

PBX3 promotes gastric cancer invasion and metastasis by inducing epithelial-mesenchymal transition

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Received July 15, 2015; Accepted September 22, 2016

DOI: 10.3892/ol.2016.5305

Abstract. The overexpression of pre-leukemia transcription factor 3 (PBX3) in tumors plays an important role in invasion, metastasis and proliferation in a variety of human cancer types. Tumor metastasis and angiogenesis significantly contribute to the progression of cancer and create challenges for cancer therapy. In the present study, reverse transcription-polymerase chain reaction demonstrated that PBX3 was upregulated in gastric cancer (GC) tissues and Transwell assay revealed that the overexpression of PBX3 promoted GC invasion and metastasis *in vitro*. In addition, a nude mouse xenograft model was established, which demonstrated that PBX3 promoted peritoneal metastases *in vivo*. Furthermore, the overexpression of PBX3 in GC promoted the tubular formation of human umbilical vein endothelial cells. Western blot analysis revealed that overexpressed PBX3 induced epithelial-mesenchymal transition (EMT) in GC, as measured by increases in the EMT protein markers N-cadherin and vimentin, while E-cadherin expression was reduced in PBX3-overexpressing GC cells. Contrasting results were observed in PBX3-knockdown GC cells. Additionally, the overexpression of PBX3 increased the levels of phosphorylated AKT (Ser473), which is involved in the progression of a variety of human cancers. Gelatin zymography assay demonstrated that the overexpression of PBX3 also elevated matrix metalloproteinase-9 activity in GC, which was closely associated with tumor metastasis and

angiogenesis. Based on these findings, it may be concluded that PBX3 enhances invasion and metastasis in GC by promoting EMT, possibly via the AKT signaling pathway.

Introduction

Gastric cancer (GC) is one of the most frequently occurring aggressive malignancies, particularly in East Asian countries (1-4), with a global incidence of 15.6 and 6.7 cases per 100,000 males and females, respectively in developed regions, and an incidence of 18.1 and 7.8 cases per 100,000 males and females, respectively, in less developed areas (4). Furthermore, GC is the second leading cause of cancer-associated mortality worldwide (1-4), accounting for 9.2 and 4.2 mortalities per 100,000 males and females, respectively, in developed areas and 14.4 and 6.5 mortalities per 100,000 males and females, respectively, in less developed regions (4). A low early GC diagnosis rate and tumor metastasis are major obstacles in GC therapy. Novel treatments are urgently required to treat this malignant cancer. Pre-leukemia transcription factor 3 (PBX3) is a member of the PBX family of three amino acid loop extension class homeodomain transcription factors, which are known to regulate gene expression with a highly conserved homologous domain (5-8). PBX proteins are also well known for their interaction with other homologous proteins, such as homeobox (HOX) proteins. The interaction between PBX and HOX increases the DNA-binding affinity and promotes the transcription of the downstream target genes (9-11).

Previously, PBX proteins have been reported to be involved in a variety of human cancer types and to play important roles in the progression of human tumors. In prostate cancer, PBX3 is upregulated and can be downregulated by lethal-7d (let-7d) through post-transcriptional regulation by androgens, and this effect may be independent of androgen receptors (12). In colorectal cancer, let-7c serves as a tumor metastasis suppressor by inhibiting PBX3 expression (13), and PBX3 expression is significantly associated with lymph node invasion, distant metastasis and poor overall survival. PBX3 can stimulate the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase signaling pathway in colorectal cancer (6). Additionally, the forced expression of PBX3 may reverse the effects caused by

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Key words: gastric cancer, PBX3, AKT, epithelial-mesenchymal transition, metastasis

microRNA-181b that promote apoptosis, inhibit proliferation and delay leukemogenesis in cytogenetically abnormal acute myeloid leukemia (14). In addition, PBX3 is well known as a critical cofactor of HOXA9 in leukemogenesis, inhibiting leukemic cell apoptosis (15). PBX3 is also overexpressed in GC and is closely correlated with invasion depth, clinical stage and differentiation. Furthermore, PBX3 accelerates cell proliferation and colony formation in GC (16). Additionally, blocking the HOX/PBX dimer has been reported to be a treatment against tumor growth in ovarian, renal and pancreatic cancer, and in non-small cell lung cancer (17-19). These findings suggest that PBX3 acts as an oncogenic gene in the progression of numerous cancer types.

