Sperm-Associated Antigen 9 Is Associated With Tumor Growth, Migration, and Invasion in Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) represents one of the most resistant tumors to radiation and chemotherapy. Current therapies for RCC patients are inefficient due to the lack of diagnostic and therapeutic markers. Our recent studies have suggested an association of sperm-associated antigen 9 (SPAG9) with ovarian carcinomas. In the present study, we investigated the clinical relevance of SPAG9 in RCC patients. RT-PCR analysis showed expression of *SPAG9* transcript in RCC tissues and RCC cell lines. *In situ* RNA hybridization and immunohistochemistry analyses confirmed the expression of SPAG9 in 88% of cancer patients, suggesting that SPAG9 participates in renal cancer. In addition, immunoblotting and ELISA analyses revealed a humoral immune response against SPAG9 in the sera of RCC patients but not in healthy individuals. Consistent with the clinical findings, knockdown of SPAG9 expression in RCC cells with specific siRNA significantly reduced cell growth and colony formation. Using *in vitro* wound healing and Matrigel invasion assays, we found that cell migration and invasive ability were also significantly inhibited. Furthermore, *in vivo* xenograft studies in nude mice revealed that administration of a SPAG9 siRNA plasmid significantly inhibited tumor growth. In conclusion, SPAG9 expression is associated with clinicopathologic features of tumors, suggesting that SPAG9 could contribute to the early spread of cancer. These results indicate that SPAG9 may have a role in tumor development and metastasis and thus could serve as a novel target for early detection and treatment of RCC. [Cancer Res 2008;68(20):8240–8]

Introduction

Renal cell carcinoma (RCC) is the most common malignant renal tumor accounting for ~2% to 3% of all malignancies. The occurrence of RCC has increased in recent years; worldwide, nearly 100,000 patients die of disease every year (1). At diagnosis, around 30% of the patients have metastasis disease and even a higher percentage of patients develop metachronous metastases after nephrectomy. RCC is resistant to conventional therapies such as radiation, hormone, and chemotherapy (2). In an advanced stage of RCC, systemic therapies are largely ineffective in affecting disease response or patient survival. RCC tumors are characterized based on histologic features as clear cell (80%), papillary (10%), chromophobe (<5%), or granular, spindle, or cyst-associated carcinomas (5–15%). Each of these histologic subtypes exhibits unique clinical behavior, with clear cell and granular types tending to show more aggressive clinical phenotypes (3). At present, surgical resection is the most effective treatment for localized RCC tumors. Therefore, novel diagnostic, prognostic, and therapeutic markers are urgently needed for this disease.

A unique class of testis proteins known as cancer testis (CT) antigens has been found to be expressed in various cancers (4, 5). Although no defined biological function of CT antigens is known thus far, it has been proposed that CT antigens are involved in signaling, transcription, translation, and chromosomal recombination (6, 7). It has also been proposed that the aberrant expression of CT antigens in tumor may contribute to various malignant properties such as immortality, migration, invasion, and metastatic capacity (6). Furthermore, tumors expressing CT antigens are able to elicit specific humoral immune responses to these antigens (5, 8). The CT antigens are, therefore, the focus of intense research for their utility as biomarkers and their potential use in immunotherapeutic strategies (4, 5).

Recently, we characterized *SPAG9* gene—a new member of CT antigen family—to be associated with ovarian malignancies (8). Sperm-associated antigen 9 (SPAG9) is involved in c-Jun-NH_2-kinase (JNK)-signaling module (9, 10) and functions as a scaffolding protein for binding to JNKs that play an important regulatory role in several physiologic processes, including cell survival, proliferation, apoptosis, and tumor development (10, 11). The scaffold proteins act by modulating the signaling strength of their cognate mitogen-activated protein kinase (MAPK) module by regulating the signal amplitude and duration (12). Recently, JLP (JNK-associated leucine zipper protein), a mammalian JNK scaffold protein, have been identified that bring MAPKs and their target transcription factors together for the execution of specific signaling pathways (13). Two different splice variants of JLP, namely JIP4 (14) and SPAG9 (10), which share common 3′ exons, but differ in the 5′ exon region, have different expression in various tissues. Although JIP4 has been shown to interact with JNKs, it also seems to be involved in the activation of p38 MAPK module involving the MAP2Ks, MKK3, and MKK6 (14). However, our studies have shown that SPAG9 interacts with JNK subgroup of MAPKs and does not interact with p38 MAPK (10). Furthermore, JLP is reported to play an important role in endothelial differentiation (15). Considering that JNK is involved in several distinct cellular responses, it is possible that JIP proteins may play a multifaceted role during oncogenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Recent studies on gene expression microarray analysis of breast tumors (16), cervix carcinoma cells (17), dermatofibrosarcoma protuberans (18), and esophageal adenocarcinoma (19) have reported *SPAG9* gene expression along with other important much studied genes. We have also shown earlier that SPAG9 is expressed in different histotypes of epithelial ovarian cancer (EOC) tissues and ovarian cancer cell lines (8). In the present study, we report the expression of SPAG9 in RCC patients and provide evidence for its role in cell migration, invasion, and tumor growth.

Materials and Methods

Tissue specimens. In the present study, a total of 52 RCC specimens (32 males, 20 females; age range, 23–72 y) were obtained from surgical tumor resections in accordance with the local Ethics Committee. The adjacent noncancerous tissue specimens were also collected from these patients. It is important to point out that these tissues cannot be regarded as healthy normal. The histotypes, stages, and grades are detailed in Table 1.

Cell lines, transient transfection, and preparation of tumor cell suspensions. Four RCC cell lines from American Type Culture Collection and three established RCC cell lines from patients with primary RCC of clear cell type were used and cultured following standard procedures. Transient transfections were performed using Lipofectamine reagent (Invitrogen Life Technologies) according to the manufacturer's instruction as described earlier (20). Established cell lines were obtained after tumor cell suspension preparation as described in Supplementary Materials and Methods.

Reverse transcription-PCR analysis. RNA was extracted from RCC cells and frozen RCC tumor tissues. Reverse transcription-PCR was performed as detailed in Supplementary Materials and Methods. The PCR product was cloned into TOPO vector (Invitrogen; Life technologies) for confirming DNA sequence.

SPAG9 antibodies, immunofluorescence microscopy, and fluorescence-activated cell sorter analysis. Antibodies against SPAG9 were raised as detailed in Supplementary Materials and Methods. SPAG9 expression in RCC cancer cells was verified by indirect immunofluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis as described earlier (see Supplementary Materials and Methods; ref. 8).

Synthesis of riboprobes and *in situ* RNA hybridization. RCC tissue specimens that showed *SPAG9* expression by RT-PCR were probed using antisense riboprobe (experimental) and sense riboprobe (control) following the protocols supplied with Digoxigenin RNA Labeling kit as described earlier (8).

Immunohistochemistry. Paraffin-embedded sections of 52 RCC tissue specimens were analyzed for the localization of SPAG9 protein using anti-SPAG9 antibody as described earlier (8). The immunohistochemistry (IHC) procedure is outlined in Supplementary Materials and Methods.

