SPANXN2 functions a cell migration inhibitor in testicular germ cell tumor cells

Fang Zhu, Hao Bo, Guangmin Liu, Ruixue Li, Zhizhong Liu and Liqing Fan

1 Institute of Reproductive & Stem Cell Engineering, School of Basic Medical Science, Central South University, Changsha, Hunan, China
2 Clinical Research Center for Reproduction and Genetics in Hunan Province, Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, Hunan, China
3 Hunan Cancer Hospital, Department of Urology, The Affiliated Cancer Hospital of Xiangya School of Medicine of Central South University, Changsha, Hunan, China

ABSTRACT

Background. SPANX family members are thought to play an important role in cancer progression. The SPANXN2 is a gene expressed mainly in normal testis, but its role in testicular germ cell tumors (TGCTs) has yet to be investigated. TGCT is one of the most common solid tumors in young men and is associated with poor prognosis; however, effective prognostic indicators remain elusive. Therefore, we investigated the role of SPANXN2 in TGCT development.

Methods. SPANXN2 expression levels were validated by quantitative real-time polymerase chain reaction (qRT-PCR) analyses of 14 TGCT samples and five adjacent normal tissue samples. SPANXN2 was transiently overexpressed in TGCT cells to study the consequences for cell function. The effects of SPANXN2 on cell migration were evaluated in transwell and wound healing assays. The effects on cloning ability were evaluated in colony formation assays. MTT assays and cell cycle analysis were used to detect the effects of SPANXN2 on cell proliferation. The expression levels of EMT- and AKT-related proteins in cells overexpressing SPANXN2 were analyzed by Western blotting.

Results. Compared with adjacent normal tissues, the Gene Expression Profiling Interactive Analysis database showed SPANXN2 expression was downregulated in TGCTs which was consistent with the qRT-PCR analysis. SPANXN2 overexpression reduced cell migration and colony formation capability and downregulated expression of EMT- and AKT-related proteins, Vimentin, Snail, AKT, and p-AKT.

Conclusion. Our results suggest that SPANXN2 regulates TGCT cell migration via EMT- and AKT-related proteins although its role in the occurrence and development of TGCT remains to be fully elucidated.

Subjects Cell Biology, Genetics

Keywords Testicular germ cell tumors (TGCTs), SPANXN2, Metastasis, Cancer/testis antigen (CTA)
INTRODUCTION

Testicular germ cell tumor (TGCT) is one of the most common solid tumors in young men aged between 15 and 40 years (Rijlaarsdam & Looijenga, 2014). The incidence of TGCTs has increased globally in recent years, to approximately 1.3/100,000 men in China and 7.8/100,000 men in Western Europe (Shanmugalingam et al., 2013; Le Cornet et al., 2014; Znaor et al., 2014). Histologically, there are two main subtypes of TGCTs, i.e., seminoma and non-seminoma (Lobo et al., 2018). Both seminoma and non-seminoma carry risks of vascular invasion and the development of distant metastases (Yilmaz et al., 2013).

Seminoma is distinctive in its sensitivity to cisplatin-based chemotherapy and radiation, and 20% of them are destined to relapse (Schmidberger et al., 1997; Classen et al., 2003; Facchini et al., 2019). Approximately 30% of patients with clinical first-stage non-seminoma have subclinical metastases in retroperitoneal lymphatic stations and lung metastasis after chemotherapy (Yilmaz et al., 2013; Albers et al., 2003). Moreover, the occurrence and treatment of TGCTs are associated with impairment of the sexual function, fertility, and quality of life, especially in young patients (Van den Belt-Dusebout et al., 2007; Albers et al., 2015). Tumor progression and poor prognosis are usually caused by metastasis; therefore, clarification of the molecular mechanisms underlying the pathogenesis and development of TGCTs is needed.

The SPANX multigene family is a representative cancer-testis antigen, which has two subfamilies: SPANX-A/D and SPANX-N (Whitehurst, 2014; Kouprina et al., 2004; Kouprina et al., 2007a; Kouprina et al., 2007b). The SPANX-N subfamily consists of five members, SPANXN1 (-N2, -N3, -N4, and -N5). Several studies of SPANX family members in breast cancer, colorectal cancer, and lung adenocarcinoma showed that their relationship with metastasis and poor prognosis in cancers (Chen et al., 2010; Maine et al., 2016; Hsiao et al., 2016). However, the role of SPANX family members in TGCTs has not yet been described (Kouprina et al., 2007a; Kouprina et al., 2007b). The SPANXN2 gene is localized on chromosome Xq27, a region of susceptibility gene localization for TGCT and prostate malignancy (Rapley et al., 2000; Kouprina et al., 2007a; Kouprina et al., 2007b; Lutke et al., 2006). In this study, we explore the role of SPANXN2 in TGCT progression to understand the importance of the SPANXN2 gene in TCGT and provide insights into the role of SPANXN2 in the progression of TGCT.