However, the mechanisms through which PBX3 affects intracellular signal transduction in GC remain to be determined, and the study of further biological behaviors of PBX3 in GC are required. The present study aimed to examine further biological effects and potential pathways of PBX3 in GC progression.

Materials and methods

Cell lines and tumor samples. The human GC cell lines, SGC-7901, AGS, BGC-823 and MKN-45, and human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were cultured at 37°C in 5% CO₂ and at saturation humidity in RPMI-1640 medium containing 10% fetal bovine serum.

Gastric tumor and adjacent non-tumorous tissues (≥3 cm from tumor) were obtained from 25 patients with GC who underwent curative surgery (D2 radical resection) at the Second Affiliated Hospital of Soochow University (Suzhou, China) between January 2014 and December 2015. Patients that had undergone radiotherapy/chemotherapy prior to surgery were excluded from the study. These tissues were used for reverse transcription polymerase chain reaction (RT-PCR). None of the patients had received radiotherapy or chemotherapy prior to surgery. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University, and all patients were fully informed of the experimental procedures.

RNA isolation and RT-quantitative PCR (RT-qPCR). Total RNA was extracted and isolated from tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Reverse transcription of RNA was performed using a reverse transcription kit (Promega, Madison, WI, USA).

In brief, 1 µg of total RNA from each sample was reverse transcribed following the manufacturer's protocol. SYBR Green reagent (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT-qPCR to analyze mRNA expression. The PCR primers were designed according to the human PBX3 and GAPDH cDNA sequences in GenBank, as follows: PBX3 forward, 5'-GAGCTGGCCAAGAAATGCAG-3' and reverse, 5'-GGGCGAATTGGTCTGTTG-3'; and GAPDH forward, 5'-GGA CCT GACCTGCCGTCTAG-3' and reverse, 5'-GTA GCCCAGGATGCCCTTGA-3'. GAPDH acted as the constitutive control. Relative expression ratios of PBX3 in each paired

tumor to non-tumor tissue sample were calculated using the 2^{-ΔΔC_q} method (20).

Vector construction and transfection. The pCMV6-PBX3 plasmid was purchased from Origene (Origene Technologies, Inc., Rockville, MD, USA). The pCMV6-PBX3 or pCMV6-vector was then transfected into SGC-7901 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol.

For depletion of endogenous PBX3 expression, short hairpin RNA (shRNA) targeting 592-613 bp was inserted into a lentivirus vector as previously described (6). Lentiviral vectors were then transfected into MKN-45 GC cells. Stably transfected cells were selected by treatment with 5 µg/ml blasticidin and were used for identification and additional research.

Western blotting. GC cells were harvested and lysed using radio-immunoprecipitation assay buffer (Solarbio, Beijing, China) containing 1% phenylmethylsulfonyl fluoride protease inhibitors. A bicinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was used to measure the total protein concentration. Equivalent amounts of protein (50 µg) were separated by 10% SDS-PAGE, and the resolved proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk for 2 h and then incubated with primary antibodies overnight at 4°C. Primary antibodies were as follows: PBX3 (polyclonal rabbit; cat. no. 12571-1-AP; dilution, 1:500; Proteintech Group, Inc., Rosemont, IL, USA); E-cadherin (monoclonal rabbit; cat. no. 3195; dilution, 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA); N-cadherin (monoclonal rabbit; cat. no. 13116; dilution, 1:500; Cell Signaling Technology, Inc.); vimentin (monoclonal rabbit; cat. no. 5741; dilution, 1:1,000; Cell Signaling Technology, Inc.); p-AKT (Ser473) (monoclonal rabbit; cat. no. 4060; dilution, 1:500; Cell Signaling Technology, Inc.); total AKT (monoclonal rabbit; cat. no. 4691; dilution, 1:500; Cell Signaling Technology, Inc.) and GAPDH (monoclonal mouse; cat. no. ab8245; dilution, 1:10,000; Abcam, Cambridge, UK). Membranes were then incubated with secondary antibody for 2 h at room temperature and were visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK) in accordance with the manufacturer's protocol.

Cell migration and invasion assays. For cell migration and invasion assays, a total number of 1×10⁵ GC cells (SGC-7901-PBX3, SGC-7901-NC, MKN-45-NC and MKN-45-PBX3/sh) were suspended in serum-free RPMI-1640 medium (HUVEC cells were suspended in supernatant from GC cells) and plated in Transwell chambers (8 µm for 24-well plate; Costar; Corning Incorporated, Corning, NY, USA) with or without Matrigel (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. For each assay, medium containing 10% FBS was added to the lower chamber as a chemoattractant. Subsequent to 24 h culture, the cells were fixed by 10% formalin and stained by 0.5% crystal violet. Finally, images of cells in the lower chamber were captured and the number of cells was counted by inverted microscopy (x200 magnification).