RNA interference cloning and plasmid sequences. The BS/U6 vector was used to construct two SPAG9-specific siRNA (SPAG9 siRNA and SPAG9 siRNA-I) and control siRNA (scrambled SPAG9) as described previously (20). The cloning strategy and protocol is described in Supplementary Materials and Methods. The efficiency of the constructs was tested through transfection into RCC cell lines and Western blot analysis of the total cell lysates with the SPAG9 antibodies.

Cell growth and colony formation assay. For plasmid transfection, cells were seeded at a density of $\sim 5 \times 10^5$ in a 35-mm dish plate and cultured for 16 h. When 60% culture confluence was reached, cells were transfected with 6 μg of SPAG9 siRNA or 6 μg of control siRNA plasmids for 3 h, after which the culture medium was replaced with complete medium. To determine cell growth, cell number was counted daily with hemocytometer.

For colony formation assay, a total of 4×10^2 to 10^3 cells transfected with indicated amount of SPAG9 siRNA or control siRNA plasmids were seeded into 6-well plates. Ten days posttransfection, the cells were fixed with 5% glutaraldehyde in PBS and stained with 5% crystal violet. The colonies were manually counted.

Table 1. Association of SPAG9 expression and humoral response with clinicopathologic status

Pathologic and clinical features	RT-PCR/IHC, positive/tested (%)		ELISA/Western blot, positive/tested (%)	
All tumors	46/52 (88)		40/52 (77)	
Tumor stage				
Early (T1 & T2)	21/22 (95)	*	20/22 (91)	†
Late (T3 & T4)	25/30 (83)		20/30 (67)	
Grade				
Low (G1 & G2)	15/16 (94)	*	15/16 (94)	†
High (G3 & G4)	31/36 (86)		25/36 (69)	
Lymph node metastasis (N stage)				
Absence (N_0)	38/40 (95)	†	32/40 (80)	*
Presence (N_1)	8/12 (67)		8/12 (67)	
Metastasis				
Absence (M0)	40/42 (95)	†	34/42 (80)	†
Presence (M1)	6/10 (60)		6/10 (60)	
Histotypes				
Clear cell	30/33		26/33	
Papillary	8/10		7/10	
Chromophobe	7/8		6/8	
Mucinous tubular & spindle cell	1/1		1/1	

*Pearson χ^2 method: statistically nonsignificant.
† $P < 0.05$ statistically significant association.

Cell invasion, migration, and wound healing assay. To study the role of SPAG9 in tumor biology, *in vitro* cell invasion, migration, and wound healing assays were performed (see Supplementary Materials and Methods).

Human cancer xenograft model and plasmid treatment. A total of 12 athymic nude mice [Nii:NIH (S; nu/nu)] at ages 5 wk were used. Briefly, Caki-1 cells were harvested, washed with PBS, and resuspended in normal culture medium, and then 1×10^7 cells were s.c. injected in the upper portion of the hind limb. Seven days after the tumor cell xenografting, the mice were randomly divided into two groups of six mice each. When a tumor reached a volume of 50 to 100 mm^3, the mouse was subjected to systemic treatment with the following treatments: control siRNA or SPAG9 siRNA plasmids. Plasmids (50 μg) in 200 μL of PBS were injected into the mouse tail vein followed by a booster injection with 25 μg of plasmid twice a week for 6 wk. The tumors were monitored for total of 49 d. One week after the last treatment, animals of each treatment group were sacrificed and their tumors were excised for immunohistochemical analyses as described in Supplementary Materials and Methods.

ELISA and Western blot analysis. ELISA was performed using recombinant SPAG9 protein according to the method described previously (10). The detail procedure for ELISA is described in Supplementary Materials and Methods. Serial dilutions of RCC patient's serum were used, and results for serum dilution (1:100) were accepted with estimated ELISA titers above the mean +2 SD of the healthy sera. SPAG9 blotting was performed as described in Supplementary Materials and Methods.

Statistical analysis. The Pearson's χ^2 test, unpaired and paired Student's *t* test, and one-way ANOVA data analyses were performed using SPSS 15.0 statistical software package (SPSS, Inc.). Results are expressed as the mean ± SE. A *P* value of <0.05 was considered statistically significant.

Results

Expression of *SPAG9* mRNA in RCC specimens and cell lines. The expression of *SPAG9* was investigated in a panel of surgically resected RCC specimens. Our RT-PCR analysis revealed that 46 of

52 (88%) RCC patients were positive for *SPAG9* mRNA. No *SPAG9* expression was detected in adjacent noncancerous renal tissue. The size of the PCR product in tumor was same as in the RCC cancer cells and in testis (Fig. 1*A*), and DNA sequence analysis showed no mutations. The relationship between *SPAG9* mRNA expression and pathologic and clinical features is shown in Table 1. The *SPAG9* expression was detected in 21 of 22 (95%) early-stage (T_1 and T_2) and in 25 of 30 (83%) late-stage (T_3 and T_4) categories of RCC tissues. Similarly, *SPAG9* expression was detected in 15 of 16 (94%) low-grade (G1 and G2) and 31 of 36 (86%) high-grade (G3 and G4) categories of RCC (Supplementary Table S1). In various RCC histotypes, *SPAG9* mRNA was observed in 30 of 33 (91%) clear cell, in 8 of 10 (80%) papillary, in 7 of 8 (88%) chromophobe, and in 1 of 1 (100%) mucinous tubular and spindle cell carcinoma. *SPAG9* expression was independent of tumor stage or grade, indicating no correlation between tumor stages ($P < 0.183$) or tumor grades ($P < 0.392$). Furthermore, significant association was found between *SPAG9* expression and lymph node metastasis (N stage; $P < 0.007$). It is important to mention that 67% N stage–positive RCC patients revealed *SPAG9* expression. In addition, significant association was also found between *SPAG9* expression and metastasis ($P < 0.009$). The data indicated that 6 of 10 (60%) metastatic RCC (*M1*) revealed *SPAG9* expression. These results suggest that *SPAG9* participate in cancer growth and invasion.

