SPAG9 expression is increased in human prostate cancer and promotes cell motility, invasion and angiogenesis *in vitro*

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Abstract. Sperm-associated antigen 9 (SPAG9) is a recently characterized oncoprotein involved in the progression of several human malignancies. To elucidate the role of SPAG9 in the development of human prostate cancer (PCa), tissue microarray (TMA) and immunohistochemistry were used to detect the clinical relevance of SPAG9 in PCa tissues. We found that SPAG9 expression was increased in the PCa tissues when compared with the level in the tumor adjacent normal prostate tissues, and increased SPAG9 staining was significantly correlated with TNM stage and tumor grade. We also examined prostate cancer cell motility, invasion and angiogenesis ability following reduced SPAG9 expression by siRNA. Our data showed that knockdown of SPAG9 in prostate cancer cell lines inhibited cell motility and invasion due to the inactivation of metalloproteinase-2 (MMP-2)/MMP-9 by upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1)/TIMP-2. Furthermore, downregulation of vascular endothelial growth factor (VEGF) secretion greatly contributed to the reduced ability of angiogenesis. Our data indicate that SPAG9 expression is significantly increased in PCa and it may be involved in the process of prostate cancer cell motility, migration and angiogenesis.

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Introduction

Human prostate cancer (PCa) is the most frequently diagnosed non-skin cancer in the United States and is the second leading cause of death among males (1). Although conventional therapies achieve a high cure rate in patients presenting with localized disease, 30% of patients develop metastatic disease (2). The lack of effective therapies for advanced PCa is related, to a large extent, to a poor understanding of the molecular mechanisms underlying the progression of the disease toward invasion and metastasis (3). Therefore, identification of new predictive biomarkers, particularly those that are indicative of invasiveness of the disease, which could serve as targets for establishing the effectiveness of therapeutic and chemopreventive interventions, will improve the clinical management of PCa.

Cancer testis (CT) antigens are a unique class of tumor antigens specifically expressed in the normal testis and show aberrant expression in various malignancies (4). It has also been suggested that the aberrant expression of CT antigens in tumors may contribute to various malignant properties, such as immortality, migration, invasion and metastatic capacity (5). Therefore, CT antigens are being vigorously pursued as targets for therapeutic cancer vaccines (4).

As a new member of the CT antigen family, sperm-associated antigen 9 (SPAG9) has been reported to be involved in the c-Jun N-terminal kinase (JNK)-signaling module and to function as a scaffold protein for binding to JNKs, thus playing an important role in cell survival, proliferation, apoptosis and tumor development. SPAG9 was first suggested to be a potential target for immunotherapy in human epithelial ovarian cancer (6). Since then, SPAG9 overexpression has been demonstrated in various human cancers including renal, breast, thyroid, cervical, colon and lung carcinomas. Moreover, SPAG9-knockdown using siRNA was found to inhibit the tumor growth of cervical cancer (7). Importantly, SPAG9 expression was also reported to be associated with circulating anti-SPAG9 antibodies in early stage and in low grade breast cancer and cervical cancer patients (8,9), suggesting its potential application in the early detection of the disease. To date, the expression pattern and biological function of SPAG9 in PCa remain unknown.

To evaluate the function of SPAG9 in PCa, we examined the expression of SPAG9 in relation to clinicopathological features using tissue microarray (TMA) of PCa tissue samples. In addition, we further investigated the involvement of SPAG9 in PCa cell motility, invasion and angiogenesis.

Materials and methods

Patients and samples. A PCa TMA was purchased from Shanghai Xinchao Biotechnology (Shanghai, China). Tumors were staged according to the 2013 revised TNM system (10) as follows: 69 cases with stages I-II and 31 with III-IV. Pathologic grades of tumors were defined according to the WHO criteria (11) as follows: 58 cases of low grade (Gleason score <6) and 42 cases of high grade (Gleason score ≥7) PCa.

Immunohistochemistry of the TMA. Immunohistochemistry was performed according to the streptavidin-peroxidase (SP) method using a standard SP kit (Zhongshan Biotech, Beijing, China). The TMA slide was incubated with monoclonal mouse anti-SPAG9 antibody (1:100) (Cell Signaling Technology, Beverly, MA, USA) overnight at 4˚C, and diaminobenzidine (DAB; Zhongshan Biotech, Beijing, China) was used to produce a brown precipitate.

Evaluation of the immunostaining of SPAG9 expression. We assessed the immunostaining of SPAG9 by counting >500 cells from 5 random fields of each specimen under x400 magnification in the best-stained tumor area of each section as previously described (12). The SPAG9 immunoreactivity score (IRS) was defined as the percentage of stained prostate tissue cells. We considered a distinct cytoplasmic positive immunoreactivity when >10% of the cancer cells stained for the SPAG9 protein.

