

## Original Article

# The role of spermatogenesis-associated protein 6 in testicular germ cell tumors

Shiwei Huo<sup>1,2</sup>, Wenyan Du<sup>3</sup>, Peng Shi<sup>2</sup>, Yuexiu Si<sup>4</sup>, Shengtian Zhao<sup>5</sup>

<sup>1</sup>Shandong University, Jinan 250100, Shandong, China; <sup>2</sup>The Central Hospital of Taian, Taian 271000, Shandong, China; <sup>3</sup>The Branch of Tai'an Central Hospital, Taian, 271000, Shandong, China; <sup>4</sup>The Fifth Hospital of Jinan, Jinan 250100, Shandong, China; <sup>5</sup>The Second Hospital of Shandong University, Jinan 250100, Shandong, China

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**Abstract:** *Objectives:* To investigate the role of spermatogenesis-associated protein 6 (SPATA6) in the testicular germ cell tumors (TGCTs). *Methods:* Human embryonic carcinoma (EC)-derived cell line NTERA2 was employed and randomly divided into normal control group, SPATA6c group, siSPATA6c group, and SPATA6c + siSPATA6c group. The recombinant expression vector pcDNA3.1 (+)-SPATA6 and target sequence for SPATA6-specific siRNA was transfected into NTERA2 cells in the SPATA6c group and siSPATA6c group, respectively. The SPATA6 protein levels in each group were determined by Western blot. Cell proliferation and apoptosis rate were assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay and flow cytometry (FCM) assay, respectively. In addition, Western blot was performed to investigate the expression of Bax and B-cell lymphoma (Bcl)-2 in each group. *Results:* Compared with control group, protein levels of SPATA6 were significantly reduced in the siSPATA6c group, but were statistically increased in the SPATA6c group ( $P < 0.05$ ). Similarly, the cell viability was significantly decreased by transfection with SPATA6 siRNA, but was increased by transfection with pcDNA3.1 (+)-SPATA6 compared with the control group. Moreover, the percentages of apoptosis cell were significantly higher in siSPATA6c group than those in the three groups. After transfection of SPATA6 siRNA, the expression of Bax was significantly increased, but the expression of Bcl-2 was markedly decreased than that in the control group and SPATA6c group. *Conclusion:* SPATA6 may play an important role in TGCTs, and down-regulation of SPATA6 could lead to apoptosis of TGCTs.

**Keywords:** Spermatogenesis-associated protein 6, testicular germ cell tumors, apoptosis

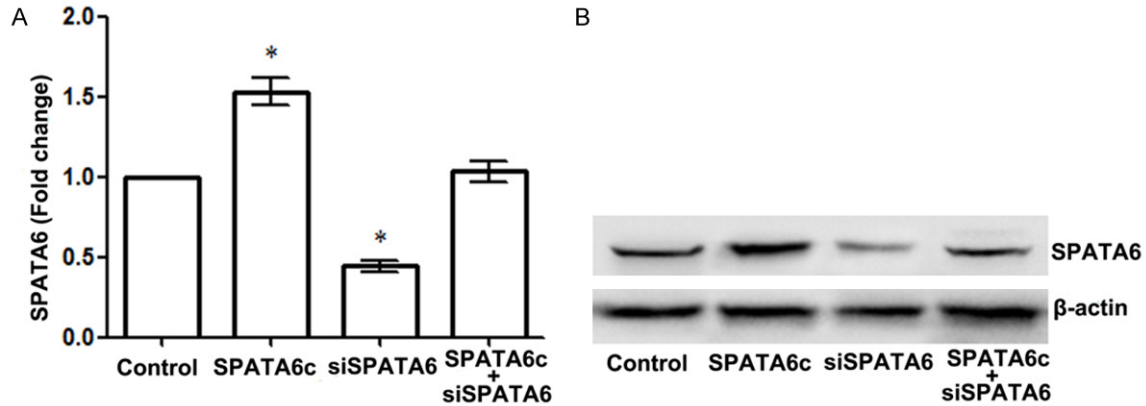
## Introduction

Testicular cancer is a frequent malignancy among young men aged from 15 to 44 years [1, 2]. The incidence of this disease has gradually increased [3, 4], and the present frequency raises 50% over 30 years ago. However, the incidence varies significantly in diverse geographical areas and ethnic groups [5]. Testicular germ cell tumors (TGCTs) represent approximately 95% of the testicular tumors and 0.005% in the general population [6, 7]. TGCTs are the leading causes of death ascribed to malignancy-related mortality and morbidity between 15 and 34 years of age [8-10]. Male patients who suffer from TGCTs have a major risk factor for developing a subsequent tumor [1]. Although TGCTs has been considered as a paradigm of a curable cancer, the exact patho-