Endothelial tube formation assay. HUVEC cells were cultured in tumor supernatant and plated in a 96-well

plate with Matrigel (BD Biosciences) at a concentration 3×10^4 cells/well. Tumor supernatant was collected from PBX3-overexpressing groups and PBX3-silenced groups subsequent to 24 h culture in RPMI-1640 medium. Following 12 h incubation at 37°C in a 5% CO₂ atmosphere, tubes were photographed by microscopy and evaluated by Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Gelatin zymography. To examine matrix metalloproteinase (MMP)-9 activity, gelatin zymography was performed using 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). In brief, GC cells were cultured in serum-free RPMI-1640 medium for 24 h and the supernatant was then collected and centrifuged at 201 x g for 5 min. The gels were washed twice in renaturation buffer (2.5% Triton X-100) for 30 min each time to remove SDS subsequent to electrophoresis and then incubated at 37°C for 24 h in a reaction buffer consisting of 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂ and 150 mM NaCl. The gels were then stained with 0.5% Coomassie brilliant blue R-250 (Sigma-Aldrich; Merck Millipore) for 2 h and destained in buffer (30% methanol; 10% acetic acid). Clear transparent bands in the background of blue staining represented gelatinase activity.

Nude mouse xenograft model. Four-week-old male BALB/c nude mice (n=10; Institute of Zoology, Chinese Academy of Sciences, Beijing, China) were used to evaluate the role of PBX3 in peritoneal spreading *in vivo*. Nude mice were housed at a specific pathogen-free environment in the Animal Laboratory Unit, School of Medicine, Soochow University. Mice received humane care and the study protocols were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Soochow University. A total number of 2×10^6 SGC-7901-PBX3 or SGC-7901-NC cells were injected into the abdominal cavity of the 5 mice of the PBX3 and NC groups, respectively. Mice were euthanized on the 30th day subsequent to injection, and the abdominal masses were imaged and photographed. All experiments were performed in accordance with the official recommendations of the Chinese Animal Community at Soochow University School of Medicine (Suzhou, China).

Statistical analysis. Student's t-test was used to examine the statistical differences between the two groups. Data are shown as mean \pm standard deviation. Statistical analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PBX3 expression is upregulated in GC tissues. The levels of PBX3 in GC and adjacent non-tumor tissues were examined by RT-qPCR assay. The results showed that the PBX3 mRNA level was markedly increased in GC tissues compared with the corresponding non-tumor tissues (Fig. 1A; $P < 0.01$). This finding suggests that PBX3 may act as an oncogenic gene in GC. Notably, a previous study showed that PBX3 expression

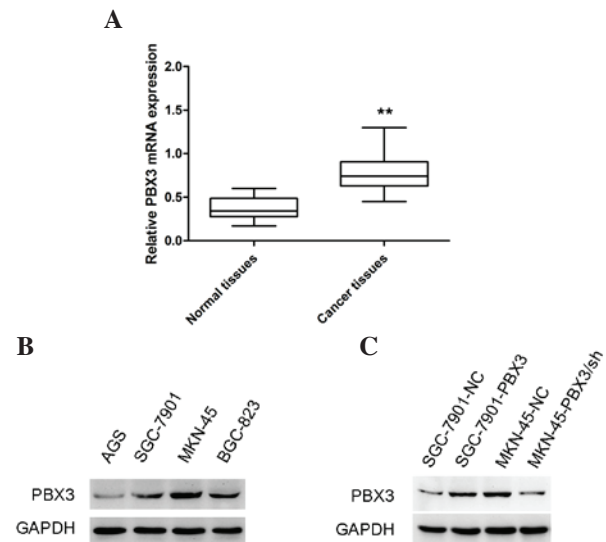


Figure 1. Expression of PBX3 in GC tissues and cells. (A) PBX3 mRNA expression in GC tissues and paired adjacent non-tumor tissues examined by reverse transcription-quantitative polymerase chain reaction. ** $P < 0.01$. (B) PBX3 expression in GC cell lines was analyzed by western blot analysis. (C) Overexpression of PBX3 was overexpressed in SGC-7901 cells and downregulated in MKN-45 cells. The effects of overexpression and knock-down were confirmed by western blotting. PBX3, pre-leukemia transcription factor 3; GC, gastric cancer.

was associated with poor tumor differentiation, invasion depth and clinical stage in GC (16).