In our study, the effect of SPANXN2 on TGCTs progression investigated in vitro. Our result showed that SPANXN2 inhibited TGCT cell migration, indicating that SPANXN2 is an inhibitor of tumor metastasis.

MATERIALS & METHODS

Human testicular samples

The adjacent normal testicular tissue and TGCTs tissue samples used in this study were obtained from the Affiliated Cancer Hospital of Central South University (Changsha, China). Five adjacent normal tissue samples had been removed during para-testicular tumor surgery and the TGCT tissue samples were obtained from 11 testicular seminomas and three non-seminomas. Fresh tissues were collected and frozen in liquid nitrogen for
storage at $-180 \, ^\circ C$. All the tissues were confirmed by histopathological examination. The patients provided written informed consent to tissue sample collection, which was performed with the authorization of the Ethics Committee of Central South University (Approve No.: LLSB-2017-002).

**Quantitative RT-PCR**

The total RNA was extracted using TRIzol Reagent (Invitrogen, USA). The amount and purity of each RNA sample were quantified by Agilent2100 (Agilent, Wilmington, DE, USA). The cDNA was synthesized from 1 µg RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). The real-time PCR system (LightCycler480, Roche, USA) was used to measure the relative expression level of the SPANXN2 gene and the $\beta$-actin was used as the housekeeping gene for normalization. Amplification was performed with the following thermo-cycling conditions: initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10s and 60 °C for 10 s, and a final extension at 72 °C for 10 s. The LightCycler480 software was used to analyze the threshold cycle (CT) values and the $2^{-\Delta\Delta CT}$ method was used to evaluated relative gene expression. The gene-specific primers used were as follows:

SPANXN2 forward: 5'-GTGTATTACTACAGGAAGCATACG-3';
reverse: 5'-CTCCTCCTCTTGGACTGGATT-3'

$\beta$–actin forward: 5'-TCACCAACTGGGACGACATG-3';
reverse: 5'-GTCACCGGAGTCCATCACGAT-3'

**Cell culture**

The human TGCT cell line NCCIT was bought from the American Type Culture Collection (ATCC, VA, USA), and the human TGCT cell line TCAM-2 was obtained from Dr. Yuxin Tang (*Peng et al., 2019; Gan et al., 2016*). NCCIT cells were cultured in RPMI-1640 medium (GIBCO, USA), and TCAM-2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO, USA). All cells were cultured in medium containing 10% fetal bovine serum (FBS, GIBCO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO) and were incubated at 37 °C under 5% CO$_2$.

**Cell transfection**

The sequence of SPANXN2 was cloned into the CMV-MCS-DsRed2-SV40-Neomycin-GV147 vector. Cells were cultured as described above and divided into negative control (NC) and test (SPANXN2) groups and transfected with the GV147 empty vector (NC) and the GV147 vectors expressing SPANXN2, respectively. Briefly, cells were seeded in a 6-well culture plate ($5 \times 10^5$ cells/well) and transfected at 70% confluence. Cells were transfected with 2.5 µg of the GV147 empty vector and 2.5 µg of the GV147 vectors expressing SPANXN2 using DNA Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions. Cells were harvested 36 h or 48 h after transfection for use in the following experiments.

**Plate colony formation assay**

Cells were seeded in a 6-well plate (300 cells/well) and incubated at 37 °C under 5% CO$_2$ for 12–14 days when most single-cell colonies consist of $>$50 cells. The cells were washed three
times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min and then stained for 15 min with 0.5% crystal violet. After staining, colonies containing >50 cells were observed under a microscope and the colony number (a single colony with >30 cells) was counted using Adobe Photoshop CC 2018.

**MTT assay**
The proliferation of TGCT cells was evaluated by MTT assay. The cells were seeded into 96-well plates at $5 \times 10^3$ cells/ml (200 µl/well). After 6 h, 20 µl MTT solution (5 mg/ml) (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT) reagent (Sigma Chemicals, St Louis, MO, USA) was added to the 96-well plates. After incubation for 6 h at 37 °C, the supernatant was discarded from each well and 200 µl DMSO was added to each well. The absorbance at 492 nm was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher, USA). This process was repeated every 24 h for 1, 2, 3, and 4 days.

**Flow cytometric cell cycle analysis**
At 48 h after transfection, cells were first enzymatically dissociated into single cells by 0.05% trypsin and then fixed with 75% cold ethanol in PBS overnight at 4 °C. Second, the cells were washed with PBS three times. Finally, the cells were double-stained with a propidium iodide solution (PI, 50 µg/mL) and treated with RNase (1 mg/mL) at 37 °C for 30 min in the dark. Subsequently, samples were analyzed using an Accuri C6 flow cytometer (BD) with the Accuri software. For each sample, data from approximately 20,000 cells were acquired in list mode using logarithmic scales. FlowJo7.6.2 was used to analyze the cell cycle distribution according to the guideline.

