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SPAG9 promotes endometrial carcinoma cell invasion through regulation of genes related to the epithelial-mesenchymal transition

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Summary

Objective: To investigate the impact of sperm-associated antigen 9 (SPAG9) on proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT) in endometrial cancer. *Materials and Methods:* The present authors' previous study demonstrated that SPAG9 is highly expressed in endometrial cancer tissues. They analyzed correlation between the levels of SPAG9 and mRNA of EMT-related genes in endometrial carcinoma tissue by using quantitative real-time PCR. They induced EMT process in ECC endometrial cancer cell lines by TGF-beta1 treatment and spheroids formation assay, and analyzed SPAG9 expression as well as correlation with EMT-related genes.In addition, they performed SPAG9 gene silencing in KLE and ECC endometrial cancer cells and evaluated the expression of genes involved in EMT, using real time PCR and Western blot analysis. Cell proliferation, colony formation, and transwell assays were employed to evaluate the functional role of SPAG9 in endometrial cancer. *Results:* The results showed that SPAG9 expression was positively correlated with Slug and N-cadherin (NcaD) in human endometrial cancer tissues. The expression of SPAG9 in ECC cells with TGF-β1 treatment and spheroids formation was increased, which was correlated with EMT-related genes. SPAG9 knockdown significantly inhibited cell growth and proliferation and reduced the motility and invasion of endometrial cancer cells. These phenotypes may partly be explained by decreased expression of EMT-related genes, including Twist, Slug, and Vimentin, after SPAG9 depletion. *Conclusions:* SPAG9 may be required for cellular invasion and migration in endometrial cancer through regulation of EMT-related genes.

Key words: Sperm-associated antigen 9 (SPAG9); Endometrial carcinoma; Epithelial–mesenchymal transition.

Introduction

Endometrial cancer is the most common gynecologic malignancy in Western countries, with an incidence of 1,566-1,948 per 100,000 women annually nation wide [1]. The American Cancer Society estimates there will be 49,560 new cases and 8,190 deaths from endometrial cancer in 2013 [2]. It is known that women with certain histological subtypes, high-grade lesions, or deep uterine invasion are at highest risk of recurrence, but the molecular mechanisms that drive endometrial cancer warrant further investigated.

Sperm-associated antigen 9 (SPAG9) belongs to the testis cancer antigen family. SPAG9 is involved in c-Jun-NH2-kinase (JNK) signaling module and functions as a scaffolding protein for binding to JNKs, thus playing an important role in cell survival, proliferation, apoptosis, and tumor development [3]. Recent studies show that SPAG9 is overexpressed in various human cancers including renal, breast, thyroid, cervical, and colon carcinomas as well as lung cancer and astrocytoma [4-8]. SPAG9 represents a promising candidate for cancer therapy owing to its expression pattern and immunogenicity in cancers of different origins [9].

The molecular pathway by which SPAG9 promotes proliferation and invasion may be related to the JNK pathway. This pathway is known to be associated with cancer metastasis and in particular the epithelial–mesenchymal transition (EMT) [10, 11]. The process of EMT is a well-described process whereby epithelial cells lose their polarity and acquire a migratory phenotype, subsequently activating a mesenchymal-like gene expression program [12]. An oncogenic type 3 EMT enables epithelial cells to acquire invasive mesenchymal phenotype characteristics, which are essential in tumor metastasis [13]. Some studies have shown that EMT process could be induced by stimulation of transforming growth factor-beta 1 or spheroids formation assay [14,15].

The present authors' previous study demonstrated that SPAG9 was highly expressed in endometrial cancer, with a high humoral immune response [16]. SPAG9 may serve as a new type of endometrial cancer marker for early detection, diagnosis, and treatment. However, the role of SPAG9 in endometrial cancer development and metastasis and related mechanism has not yet been studied. Furthermore, both SPAG9 and EMT are associated with the JNK path-

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Table 1. — *Sequences of primers and shRNAs.*

Primer sequence	Forward	Reverse
SPAG9	GGTCAAGTCATCAGCCCACA	CATCACCCATTCGGAAGTCG
β-Acitn	CTGGGACGACTGGAGAAAA	AAGGAAGGCTGGAAGAGTGC
TWIST	GGACAGTGATTCCCAGACGG	GTGGCTGATTGGCACGACC
ECAD	GAACGCATTGCCACATACACTC	GCACCTTCCATGACAGACCC
NCAD	ATCCTACTGGACGGTTCG	TTGGCTAATGGCACTTGA
SLUG	CCGCAAACATGTCTATGAGG	ATTGGGTAGTTCGGCATTG
shRNA sequence		
SPAG9ShRNA1	GCTCGAGATGGATTGCTTACA	
SPAG9ShRNA2	GGAGCAGATTTACTAGGAATG	
Control shRNA	GTTCTCCGAACGTGTCACGT	

way, and EMT has been extensively described in other types of cancer but poorly studied in endometrial cancer. The present authors hypothesized that SPAG9 may promote endometrial carcinoma cell migration and invasion through regulation of EMT-related genes such as Slug, Twist, N-cadherin (Ncad), E-cadherin (Ecad), and Vimentin.

Materials and Methods

The study protocol was approved by an institutional review board (IRB) of the Shandong Provincial Hospital affiliated to Shandong University.