Cell lines and siRNA transfection. Human PCa cell lines were used as previously described (12). Cells were grown to 50% confluency before small interfering RNA (siRNA) transfection. Nonspecific control siRNA (Qiagen, Mississauga, ON, Canada) or SPAG9 siRNA (GenePharma, Shanghai, China) was transfected by siLentFect lipid reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Would healing assay. Wound-healing assay was employed as previously described (13). The migration of cells and closing of the scratch wound were observed, and microphotographs were captured every 4 h. Within each assay the experiments were performed in triplicate and the whole assay was repeated three times.

Migration and invasion assays. The migration and invasion assays were performed as described previously (14), with the exception of 10% serum-containing medium added to the bottom chamber. Serum-free medium was used in the top chamber in cells that were transfected with control siRNA or SPAG9 siRNA.

HUVEC growth and tube formation assay. The HUVEC growth and tube formation assays were used as described previously (14). The number of capillary-like tubes from three randomly chosen fields was counted and photographed under a Nikon inverted microscope (Japan).

ELISA for vascular endothelial growth factor (VEGF). The ELISA assay was employed as described previously (12). The VEGF concentration was determined using Quantikine ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Gelatin zymography. Gelatin zymography was carried out as previously described (12). Sixty hours after transfection, cells were incubated in serum-free medium for 24 h. The proteins in the conditioned medium were concentrated with Ultracel-30 k centrifugal filters (Millipore, Billerica, MA, USA). Fifty micrograms of the proteins was loaded for gelatin zymography.

Western blot analysis. Forty-eight hours after transfection, cells were harvested from the plates, and aliquots of the cell extracts were separated on SDS-polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and incubated overnight at 4˚C with the following antibodies: mouse anti-SPAG9, rabbit anti-MMP-2, -MMP-9, -c-Jun N-terminal kinase (JNK) and -p-JNK (Cell Signaling Technology) and mouse anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed four times with TBST (TBS containing 0.1% Tween-20), incubated simultaneously with IRDye® 800CW goat anti-rabbit and IRDye® 680CW goat anti-mouse secondary antibodies (1:15,000) (Li-Cor Biosciences, Lincoln, NE, USA) for 1 h at room temperature, followed by three washes with TBST and one wash with TBS. Immunoblots were imaged and the bands were quantified by densitometry using Odyssey Infrared imaging system software (Li-Cor).

Statistical analysis. Numerical data are expressed as means ± SD. Statistical differences between the mean values for the different groups were evaluated with Instat 5.0 (GraphPAD Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA). For the TMA, statistical analysis was performed using SPSS 11.5 software (SPSS). The associations between SPAG9 staining and the clinicopathological parameters of the PCa patients, including age, tumor size, tumor grade and TNM stage, were evaluated by the Chi-square test. The t-test was used for cell proliferation assays. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SPAG9 expression is overexpressed in human PCa tissues. We first determined whether SPAG9 expression is altered in human PCa. Representative images are shown in Fig. 1A. A significantly higher level of SPAG9 expression was noted in the PCa tumor tissues than that in the tumor adjacent normal prostate tissues ($P<0.01$, Fig. 1B). These findings suggest that SPAG9 is commonly expressed in PCa tissues but decreased or absent in human prostate tissues.

SPAG9 expression is correlated with tumor grade and TNM stage in human PCa. In all 100 PCa patients, the relationship between SPAG9 expression and pathologic and clinical