Western blot analysis was then used to examine the expression of PBX3 at the protein level in GC cell lines. It was found that the expression levels were different in different GC cell lines (Fig. 1B). The results indicated that MKN-45 GC cells inherently express high levels of PBX3, whereas SGC-7901 GC cells express low levels of PBX3. Overexpression of PBX3 in SGC-7901 and attenuation of PBX3 expression in MKN-45 GC cells were confirmed at the protein level by western blot analysis (Fig. 1C). Stably transfected cells were used for further experiments.

PBX3 promotes the migration and invasion of GC cells. Cancer metastasis is the leading cause of cancer-associated mortality and it has been challenging to study due to a series of rare, stochastic events involved in cancer metastasis (21). Therefore, the present study examined the effects of PBX3 on GC mobility by Transwell assay. It was observed that the cell migration rate and invasion rate were each increased in PBX3 overexpressing groups compared with their respective control groups (Fig. 2A-C; $P < 0.01$). The opposing results were obtained subsequent to silencing of PBX3 in MKN-45 cells (Fig. 2A-C; $P < 0.01$). This finding suggests that the mobility of GC cells changed with the expression of PBX3.

Overexpression of PBX3 in GC promotes tubular formation of HUVEC cells. It is well known that tumor angiogenesis is a key role in cancer progression. To examine the effects of PBX3 on tubular formation of HUVEC cells, tumor supernatant was collected from GC cells to suspend HUVEC cells. In total, 3×10^4 HUVEC cells were plated onto a 96-well plate that was coated with Matrigel. The 96-well plate was