Immunohistochemistry was performed on endometrial cancer tissue samples obtained from 47 patients as described previously [16]. Follow-up on survival outcome was performed by telephone. Survival analysis of patients with SPAG9 expression and those without SPAG9 expression was performed. Fresh tumor tissues from 17 patients with endometriod endometrial cancer were collected for RNA extraction and RT-PCR.

KLE and ECC cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 mg/ml of penicillin, and 100 mg/ml of streptomycin at 37°C in a humidified environment with 5% CO2 in air.

For TGF-β1 treatment, 1×10^5 ECC cells were seeded onto each well of a 24-well plate and cultured in RPMI-1640 medium supplemented with 10% FBS overnight. The cells were treated with recombinant human TGF-β1 in medium at a concentration of ten ng/ml. For the control, an equal volume of double distilled water was added. Cells were harvested after 72 hours of treatment for extraction of RNA and protein.

1×10^5 ECC cells were seeded onto each well of an ultra low cluster 24-well plate and cultured for three days. ECC spheroids generated on ultra low cluster plate can be utilized for downstream analyses.

KLE cells were transfected with plasmids expressing SPAG9 shRNA or a negative control shRNA (Table 1) for stable knockdown, following the manufacturer's protocol. Briefly, 1×10^5 KLE cells were plated on 24-well plate and maintained in RPMI-1640 containing 10% FBS. Cells were transfected with 0.8 μg negative control plasmid or 0.8 μg shSPAG9 plasmid using 1.6 μl lipofectamine TM 2000 transfection reagent/well. Twenty-four hours after transfection, the cells were placed under geneticin selection at 800 ng/μl for five days and 200 ng/ml for 30 days. Individual colonies were removed by trypsinization and expanded and verified by real time PCR and Western blot.

ECC cells were transfected with plasmids expressing SPAG9-shRNA1 or shRNA2 or a negative control shRNA (Table 1) following the manufacturer's protocol. Briefly, 1×10^5 ECC cells were plated on 24-well plate and maintained in RPMI-1640 containing 10% FBS. Cells were transfected with 0.8 μg negative control plasmid or 0.8μg shSPAG9 plasmid using 1.6 μl lipofectamine TM 2000 transfection reagent/well. After 48 or 72 hours in a 37°C incubator with 5% CO2,the cells were used for RNA or protein extraction.

Total RNA was isolated using Trizol according to the manufacturer's instructions. Real time PCR was carried out using a light cycler 480 system. Primers used in this study are shown in Table 1. Reactions were carried out for one cycle at 94°C for five minutes; 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; one cycle at 94°C for one minute, and one cycle at 55°C for 30 seconds. The transcript level of each specific gene was normalized to human β-actin amplification.

Protein extraction and Western blot analysis

Cells were harvested by centrifugation and washed with phosphate buffered solution (PBS). Preparation of cell extractions, electrophoresis, transfer and blocking were performed as described previously [16]. Then the membranes were incubated overnight at 4 degrees with anti-human SPAG9, Ncad, Ecad, Vimentin, Slug, and β-actin primary antibodies in 5% milk. The immunoblots were developed with an enhanced chemiluminescence system (ECL) and exposed by chemiluminescence imaging analyzer LAS-4000 mini imaging. β-actin was used as the loading control.

For the cell counting kit-8 (CCK-8) assay, control shRNA and SPAG9 shRNA stably transfected cells were plated in 96-well plates in a medium containing 10% FBS at 3,000 cells per well. For quantitation of cell viability, ten μl of CCK solution with 100 μl complete medium was added to each well at 12, 24, 48, and 72 hours after seeding. Each solution was measured spectrophotometrically at 450 nm 1.5 hours after adding CCK-8.

Cells (4×10^2 per well) was plated on a six-well plate and allowed to attach overnight and incubated undisturbed at 37°C for ten days. Following incubation, the medium was removed and colonies that contained more than 50 cells were fixed with methanol and stained with crystal violet. Photographs were taken with a digital camera. The experiment was carried out in triplicate.

Stable transfected KLE cells and transient transfected ECC cells were serum starved for 12 hours and 1×10^5 cells were resuspended in 200 μl serum free medium and plated into the upper chamber (coated *vs.* non-coated with Matrigel, and incubated for 48 hours for invasion assay and 24 hours for migration assay, respectively. Cells inside the chamber were removed with cotton swabs, and migratory cells on the lower membrane