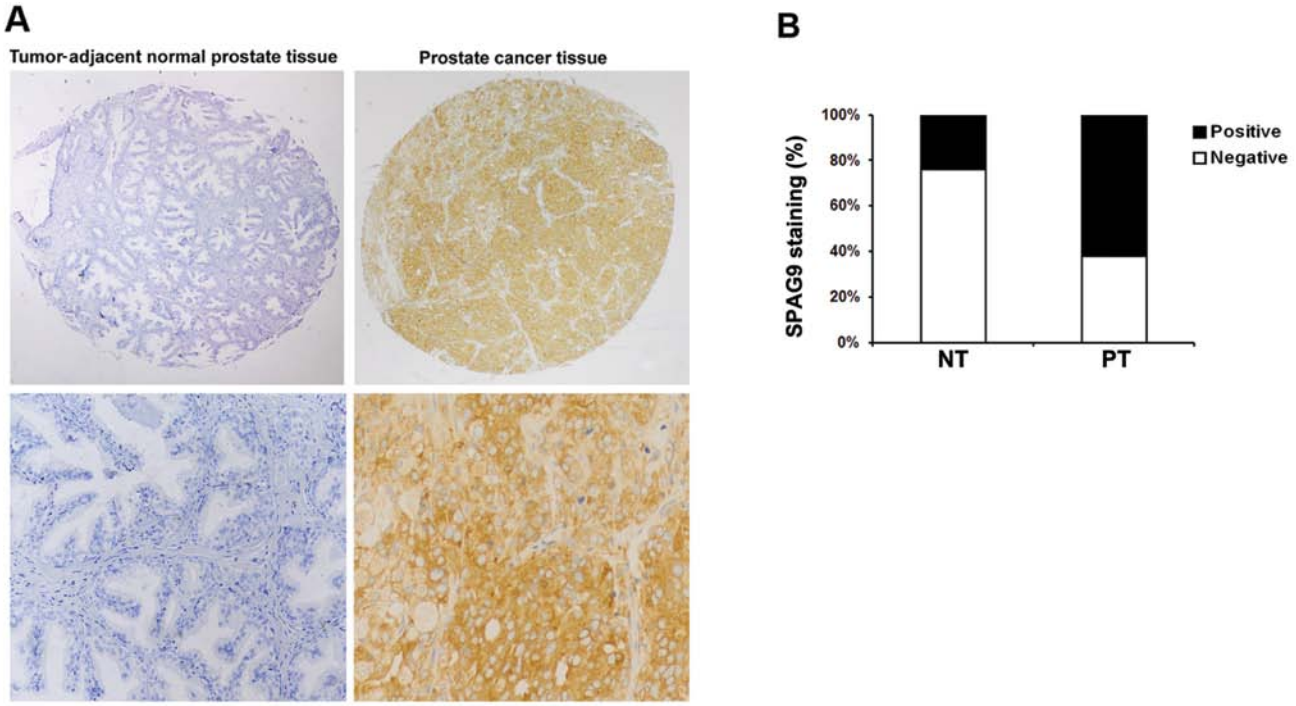

Figure 1. SPAG9 protein expression in tumor-adjacent normal prostate and prostate cancer tissues. (A) Representative immunohistochemical images were captured at a different magnification in tumor adjacent normal prostate and prostate cancer tissues (top panel x100, bottom panel x400). (B) Compared with that in the tumor-adjacent normal prostate tissues, the overall expression level of SPAG9 in the prostate cancer tissues was significantly higher (P<0.01, Chi-square test).

Table I. Patient characteristics and SPAG9 expression.

Variables	SPAG9 staining		Total	P-value[a]
	Negative n (%)	Positive n (%)		
Age (years)				0.161
≤60	17 (35.4)	31 (64.6)	48	
>60	26 (50.0)	26 (50.0)	52	
Tumor size (cm)				0.193
≤2.5	31 (64.6)	17 (35.4)	48	
>2.5	40 (76.9)	12 (23.1)	52	
TNM stage				<0.01
I	29 (67.4)	14 (32.6)	43	
II	14 (53.8)	12 (46.2)	26	
III	3 (30.0)	7 (70.0)	10	
IV	8 (38.1)	13 (61.8)	21	
Grade				<0.05
G1	21 (67.7)	10 (32.3)	31	
G2	15 (55.6)	12 (44.4)	27	
G3	9 (33.3)	18 (66.7)	27	
G4	7 (46.7)	8 (53.3)	15	

[a]P-values are obtained from Chi-square test.

features is shown in Table I. We found that SPAG9 expression was significantly correlated with tumor stage (comparing

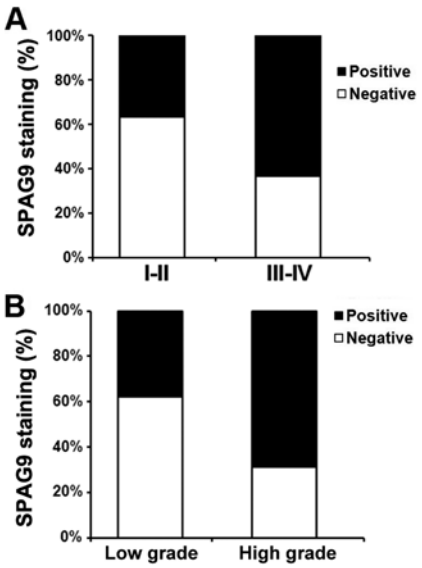

Figure 2. Correlation between SPAG9 expression and TMA stage and tumor grade in the PCa tissues. (A) Increased SPAG9 expression was correlated with TNM stage (P<0.01, Chi-square test, comparing I-II vs. III-IV). (B) Increased SPAG9 expression was correlated with tumor grade (P<0.05, Chi-square test, comparing G1-G2 vs. G3-G4).

I-II vs. III-IV) (P<0.01, Fig. 2A). In addition, SPAG9 staining was also markedly increased in high grade when compared with low stage disease (P<0.05, Fig. 2B). However, we did not find significant correlations between SPAG9 expression and other clinicopathological variables of the PCa patients, including age (P<0.161) and tumor size (P<0.193).