SPAG9 protein is expressed in RCC cell lines. The expression of endogenous SPAG9 protein in RCC cancer cells, A704, ACHN, Caki-1, Caki-2, NII-AKS395, NII-AKS413, and NII-AKS414 was confirmed and observed in virtually all cells using indirect immunofluorescence. Representative immunostaining as shown in Fig. 1*B* revealed strong SPAG9 immunoreactivity in all the RCC

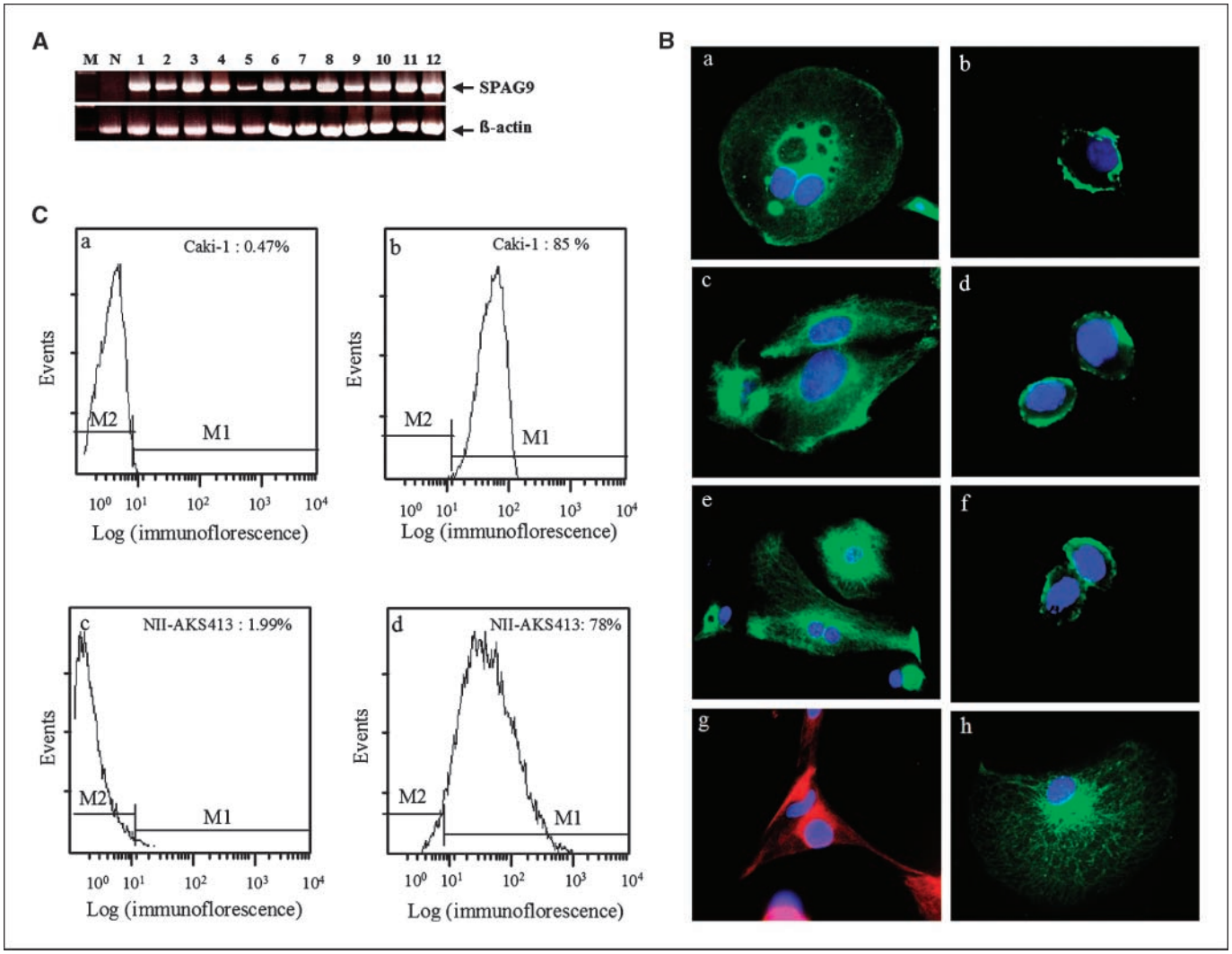

Figure 1. Expression of *SPAG9* in RCC tissues and cell lines. *A,* RT-PCR analysis of mRNA expression of *SPAG9* showing specific 1.2 kb product from clear cell (*lane 1*), papillary (*lane 2*), chromophobe (*lane 3*), and mucinous tubular and spindle cell carcinoma (*lane 4*) tissues; four RCC cell lines: A704 (*lane 5*), ACHN (*lane 6*), Caki-1 (*lane 7*), and Caki-2 (*lane 8*); three established RCC cell lines: NII-AKS395 (lane 9), NII-AKS413 (lane 10), NII-AKS414 (*lane 11*), and testis (*lane 12*; positive control); and no SPAG9 expression was detected in adjacent noncancerous renal tissue *M*, molecular size marker; *N,* negative control. β-*actin* expression was used as an internal control. *B,* indirect immunofluorescence of SPAG9 protein expression in fixed/permeabilized (*a, c*, and *e*) and live RCC cells (*b, d*, and *f*); Caki-1 (*a* and *b*), NII-AKS413 (*c* and *d*), and NII-AKS414 cells (*e* and *f*). EMA (*g*) and cytokeratin 7 (*h*) expression is shown in NII-AKS413 cells as epithelial markers. *C,* FACS analysis of SPAG9 expression in Caki-1 and NII-AKS413. The percentile fluorescence (displacement of fluorescence on *X*-axis) of control Caki-1 (*a*) and NII-AKS413 (*c*) cells stained with secondary antibody only. Percentile fluorescence of SPAG9 expressing Caki-1 (*b*) and NII-AKS 413 (*d*) cells probed with anti-SPAG9 antibodies as indicated by M1 (*M1,* the gating of fluorescence-positive cells; *M2,* the gating of fluorescence-negative cells). All these experiments were done at least thrice. Representative results are shown. Side scatter versus forward scatter gate analysis was carried out for all the experiments to avoid debris.