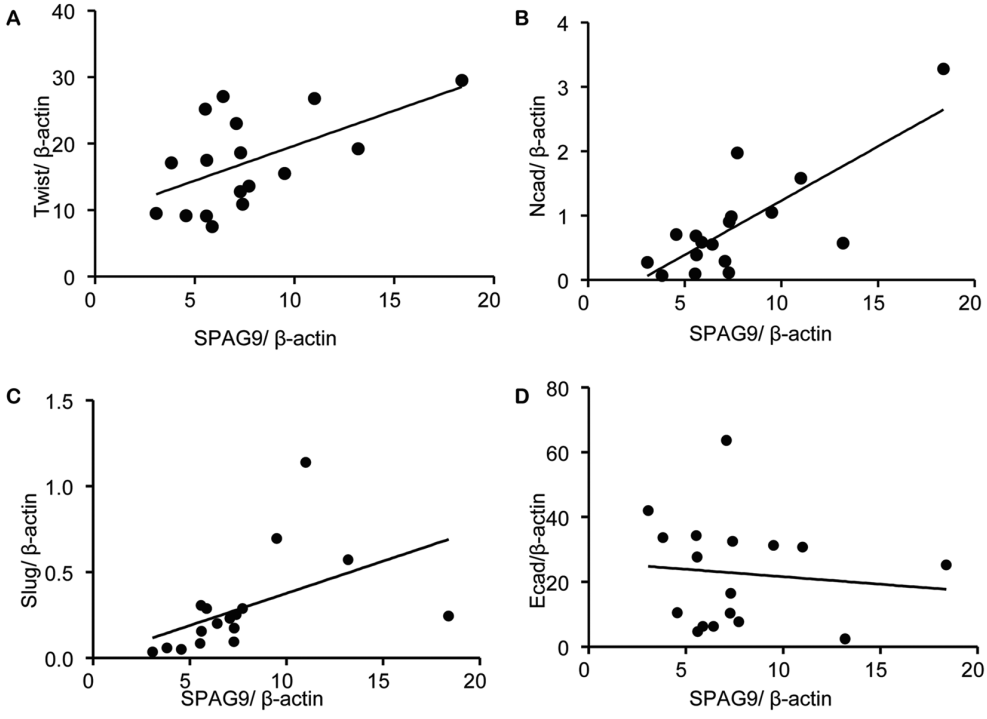

Figure 1. — Positive correlations of SPAG9 and EMT associated genes in human endometrial carcinoma tissues. (A) Twist, (B) Ncad, (C) Slug, (D) Ecad mRNA levels in endometrial carcinoma tissue samples (n =17) were quantified using reverse transcription and real-time PCR. The transcriptional levels (ΔCt of gene level/βactin) of the EMT associated genes were compared to that of SPAG9 using the Spearman correlation analysis to determine statistical correlations.

surface were washed by PBS two times prior to fixation in 4% paraformaldehyde. Cells were stained with DAPI or crystal violet and counted (five random ×100 fields per well). Cell counts are expressed as mean number of cells per field of view. Three independent experiments were performed in triplicate for each experiment.

Statistical analyses were performed using SPSS 17.0. Values are expressed as mean ± SD. Differences between two groups were determined by *t*-test. Spearman correlation analysis was used to examine the correlation between SPAG9 and EMT-associated genes. The Kaplan–Meier method was used to estimate the probability of patient survival, and difference in survival were compared by using Mantel's log-rank test. A p-value < 0.05 was considered statistically significant.

Result

SPAG9 is positively correlated with EMT-associated genes in human endometrial cancer tissues.

To examine correlations among the transcriptional levels of SPAG9 and EMT associated genes, fresh tumor tissues from 17 endometrial cancer patients were collected and analyzed for levels of Twist, Ncad, Ecad, and Slug mRNAs using real-time PCR. All 17 patients had endometrioid endometrial cancer (eight in Grade 1, eight in Grade 2, and one in Grade 3).

As shown in Figure 1, the transcriptional level of SPAG9 was positively correlated ($p < 0.001$) with levels of invasion-related genes including Slug (p = 0.001) and Ncad ($p = 0.002$). Levels of SPAG9 showed no correlation with Ecad ($p = 0.279$) or Twist ($p = 0.073$). These correlations suggest that SPAG9 expression may influence the transcriptional regulation of EMT-associated genes in human endometrial cancer tissues.

To evaluate whether protein levels of SPAG9 could predict prognosis in endometrial cancer patients, the present authors constructed survival curves for the 47 endometrial cancer patients reported in their previous paper [16]. Kaplan–Meier survival analysis did not demonstrate significantly lower overall survival in the 33 SPAG9-positive patients as compared to the 14 SPAG9-negative patients (as defined by immunohistochemistry analysis of endometrial carcinoma tissues) ($p = 0.373$, log rank test, Figure 2), although there was a trend toward lower survival rate in the SPAG9-positive group.

TGF-β1 treatment and spheroids formation significantly increased the level of SPAG9 expression.

To elucidate the relationship between SPAG9 expression and EMT, the present authors performed a study using ECC cells with TGF-β1 treatment and spheroids formation to induce EMT process. They found the transcriptional level of SPAG9 expression was significantly increased in ECC cells with TGF-β1 treatment and spheroids formation ($p = 0.000$), while the expression of the key EMT markers, such as Ncad, Twist, Vimentin, and Slug was increased accordingly (Figure 3A, C, $p < 0.05$). The protein levels of SPAG9, Ncad, and vimentin were significantly higher after TGF-β1 treatment and spheroids formation (Figure 3B, D, $p < 0.05$).