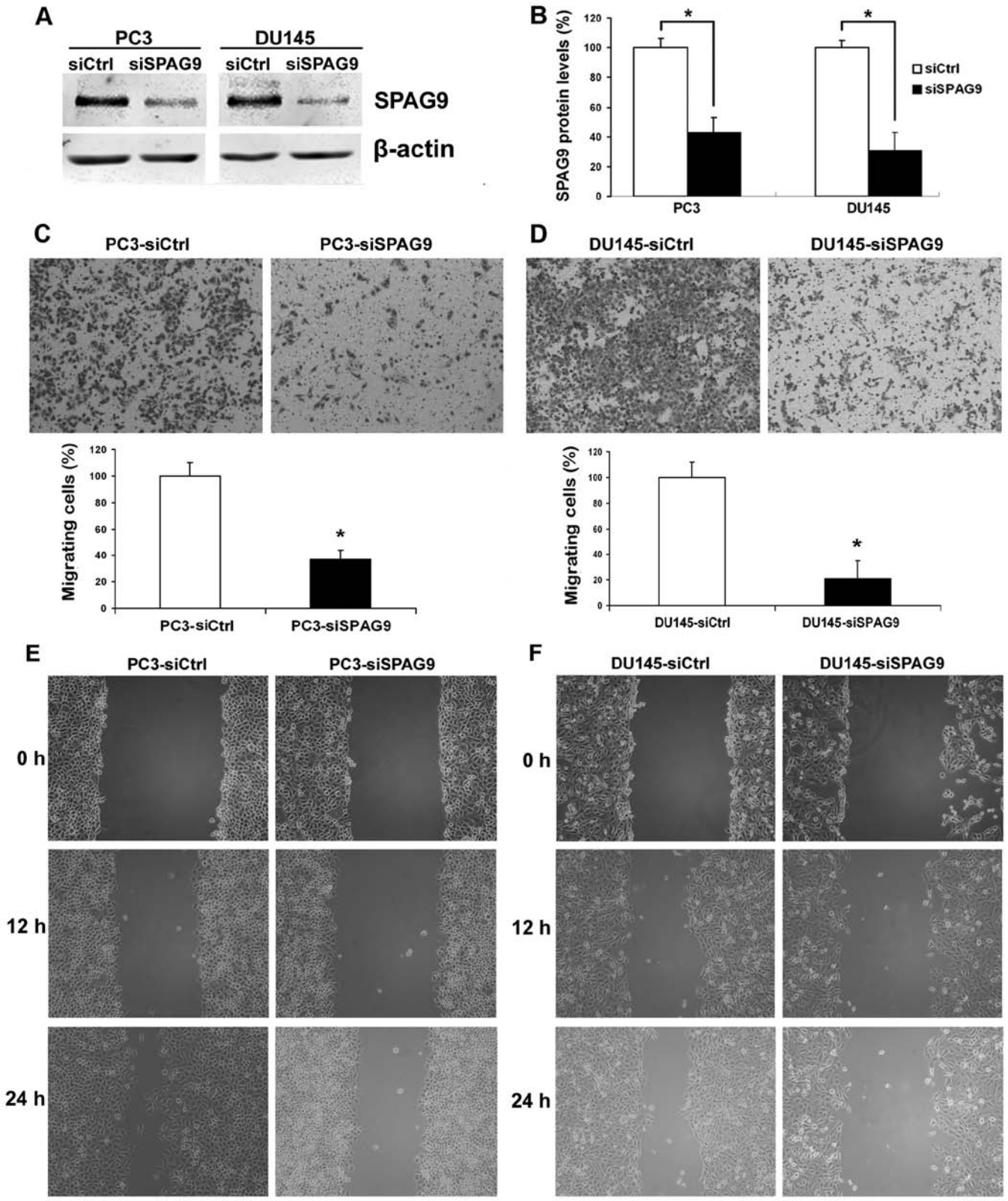

Figure 3. Effect of the reduction in SPAG9 expression on the abilities of cell motility *in vitro*. (A and B) Forty-eight hours after transfection, the expression of SPAG9 in the PC3 and DU145 cells was evaluated by western blotting. β-actin was used as an internal control. (C and D) Cell migration assay. Representative fields of migrating cells on the membrane (magnification, x200). Average percentage of migrating cells per field. (E and F) Wound healing assay revealed that there was a significant delay in wound closure after knockdown of SPAG9 expression when compared with the rate of wound closure in the control group.

Knockdown of SPAG9 by small interfering RNA (siRNA) in DU145 and PC3 cells inhibits cell motility and invasion. Since SPAG9 expression is related to the tumor grade and TNM stage of PCa, SPAG9 may play important roles in one or more steps of PCa metastasis. We first examined the effect of siSPAG9 on PCa cell motility. To validate whether SPAG9 functions in the regulation of cell motility, PCa cell lines DU145 and PC3 were used to examine changes in cellular phenotypes after SPAG9