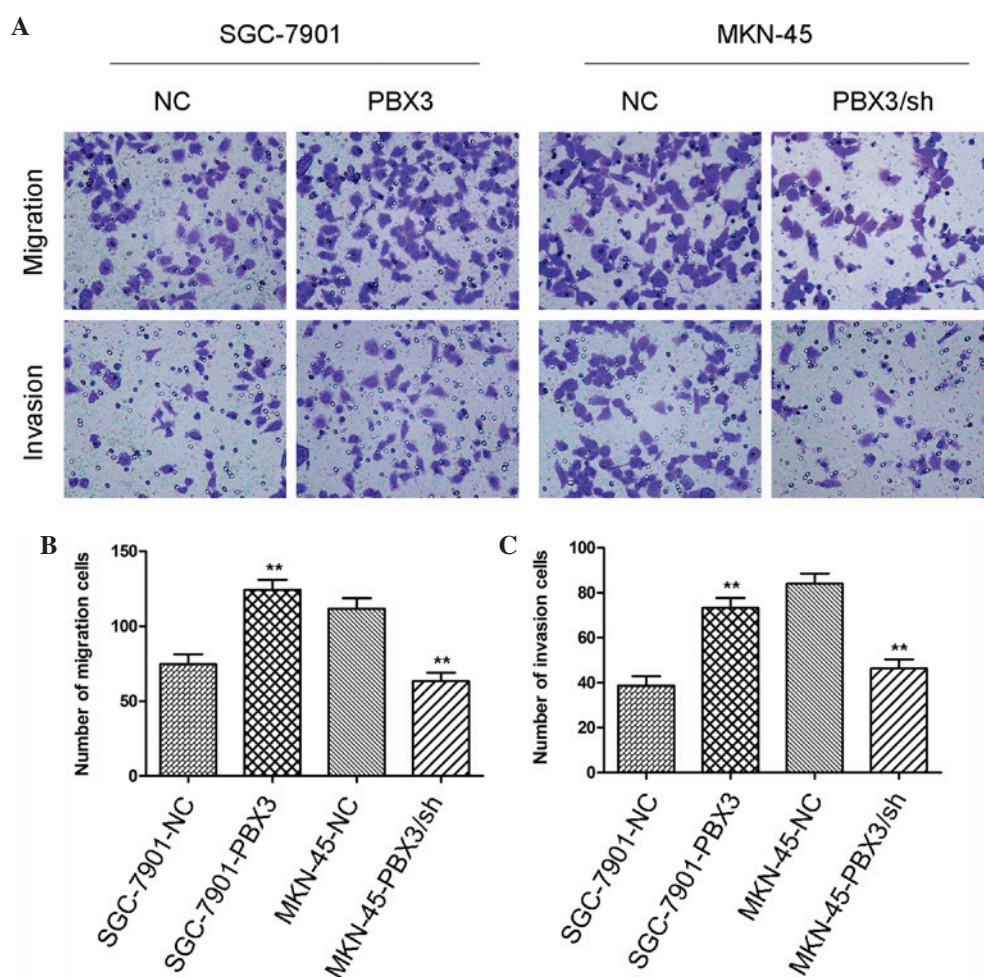


Figure 2. Effects of PBX3 on cell migratory and invasive ability in GC cells. (A) Transwell assay. PBX3 promotes GC cells migration and invasion. Images show cells that had traveled through the micropore membrane with or without Matrigel (magnification, x200). (B) Histograms showed the numbers of migrating cells. (C) Histograms showed the numbers of invading cells. The results are means of three independent experiments in the bar graphs. **P<0.01. PBX3, pre-leukemia transcription factor 3; GC, gastric cancer.

then incubated for 12 h at 37°C in 5% CO₂. Following 12 h incubation, increased tubule forming ability was observed in PBX3-overexpressing groups compared with the control groups (Fig. 3A), such as number of tubules (Fig. 3B), and number of intersections (Fig. 3C). Converse results were achieved in PBX3-knockdown groups compared with the control groups (Fig. 3A-C).

Tubular formation of HUVEC cells was associated with the migration of HUVEC cells. Therefore, the effect of PBX3-overexpressing GC cells on the migration of HUVEC cells. It was observed that supernatant from PBX3-overexpressing GC cells could stimulate the migration of HUVEC cells. The mechanism of the promotion of HUVEC cell migration by PBX3-overexpressing GC cells remains unclear.

PBX3 induces epithelial mesenchymal transition (EMT) in GC cells. EMT is known to play important roles in the progression of tumors, particularly in cancer metastasis. It was found that overexpression of PBX3 elevated the migration and invasion rates of GC cells. EMT has an unelucidated effect on these processes. To clarify this, the EMT-associated proteins in GC cells were examined by western blot analysis. It was observed that the N-cadherin and vimentin protein levels were

increased, while E-cadherin was decreased in PBX3-overexpressing GC cells compared with their respective control cells (Fig. 4A and B). Converse findings were observed after PBX3 was knocked down in MKN-45 GC cells (Fig. 4A and B).

Furthermore, AKT signaling is strongly associated with EMT, invasion and metastasis in a variety of human tumors. Therefore, AKT activity was evaluated in PBX3-overexpressing GC cells. Western blot analysis showed that the level of p-AKT (ser473) was increased in PBX3-overexpressing GC cells, whereas opposing results were observed in PBX3-silencing GC cells (Fig. 4A and C). Additionally, it was observed that overexpression of PBX3 elevated the activity of MMP-9 in GC cells by gelatin zymography assay (Fig. 4D and E). However knockdown of PBX3 made no significant change in the activity of MMP-9 in GC cells. These findings suggest that PBX3-induced EMT may be via the AKT signaling pathway in GC cells.

PBX3 promotes peritoneal spreading in vivo. To examine the effects of PBX3 on peritoneal spreading and metastasis *in vivo*, SGC-7901-PBX3 and SGC-7901-NC GC cells were injected into the abdomens of nude mice. Extensive peritoneal spreading was observed in the PBX3-overexpressing