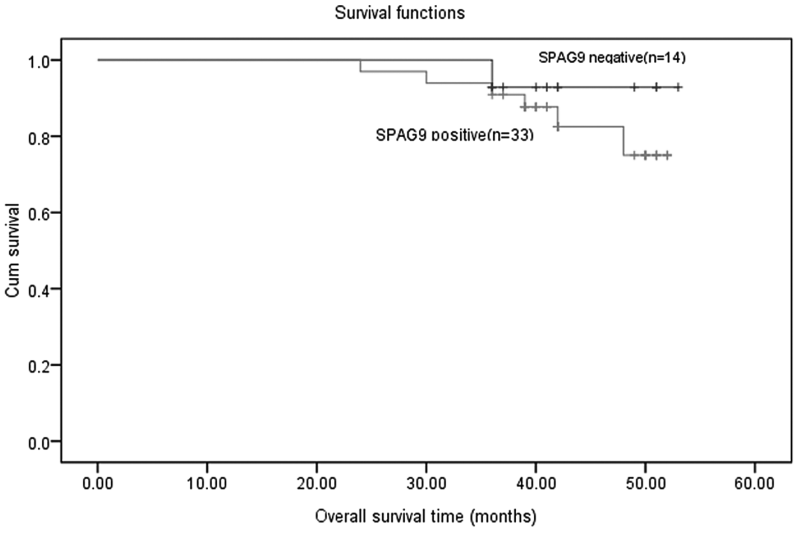

Figure 2. — Survival analysis of endometrial cancer patients with or without SPAG9 expression. The overall survival rate was not significantly lower in patients with SPAG9-positive endometrial cancer (n=33) than in patients with SPAG9-negative cancers (n=14).

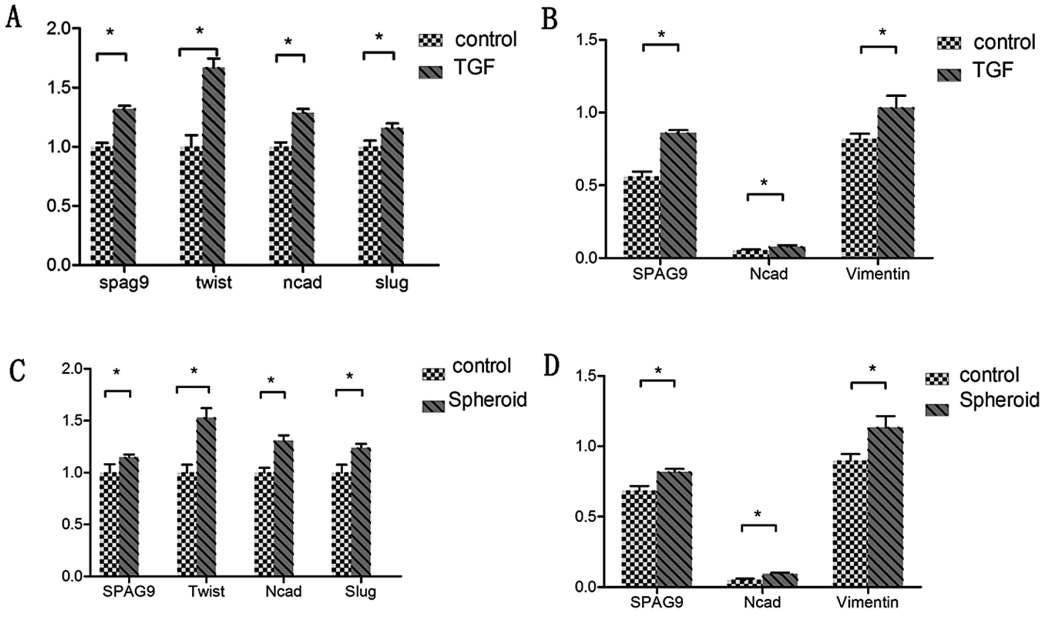

Figure 3. — TGF-β1 treatment and spheroids formation increased the level of SPAG9 expression.(A)The relative transcriptional levels of SPAG9. Twist, Ncad, and Slug in ECC cells with or without TGF-β1 treatment were determined using reverse transcription and quantitative real-time PCR. (B) The relative protein levels of SPAG9, Ncad, and Vimentin in ECC cells with or without TGF-β1 treatment were analyzed by Western blotting. (C)The relative transcriptional levels of SPAG9. Twist, Ncad, and Slug in spheroids formation or adherent ECC cells were measured using reverse transcription and quantitative real-time PCR. (D)The relative protein levels of SPAG9, Ncad, and Vimentin in spheroids formation or adherent ECC cells were analyzed by Western blotting. β-actin was the internal control. Data are representative of at least three independent experiments. *$p < 0.05$.

SPAG9 knockdown decreases the expression of EMT related genes

To evaluate the potential role of SPAG9 in regulating the expression of EMT-related genes, KLE cells were transfected with SPAG9-shRNA or control shRNA and stable cell lines were formed. A dramatic reduction in SPAG9 mRNA expression level ($p = 0.000$) and protein level ($p = 0.010$) was found as compared to negative control-shRNA-treated cells. Next, the present authors investigated the effect of SPAG9 knockdown on the expression of key EMT-related markers such as Twist, Ncad, Ecad, and Slug (Figure 4A). They noted that Twist ($p = 0.007$, Ncad ($p = 0.032$), and Slug ($p = 0.031$) expressions were significantly decreased. Western blot results confirmed alterations in