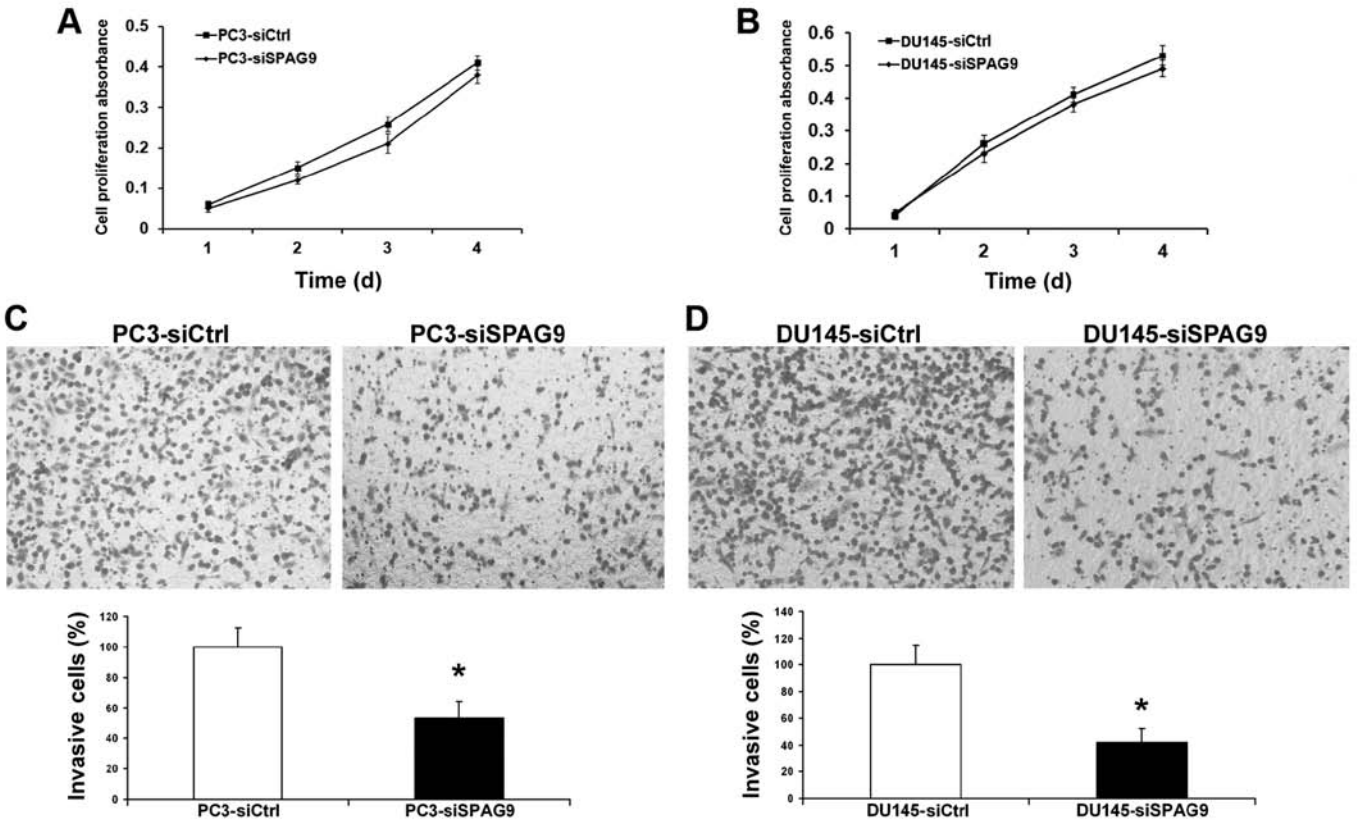

Figure 4. Reduction in SPAG9 expression suppresses cell invasion but not cell proliferation in prostate cancer cells. (A and B) CCK-8 cell proliferation assay was performed to detect prostate cancer cell proliferation. (C and D) Matrigel cell invasion assays. Representative images of the cells that invaded through the Matrigel following transfection with siSPAG9 or control siRNA. Representative histograms of the percentage of invaded tumor cells are displayed and the number of invaded tumor cells was quantified. *Significant difference from the controls (P<0.05, ANOVA).

knockdown by RNA interference. In these experiments, treatment with SPAG9 siRNA revealed ablation of SPAG9 protein expression (Fig. 3A and B). Therefore, the subsequent experiments were restricted to SPAG9 siRNA in all cellular motility experiments. In the cell migration assay, we found that transfection of SPAG9 siRNA in DU145 and PC3 cells decreased their ability to migrate through a Boyden chamber by 76 and 72%, respectively (Fig. 3C and D). In addition, in the wound healing assay, our data revealed that there was a significant delay in wound closure after treatment with SPAG9 siRNA (Fig. 3E and F).

Invasion is a crucial step in the progression of cancer metastasis. We assessed the capacity of PCa cells to invade through Matrigel, an artificial extracellular matrix (ECM), after transfection with control siRNA or SPAG9 siRNA. Knockdown of SPAG9 expression led to the inhibition of cell invasion by 78 and 55% (Fig. 4C and D), and histograms show that a significantly lower percentage of cells (P<0.05) passed through the filters coated with the artificial ECM, suggesting that the invasive potential of the SPAG9 siRNA-transfected prostate cancer cells was severely impaired. However, inhibition of SPAG9 had no effect on the proliferation of PCa cells (Fig. 4A and B).