**Wound healing assay**
Cells were seed in a 6-well culture plate ($5 \times 10^5$ cells/well) and the next day, transfected at 70% confluence. At 48 h after transfection, we drew three straight lines with an average interval in the layer of cells with a 100 µl of the tip. Cells were then washed three times with PBS to remove the scraped suspended cells. Cells were then washed cultured in medium containing 2% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C under 5% CO₂. The wound healing areas were recorded by observation under a microscope at 0 h, 24 h and 48 h, and Image-Pro Plus 6.0 software was used to analyze the width of wound healing.

**Transwell migration assay**
Transwell Cell Culture Inserts (8 µm pore size, FALCON, USA) were used to measure cell migration capacity in 24-well plates. In brief, TCAM-2 and NCCIT cells ($2 \times 10^4$ cells in 200 µl of 2% FBS medium) were added to the upper chamber, and 800 µl 15% FBS medium was added to the lower chamber. After incubation at 37 °C under 5% CO₂ for 48 h, the cells on the upper surface were wiped with cotton swabs, while the cells that migrated through the filter pores were fixed in 4% paraformaldehyde for 30 min. After staining with 0.5% (w/v) crystal violet (Sigma) in PBS (GIBCO) for 15 min, cells were observed and photographed under an inverted microscope. Cells were counted in five randomly selected fields of view.
Western Blot analysis
Cells were collected and lysed with RIPA lysis buffer (Co Win Biosciences, China) according to the manufacturer’s instructions, and proteins were harvested. Protein quantification was performed using a BCA Protein Quantification Kit (Thermo, USA), and samples were adjusted to the same concentration. After boiling in 6× Loading Buffer for 5 min, the proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membrane was incubated overnight at 4 °C with the appropriate primary detection antibodies: GAPDH (Sigma, 1:1,000), α-Tubulin (Sigma, 1:600), Snail (Cell Signaling Technology, 1:1,000), and Vimentin (Cell Signaling Technology, 1:1,000); AKT (ProteinTech, 1:1,000) and p-AKT (ProteinTech, 1:1,000). After washing three times with TBST for 5 min, the membrane was incubated with secondary antibodies (Cell Signaling Technology, 1:1,000) for 1 h at 37 °C. After washing three times with TBST for 15 min each time, the protein bands were visualized by fluorography using an enhanced chemiluminescence system (Millipore, USA); the expression levels of α-Tubulin and GAPDH were used as internal references.

Statistical analysis
All assays were repeated three times and all samples were analyzed in triplicate. The data were expressed as the mean ± SD or mean ± SEM of at least three independent experiments. Student’s t-test or a two-way ANOVA was used to determine the significance of differences between groups, and P < 0.05 was deemed statistically significant. Curve fitting analyses were performed with GraphPad Prism Software 5.0 (GraphPad Software, USA).

RESULTS
Validation of SPANXN2 expression in TGCTs
To verify the reliability of differential SPANXN2 expression, we analyzed its expression in TGCTs in the GEPIA database (Tang et al., 2017) (http://gepia.cancer-pku.cn/index.html), and the results showed that SPANXN2 was significantly downregulated in TGCT tissues (Fig. 1A). Subsequent qRT-PCR analysis of TGCT samples and normal samples also confirmed the differential expression of SPANXN2 (Fig. 1B). These results revealed that TGCT tissues express a lower level of SPANXN2 relative to those detected in normal tissues.

Transfection of TGCT cell lines
As shown in Figs. 2A, 2B, most cells (>75%) showed red fluorescence that confirmed transfection with plasmids targeting SPANXN2, or an empty-plasmid control. Quantitative RT-PCR was used to detect the relative expression of SPANXN2 in these two transfected TGCT cell lines (TCAM-2, NCCIT), using β-actin as a reference (Figs. 2C and 2D). The results confirmed the relative overexpression of SPANXN2 in the SPANXN2 group compared with that in the NC group, laying the foundation for the study of its function.

SPANXN2 upregulation inhibited colony formation ability with no effect on cell proliferation
The effect of SPANXN2 on the ability of TGCT cells to form colonies was investigated in colony formation assays. The colony formation ability of TGCT cells overexpressing
SPANXN2 was significantly decreased compared with that of the cells in the NC group \((P < 0.05, \text{Figs. 3A and 3B})\).

To investigate the effect of SPANXN2 overexpression on TGCT cell proliferation, NCCIT and TCAM-2 cells were transfected with the SPANXN2 plasmid and their negative control plasmid. The MTT assay results revealed no significant difference in the cell proliferation rates of the NC and SPANXN2 groups \((P > 0.05, \text{Figs. 3C and 3D})\). Furthermore, flow cytometric analysis carried out 48 h after transfection showed that there were no significant differences in the cell cycle distribution of TGCT cells overexpressing SPANXN2 compared with the cells in the NC group \((P > 0.05, \text{Figs. 4A–4F})\). In addition, no marked cell apoptosis was observed at 24 h and 48 h after transfection with the SPANXN2 plasmid. These results indicated that SPANXN2 overexpression inhibited the colony formation ability of TGCT cells, but has no significant effect on the cell cycle distribution and apoptosis.