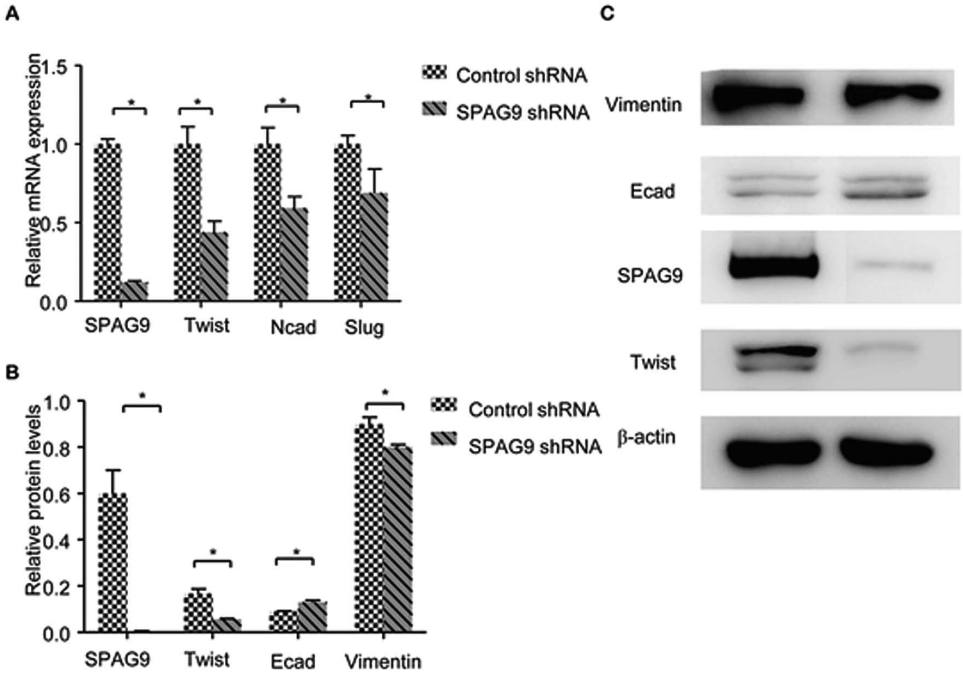

Figure 4. — SPAG9 knockdown decreases the expression of EMT related genes. (A) The relative transcriptional levels of SPAG9. Twist, Ncad, and Slug in KLE cells with or without SPAG9 depletion were determined using reverse trans- cription and quantitative real-time PCR. β-actin was the internal control. (B) The relative protein levels of SPAG9, Twist, Ecad, and Vimentin in KLE cells with or without SPAG9 depletion were analyzed by Western blotting. β-actin was the internal control. Data are representative of at least three independent experiments. (C) Bands of proteins in (B).*< 0.05.