Silencing of SPAG9 inhibits PCa cell angiogenesis in vitro. To further determine and study the functional role of SPAG9 in the angiogenic potential of human PCa cells, HUVEC growth and tube formation *in vitro* were investigated. The growth of HUVECs in conditioned medium from SPAG9 siRNA-transfected cells was inhibited by 48 and 51% compared with the corresponding controls (Fig. 5A and B). The average number of complete tubular structures formed by HUVECs was significantly decreased in the conditioned medium from the SPAG9-silenced DU145 and PC3 cells when compared with the control group (Fig. 5C and D).

Knockdown of SPAG9 suppresses MMP-2/MMP-9 expression and activities by upregulation of TIMP-1/TIMP-2 in PCa cells. Extensive data reveal that MMPs, in particular MMP-2 and MMP-9, play important roles in PCa cell invasion and metastasis. Thereby, western blot and gelatin zymography analyses were employed to determine MMP-2 and MMP-9 expression and activity in the siSPAG9-transfected cancer cells. Our data showed that MMP-2 and MMP-9 expression and activities were significantly suppressed after inhibition of SPAG9 expression in the DU145 and PC3 cells (Fig. 6A and B). The activity of MMPs is controlled by interaction with the TIMPs. We performed western blotting and the data revealed that TIMP-1 and TIMP-2 proteins were increased after silencing SPAG9 (Fig. 6B).

SPAG9 regulates VEGF secretion in PCa cells. VEGF is an important mitogen and survival factor for endothelial cells. In response to angiogenic stimulation, endothelial cells enter