**SPANXN2 negatively regulates TGCT cell migration**

The effect of SPANXN2 overexpression on the migration of TGCTs cells was investigated in wound healing and Transwell assays. As shown in Fig. 5, the wound healing gap in the NC group was significantly smaller than that in the SPANXN2 group. At 48 h after transfection, there were significantly fewer migrated cells in the SPANXN2 group compared with that in the NC group \((P < 0.05, \text{Figs. 6A and 6B})\). The results indicated that SPANXN2 overexpression inhibited TGCT cell migration.

**SPANXN2 influences the expression of EMT- and AKT-related proteins**

EMT describes the transition of epithelial cells into mesenchymal cells, some molecules of which will be changed during the process of metastasis of many tumors. To explore how SPANXN2 inhibited TGCT cell migration, the expression levels of EMT-related proteins...
were detected by western blot analysis. These results revealed that the expression levels of the EMT related proteins, Vimentin, and Snail were decreased (Fig. 6C), suggesting that SPANXN2 regulates the EMT-related proteins. Similarly, the AKT-related molecules were also detected. AKT and p-AKT protein expression levels were lower after SPANXN2 overexpression (Fig. 6D). These results showed that SPANXN2 overexpression regulated EMT- and AKT-related proteins in TGCT cells.

**DISCUSSION**

TGCTs are one of the major male reproductive tumors in young men (Rijlaarsdam & Looijenga, 2014). The clinical treatment of TGCTs is mainly orchiectomy supplemented with chemotherapy and radiotherapy (Greene et al., 2010). Data show that the incidence of TGCTs has increased globally in recent years (Shanmugalingam et al., 2013; Le Cornet et al., 2014; Znaor et al., 2015). Despite encouraging progress in the diagnosis and treatment, TGCT patients still have a high risk of relapse with poor prognosis (Qin et al., 2020). The mechanisms underlying the development of TGCT have yet to be fully elucidated (Kalavska et al., 2017).

The results of our analysis of clinical TGCT specimens were consistent with those of the GEPIA database, in which SPANXN2 expression was lower in TGCTs at the mRNA
Studies of the role of non-coding RNAs in TGCTs have been conducted. A profiling study of the small RNA populations in TGCTs showed the global loss of piwi-interacting RNA (Rounge et al., 2015). In a gene expression profiling study of TGCT samples, Siska et al., (2017) demonstrated that activated T-cell infiltration and PD-1/PD-L1 interaction are closely correlated with seminoma histology and good prognosis. To our knowledge, in vitro studies of the role of SPANXN2 in TGCT have not yet been reported and it is not clear if SPANXN2 acts as a tumor suppressor gene or oncogene in TGCT. Previous studies have shown abnormal SPANX gene expression in many cancers, which may promote or inhibit tumor invasion (Yilmaz-Ozcan et al., 2014; Maine et al., 2016). However, the role of SPANXN2 in TGCTs progression has not yet been elucidated. The differential expression of SPANXN2 between TGCTs tissues and adjacent normal testicular tissues suggests that SPANXN2 plays a role in the progression of TGCT.

In our study, no significant effects of SPANXN2 on proliferation activity and cell cycle distribution in TGCT cell lines were observed. However, we found that SPANXN2 significantly inhibited the colony formation and migration abilities of TGCT cells. These findings suggested that the inhibitory effects of SPANXN2 on the colony formation and
migration of TGCT cell lines are not influenced by cell proliferation and may be regulated by other pathways.

Metastasis is an important aspect of cancer progression and may lead to a poor prognosis in tumor patients. In addition to SPANXN2, other SPANX gene family members also play important roles in tumors metastasis. Research showed that SPANX expression was significantly correlated with the migration of colorectal cancer to the liver with poor prognosis (Chen et al., 2010). SPANXA/D was required for spontaneous metastasis of breast cancer cells to the lung and elevated SPANXA/D expression in breast cancer patient tumors correlated with poor outcome (Maine et al., 2016). Similar to our study, SPANXN2 inhibited the migration of TCGT cells, suggesting that SPANXN2 might be a tumor suppressor gene in TGCT and related to the poor prognosis of TGCT patients. Functional investigations of SPANXN2 are restricted by the high homology among SPANX family members. The mechanism of SPANXN2 dysregulation in TGCT requires further research, although our results showed that SPANXN2 inhibited the colony formation and migration abilities of TGCT cell lines. To our knowledge, our study is the first to characterize the functional role of SPANXN2 in TGCTs.