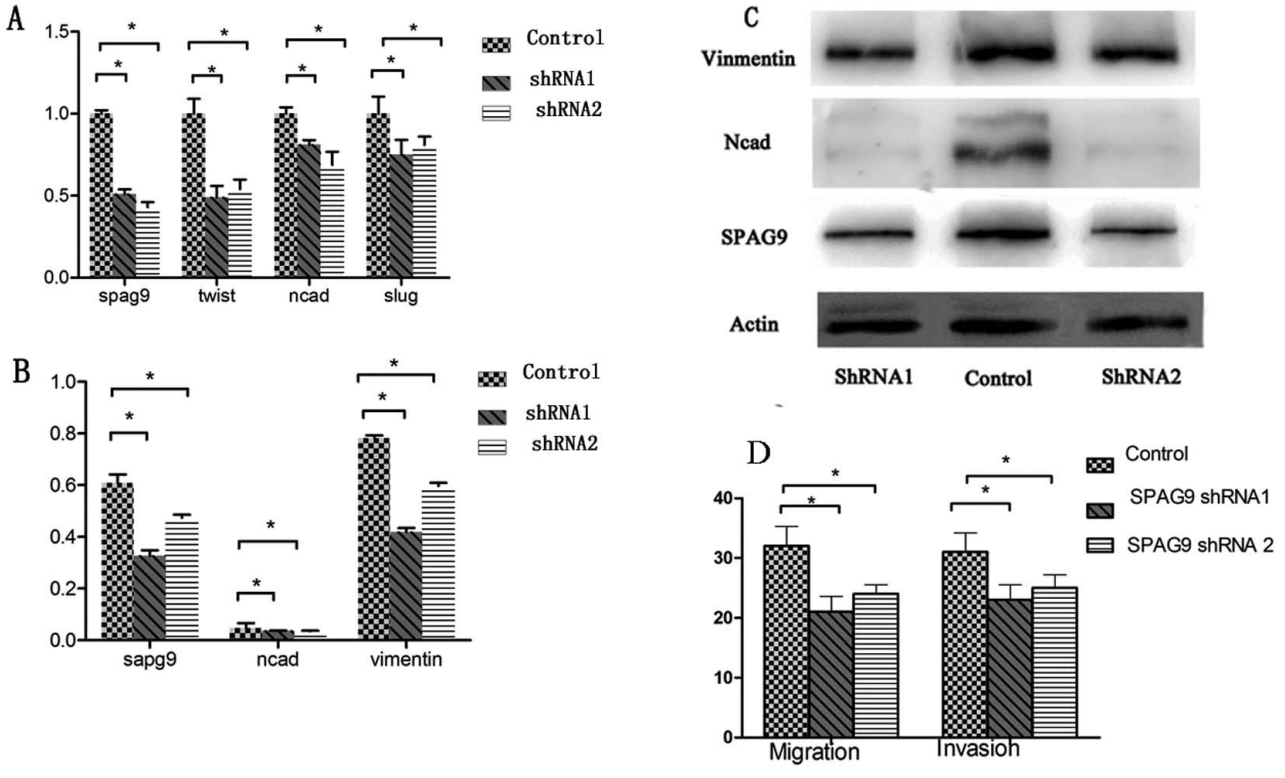

Figure 5. — Transient transfection with SPAG9shRNA decreases the expression of EMT related genes. (A) The relative transcriptional levels of SPAG9. Twist, Ncad, and Slug in ECC cells with or without transient transfection with SPAG9shRNA were determined using reverse transcription and quantitative real-time PCR. β-actin was the internal control. (B) The relative protein levels of SPAG9, Ncad, and Vimentin in ECC cells with or without transient transfection with SPAG9shRNA were analyzed by Western blotting. β-actin was the internal control. Data are representative of at least three independent experiments. (C) Bands of proteins in (B)*$p < 0.05$. (D) Migration and matrigel invasion assay showed that transient transfection with SPAG9shRNA inhibited cell migration and invasion in ECC cell lines ($p = 0.000$).

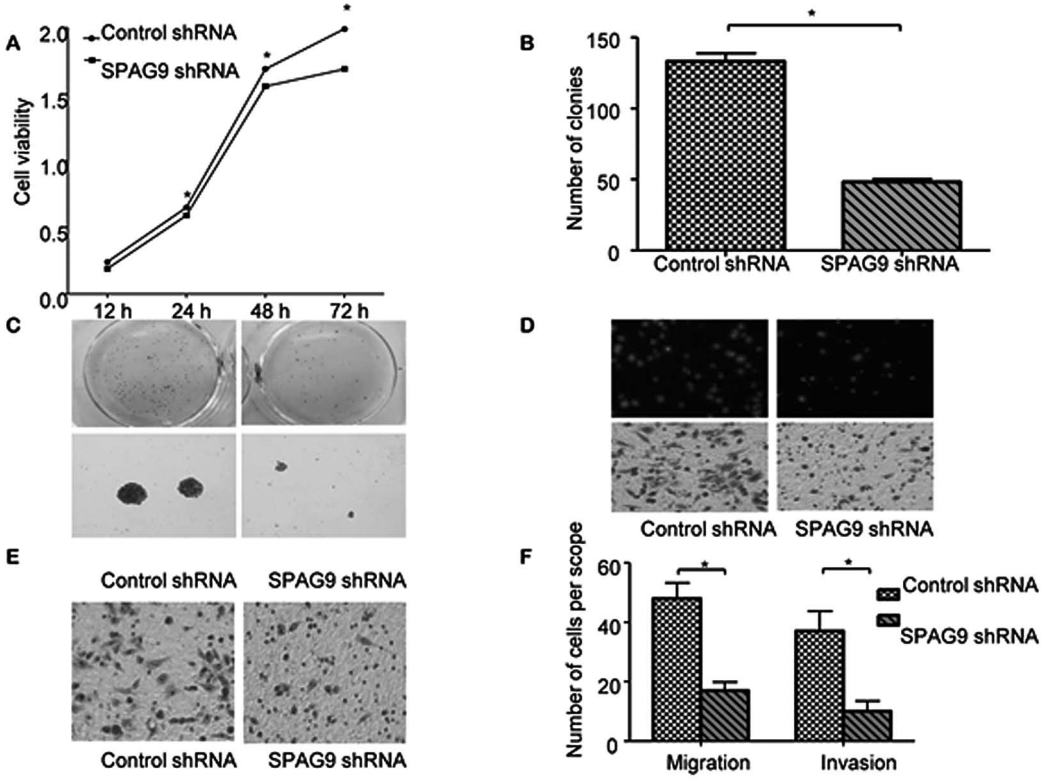

Figure 6. — SPAG9 knockdown inhibited cell proliferation and invasion in KLE cell lines. (A) CCK8 assay showed that SPAG9 knockdown inhibited cell proliferation. There was a time dependent decrease in cell proliferation after SPAG9 depletion compared with control. (B) There was significant difference in colonies' formation number between the two groups. (C) Stable cell lines were plated in six-well plates. The surviving fraction of cells (visible colonies) was stained with Gentian violet, photographed, and counted manually. There were more colonies in the left lanes (control shRNA) and the mean diameter of colonies of the left lane was larger than the right lane (SPAG9 shRNA). (D) Migration assay (24 hours) showed that SPAG9 knockdown decreased cell migration. (E) Matrigel invasion assay showed that SPAG9 depletion decreased cell invasion in KLE cell line. (F) Quantitative analysis of migration and invasion assays.. *$p < 0.05$.

Twist ($p = 0.000$), and Ecad ($p = 0.000$) expression (Figures 4B, C). The protein level of Vimentin was significantly lower after SPAG9 knockdown ($p = 0.009$). Similar results were also found in ECC cells after transient transfection with SPAG9shRNA1 or shRNA2 (Figure 5). These data suggest that SPAG9 may mediate the EMT process in endometrial cancer cell lines.

shRNA-mediated knockdown of SPAG9 inhibits cell growth and colony formation, migration and invasion.