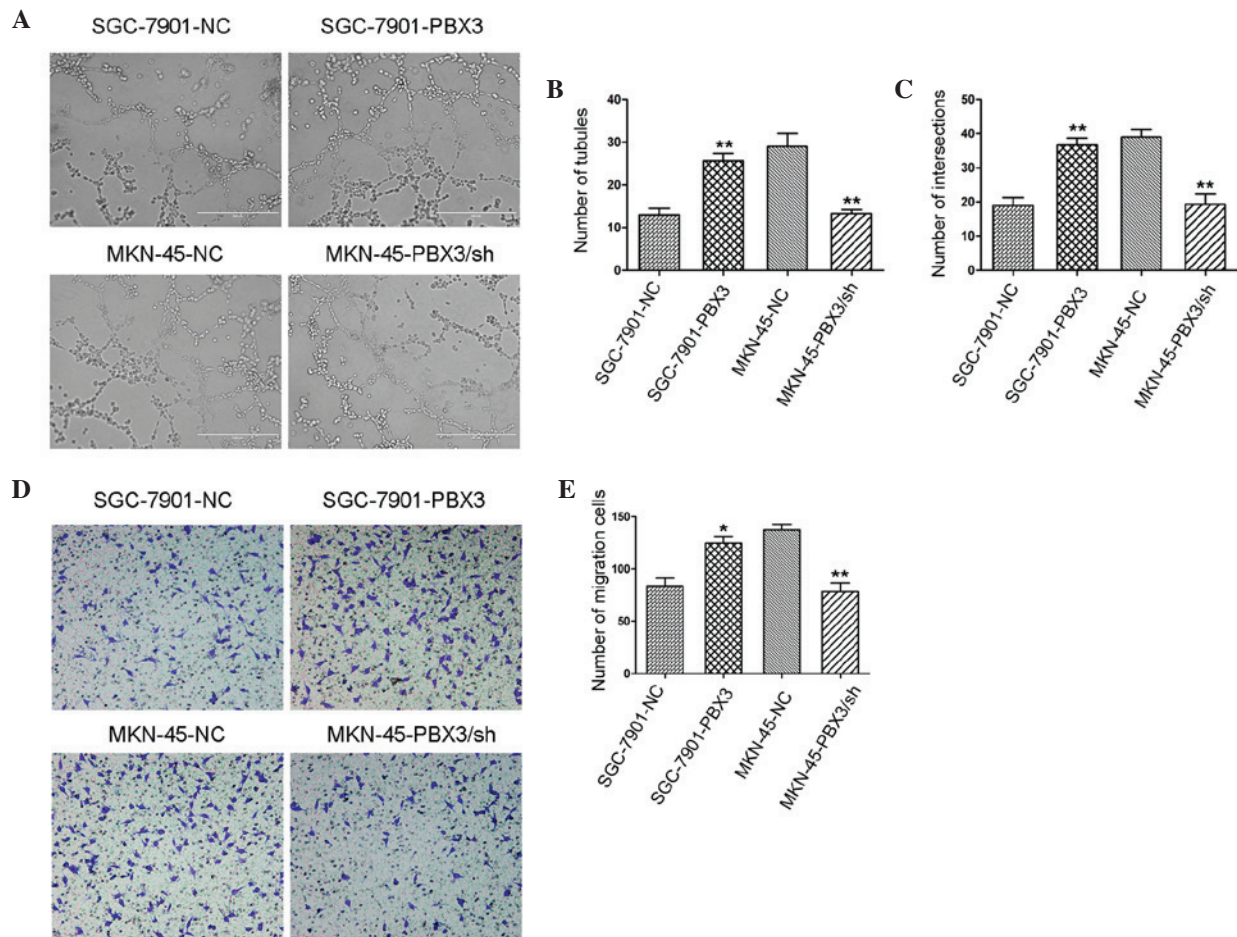


Figure 3. Effect of PBX3 on tubular formation *in vitro*. (A) An increased number of tubes was observed in the SCG-7901-PBX3 groups compared with the control groups, whereas the MKN-45-PBX3/sh groups formed less tubes than the control groups (magnification, x200). Bar charts show (B) the number of tubes, and (C) the number of intersections between different groups. (D) The supernatant of GC cells was collected after 24 h culture. Overexpression of PBX3 increased the migration of HUVEC cells, whereas knockdown of PBX3 decreased the migration of HUVEC cells (magnification, x100). (E) Histograms showed the number of migratory cells. * $P < 0.05$; ** $P < 0.01$. Data are represented as mean \pm standard deviation of three independent experiments. PBX3, pre-leukemia transcription factor 3; GC, gastric cancer.

group compared with the control group (Fig. 5A). There were significantly more visible peritoneal nodules in the PBX3-overexpressing group than in the control group (4.600 ± 0.748 vs. 2.200 ± 0.374 ; $P < 0.05$; Fig. 5B). Thus overexpression of PBX3 promotes GC invasion and metastasis *in vivo* as well as *in vitro*.

Discussion

GC is a major health burden in the world and additional studies should be performed to investigate ways to prevent gastric cancer progression. Increasing evidence indicates that PBX3 plays an important role in human cancer progression. The aim of the present study was to further examine the contribution of the transcription factor PBX3 to GC progression.