EMT is critical for local invasion and cell dissemination, which in cancer, are associated with tumor initiation and progression, stemness, survival, and resistance to therapy (Mitschke, Burk & Reinheckel, 2019). Epithelial cells convert to interstitial phenotypes, characterized by the upregulation of interstitial proteins, such as Vimentin (Mitschke, Burk & Reinheckel, 2019; Jolly et al., 2019) and EMT transcriptional drivers such as Snail (Thiery et al., 2009). In our study, we found that SPANXN2 suppressed TGCT cell migration and altered the expression of the EMT-related proteins, Vimentin and Snail. These findings are in accordance with those reported by Hsiao et al., showing that SPANXA inhibits the
invasion and metastasis of lung cancer cells \textit{in vitro} and \textit{in vivo} by inhibiting the EMT pathway in lung adenocarcinoma (Hsiao et al., 2016). Previous studies have also indicated that CTAs, such as the SPANX protein family, activate the EMT signaling pathway in cancer stem cell-like cells, and leading to the occurrence of cancer (Yang et al., 2015). Therefore, we speculated that SPANXN2 suppressed the metastasis of TGCT cells by regulating the EMT-related proteins in TGCTs.

The AKT signaling pathway is activated in many cancers, such as thyroid carcinomas (Chen et al., 2018). A previous study showed that activation of AKT expression drives the EMT phenotype and enhances the proliferation and invasion of seminoma (Chen et al., 2018). The AKT pathway is involved in cellular processes, particularly in cell proliferation and migration, and has a pivotal role in human tumorigenesis (Chen et al., 2018; Minna
Effects of SPANXN2 on cell migration and the related proteins of EMT and AKT. (A) Image showing migration of TGCT cells overexpressing SPANXN2. (B) The relative number of migrated cells in the NC and SPANXN2 groups; *P<.05 compared with the NC group. (C) Western blot analysis of Vimentin and Snail proteins. (D) Western blot analysis of AKT and p-AKT proteins. Scale bar in A, 100 µm.

In our study, the AKT proteins were downregulated in the TGCT cells overexpressing SPANXN2. Above all, these results suggested that SPANXN2 suppresses tumor cell migration and colony formation via regulation of EMT- and AKT-related proteins.

Certain limitations of qRT-PCR analysis should be noted. Since clinical specimens are hard to obtain, we included biological tissues from only 14 cases, comprising a population including both metastatic and chemotherapy-refractory tumors, and all were obtained from a single institution. In addition, further studies are required to confirm the molecular mechanisms by which SPANXN2 inhibited tumor cell migration by regulating the EMT and AKT/p-AKT signaling pathways. On the other hand, we were unable to determine the SPANXN2 expression at the protein level is failed detected due to its sufficient homology among SPANX gene family members resulting in a lack of specificity of the SPANXN2-binding antibody. Third, the population of TGCT cells overexpressing SPANXN2 used for clonal analysis contained some wild-type cells, which may weaken the effect of SPANXN2 on clone formation; however, our study showed that SPANXN2 inhibited TGCT cell clone formation, thus verifying that the observation that SPANXN2 inhibited TGCT cell clone formation. Finally, the functions of these molecules in the progression of TGCT remain to be elucidated. According to large-scale sequencing studies of testicular germ cell tumors, there is no major high-penetrance TGCT predisposition gene (Crockford et al., 2016).
It is likely to be due to the involvement of many genes in the occurrence and development of TGCT.

CONCLUSIONS

In summary, this is the first report describing the role of SPANXN2 in TGCTs. *In vitro* studies demonstrated that SPANXN2 significantly inhibited the migration and colony formation of TGCT cells and regulated the expression of EMT- and AKT-related proteins. Further studies are required to confirm that SPANXN2 suppresses tumor cell migration and colony formation by regulating EMT and AKT signaling pathways. Our results indicated that SPANXN2 is a potential therapeutic target for TGCTs and will facilitate accurate and effective treatment of TGCTs.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This research was funded by the National Natural Science Foundation of China (Grant No. 31472054), the National Key Research and Development Program of China (Nos. 2016YFC1000600), the Fundamental Research Funds for the Central Universities of Central South University (Nos. 2019zzts715), and the Fundamental Research Funds for Health Commission of Hunan Province (Grant Number: C2019073). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31472054.
National Key Research and Development Program of China: 2016YFC1000600.
Fundamental Research Funds for the Central Universities of Central South University: 2019zzts715.
Fundamental Research Funds for Health Commission of Hunan Province: C2019073.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
- Fang Zhu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Hao Bo conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Guangmin Liu and Ruixue Li analyzed the data, prepared figures and/or tables, and approved the final draft.
- Zhizhong Liu performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
Liqing Fan conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

**Human Ethics**
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All patients provided written informed consent to tissue sample collection. This study was carried out with the approval and under the supervision of Ethic Committee of Basic Medical Science School, Central South University (Approval No: LLSB-2017-002).