To investigate the biological function of SPAG9 in endometrial cancer cells, the present authors assessed various cellular functions such as proliferation, migration, and invasion after treatments of non-silencing shRNA and SPAG9 shRNA. CCK assay revealed significantly decreased proliferation in SPAG9 shRNA transfected cells as compared to non-transfected cells at 24, 48, and 72 hours after cell seeding (Figure 6A, $p = 0.006$, $p = 0.006$, $p = 0.004$, respectively). Colony formation assay also showed that the number of colonies formed was significantly greater in control than in SPAG9 shRNA transfected cells at 24, 48, and 72 hours (Figure 6B, 6C, $p = 0.000$). They next performed transwell assays to determine whether attenuated SPAG9 levels might affect cellular migration and invasion. As shown in Figures 6D, 6E, and 6F, SPAG9 knockdown suppressed cell migration; additionally, weakened matrigel invasive capacity was observed as compared to cells transfected with non-silencing control shRNA ($p = 0.000$, $p = 0.000$, respectively). Similar results were also found in ECC cells after transient transfection with SPAG9shRNA1 or shRNA2 (Figure 5D, $p = 0.000$).

Discussion

The molecular pathway by which SPAG9 promotes tumor cell proliferation and invasion has thus far remained unclear. In this study, the authors explored the potential role of SPAG9 expression in endometrial cancer

cell proliferation, invasion and the EMT process. They found that SPAG9 was positively correlated with expression of EMT-related genes in human endometrial cancer tissues. Down-regulation of SPAG9 gene expression inhibited EMT-related gene expression and also inhibited cell growth, migration and invasion in the KLE endometrial cancer cell line. They also stimulated EMT process via TGF-β1 treatment and spheroids formation in ECC endometrial cancer cells. The increased SPAG9 expression,which was correlated with EMT-related genes, also be detected.This is the first study to demonstrate a role for SPAG9 in the biological characteristics of endometrial cancer cells as well as in regulation of expression of EMT-related genes.

EMT is recognized as an important phenomenon associated with invasion and metastasis [12]. There are a number of transcription factors, known as EMT-related genes, which are known to be involved in the regulation of EMT, including Snail, Slug, Ncad, Ecad, and Twist. Increased expression of the Ecad transcription repressors Twist, Snail, and Slug has been demonstrated in endometrial carcinoma cell lines and in tumor samples, and downregulation of Ecad immuno-reactivity has been described in endometrial cancer [18]. The present data showed that both Slug and Ncad, and Twist were all positively correlated with SPAG9 with Twist had a similar trend. The finding that Ecad expression did not negatively correlate with SPAG9 as expected may be due to small sample size; additionally, the tissues we collected were mixed rather than pure cancer cells. The co-expression data indicates that SPAG9 and EMT-related genes may show regulatory interactions *in vivo*.

Expression of EMT-related genes such as Twist, Slug, Vimentin, Snail, Ncad, and Ecad was correlated with patient survival. Importantly, EMT status is a useful prognostic factor for cancers [19-22]. Wang *et al.* [8] reported that the overall survival of lung cancer patients was significantly lower in patients with SPAG9-positive cancers than in patients with SPAG9-negative disease. Based on the present survival analysis, patients with higher SPAG9 expression did not demonstrate a significantly poorer prognosis than those with lower scores. This may be because of the small sample size and the relatively better prognosis of endometrial cancer as compared to lung cancer. Ultimately, however, the present authors did note a trend toward lower survival rate in the SPAG9 positive group.

Apart from being expressed in a majority of cancers, SPAG9 has also been found to be associated with cellular proliferation, migration, and invasiveness in some kinds of cancer cells [4,7,8]. *In vivo* xenograft studies in nude mice revealed that administration of a SPAG9 shRNA plasmid significantly inhibited cervical and renal cancer growth [4, 6]. In the present study, the authors also showed that SPAG9 depletion could inhibit endometrial cancer cell proliferation, colony formation, migration, and invasion, and could also trigger decreased expression of several EMT-related genes. Slug and Twist are well known EMT-related genes and are associated with cell migration and invasion. Vimentin and Ncad are mesenchymal markers. The present study demonstrates the role of SPAG9 in regulating those genes.

Previous research demonstrated that SPAG9 interacts with the JNK pathway; specifically, SPAG9 depletion decreased JNK phosphorylation in lung cancer cell lines [8]. For future studies, to further prove that SPAG9 regulates EMT-related genes and tumor invasiveness, the present authors plan to construct a SPAG9 overexpression plasmid and cause inhibition of the JNK pathway after SPAG9 plasmid transfection.

In conclusion, the authors demonstrated in the present study that a significant positive correlation exists between SPAG9 and expression of two EMT-related genes (Slug and Ncad) in endometrial cancer tissues. In addition, their in vitro experiments revealed that suppression of SPAG9 expression induces a dramatic reduction of certain EMT-related genes (Twist, Slug, and Vimentin) expression in KLE cell lines, which in turn leads to decreased rates of cell proliferation, invasion, and migration. Whether overexpression of SPAG9 is accompanied by simultaneous increase in EMT-related genes and promotion of cell invasiveness and metastasis in cancer patients requires further study.

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References

[1] "Netherlands Cancer Registry: 2001-2011", based on April 2011 NCR data submission, posted to the NCR web site, 2011. Available at: http://www.cijfersoverkanker.nl/selecties/dataset_1/img56c32cc90a2bc?row=0&direction=down#table

[2] Siegel R., Naishadham D., Jemal A.: "Cancer statistics, 2013". *CA Cancer J. Clin.,* 2013, *63*, 11-30.