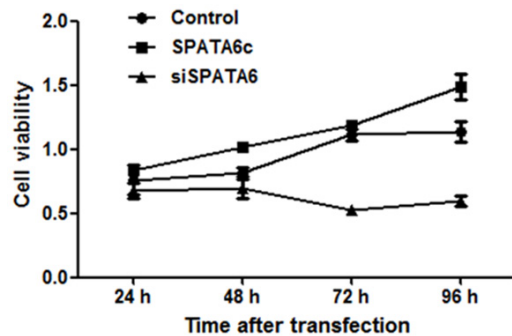
genesis of TGCTs still remains unclear because it is occurred during fetal development [11, 12]. Besides, cardiac problems and second cancers, such as stomach cancer [13], are still common in the cured patients, as well as chronic side effects of chemotherapy and radiotherapy (e.g. hearing loss, and renal impairment) [14]. Therefore, it is imperative to understand the pathogenesis of TGCTs, and search for a more effective treatment of TGCTs.

It has been reported that male infertility is one of clinical risk factors for TGCTs [15, 16]. Recently, a study conducted by Yuan *et al.* suggested that inactivation of spermatogenesis-associated protein 6 (SPATA6) could result in male sterility and acephalic spermatozoa [17], which is attributable to the interrupted myosin based microfilament transport regulated by

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**Figure 1.** Effects of transfection on SPATA6 expression in NTERA2 cells. A. Fold change of SPATA6 in the four groups; B. The picture of SPATA6 analyzed by Western blot; SPATA6, spermatogenesis-associated protein 6.



**Figure 2.** Effect of transfection on cell viability in NTERA2 cells. SPATA6, spermatogenesis-associated protein 6.

SPATA6. Because SPATA6 plays an important role in the formation of segmented columns during the development of the connecting piece. SPATA6, also known as spermatogenesis-related factor-1 (SRF-1), is a spermatogenesis associated gene. It is specifically expressed in haploid germ cells [18]. It was first reported by Yamano *et al.* in rat [19]. SPATA6 is localized to chromosome 1, region p32-35 in the human, and localized to chromosome 5, region q34-35 in the rat. However, this gene has rarely been reported in the recent years. So far, little information is available regarding the role of SPATA6 in TGCTs.

Therefore, we hypothesized that SPATA6 may be involved in TGCTs. To confirm the hypothesis, our study is aimed to explore the role of SPATA6 in TGCTs. Our results may provide a fundamental research for searching a new target

gene of TGCTs, as well as a potential drug therapy.

### Materials and methods

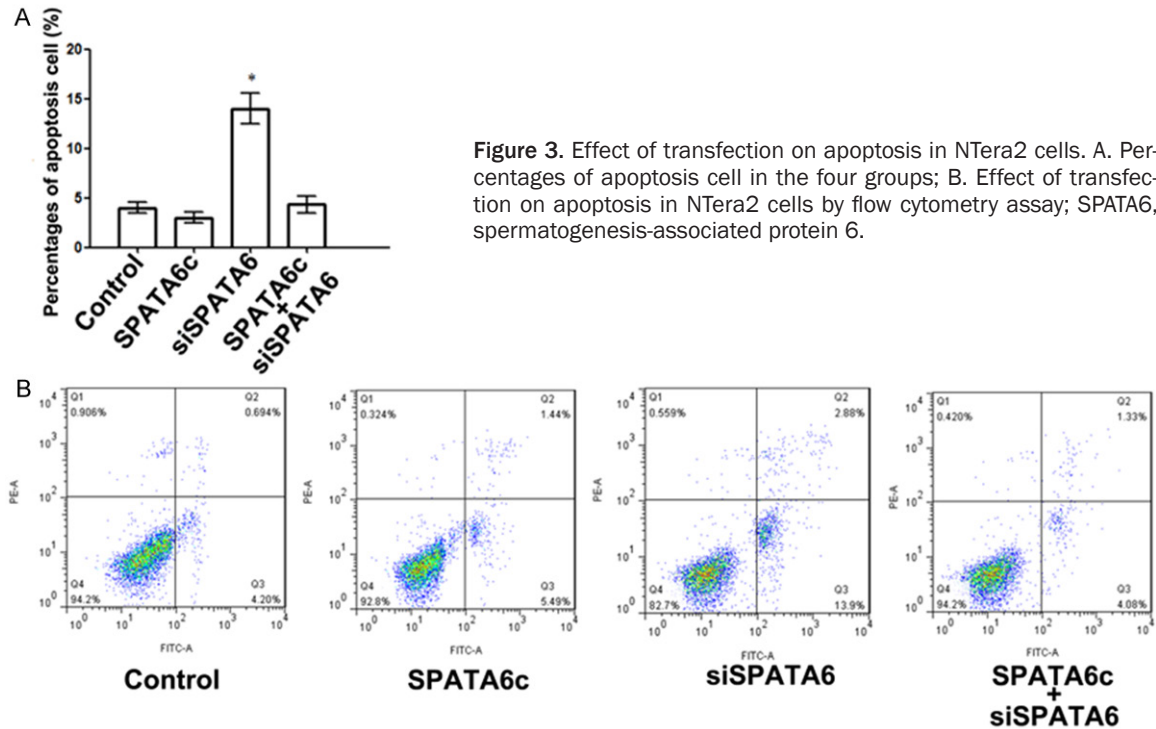
#### Cell culture and experimental protocols