**DNA Deposition**
The following information was supplied regarding the deposition of DNA sequences:

The SPANX2 sequence is available at GenBank: NM_001009615.2.

**Data Availability**
The following information was supplied regarding data availability:

Raw data and pictures are available in the Supplemental Files.

**Supplemental Information**
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.9358#supplemental-information.

**REFERENCES**

Albers P, Albrecht W, Algba F, Bokemeyer C, Cohn-Cedermark G, Fizazi K, Horwich A, Laguna MP, Nicolai N, Oldenburg J. 2015. Guidelines on testicular cancer: 2015 update. *European Urology* 68:1054–1068 DOI 10.1016/j.eururo.2015.07.044.

Albers P, Siener R, Kliesch S, Weissbach L, Krege S, Sparwasser C, Schulze H, Heidenreich A, de Riese W, Loy V, Bierhoff E, Wittekind C, Fimmers R, Hartmann M. 2003. Risk factors for relapse in clinical stage I nonseminomatous testicular germ cell tumors: results of the German Testicular Cancer Study Group Trial. *Journal of Clinical Oncology* 21:1505–1512 DOI 10.1200/JCO.2003.07.169.

Chen Y, Lu J, Xia L, Xue D, Yu X, Shen D, Xu L, Li G. 2018. Testicular orphan receptor 4 promotes tumor progression and implies poor survival through AKT3 regulation in seminoma. *Cancer Science* 109:384–394 DOI 10.1111/cas.13461.

Chen Z, Li M, Yuan Y, Wang Q, Yan L, Gu J. 2010. Cancer/testis antigens and clinical risk factors for liver metastasis of colorectal cancer: a predictive panel. *Diseases of the Colon & Rectum* 53:31–38 DOI 10.1007/DCR.0b013e3181bdca3a.

Classen J, Schmidberger H, Meisner C, Souchon R, Sautter-Bihl ML, Sauer R, Weinknecht S, Kohrmann KU, Bamberg M. 2003. Radiotherapy for stages IIA/B testicular seminoma: final report of a prospective multicenter clinical trial. *Journal of Clinical Oncology* 21:1101–1106 DOI 10.1200/JCO.2003.06.065.

Crockford GP, Linger R, Hockley S, Dudakia D, Johnson L, Huddart R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G,
Richard S, Chompret A, Bonaiti-Pellie C, Heidenreich A, Albers P, Olah E, Geczi L, Bodrogi I, Ormiston WJ, Daly PA, Guilford P, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Forman D, Oliver T, Einhorn L, McMaster M, Kramer J, Greene MH, Weber BL, Nathanson KL, Cortessis V, Easton DF, Bishop DT, Stratton MR, Rapley EA. 2006. Genome-wide linkage screen for testicular germ cell tumour susceptibility loci. *Human Molecular Genetics* **15**:443–451 DOI 10.1093/hmg/ddi459.

Facchini G, Rossetti S, Berretta M, Cavaliere C, D’Aniello C, Iovane G, Mollo G, Capasso M, Della PC, Pesce L, Facchini S, Imbimbo C, Pisconti S. 2019. Prognostic and predictive factors in testicular cancer. *European Review for Medical and Pharmaceutical Sciences* **23**:3885–3891 DOI 10.26355/eurrev_201905_17816.

Gan Y, Wang Y, Tan Z, Zhou J, Kitazawa R, Jiang X, Tang Y, Yang J. 2016. TDRG1 regulates chemosensitivity of seminoma TCam-2 cells to cisplatin via PI3K/Akt/mTOR signaling pathway and mitochondria-mediated apoptotic pathway. *Cancer Biology & Therapy* **17**:741–750 DOI 10.1080/15384047.2016.1178425.

Greene MH, Kratz CP, Mai PL, Mueller C, Peters JA, Bratslavsky G, Ling A, Choyke PM, Premkumar A, Bracci J, Watkins RJ, McMaster ML, Korde LA. 2010. Familial testicular germ cell tumors in adults: 2010 summary of genetic risk factors and clinical phenotype. *Endocrine-related Cancer* **17**:R109–R121 DOI 10.1677/ERC-09-0254.

Hsiao YJ, Su KY, Hsu YC, Chang GC, Chen JS, Chen HY, Hong QS, Hsu SC, Kang PH, Hsu CY, Ho BC, Yang TH, Wang CY, Jou YS, Yang PC, Yu SL. 2016. SPANXA suppresses EMT by inhibiting c-JUN/SNAI2 signaling in lung adenocarcinoma. *Oncotarget* **7**:44417–44429 DOI 10.18632/oncotarget.10088.

Jolly MK, Somarelli JA, Sheth M, Biddle A, Tripathi SC, Armstrong AJ, Hanash SM, Bapat SA, Rangarajan A, Levine H. 2019. Hybrid epithelial/mesenchymal phenotypes promote metastasis and therapy resistance across carcinomas. *Pharmacology and Therapeutics* **194**:161–184 DOI 10.1016/j.pharmthera.2018.09.007.