[3] Jagadish N., Rana R., Selvi R., Mishra D., Garg M., Yadav S., *et al.*: "Characterization of a novel human spermassociated antigen 9 (SPAG9) having structural homology with c-Jun N-terminal kinase-interacting protein". *Biochem.,* 2005, *389*, 73.

[4] Garg M., Kanojia D., Khosla A., Dudha N., Sati S., Chaurasiya D., *et al.*: "Sperm associated antigen 9 is associated with tumor growth, migration and inv asion in renal cell carcinoma". *Cancer Res.,* 2008, *68*, 8240.

[5] Kanojia D., Garg M., Gupta S., Gupta A., Suri A.: "Sperm associated antigen 9 (SPAG9), a novel biomarker for early detection of breast cancer". *Cancer Epidemiol. Biomarkers Prev.,* 2009, *18*, 630.

[6] Kanojia D., Garg M., Gupta S., Gupta A., Suri A.: "Sperm-associated antigen 9 is a novel biomarker for colorectal cancer and is involved in tumor growth and tumorigenicity". *Am. J. Pathol.,* 2011, *178*, 1009.

[7] Yi F., Ni W., Liu W., Pan X., Han X., Yang L., *et al.*: "SPAG9 is overexpressed in human astrocytoma and promotes cell proliferation and invasion". *Tumour Biol.,* 2013, *34*, 2849.
[8] Wang Y., Dong Q., Miao Y., Fu L., Lin X., Wang E.: "Clinical significance and biological roles of SPAG9 overexpression in non-small cell lung cancer". *Lung Cancer,* 2013, *81*, 266.
[9] Suri A., Saini S., Sinha A., Agarwal S., Verma A., Parashar D., *et al.*: Cancer testis antigens: a new paradigm for cancer therapy". *Oncoimmunology,* 2012, *1*, 1194.
[10] Van Slambrouck S., Grijelmo C., De Wever O., Bruyneel E., Emami S., Gespach C., *et al.*: "Activation of the FAK-src molecular scaffolds and p130Cas-JNK signaling cascades by alpha1-integrins during colon cancer cell invasion". *Int. J. Oncol.,* 2007, *31*, 1501.
[11] Chen H.H., Zhou X.L., Shi Y.L.,Yang J.: "Roles of p38 MAPK and in TGF-β1-induced human alveolar epithelial to mesenchymal transition". *Arch. Med. Res.,* 2013, *44*, 93.
[12] Kang Y., Massagué J.: "Epithelial–mesenchymal transitions:Twist in development and metastasis". *Cell,* 2004, *118*, 277.
[13] Thiery J.P.: "Epithelial-mesenchymal transitions in tumour progression". *Nat. Rev. Cancer,* 2002, *2*, 442.
[14] Yin G., Alvero A.B., Craveiro V., Holmberg J.C., Fu H.H., Montagna M.K., *et al.*: "Constitutive proteasomal degradation of TWIST-1 in epithelial--ovarian cancer stem cells impacts differentiation and metastatic potential". *Oncogene,* 2012, *32*, 39.
[15] Noh H., Oh E.Y., Seo J.Y., Yu M.R., Kim Y.O., Ha H., Lee H.B.: "Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-1 induced renal injury". *Am. J. Physiol. Renal Physiol.,* 2009, *297*, F729.
[16] Yu P., Yan L., Zhang H., Lin X., Zhao X.: "Expression and clinical significance of sperm-associated antigen 9 in patients with endometrial carcinoma". *Int. J. Gynecol. Cancer,* 2012, *22*, 87.
[17] Zhang H., Li M., Zheng X.,Wen Z., Zhao X.: "Endometriotic stromal cells lose the ability to regulate cell-survival signaling in endometrial epithelial cells in vitro". *Mol. Hum. Reprod.,* 2009, *15*, 653.
[18] Colas E., Pedrola N., Devis L., Ertekin T., Campoy I., Martínez E., *et al.*: "The EMT signaling pathways in endometrial carcinoma". *Clin. Transl. Oncol.,* 2012, *14*, 715.
[19] Kyo S., Sakaguchi J., Ohno S., Mizumoto Y., Maida Y., Hashimoto M., *et al.*: "High Twist expression is involved in infiltrative endometrial cancer and affects patient survival". *Hum. Pathol.,* 2006, *37*, 431.
[20] Yamada S., Fuchs B.C., Fujii T., Shimoyama Y., Sugimoto H., Nomoto S., *et al.*: "Epithelial-to-mesenchymal transition predicts prognosis of pancreatic cancer". *Surgery,* 2013, *154*, 946.
[21] Toiyama Y., Yasuda H., Saigusa S., Tanaka K., Inoue Y., Goel A., Kusunoki M.: "Increased expression of Slug and Vimentin as novel predictive biomarkers for lymph node metastasis and poorprognosis in colorectal cancer". *Carcinogenesis,* 2013, *34*, 2548.
[22] Whiteland H., Spencer-Harty S., Thomas D.H., Davies C., Morgan C., Kynaston H., *et al.*: "Putative prognostic epithelial-to-mesenchymal transition biomarkers for aggressive prostate cancer". *Exp. Mol. Pathol.,* 2013, *95*, 220.

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