehensive molecule in diverse types of cancer, including prostate, breast and pancreatic cancer. Although the significance of maspin expression varies in different types of cancer, it is usually believed that the decreased maspin expression is associated with an unfavorable prognosis in lung cancer, particularly in NSCLC (5,30,31).

The maspin expression was examined in PC-9, A549, SPC-A1 and H460 cell lines. All four cell lines were derived from NSCLC patients, in which the first three cell lines were from adenocarcinoma and the H460 cell line was from large cell cancer of lung. A clear heterogeneity was observed among the four cell lines. The maspin expression was high in PC-9 and H460 cells; however, the expression was barely detected in the A549 and SPC-A1 adenocarcinoma cell lines, suggesting that the behaviors of maspin were also extremely complicated in lung cancer.

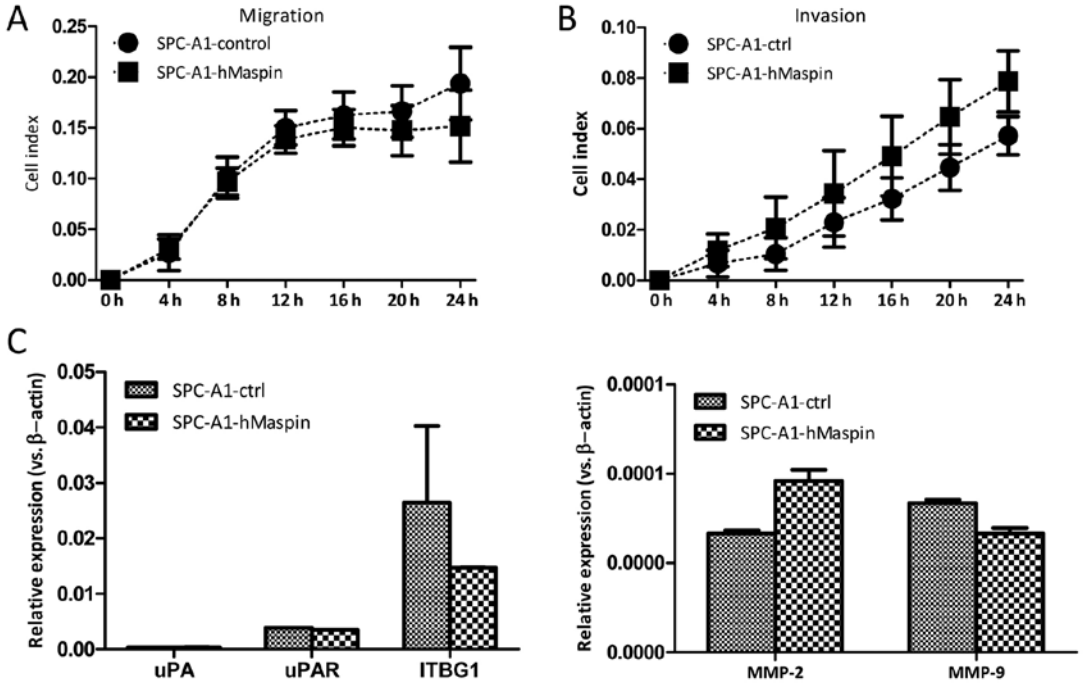


Figure 6. Maspin does not affect the migration and invasion abilities of SPC-A1 cells. (A) The migration ability of SPC-A1-ctrl and SPC-A1-hMaspin cell lines was detected by the real-time xCELLigence system using CIM-plates. (B) The invasion ability of SPC-A1-ctrl and SPC-A1-hMaspin cell lines was detected by the real-time xCELLigence system using Matrigel (1:30 dilution)-coated CIM-plates. (C) The mRNA expression of uPA, uPAR, *ITGB1*, *MMP-2* and *MMP-9* genes was examined by reverse transcription-quantitative polymerase chain reaction.

Maspin was also ectopically expressed in the A549 and SPC-A1 cells, which were almost void of maspin expression, and identified that maspin functioned in a different way in the two cell lines. Overexpression of maspin suppressed the cell migration and invasion abilities of A549 cells, which was associated with the downregulation of integrin β1 and inactivation of MMP-2. However, maspin had no effect on the cell growth of A549 cells. When overexpression of maspin occurred in the SPC-A1 cells, reduced cell growth was observed, accompanied by the reduced phosphoyration of AKT. In contrast to that of the A549 cells, maspin overexpression had no effect on cell migration and invasion of the SPC-A1 cells.