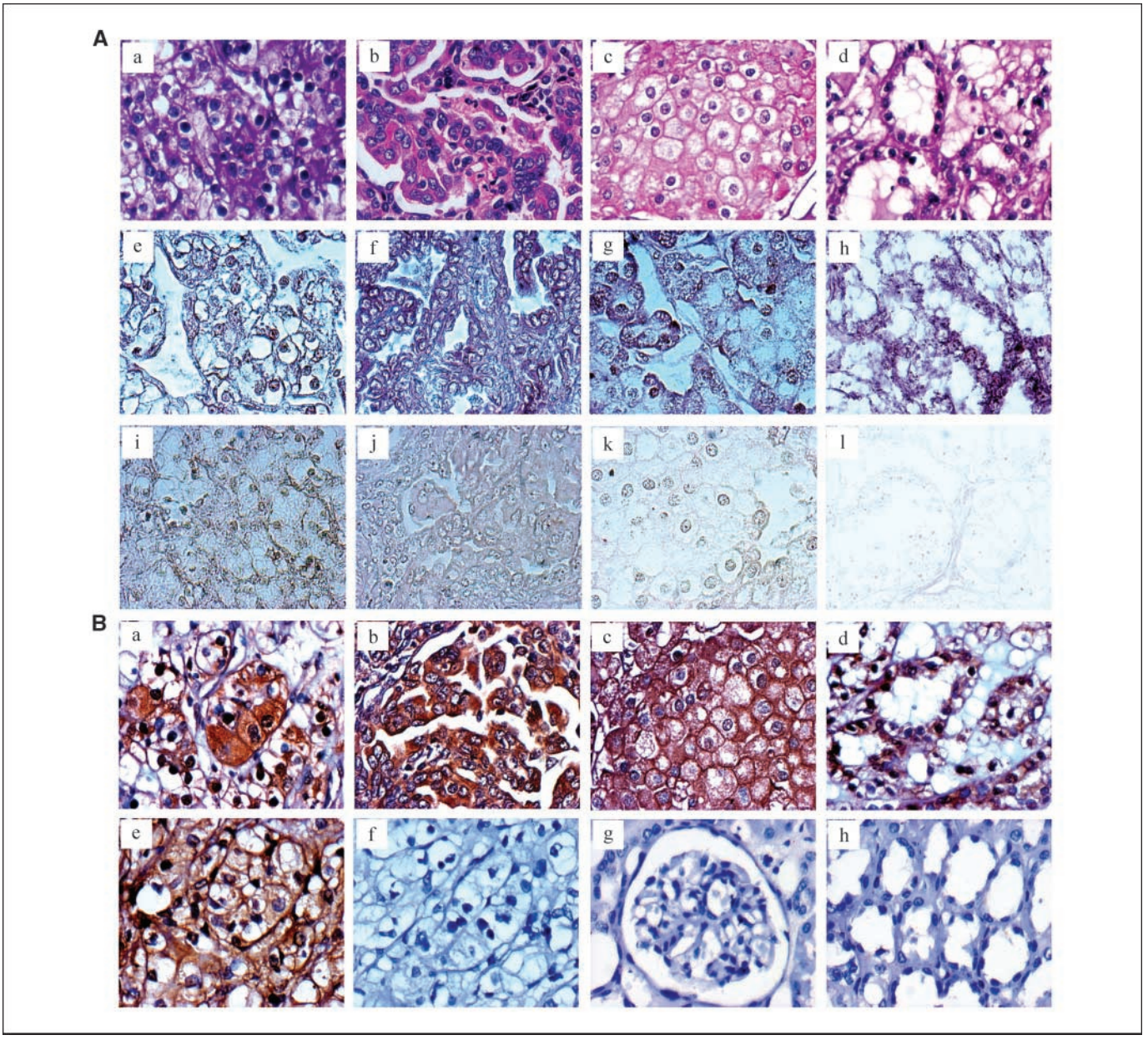

Figure 2. *A, in situ* RNA hybridization showing *SPAG9* mRNA expression in different histotypes: H&E-stained sections (*a–d*), antisense riboprobe (*e–h*), and sense riboprobe (*i–l*), respectively, in clear cell (*a, e*, and *i*), papillary (*b, f*, and *j*) chromophobe (*c, g*, and *k*) and mucinous tubular and spindle cell carcinoma (*d, h*, and *l*). SPAG9 antisense riboprobe showed a strong chocolate brown signal in all histotypes (*e–h*), whereas sense riboprobe as expected failed to show any signal (*i–l*). *B,* immunohistochemical staining of SPAG9 in different RCC histotypes using anti-SPAG9 antibodies: (*a*) clear cell, (*b*) papillary, (*c*) chromophobe, and (*d*) mucinous tubular and spindle cell carcinoma. *e,* clear cell RCC probed with anti-SPAG9 antibody without recombinant SPAG9 protein. *f,* preincubation of anti-SPAG9 antibody with recombinant SPAG9 protein (15 μg/mL) completely eliminated immunoreactivity against SPAG9 in matched RCC serial tissue section. Immunostaining of adjacent noncancerous kidney glomerulus (*g*) and distal convoluted tubules (*h*) revealed no reactivity. All images: original magnification, ×400.

cancer cells with predominant expression in cytoplasm in fixed and permeabilized cells (Fig. 1*B, a, c*, and *e*), whereas a surface localization of SPAG9 was observed in live cells (Fig. 1*B, b, d*, and *f*). All the three established RCC cell lines NII-AKS395, NII-AKS413, and NII-AKS414 revealed distinct reactivity with epithelial markers (EMA and cytokeratin 7; Fig. 1*B, g* and *h*). The surface localization of SPAG9 was reconfirmed by flow cytometry in Caki-1 and NII-AKS413 cells as shown in Fig. 1*C*.

Analysis of SPAG9 expression by *in situ* RNA hybridization and IHC. *In situ* hybridization studies with DIG-labeled riboprobes showed *SPAG9* mRNA expression in different RCC histotypes (Fig. 2*A*). Antisense riboprobe hybridization confirmed the expression of *SPAG9* mRNA in all histotypes (Fig. 2*A, e–h*) as against sense riboprobe control (Fig. 2*A, i–l*). Furthermore, the expression of SPAG9 protein was investigated by IHC in RCC and adjacent noncancerous kidney tissues (Fig. 2*B*). Examination of immunostained section revealed a positive diffused cytoplasmic SPAG9 expression (>10% of cancer cells were stained) in various histotypes of RCC (Fig. 2*B, a–e*). Furthermore, neutralization experiment was performed by including recombinant SPAG9 protein in the incubation with primary antibody, which resulted in loss of immunoreactivity with SPAG9 (Fig. 2*B, f*). However, no