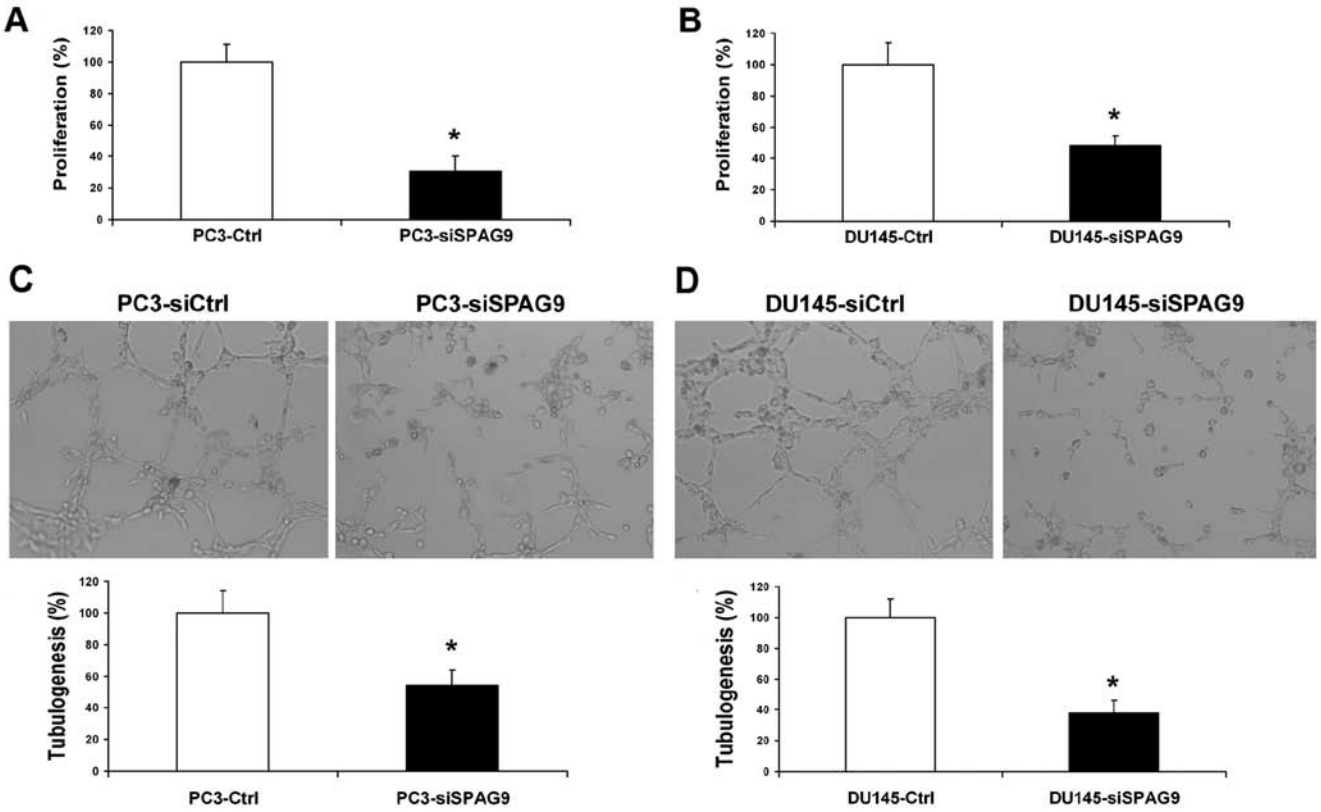

Figure 5. Inhibition of angiogenesis induced by SPAG9. (A and B) CCK-8 cell proliferation assay was performed to detect HUVEC proliferation. (C and D) Representative images were captured *in situ* for tube formation in the supernatant of PC3 and DU145 cells transduced with siRNA control and siSPAG9. All experiments were carried out in triplicate.

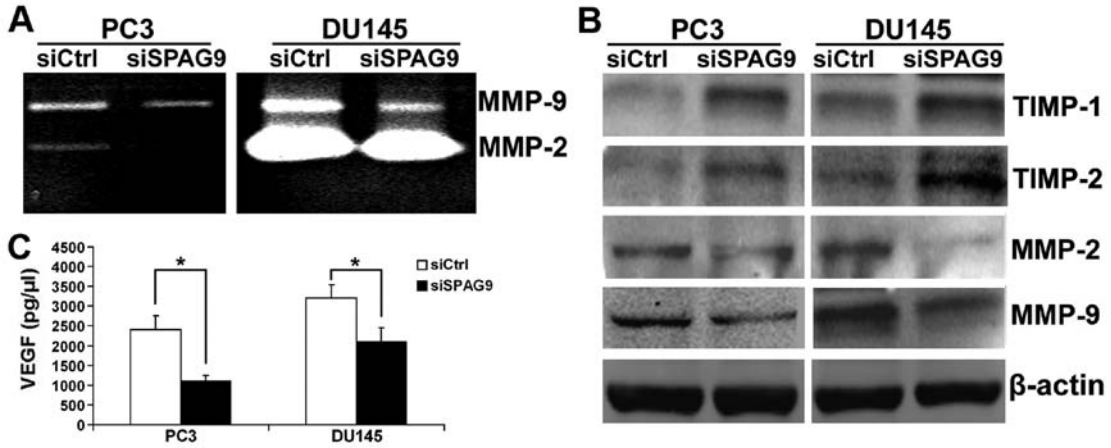

Figure 6. Target genes regulated by SPAG9. (A) The activity of MMP-2 and MMP-9 was evaluated by gelatin zymography. (B) Western blot analysis of relative protein levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and β-actin following silencing of SPAG9 and the control group for both PC3 and DU145 cell lines. (C) ELISA for the secretion of VEGF in PC3 and DU145 cells transduced with siSPAG9 and control siRNA. Data are shown as mean ± SD; $^{*}P<0.05$.

into an active proliferative state. To evaluate whether depletion of SPAG9 contributes to VEGF secretion in PCa cells, conditioned medium from DU145 and PC3 cells transfected with SPAG9 siRNA were used to measure the secretion of proangiogenic factor VEGF by ELISA analysis. As shown in Fig. 6C, a significant reduction in VEGF secretion was observed in the conditioned medium from DU145 and PC3 cells transfected with SPAG9 siRNA when compared with the control cells.

Discussion

SPAG9 is localized on the surface of sperm, and is only expressed in haploid germ cells during spermatogenesis (15). It may promote the process of sperm-egg fusion which is characterized by an increase in intracellular Ca^{2+} and pH[1] and the tyrosine phosphorylation of several proteins (16,17). Recently, the role of SPAG9 in cancer has been studied due to its high expression in cancer tissues. It is considered as a tumor

marker in several types of human cancers, such as breast cancer, thyroid cancer, colorectal cancer and renal cell carcinoma (6,8,9,18). In addition, SPAG9-knockdown was found to inhibit the tumor growth of renal cell carcinoma *in vivo* indicating its potential role in regulating tumor development and metastasis (18). These observations indicate that SPAG9 may play an important role in the tumorigenesis of human cancer. However, the relationship between SPAG9 and PCa has not yet been examined. To better understand the role of SPAG9 in PCa development, we used TMA technology and *in vitro* cell modeling to investigate whether SPAG9 has a function in the development of PCa. Our clinical results showed that SPAG9 expression was upregulated in PCa and was correlated with TNM stage and tumor grade. Our *in vitro* studies revealed that knockdown of SPAG9 by siRNA in PCa cells reduced the abilities for cell migration, invasion and angiogenesis.