Human embryonic carcinoma (EC)-derived cell line NTERA2 was employed in our study. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 58.5 mg/ml glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in humidified atmosphere of 5% CO<sub>2</sub>. All cell media and reagents were obtained from Life Technologies. After post-culturing in 10% FCS/DMEM for 72 h, the cultures were randomly divided into four groups: (1) normal control group, the cultures were continuously maintained in 10% FBS/DMEM for 24 h; (2) SPATA6c group, the cultures were subjected to plasmids construction; (3) siSPATA6c group, the cultures were subjected to small interfering RNA (siRNA) silencing approach; (4) SPATA6c + siSPATA6c group, combination (2) and (3).

#### Plasmids and siRNA transfection

SPATA6 gene was amplified by polymerase chain reaction (PCR) with NTERA2's cDNA, and the template and the fragment was combined with pcDNA3.1 (+). The recombinant expression vector pcDNA3.1 (+)-SPATA6 was transfected into NTERA2 cells. Besides, SPATA6 expression was suppressed using the siRNA silencing approach with the target sequence for SPATA6-specific siRNA (Shanghai, China). Cell transfections were carried out using

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**Figure 3.** Effect of transfection on apoptosis in Ntera2 cells. A. Percentages of apoptosis cell in the four groups; B. Effect of transfection on apoptosis in Ntera2 cells by flow cytometry assay; SPATA6, spermatogenesis-associated protein 6.

Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

### Cell proliferation assay

After transfection 24 h, 48 h, 72 h, and 96 h, the cells were harvested. Cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the manufacturer's protocol. In brief, Ntera2 cells were washed with phosphate buffer saline (PBS) and subsequently seeded in 96-well plate at a final concentration of  $2 \times 10^4$  per mL. Then the plates were incubated at 37°C in humidified atmosphere of 5% CO<sub>2</sub>. After incubation, MTT (10 µl) was added to each well, and the plates were incubated at 37°C for another 2 h. The absorbance at 595 nm was determined using an ultraviolet spectrophotometer (SpectraMax M5, Molecular Device, USA). Experiments were performed at least 3 times.

### Flow cytometry (FCM) assay

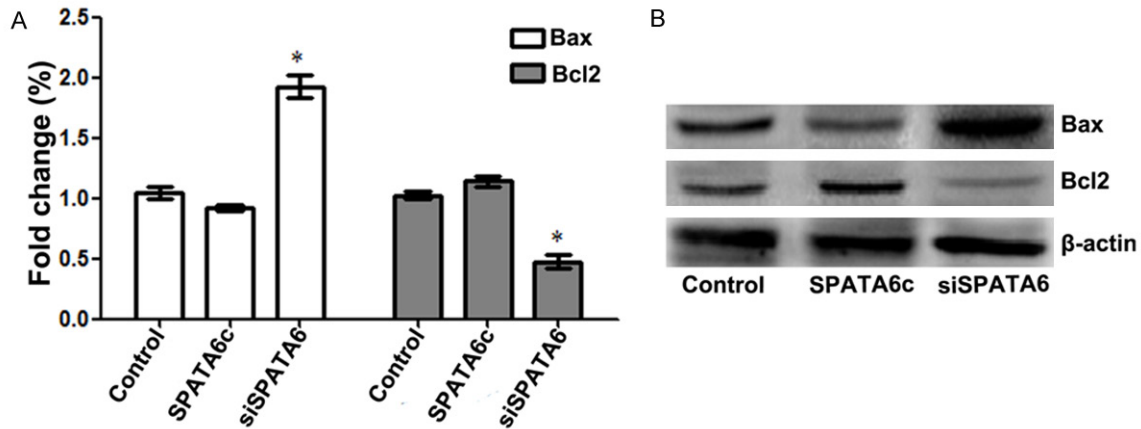
An Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (BD Pharmingen) was used to assess the apoptosis rate according to the manufacturer's protocol. Briefly, cells ( $1 \times 10^6$  cells/ml) were harvested