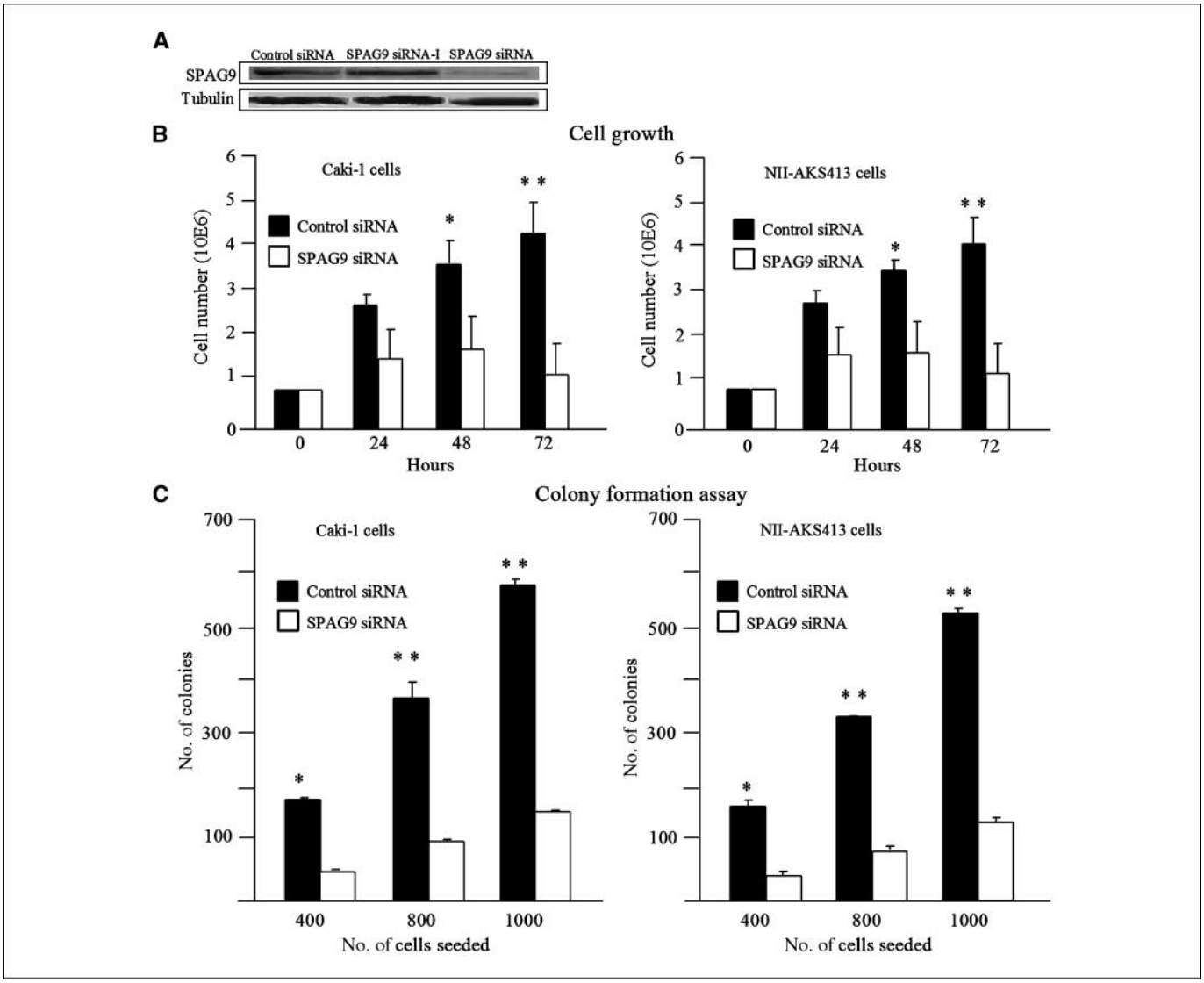

Figure 3. Effect of SPAG9 expression on cell growth and colony formation. *A,* Western blot analysis of SPAG9 in NII-AKS413 cells in the presence of control siRNA, SPAG9 siRNA-I, and SPAG9 siRNA. SPAG9 siRNA–treated cells revealed ablation of SPAG9 protein as compared to control siRNA or SPAG9 siRNA-I–treated cells. Protein loading was normalized using anti-tubulin antibodies (Sigma). *B,* SPAG9 knockdown inhibits cell growth. For each experiment, $\sim 5 \times 10^5$ of Caki-1 or NII-AKS413 cells were seeded in a 35-mm dish plate, transfected with control siRNA or SPAG9 siRNA, and cultured for 72 h. Viable cell numbers were determined every 24 h by staining with 0.4% trypan blue. *C,* quantitative determination of colony numbers in control siRNA or SPAG9 siRNA–treated cells. After transfection with control siRNA or SPAG9 siRNA, various numbers of Caki-1 or NII-AKS413 cells (4×10^2–10^3) were seeded in a 6-well plate and incubated for 10 d to allow colony formation. Cell colonies were counted by 5% crystal violet staining. $n = 3$ independent experiments; each experiment was performed in triplicate. *Points,* mean; *bars,* SE. The difference in growth ratio and colony formation between control siRNA or SPAG9 siRNA transfectant cells achieved statistical significance.
*, $P < 0.01$; **, $P < 0.001$.

positive staining of SPAG9 was observed in adjacent noncancerous kidney tissues (Fig. 2*B, g* and *h*).

siRNA-mediated knockdown of SPAG9 in Caki-1 and NII-AKS413 cells inhibits cell growth and colony formation. To validate whether SPAG9 functions in cell growth regulation, RCC cell lines were used to examine changes in cellular phenotypes after SPAG9 knockdown by RNA interference. In these experiments, treatment with SPAG9 siRNA revealed ablation of SPAG9 protein expression (Fig. 3*A*), although there was always some residual expression of SPAG9. Therefore, the subsequent experiments were restricted to SPAG9 siRNA in all cellular motility experiments. The treatment of SPAG9 siRNA resulted in a gradual and significant decrease in cell growth in Caki-1 and NII-AKS413 cell lines tested (Fig. 3*B*). After 72 h, cell growth was reduced to 30% and 33%, respectively, for Caki-1 and NII-AKS413. Similarly, cellular colony formation was significantly suppressed by SPAG9 siRNA (Fig. 3*C*). The numbers of cell colonies of SPAG9 knockdown cells were significantly reduced for various cell numbers seeded for Caki-1 (range of 28–30% for 400–1,000 cells) and NII-AKS413 (range of 29–32% for 400–1,000 cells) cells. Suppression of SPAG9 expression apparently reduces cell growth and colony-forming ability in RCC cells.

SPAG9 knockdown suppresses cell invasion, migration, and wound healing properties *in vitro*. Our observation raised another question of whether substantial amounts of endogenous expressed SPAG9 plays any functional role(s) in cell motility and invasion. To determine if SPAG9 siRNA could reduce protumorogenic cellular behaviors associated with SPAG9 expression, we first

determined the effect of decreased SPAG9 expression on tumor cell invasion. We measured the capacity of RCC cells to invade through Matrigel, an artificial extracellular matrix, after transfection with a control siRNA or SPAG9 siRNA. Decreased SPAG9 expression led to the inhibition of invasion by 78% (Fig. 4*A*), and histogram shows that a significantly lower number of cells (P = 0.000) passed through coated filters with an artificial extracellular matrix, suggesting that the invasive potential of SPAG9 siRNA transfected RCC cells was severely impaired. Subsequently, transwell migration assay was carried using 8-μm insert. Migration assay revealed 77% inhibition in motility potential of SPAG9 siRNA–transfected RCC cells (Fig. 4*B*). The histogram shows that a significantly lower number of cells (P = 0.000) migrated through inserts. In addition, in wound healing assay, similar results were obtained. Figure 4*C* shows the decreased motility of SPAG9 siRNA–transfected RCC cells. The closing of the scratch wound in SPAG9 siRNA–transfected cells was not complete even after 48 hours, whereas cells transfected with control siRNA as well as untreated cells (data not shown) successfully closed the entire wound within 24 hours.

Growth inhibition of Caki-1 cell xenografts with SPAG9 siRNA. To investigate the effect of SPAG9 siRNA treatment on tumor growth *in vivo*, we established a xenograft tumor in nude mice. After injection of Caki-1 cells, SPAG9 siRNA or control siRNA were administered into 6 animals per treatment group and continuously monitored for 49 days. Figure 5*A* shows the average tumor volume over the total 49 days of study between these two groups. Tumors in mice given SPAG9 siRNA had sustained a significant growth arrest compared with the controls. A representative photograph showing tumor growth in control siRNA and SPAG9 siRNA–treated mice (Fig. 5*B*). On average, SPAG9 siRNA treatment decreased tumor growth by 60% at day 36 (P < 0.03) and by 70% at day 49 (P < 0.001).