It is generally accepted that surgical resection is the most powerful tool for improving patient prognosis when early diagnosis of PCa is successful (2). Unfortunately, most PCa patients are diagnosed late at a locally advanced stage (19). Thus, it is essential to predict the risk of recurrence in order to minimize the adverse effects and maximize the therapeutic effect of PCa treatment. However, among the available prognostic factors for PCa, one of the most important is the International Union Against Cancer TNM stage as determined by the depth of invasion, involvement of lymph nodes, and presence of distant metastasis (10). Apart from that, the Gleason grade is also considered to be highly associated with aggressiveness and progression of PCa; its accurate determination is crucial in deciding the best treatment for each patient (20). In the present study, we found that SPAG9 expression was increased in PCa tissues. Moreover, SPAG9 protein was significantly reduced in late stage and high tumor grade disease. Our clinical evidence clearly supports the notion that altered expression of SPAG9 contributes to PCa development and metastasis.

Metastasis is a multistep process that involves the detachment from the primary tumor mass, migration through the extracellular matrix (ECM) and colonization of surrounding sites. During this process, cell motility and invasion are essential for metastasis (21). In the present study, we found that SPAG9 knockdown suppressed PCa cell motility and invasive ability through wound healing and Transwell assays. One of the key factors in cancer invasion and metastasis is the degradation of ECM allowing cancer cells to invade and migrate. MMPs have been demonstrated to play important roles in this process (22). Among the MMP family, MMP-2 and MMP-9 are postulated to promote invasion and lymph node metastasis of PCa cells (23). To determine how SPAG9 siRNA inhibits PCa cell motility and invasion, we focused on elucidating the effect of SPAG9 siRNA on MMP-2 and MMP-9 activities. Here, we found that knockdown of SPAG9 expression significantly inhibited the bioactivities of MMP-2 and MMP-9 in DU145 and PC3 cells. The activity of MMPs is controlled by interaction with the TIMPs (24). There has been evidence that the imbalance between MMPs and TIMPs is responsible for cancer metastasis (25). Chen *et al* showed that MMP-9-inhibiting activity of RUNX3 is due to the direct interaction of RUNX3 with the TIMP-1 promoter (26). Furthermore, it has been suggested that SPAG9 can modulate MMP-9 transcription independent of TIMP-1 and TIMP-2 (27). However, in our study, we noted that knockdown of SPAG9 simultaneously upregulated TIMP-1 and TIMP-2 expression, which are the negative regulators of MMP-2 and MMP-9. The results showed that reduced cell motility and invasive abilities were due to the low activity of MMP-2 and MMP-9 and the high level of TIMP-1 and TIMP-2 protein expression after SPAG9 silencing. Therefore, the balance of TIMP-1/-2 and MMP-2/-9 was disrupted by SPAG9 siRNA, which eventually resulted in the reduced cell motility and invasive abilities of the PCa cells.

Angiogenesis is essential for the growth and metastasis of solid tumors, and inhibition of angiogenesis is emerging as a promising strategy for cancer treatment (28). Yet, the effect of SPAG9 on angiogenesis has not been reported. In our study, we found that transfection of SPAG9 siRNA reduced the capacity of the PCa cell supernatant to stimulate tube formation and proliferation of human endothelial cells compared with this capacity in the control cells, suggesting that knockdown of SPAG9 expression significantly impaired the angiogenic potential of PCa cells *in vitro*. However, its molecular bases are unclear. Among several potential mechanisms, influences on the expression of various angiogenic molecules have been shown (29,30). Obviously, one of the most essential angiogenic factors is VEGF, which exerts its mitogenic activity particularly on endothelial cells. VEGF has been identified as a key mediator of tumor angiogenesis involved in the development of tumor blood supply in the progression of solid tumors (31). VEGF expression is suppressed by tumor-suppressor genes p53, p75 and von Hippel-Lindau, which is most likely due to formation of complexes with Sp1 and inhibition of its binding to and transcriptional activation of the VEGF promoter (32,33). We, thus, investigated whether SPAG9 regulates VEGF activity *in vitro*. ELISA analysis showed that VEGF secretion was decreased by reduction of SPAG9. These results suggest that the decrease in SPAG9 suppresses blood-vessel formation by regulating VEGF activity. It has been reported that SPAG9 participates in JNK pathway activation, and JNK signaling could induce c-Jun phosphorylation at the VEGF promoter resulting in its activation (18,34). Thus, it is possible that JNK signaling is involved in the angiogenesis promoting function of SPAG9.

The present study provides evidence that SPAG9 is expressed at high levels in PCa tissues. Increased SPAG9 expression is significantly associated with prostate progression. To the best of our knowledge, this is the first report showing that CT protein is involved in promoting PCa cell motility, invasion and angiogenesis. Sliencing of SPAG9 leads to the inhibition of PCa cell motility and invasion due to the imbalance between MMP-2/MMP-9 and TIMP-1/TIMP-2. In addition, we further demonstrated that knockdown of SPAG9 reduced blood-vessel formation and proliferation of HUVECs by decreasing the secretion and expression of VEGF. Thus, these findings identify SPAG9 as a promising novel diagnostic and therapeutic target for PCa.

Acknowledgements

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