and washed twice with cold PBS. After resuspension with 0.5 ml binding buffer, the mixture was incubated with 5 µL Annexin V-FITC and 5 µL propidium iodide (PI). Then the cells were incubated at room temperature in the dark for 15 min. Thereafter, the cells were read by FCM (Becton Dickinson, San Jose, CA, USA). The results were determined using CELLQuest 3.0 software (BD Biosciences, San Jose, CA, USA).

### Western blot analysis

Twenty-four hours after transfection, cells in each group were harvested for protein extraction. The protein concentration was determined using Bio-Rad DC protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (20 µg) were resolved with 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and blotted onto polyvinylidene difluoride (PVDF) membranes (Milipore, Bedford, MA), blocked in 5% defatted milk powder for 2 h at room temperature and incubated with the following primary antibodies overnight at 4°C: anti-SPATA6 monoclonal antibody (K-23, Santa Cruz Biotech), anti-Bax monoclonal antibody (Santa Cruz Biotech), and anti-B-cell lymphoma (Bcl)-2 monoclonal antibody (Santa Cruz CA). Then an appropriate secondary antibody was employed. An anti-human β-actin antibody (Santa, Cruz

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**Figure 4.** Effect of transfection on expression of Bax and Bcl-2. A. Fold change of SPATA6 in the three groups; B. The picture of SPATA6 analyzed by Western blot; SPATA6, spermatogenesis-associated protein 6; Bcl, B-cell lymphoma.

Biotechnology) was considered as a loading control. Finally, the immunoreactive protein bands were subjected to enhanced chemiluminescence.

### Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using statistical package for the social sciences (SPSS) (version 17.0; SPSS Inc., Chicago, IL). Student's t test was performed to statistical comparisons. One-way analysis of variance (ANOVA) was used to multiple comparisons. A statistical significance was defined when  $P < 0.05$ .

### Results

#### Effects of transfection on SPATA6 expression in NTERA2 cells

As shown in **Figure 1A** and **1B**, the expression of SPATA6 protein in the SPATA6c group was significantly increased after transfection 24 h than that in the control group and SPATA6c + siSPATA6 group ( $P < 0.05$ ). However, the expression of SPATA6 protein in NTERA2 cells was markedly diminished after by transfection with SPATA6 siRNA.

#### Effect of transfection on cell viability in NTERA2 cells

There were no significant differences among three groups after transfection 24 h ( $P > 0.05$ ). After 24 h transfection, the results of MTT showed that the cell viability was significantly

decreased by transfection with SPATA6 siRNA, but was increased by transfection with pcDNA3.1 (+)-SPATA6 compared with the control group (**Figure 2**).

#### Effect of transfection on apoptosis in NTERA2 cells

As shown in **Figure 3A** and **3B**, no significance was observed among the control group, SPATA6c group, and SPATA6c + siSPATA6 group in percentages of apoptosis cell ( $P > 0.05$ ). But the percentages of apoptosis cell were significantly higher in the siSPATA6 group than those in the three groups.

#### Effect of transfection on expression of Bax and Bcl-2

After transfection of SPATA6 siRNA, the expression of Bax was significantly increased, but the expression of Bcl-2 was markedly decreased than that in the control group and SPATA6c group. But no significant differences were found between the control group and the SPATA6c group (**Figure 4A** and **4B**).

### Discussion

SPATA6 is an evolutionarily conserved gene of testicle, and was expressed higher in spermatids than in spermatocytes and spermatogonia. Inactivation of SPATA6 leads to male infertility and teratospermia [17]. Here we show for the first time that down-regulation of SPATA6 can induce apoptosis in TGCTs cell lines in vitro, by increasing the expression of apoptosis-related

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protein, and by decreasing cell viability and cell survival. Our findings demonstrate that SPATA6 plays an important role in TGCTs, and down-regulation of SPATA6 may be a potential effective treatment of TGCTs.