To examine whether the suppression of tumor growth is associated with the effect of injected SPAG9 siRNA, the xenograft

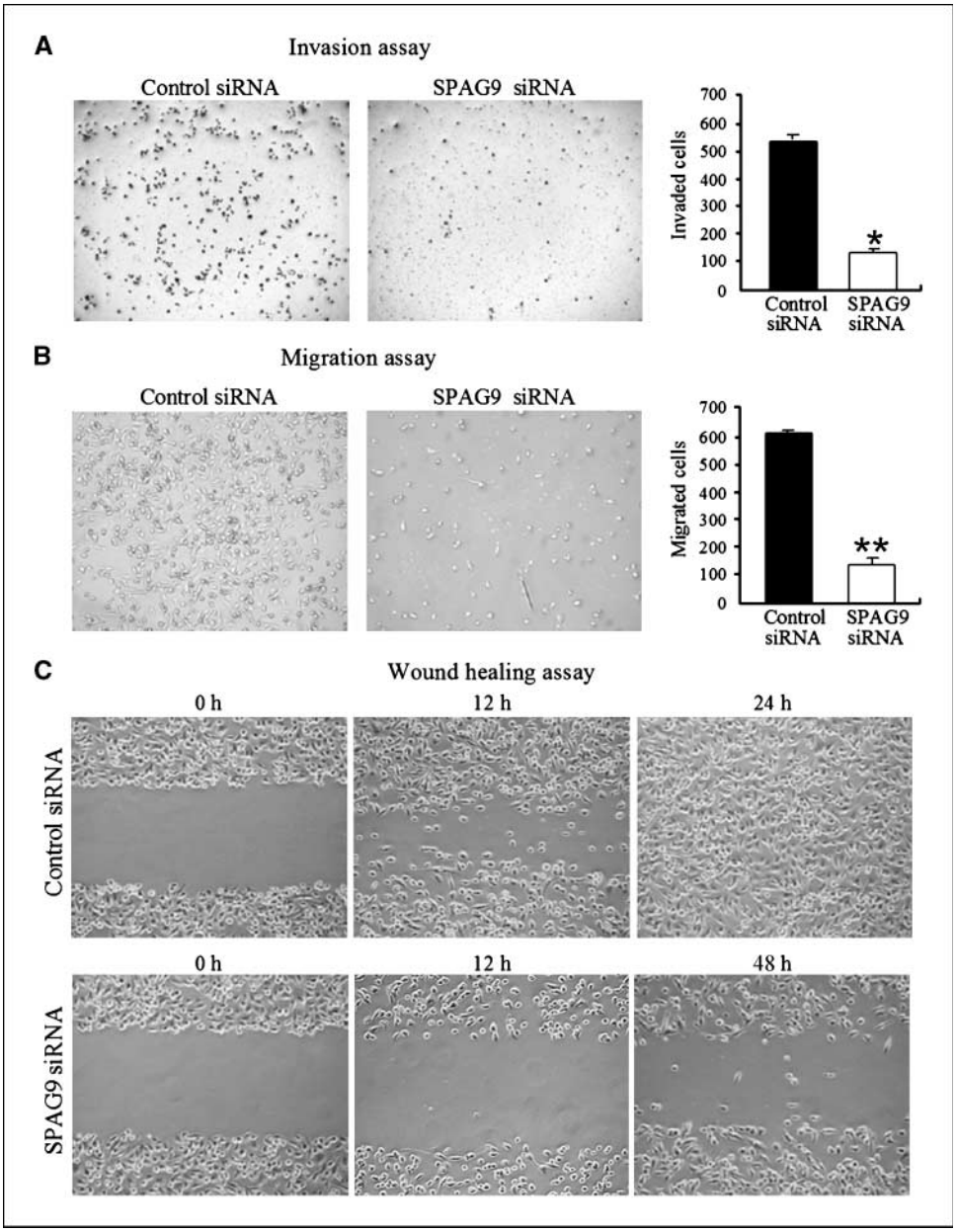

Figure 4. Role of SPAG9 in RCC tumor cell motility, invasion, and migration. *A* to *C,* invasion, migration, and wound healing assays after blocking SPAG9 expression with SPAG9 siRNA. *A,* the results of the Matrigel invasion assay: representative images show the cells that invaded through the Matrigel when transfected with control siRNA or SPAG9 siRNA. Representative histograph of invaded tumor cells is displayed and number of invaded tumor cells quantified. *, P = 0.000, control siRNA compared with SPAG9 siRNA. *B,* the results of the Transwell migration assay: representative images show the cells that migrated through 8-μm pore inserts when transfected with control siRNA or SPAG9 siRNA. Representative histograph of migrated tumor cells is displayed and numbers of migrated cells quantified. **, P = 0.000, control siRNA compared with SPAG9 siRNA. *C,* wound healing assay shows that closing of scratch wound in SPAG9 siRNA–transfected cells was not complete even after 48 h, whereas cells transfected with control siRNA successfully closed the scratch wound within 24 h.