Kalavska K, Cierna Z, Chovanec M, Takacova M, Svetlovska D, Miskovska V, Obertova J, Palacka P, Rajec J, Sycova-Mila Z, Machalekova K, Kajo K, Spanik S, Mardiak J, Babal P, Pastorekova S, Mego M. 2017. Prognostic value of intratumoral carbonic anhydrase IX expression in testicular germ cell tumors. *Oncology Letters* **13**:2177–2185 DOI 10.3892/ol.2017.5745.

Kouprina N, Mullokandov M, Rogozin IB, Collins NK, Solomon G, Otstot J, Risinger JI, Koonin EV, Barrett JC, Larionov V. 2004. The SPANX gene family of cancer/testis-specific antigens: rapid evolution and amplification in African great apes and hominids. *Proceedings of the National Academy of Sciences of the United States of America* **101**:3077–3082 DOI 10.1073/pnas.0308532100.

Kouprina N, Noskov VN, Pavlichek A, Collins NK, Schoppee BP, Ottolenghi C, Loukinov D, Goldsmith P, Risinger JI, Kim JH, Westbrook VA, Solomon G, Sounders H, Herr JC, Jurka J, Lobanenko V, Schlessinger D, Larionov V. 2007a. Evolutionary diversification of SPANX-N sperm protein gene structure and expression. *PLOS ONE* **2**:e359 DOI 10.1371/journal.pone.0000359.
Kouprina N, Noskov VN, Solomon G, Otstot J, Isaacs W, Xu J, Schleutker J, Larionov V. 2007b. Mutational analysis of SPANX genes in families with X-linked prostate cancer. *Prostate* 67:820–828 DOI 10.1002/pros.20561.

Le Cornet C, Lortet-Tieulent J, Forman D, Beranger R, Flechon A, Fervers B, Schuz J, Bray F. 2014. Testicular cancer incidence to rise by 25% by 2025 in Europe? Model-based predictions in 40 countries using population-based registry data. *European Journal of Cancer* 50:831–839 DOI 10.1016/j.ejca.2013.11.035.

Litchfield K, Loveday C, Levy M, Dudakia D, Rapley E, Nsengimana J, Bishop DT, Reid A, Huddart R, Broderick P, Houlston RS, Turnbull C. 2018. Large-scale Sequencing of Testicular Germ Cell Tumour (TGCT) cases excludes major TGCT predisposition gene. *European Urology* 73:828–831 DOI 10.1016/j.eururo.2018.01.021.

Lobo J, Costa AL, Vilela-Salgueiro B, Rodrigues A, Guimaraes R, Cantante M, Lopes P, Antunes L, Jeronimo C, Henrique R. 2018. Testicular germ cell tumors: revisiting a series in light of the new WHO classification and AJCC staging systems, focusing on challenges for pathologists. *Human Pathology* 82:113–124 DOI 10.1016/j.humpath.2018.07.016.

Lutke HM, Hoekstra HJ, Sijmons RH, Sonneveld DJ, Van der Steege G, Sleijfer DT, Nolte IM. 2006. Re-analysis of the Xq27-Xq28 region suggests a weak association of an X-linked gene with sporadic testicular germ cell tumour without cryptorchidism. *European Journal of Cancer* 42:1869–1874 DOI 10.1016/j.ejca.2006.03.009.

Maine EA, Westcott JM, Prechtl AM, Dang TT, Whitehurst AW, Pearson GW. 2016. The cancer-testis antigens SPANX-A/C/D and CTAG2 promote breast cancer invasion. *Oncotarget* 7:14708–14726 DOI 10.18632/oncotarget.7408.

Minna E, Romeo P, Dugo M, De Cecco I, Todoerti K, Pilotti S, Perrone F, Seregni E, Agnelli I, Neri A, Greco A, Borrello MG. 2016. miR-451a is underexpressed and targets AKT/mTOR pathway in papillary thyroid carcinoma. *Oncotarget* 7:12731–12747 DOI 10.18632/oncotarget.7262.

Mitschke J, Burk UC, Reinheckel T. 2019. The role of proteases in epithelial-to-mesenchymal cell transitions in cancer. *Cancer and Metastasis Reviews* DOI 10.1007/s10555-019-09808-2.

Peng D, Wei J, Gan Y, Yang J, Jiang X, Kitazawa R, Xiang Y, Dai Y, Tang Y. 2019. Testis developmental related gene 1 regulates the chemosensitivity of seminoma TCam-2 cells to cisplatin via autophagy. *Journal of Cellular and Molecular Medicine* 23:7773–7784 DOI 10.1111/jcmm.14654.

Qin G, Mallik S, Mitra R, Li A, Jia P, Eischen CM, Zhao Z. 2020. MicroRNA and transcription factor co-regulatory networks and subtype classification of seminoma and non-seminoma in testicular germ cell tumors. *Scientific Reports* 10:852 DOI 10.1038/s41598-020-57834-w.