Testis malignant tumors are infrequent [11], but TGCTs are responsible for cancer-related mortality and morbidity in ages between 15 to 34 years. Besides, the incidence is expected to continue to increase. The pathogenesis of TGCTs remains unclear, and the reported clinical risk factors for TGCTs mainly include a history of cryptorchidism [20, 21] and male infertility [16]. These three disorders share the common characteristic of fetal developmental origins [22]. Moreover, TGCTs often in line with dysgenetic tubules exhibiting spermatogenic arrest and microcalcifications showed by histological investigations [23]. A previous study has suggested that inactivation of SPATA6 results in male sterility and acephalic spermatozoa [17]. Hence, we speculated SPATA6 may also be participated in the development of TGCTs. SPATA6 encodes a predicted protein of 488 amino acids, and consists of 15 exons ranging from 40 bp to 596 bp. In addition, it encodes -2.6, -1.8 and -1.2 kb mRNAs [18]. The characteristics of SPATA6 are similar to the motor domain of kinesin related proteins and the *Caenorhabditis elegans* neural calcium sensor protein (NCS-2). The 2.6 kb transcripts are expressed in testis, ovary, thymus and placenta, but with low levels, while the expression of the 1.8 and 1.2 kb mRNA are exclusively detected in testis. The expression of SPATA6 transcript is detected during embryonic development, and the localization of SPATA6 transcript is found in neural tube, somites and limb buds of mouse embryo. In an effort to demonstrate the more function of SPATA6, we up-regulated and down-regulated the expressions of SPATA6 in human EC-derived cell line Ntera2 through transfection techniques using the recombinant expression vector pcDNA3.1 (+)-SPATA6 and target sequence for SPATA6-specific siRNA, respectively. The SPATA6 protein levels were determined by Western blot. The results showed that the levels of SPATA6 were significantly reduced after transfection of pcDNA3.1 (+)-SPATA6 compared with control group, but were statistically increased after transfection of SPATA6 siRNA, suggesting that the recombinant plasmid was successfully expressed. To explore the effect of SPATA6

after transfection on cell proliferation and apoptosis rate, MTT and FCM assay were performed. We found that the cell viability was significantly reduced after transfection of SPATA6 siRNA, but was raised after transfection of pcDNA3.1 (+)-SPATA6 compared with the control group. Moreover, the percentages of apoptosis cell were the highest after transfection of SPATA6 siRNA than other groups. The results indicated that down-expression of SPATA6 inhibited the cell proliferation and induced cell apoptosis.

In addition, in order to confirm the cell apoptosis caused by down-expression of SPATA6, we explored the expression of apoptosis-related protein after transfection of pcDNA3.1 (+)-SPATA6 and SPATA6 siRNA. Cell apoptosis is regulated by Bcl-2 family protein in many cell types [24-26]. Bcl-2 family protein consists of anti-apoptotic and pro-apoptotic members [27, 28]. Bcl-2 is an anti-apoptotic protein, which controls the mitochondrial membrane potential and inhibits the release of cytochrome c (Cyt-c) and apoptosis-inducing factor into the cytoplasm [29]. Bax, a pro-apoptotic protein, belongs to Bcl-2 family protein and acts by blocking Bcl-2 [30]. It initiates the release of Cyt-c from mitochondria both in vitro and in vivo [31]. Cell death or survival is determined by the balance between pro-apoptotic and anti-apoptotic members [32]. Our results demonstrated that the expression of Bax was significantly increased, but the expression of Bcl-2 was markedly decreased after transfection of SPATA6 siRNA, indicating that down-expression of SPATA6 could induce the cell death of TGCTs.

However, there are some limitations in our study. TGCTs consists of two major groups, pure seminoma and nonseminoma, however, we only selected human EC-derived cell line Ntera2. Moreover, the possible signaling pathways of SPATA6 on TGCTs were not explored. Furthermore, the exact mechanism of apoptosis induced by down-regulation of SPATA6 on TGCTs was unclear. Therefore, further studies should be carried out to confirm the function and mechanism of SPATA6 on TGCTs, as well as the corresponding signaling pathways.

In conclusion, our results showed that SPATA6 may play an important role in the TGCTs, and down-regulation of SPATA6 could lead to apoptosis of TGCTs.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Shengtian Zhao, Department of Urology, The Second Hospital of Shandong University, 247 Beiyuan Street, Tianqiao District, Jinan 250100, Shandong, China. Tel: +86-532-89751558; Fax: +86-532-89751558; E-mail: zhaoshengtian963@126.com

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