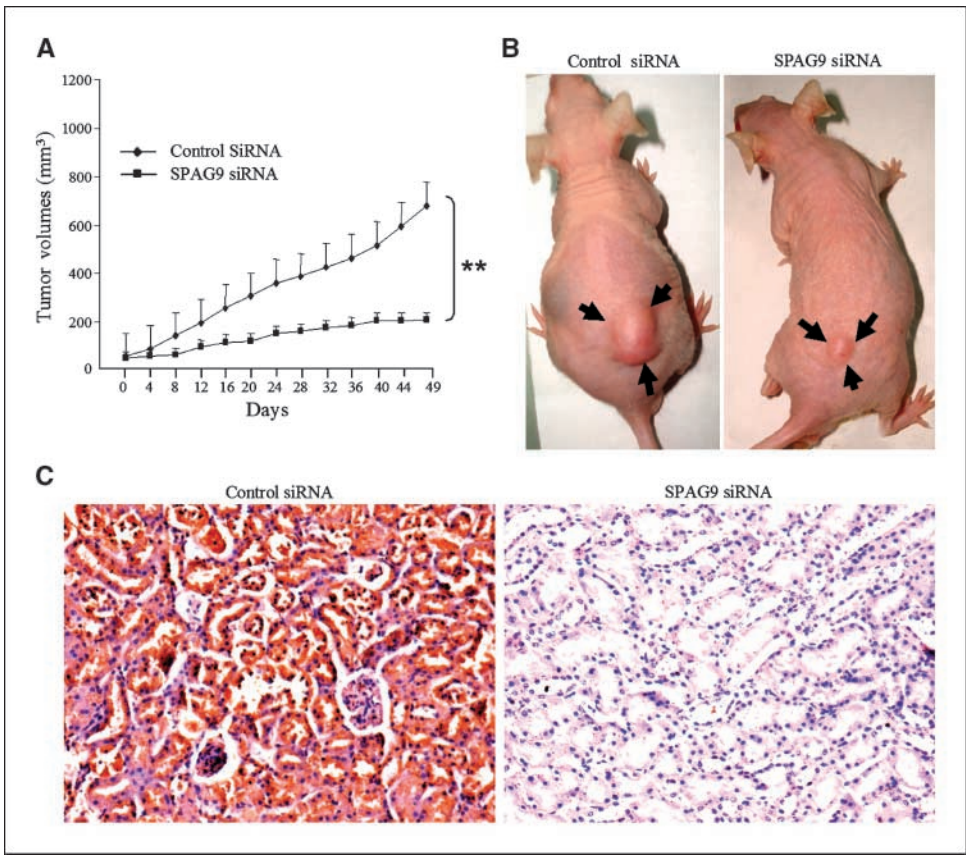

Figure 5. SPAG9 knockdown inhibits growth in tumor xenograft. *A*, the mice were s.c. injected with 1×10^7 Caki-1 cells. When tumor reached volume of 50 to 100 mm^3, the mouse was subjected to systemic treatment with control siRNA or SPAG9 siRNA plasmids. Fifty micrograms of either control siRNA or SPAG9 siRNA plasmids in a total of 200 μL PBS solution were injected i.v., followed by a booster injection with 25 μg of plasmid twice a week for 6 wk. The tumors were monitored for total of 49 d. Tumor diameter was measured with a caliper every 4 d, and tumor volume was calculated by the formula $V = \pi/6 \times D_l \times D_s^2$, where V is volume, D_l is the largest diameter, and D_s is the smallest diameter. Mean tumor diameters and their 95% confidence intervals were calculated. The tumor volume was significantly greater at 49 d in mice given control siRNA compared with those given with experimental SPAG9 siRNA (**, $P < 0.001$). *B,* representative mice bearing tumors (*arrows*) treated with control siRNA compared with SPAG9 siRNA. *C,* 1 wk after the last treatment, tumors were removed and subjected to immunohistochemical analysis. Control siRNA–injected tumor revealed strong immunoreactivity against SPAG9 protein (reactivity shown as brown color), whereas no or very weak reactivity was observed in SPAG9 siRNA–injected tumor. After immunostaining, the sections were counterstained with hematoxylin (*blue color*).

tumors were dissected to examine SPAG9 expression by immunohistochemical analysis. As shown in the Fig. 5*C*, the SPAG9 protein was knockdown in SPAG9 siRNA–injected xenograft compared with high SPAG9 expression in the control siRNA–injected tumors. These results further strengthen the significant effect of SPAG9 suppression on RCC growth and suggest that SPAG9 may be a molecular target for treatments aimed at decreasing oncogenesis.

Humoral response against SPAG9 in RCC patients. We tested the sera of RCC patients for the presence of circulating anti-SPAG9 antibody using ELISA method. Seventy-seven percent of RCC patient's sera showed IgG antibodies directed at SPAG9 protein irrespective of disease stages, grades, and histotypes (Supplementary Fig. S1*A*; Table 1). It is interesting to note that higher number of RCC patients with early stages (91%) and low grades (94%) revealed circulating anti-SPAG9 antibodies in their sera compared with late-stage (67%) and high-grade (69%) RCC patients, suggesting that the occurrence of antibodies was not a feature of advanced stage disease. Furthermore, statistical analysis revealed that there was significant association between circulating anti-SPAG9 antibodies and T stage ($P < 0.040$), tumor grade ($P < 0.053$), and metastasis ($P < 0.009$). The humoral response in various stages and grades are detailed in Supplementary Table S1.

Furthermore, we examined the immunologic identification of circulating anti-SPAG9 antibody present in the sera of cancer patients by Western blotting as shown in Supplementary Fig. S1*B*. Seventy-seven percent of RCC patient's sera exhibited immunoreactivity toward the purified SPAG9 protein irrespective of malignant histotypes as shown in representative Western blot (Supplementary Fig. S1*B, lane 3–6*), whereas no such immunoreactivity for SPAG9 protein was observed in normal sera (Supplementary Fig. S1*B, lane 11–12*). Furthermore, the specificity of SPAG9 antibody from patient's sera was evaluated by carrying out neutralization experiment by including recombinant SPAG9 (15 μg/mL) in the incubation with the patient's serum, which resulted in loss of immunoreactivity with SPAG9 protein (Supplementary Fig. S1*B, lane 7–10*).

Discussion

Numerous candidate RCC biomarkers have been identified to date by "ome"-based technologies, cDNA microarrays (21), and tissue microarrays (22). However, for the vast majority of these genes, neither the expression pattern of the protein product, nor its localization and function in the tumor tissues, has been investigated. An important issue in tumorigenesis is development of distant metastasis. In this regard, identification of specific genetic markers that are associated with tumor aggressiveness may prove to be powerful new biomarkers to assess the progression of disease. Cell migration is a critical feature of numerous physiologic and pathologic phenomena, including development, wound repair, angiogenesis, and metastasis (23). In this study, we have evaluated the clinical relevance and the role of SPAG9 in RCC. SPAG9 is widely expressed in all the renal cancer cell lines tested. SPAG9 was observed to be expressed in RCC, and its expression level was correlated with the characteristics of the tumors. As the invasiveness of cancer cells is dependent on migratory and invasive properties, we investigated whether SPAG9 expression in RCC cell lines will promote the migration and invasion of RCC cells. Small interfering RNA–mediated knockdown of SPAG9 expression in RCC cell reduced significantly cell migration and invasion. The

association of SPAG9 with clinical T stage (Table 1) may be explained on a cellular level by role of SPAG9 in the regulation of growth and colony formation (Fig. 3). SPAG9 correlation with N stage and metastasis (Table 1) corresponds on the cellular level to its participation of SPAG9 in the regulation of cell migration and invasion (Fig. 4). The fact that knockdown of SPAG9 significantly inhibits xenograft tumor growth suggests that it may function in RCC carcinogenesis (Fig. 5). In addition, immunoblotting and ELISA analyses revealed a humoral response against SPAG9 protein in RCC patients and not in healthy donors. Furthermore, sequence analysis of the RT-PCR products from various RCC histotypes revealed homologous nucleotide sequence as *SPAG9* cDNA and did not show any mutation within *SPAG9* cDNA. Therefore, the humoral responses were directed against endogenously expressed SPAG9 protein. These findings further support that the *SPAG9* is not only expressed at the mRNA level but is also translated to its protein as evident from IHC analysis of various RCC histotypes. Because RCC represents a disease encompassing several histopathologic subtypes (3), it is important to note that our results seem applicable to patients diagnosed with the most prevalent form of RCC that is clear cell as well as more rare forms of the disease, papillary, chromophobe, and mucinous tubular and spindle cell carcinoma. The key finding of this study is that SPAG9 expression in RCC tumors is associated with cell migration, invasion, and cellular growth.