The human MMP family consists of ≥26 proteases, which are subdivided into collagenases, gelatinases, stromelysins and matrilysins. MMP-2 and MMP-9 are well-known gelatinases and are involved in cancer invasion and metastasis due to the strong proteolytic activity of the extracellular matrix. While MMP-2 promotes cleavage of the extracellular matrix proteins, MMP-9 modulates permeability of the vascular endothelium. The critical roles of MMPs and their inhibitors in the growth and progression of lung cancer have been reported (32). In the present study, overexpression of maspin in the A549 cells led to a clear reduction of the *MMP-2* mRNA level, whereas that of *MMP-9* was unchanged. The decreased MMP-2 activity was also observed in the maspin-overexpressing A549 cells, indicated by the gelatinase zymography assay. The migration and invasion abilities of the A549 cells were largely inhibited by the maspin overexpression. These data are in line with the previous data that maspin overexpression suppresses the invasion ability and inhibits the expression of MMP-2 in malignant melanoma cells (33). Although several reports indicate that maspin could target the extracellular uPA/uPAR complex (34,35), which further affects the migration and invasion abilities of certain cancer cells, the expression of uPA/uPAR was not affected by the overexpression of maspin in A549 cells. In the present study, the expression of integrin β1 was downregulated in the maspin-overexpressing A549 cells. Integrin β1, encoded by the *ITGB1* gene, belongs to the family of heterodimeric transmembrane cell surface receptors that contain 18 α and 8 β subunits. There is a direct interaction between maspin and integrin β1 by the reactive centre loop of maspin (23). Maspin could integrate with the plasminogen activation system and integrin β1, thus, regulating cell adhesion and migration (23,36). Integrin β1-silencing suppresses lung cancer cell invasion and metastasis *in vitro* and *in vivo* (37). Integrin β1-silencing in A549 cells causes a defective activation of the EGFR signaling cascade, leading to impaired cell proliferation, migration and invasive behavior *in vitro*. Integrin β1 overexpression in lung cancer cells also has a key role in chemoresistance (38,39). In the present study, in the A549 cells, maspin negatively regulated the integrin β1 expression. Taken together, the decreased activities of MMP-2 together with the downregulated integrin β1 contributed to the diminished migration and invasion abilities of A549 lung adenocarcinoma cells with high expression of maspin.

The overexpression of maspin affected the migration and invasion abilities of A549 cells; however, these observations were not found in the SPC-A1 cells. Rather, the high maspin expression suppressed the cell growth of SPC-A1 cells. Several studies suggest that maspin inhibits the survival pathway by influencing the response to cell death in lung cancer cells. The PI3K/Akt signaling pathway has essential roles in lung cancer cell proliferation and survival (40). Maspin modulates the prostate cancer cell apoptotic and angiogenic response to hypoxia

through targeting Akt signaling (19). In lung cancer cells, maspin also modulates Akt phosphorylation and chemoresistance (41). In the present study, a slow cell growth was detected in the SPC-A1 cells overexpressing maspin by a real-time xCELLigence system. In addition, reduced phosphorylation of Akt was identified in the SPC-A1 cells overexpressing maspin. However, a clear change in the cell proliferation and apoptosis was not detected by the Ki-67 and TUNEL assays (data not shown), compared to the control cells.

Overexpression of maspin in A549 cells did not affect the phosphorylation of Akt. A549 cells harbor the *KRAS* gene mutation (p.G12S), while SPC-A1 cells are wild-type for the *K-ras* gene. Following EGF binding to its receptor and activation of tyrosine kinases, the K-ras protein becomes activated and transduces the activation signals to the nucleus by mitogen-activated protein kinases and PI3K/AKT-mediated cascades. The *K-ras* gene mutation in the NSCLC cells leads to the aberrant activation of the Akt signaling pathway (42). It appears that the constitutively activated Akt due to the *K-ras* gene mutation was not affected by the overexpression of maspin in A549 cells.

Taken together, maspin overexpression in lung adenocarcinoma cells affected several aspects of malignant phenotypes, including cell growth, migration and invasion. In the A549 cells carrying the *K-ras* gene mutation, maspin negatively regulated the expression of MMP-2 and integrin β1, and influenced the migration and invasion abilities. In SPC-A1 cells carrying the wild-type *K-ras* gene, maspin inhibited phosphorylation of Akt, and mainly influenced the cell growth. Maspin functioned differently in lung adenocarcinoma cells due to the diverse genetic background.

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