In the present study, it was observed that PBX3 expression was overexpressed in GC tissues measured by RT-qPCR assay, which was consistent with a previous study (16). PBX3 was silenced and overexpressed in GC cells to examine the effects of PBX3 in GC. It was found that overexpression of PBX3 promoted GC cell invasion and migration compared with the control groups, while knockdown of PBX3 reduced GC cell invasion and migration. These observations demonstrated that

PBX3 may induce something changing in GC that contributing to accelerate the mobility of GC cells. To further study the effects of PBX3 in GC metastasis *in vivo*, a nude mouse xenograft model was constructed and it was observed that there were significantly more visible peritoneal nodules in the PBX3-overexpressing group compared with the control group. According to these observations, it was confirmed that PBX3 promotes GC invasion and metastasis *in vivo* as well as *in vitro*.

Tumor progression is not only associated with cancer cell metastasis, but also with tumor angiogenesis (3). There must be angiogenesis tubular formation in tumors with a diameter of ~ 2 mm (22). Notably, it was observed that overexpression of PBX3 promoted tubular formation of HUVEC cells in GC compared with the control groups. As an attempt to understand the observations that promoted tubular formation through PBX3, gelatin zymography for determining MMP-9 activity and HUVEC cells migration assays were performed. It was detected that MMP-9 activity was increased in PBX3-overexpressing GC cells and PBX3 promoted HUVEC cell migration. MMP-9 can degrade extracellular matrix components and plays a critical role in tissue remodeling during development in pathological process, including

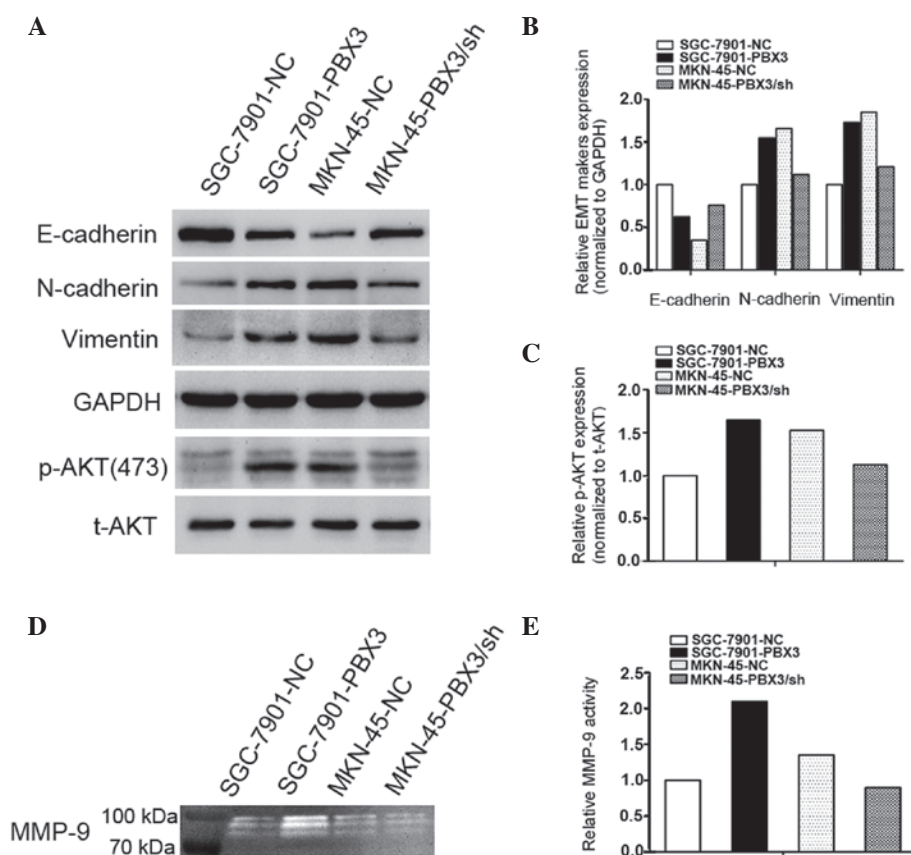


Figure 4. PBX3 induces EMT in GC. (A) The protein levels of EMT markers and p-AKT (Ser473) were measured by western blot analysis in GC cells. (B) Relative EMT markers expression in GC cells were calculated by gray scale ratio. Normalized to GAPDH. (C) Relative p-AKT expression in GC cells. Normalized to t-AKT. (D) The supernatant of GC cells was collected after 24 h. Then the MMP-9 activity was examined by gelatin zymography. (E) Relative MMP-9 activity in GC cells. GC, gastric cancer; EMT, epithelial-mesenchymal transition; MMP-9, matrix metalloproteinase 9.