A number of RCC associated antigens have been defined via cellular, humoral, or *in situ* immunity; however, no prominent CT antigen expression has been reported thus far. RCC CT genes are among the least studied. Recently, we characterized an evolutionarily conserved *SPAG9* gene, a new member of CT antigen family (8) expressed in haploid spermatid cells during spermatogenesis in testis (9) and in EOC patients (8). CT antigens can be divided based on genes transcribed from X-chromosome (CT-X antigens) or other than X-chromosomes (non–X CT antigens; ref. 6). Because *SPAG9* gene is transcribed from chromosome 17 and is expressed in spermatids, it may be classified in the category of non–X CT antigen. The present investigation reports the predominant *SPAG9* expression in RCC patients, which is interesting when compared with other known potential CT antigens. A well-characterized CT antigen *NY-ESO-1* mRNA expression was not detected in 39 RCC specimens analyzed (24). Another CT antigen *MAGE-2* expression was only found in 1 of 39 RCC tumor specimens but not in any RCC cell line analyzed (24). However, CT antigens *PRAME* and *RAGE-1* mRNA were reported in 40% and 21% of RCC specimens, respectively. In contrast, *SPAG9* mRNA and protein expression was observed in majority of RCC tissues analyzed irrespective of disease stages, grades, and histotypes compared with 76% of RCC tissues analyzed for MAGE-3 (25). This is one of the important criteria toward identifying tumor-specific protein targets for immunotherapy and development of cancer biomarker.

At present, no ideal noninvasive tumor markers with high sensitivity and specificity currently exist for early detection, monitoring, or prognostic prediction of RCC. Our demonstration of *in vivo* immunogenicity of SPAG9 in RCC patients with SPAG9-expressing tumor is an important lead toward developing a noninvasive tumor marker. Moreover, this is consistent with the known immunogenicity of SPAG9 protein. Recently, we have shown that SPAG9 is highly immunogenic *in vivo* in EOC patients (8). In addition, our earlier immunogenicity studies in rodents and nonhuman primate showed the generation of antibodies against SPAG9 (26, 27). In this context, it is relevant to mention here that a similar humoral response against SPAG9 was observed in the sera of RCC patients with 77% sample exhibiting circulating anti-SPAG9 antibody, suggesting its strong immunogenicity. When compared with other sperm protein, it is important to note that a well-characterized sperm protein synaptonemal complex protein (SCP-1), only 1 of 31 RCC patients (3%) had serum antibody response to recombinant SCP-1 protein (28). Here, we showed a significant association of SPAG9 immune response of RCC patients (Table 1) with early stages (T_1 and T_2; $P < 0.040$) and low grades (G1 and G2; $P < 0.053$). This is an important finding where we show a specific humoral response against SPAG9 in RCC patients with early stages and low grades of RCC, suggesting its important role in early diagnostics of RCC malignancies for surgical resection.

MAPKs regulate critical signaling pathways involved in cell growth, differentiation, and apoptosis. Our earlier MAPK interaction studies showed that SPAG9 functions as a scaffolding protein exhibiting higher binding affinity to JNK2 and JNK3 compared with JNK1 (10). The interaction is important in view of the pleiotropic end points of JNK signaling pathways leading to cell proliferation, differentiation, apoptosis, immune cell function, and embryonic morphogenesis (29). Although the functional consequences of interaction between JNKs and scaffolding proteins are not fully understood, they are likely to be important for the regulation of JNK signaling pathways toward specific physiologic events. For example, IB1/JIP1 interaction facilitates the signal transduction in pancreatic β-cell lines mediated by other proteins. IB1/JIP-1 interacts with JNK through the JNK binding domain (JBD), a domain able to prevent apoptosis of pancreatic β-cell lines induced by IL-1β and thus act as a crucial regulator of survival in insulin-secreting cells (30). Similarly, SPAG9 interacts with JNKs through its JBD, which exhibits a significant sequence identity to JBD of JIP1, JIP2, and JIP3. Our earlier studies have shown that a SPAG9 mutant that lacks JBD (SPAG9ΔLZΔT) also fails to interact with JNK pathway, suggesting that JBD of SPAG9 is involved in JNK interaction (10). Thus far, various studies have revealed the functional roles of scaffold proteins with which these proteins accelerate the kinetics of signaling by the respective MAPK signaling modules (31). However, defining the spatiotemporal mechanisms by which these scaffolding proteins finely regulate the signaling modules in different aspects of cell growth and oncogenesis needs to be investigated. The mechanism underlying SPAG9 association with carcinogenesis is still unclear. Perhaps, SPAG9 expression indicates the need of cancer cells where overexpression of this gene product may disrupt the stability or kinetics of MAPK signaling pathways leading to facilitation of cell invasion and migration and warrants further studies.

We were intrigued by our *in situ* RNA hybridization study, which showed that *SPAG9* gene was expressed in all histotypes. Several mechanisms have been suggested as possible causes of an aberrant CT antigen gene expression program in carcinomas. In various cancers, it has been shown that CT-X gene expression is coordinated through hypomethylation of their promoters (32, 33). However, non–X CT gene expression has not been well studied thus far. Although, the function of CT-X and non–X CT genes are not well-defined, it has been documented that their gene products are involved in various pathways involving cellular differentiation, signaling, transcription, translation, and chromosomal recombination. SPAG9 protein, which acts as a scaffolding protein in MAPK signaling module during cell-cell interactions (10), is a non–X CT antigen. The mechanism for SPAG9 expression in RCC is still remains to be determined.

In recent years, efforts have been made to correlate p53 malfunctioning with human cancer. However, RCC belongs to the type of tumors with low incidence of p53 mutations (34). In this regard, a study suggested that the wild-type p53 expression uses a unique tissue-specific mechanism of p53 repression, which may be the cause of RCC tumors (35). Furthermore, yet another study strongly suggested that loss of p53 function is a critical event in the evolution of the RCC (36). Interestingly, a recent study in breast cancer patients, reported that p53–down-regulated genes are expressed at lower levels in most of the p53-wild-type tumors relative to the p53 mutant tumors (37). The data further indicated the higher expression of p53-repressed genes, which included antiapoptotic (*BCL2A1* and *TNFAIP8*) and *SPAG9* gene in p53-mutants tumors. This was the first report where dysregulation of target genes was, in each case, significantly linked to the development of distant metastasis within 5 years of diagnosis of breast cancer patients (37). Therefore, *SPAG9* expression characteristics in RCC tissues could be potentially used as a molecular gauge of tumor aggressiveness and progression of disease and warrants further investigation.

Understanding the mechanisms involved in oncogenesis has wide-ranging implications for targeting the treatment of cancer. In particular, treatment directed at molecules such as SPAG9 that are overexpressed in tumor tissues may minimize cytotoxic effects on normal cells. Furthermore, the potential use of SPAG9 as a biomarker in tissue biopsies and monitoring patients after surgery should be explored. To the best of our knowledge, this is the first report showing CT protein involved in promoting RCC cell migration, invasion, and cellular growth. Moreover, SPAG9 as a novel gene product involved in tumor cell motility and invasion could represent a molecular target for RCC and could eventually provide new leads for improved therapeutic approaches against RCC. In summary, our study provides a foundation for further investigation into the manipulation of SPAG9 in the treatment of RCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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