Rapley EA, Crockford GP, Teare D, Biggs P, Seal S, Barfoot R, Edwards S, Hamoudi R, Heimdal K, Fossa SD, Tucker K, Donald J, Collins F, Friedlander M, Hogg D, Goss P, Heidenreich A,Ormiston W, Daly PA, Forman D, Oliver TD, Leahy M, Huddart R, Cooper CS, Bodmer JG, Easton DF, Stratton MR, Bishop DT. 2000.
Localization to Xq27 of a susceptibility gene for testicular germ-cell tumours. *Nature Genetics* 24:197–200 DOI 10.1038/72877.

Rijlaarsdam MA, Looijenga LH. 2014. An oncofetal and developmental perspective on testicular germ cell cancer. *Seminars in Cancer Biology* 29:59–74 DOI 10.1016/j.semcancer.2014.07.003.

Rounge TB, Furu K, Skotheim RI, Haugen TB, Grotmol T, Enerly E. 2015. Profiling of the small RNA populations in human testicular germ cell tumors shows global loss of piRNAs. *Molecular Cancer* 14:153 DOI 10.1186/s12943-015-0411-4.

Schmidberger H, Bamberg M, Meisner C, Classen J, Winkler C, Hartmann M, Templin R, Wiegel T, Dornoff W, Ross D, Thiel HJ, Martini C, Haase W. 1997. Radiotherapy in stage IIA and IIB testicular seminoma with reduced portals: a prospective multicenter study. *International Journal of Radiation Oncology, Biology, Physics* 39:321–326 DOI 10.1016/s0360-3016(97)00155-7.

Shanmugalingam T, Soultati A, Chowdhury S, Rudman S, Van Hemelrijck M. 2013. Global incidence and outcome of testicular cancer. *Clinical Epidemiology* 5:417–427 DOI 10.2147/CLEP.S34430.

Siska PJ, Johnpulle R, Zhou A, Bordeaux J, Kim YJ, Dabbas B, Dakappagari N, Rathmell JC, Rathmell WK, Morgans AK, Balko JM, Johnson DB. 2017. Deep exploration of the immune infiltrate and outcome prediction in testicular cancer by quantitative multiplexed immunohistochemistry and gene expression profiling. *OncolImmunology* 6:e1305535 DOI 10.1080/2162402X.2017.1305535.

Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. 2017. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Research* 45:W98–W102 DOI 10.1093/nar/gkx247.

Thiery JP, Acloque H, Huang RY, Nieto MA. 2009. Epithelial-mesenchymal transitions in development and disease. *Cell* 139:871–890 DOI 10.1016/j.cell.2009.11.007.

Van den Belt-Dusebout AW, De Wit R, Gietema JA, Horenblas S, Louwman MW, Ribot JG, Hoekstra HJ, Ouwens GM, Aleman BM, Van Leeuwen FE. 2007. Treatment-specific risks of second malignancies and cardiovascular disease in 5-year survivors of testicular cancer. *Journal of Clinical Oncology* 25:4370–4378 DOI 10.1200/JCO.2006.10.5296.

Whitehurst AW. 2014. Cause and consequence of cancer/testis antigen activation in cancer. *Annual Review of Pharmacology and Toxicology* 54:251–272 DOI 10.1146/annurev-pharmtox-011112-140326.

Yang P, Huo Z, Liao H, Zhou Q. 2015. Cancer/testis antigens trigger epithelial-mesenchymal transition and genesis of cancer stem-like cells. *Current Pharmaceutical Design* 21:1292–1300 DOI 10.2174/1381612821666141211154707.

Yilmaz A, Cheng T, Zhang J, Trpkov K. 2013. Testicular hilum and vascular invasion predict advanced clinical stage in nonseminomatous germ cell tumors. *Modern Pathology* 26:579–586 DOI 10.1038/modpathol.2012.189.

Yilmaz-Ozcan S, Sade A, Kucukkaraduman B, Kaygusuz Y, Senses KM, Banerjee S, Gure AO. 2014. Epigenetic mechanisms underlying the dynamic expression of
cancer-testis genes, PAGE2, -2B and SPANX-B, during mesenchymal-to-epithelial transition. *PLOS ONE* 9:e107905 DOI 10.1371/journal.pone.0107905.

**Znaor A, Lortet-Tieulent J, Jemal A, Bray F. 2014.** International variations and trends in testicular cancer incidence and mortality. *European Urology* 65:1095–1106 DOI 10.1016/j.eururo.2013.11.004.

**Znaor A, Lortet-Tieulent J, Laversanne M, Jemal A, Bray F. 2015.** International variations and trends in renal cell carcinoma incidence and mortality. *European Urology* 67:519–530 DOI 10.1016/j.eururo.2014.10.002.