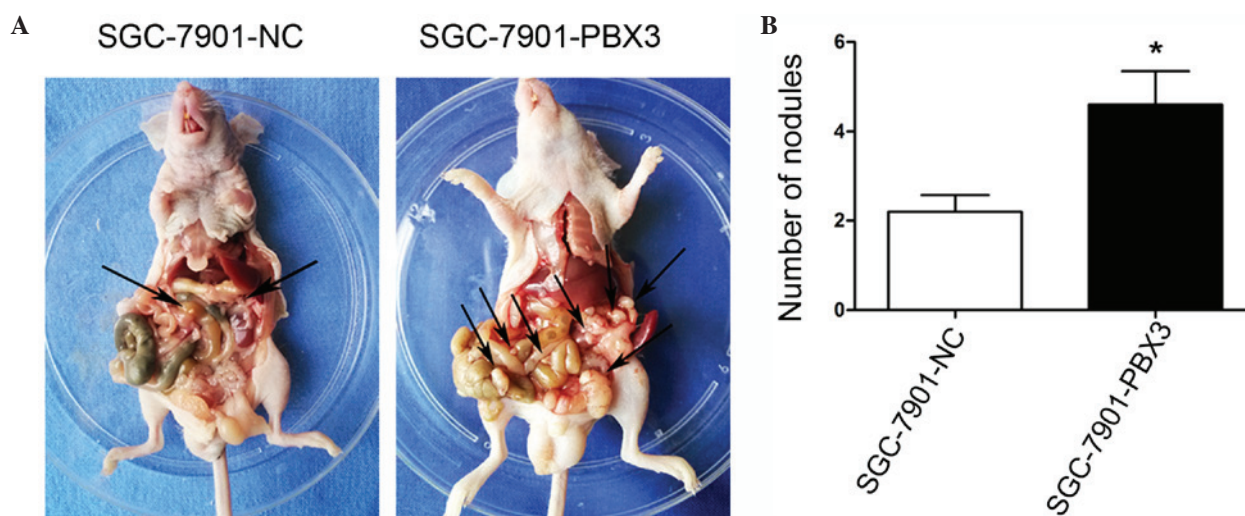


Figure 5. Effect of PBX3 on peritoneal spread and metastasis. (A) Increased numbers of metastatic nodules were observed in the SGC-7901-PBX3 group than the SGC-7901-NC group, as indicated by the black arrows. (B) Average number of peritoneal nodules in two groups. More nodules occurred in the SGC-7901-PBX3 group than that in the control group. * $P < 0.05$. PBX3, pre-leukemia transcription factor 3.

inflammation, tumor invasion and metastasis, and tumor angiogenesis (23-25). These observations may contribute to account for the effects of PBX3 in GC that promoting invasion, metastasis and tubular formation.

EMT is implicated in a pathological element in cancer progression as well as in a physiological process during

embryonic development (26,27). Tumor metastasis is always associated with EMT process in lots of tumor progression. Therefore, the present study examined the association between PBX3 and EMT in GC cells. It was then observed that PBX3-overexpressing in GC cells decreased E-cadherin expression, which is a biomarker of epithelial cells, and

increased N-cadherin and vimentin expression, which are biomarkers of mesenchymal cells. These observations suggest that overexpression of PBX3 promotes invasion and metastasis by inducing EMT in GC. Numerous mechanisms involved in EMT initiation have been documented, containing the transforming growth factor β , focal adhesion kinase, interleukin-6, phosphoinositide 3-kinase/AKT, RAF/MAPK signaling pathways (25,28,29). Therefore, the present study examined the effects of PBX3 overexpression on the levels of AKT phosphorylation in GC cells. Notably, overexpression of PBX3 elevated the phosphorylation of AKT (ser473) in GC cells. Based on the aforementioned findings, it was concluded that PBX3 may promote GC invasion and metastasis by inducing EMT via the AKT signaling pathway.

In conclusion, the present study illustrated that PBX3 acts as an oncogene by promoting EMT via AKT signaling in GC. Furthermore, PBX3 promotes GC invasion and metastasis *in vitro* and *in vivo*, and may act as a potential new diagnostic and prognostic marker and novel target for GC therapy.

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