SPANXN2 is a cell migration inhibitor in testicular germ cell tumor cell

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Background: Increasing evidence has revealed that SPANX family members have been proved to play an important role in cancer progression. SPANXN2, a cancer-testis antigen (CTA), is a testis-specific gene. There was no study about the role of SPANXN2 in testicular germ cell tumors (TGCTs). TGCT is one of the most common solid tumors among young men, which has increasing incidence and could occur distant metastases easily in men with poor prognosis. Therefore the aim of our study is to clarify the role of SPANXN2 in TGCT development. **Methods**: In order to clarify the role of SPANXN2 in TGCT development, firstly, we performed quantitative real-time polymerase chain reaction (qRT-PCR) analyses of fourteen TGCTs samples and five adjacent normal tissue samples to validate the expression level of SPANXN2 in TGCTs. Secondly, the experiments in vitro were used to explore the function of SPANXN2 in TGCT cells. Transwell assay was used to detect the effect of SPANXN2 on cell migration. Colony formation assay was used to detect the effect of SPANXN2 on cell clone ability. MTT assay and cell cycle analysis were used to detect the effects of SPANXN2 on cell proliferation. Lastly, western blot was performed to detect the expression levels of EMT-related proteins and AKT-related proteins in SPANXN2 overexpression cells. **Results**: SPANXN2 expression level was lower in TGCTs than in adjacent normal tissues, which was consistent with the result of the Gene Expression Profiling Interactive Analysis database. Experiments *in vitro* demonstrated that SPANXN2 overexpression significantly inhibited the TGCTs cell migration and colony formation capability. SPANXN2 inhibited the expression levels of EMT-related proteins and AKT-related proteins, Vimentin, Snail, AKT, and p-AKT. **Conclusion**: Our results showed that SPANXN2 inhibited migration and colony formation of TGCT cells. And in TGCT cell overexpressing SPANXN2, the expression levels of EMT-related proteins and AKT-related proteins have been changed. It suggested that the migration of TGCT cells may regulate by EMT and AKT related molecules in TGCT cells overexpressing SPANXN2. Further

research should be conduct to verify whether SPANXN2 would be a potential diagnostic biomarker or novel therapeutic target in TGCTs.

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Abstract

Background: Increasing evidence has revealed that SPANX family members have been proved to play an important role in cancer progression. SPANXN2, a cancer-testis antigen (CTA), is a testis-specific gene. There was no study about the role of SPANXN2 in testicular germ cell tumors (TGCTs). TGCT is one of the most common solid tumors among young men, which has increased incidence and could occur distant metastases easily in men with poor prognosis. Therefore the aim of our study is to clarify the role of SPANXN2 in TGCT development.

Methods: In order to clarify the role of SPANXN2 in TGCT development, firstly, we performed quantitative real-time polymerase chain reaction (qRT-PCR) analyses of fourteen TGCTs samples and five adjacent normal tissue samples to validate the expression level of SPANXN2 in TGCTs. Secondly, the experiments in vitro were used to explore the function of SPANXN2 in TGCT cells. Transwell assay was used to detect the effect of SPANXN2 on cell migration. Colony formation assay was used to detect the effect of SPANXN2 on cell clone ability. MTT assay and cell cycle analysis were used to detect the effects of SPANXN2 on cell proliferation. Lastly, western blot was performed to detect the expression levels of EMT-related proteins and AKT-related proteins in SPANXN2 overexpression cells.

Results: SPANXN2 expression level was lower in TGCTs than in adjacent normal tissues, which was consistent with the result of the Gene Expression Profiling Interactive Analysis database. Experiments *in vitro* demonstrated that SPANXN2 overexpression significantly inhibited the TGCTs cell migration and colony formation capability. SPANXN2 inhibited the expression levels of EMT-related proteins and AKT-related proteins, Vimentin, Snail, AKT, and p-AKT.
Conclusion: Our results showed that SPANXN2 inhibited migration and colony formation of TGCT cells. And in TGCT cell overexpressing SPANXN2, the expression levels of EMT-related proteins and AKT-related proteins have been changed. It suggested that the migration of TGCT cells may regulate by EMT and AKT related molecules in TGCT cells overexpressing SPANXN2. Further research should be conduct to verify whether SPANXN2 would be a potential diagnostic biomarker or novel therapeutic target in TGCTs.

Introduction

Testicular germ cell tumor (TGCT) is one of the most common solid tumors in young men aged between the ages of 15 and 40 *(Rijlaarsdam & Looijenga, 2014)*. The incidence of TGCTs has increased globally in recent years, that approximately 1.3/100,000 men in China and about 7.8/100,000 men in Western Europe *(Shanmugalingam et al., 2013; Le Cornet et al., 2014; Znaor et al., 2014)*. Histologically, TGCTs include two main subtypes, 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 1/5 of them are destined to relapse *(Schmidberger et al., 1997; Classen et al., 2003; Facchini et al., 2019)*. About 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 severe opposite effects on the sexual function, fertility, and quality of life, especially in young patients *(van den Belt-Dusebout et al., 2007; Albers et al., 2015)*. However, the underlying mechanism of TGCTs development remains unclear. Therefore to further clarify the molecular mechanisms referring to the pathogenesis and development of TGCTs is critical.

Cancer-testis antigens (CTAs), the expression in normal tissues seems to be testis-specific, have been widely investigated as targets for anticancer vaccines *(Whitehurst, 2014)*. SPANX multigene family is a representative CTA, which has two subfamilies, SPANX-A/D and

SPANXN *(Kouprina et al., 2004; Kouprina et al., 2007)*. SPANX-N genes consist of five members, SPANXN1 (-N2, -N3, -N4, and -N5). Even though there have been several studies on SPANX family members in cancer, such as breast cancer, colorectal cancer and lung adenocarcinoma *(Chen et al., 2010; Maine et al., 2016; Hsiao et al., 2016)*. No researches on SPANX family members in TGCTs at present. SPANX-N can be detected in human sperm cell precursors *(Kouprina et al., 2007)*. The *SPANXN2* gene is localized on chromosome Xq27, a region of susceptibility gene localization for TGCTs and prostate malignancy *(Rapley et al., 2000; Kouprina et al., 2007; Lutke et al., 2006)*. So in this study, we aimed to explore the role of SPANXN2 in TGCTs progression. It was significant to understand the importance of the SPANXN2 gene in TCGTs and provided a novel insight concerning the role of SPANXN2 in the progression of TGCTs.

In our study, we had validated the expression level of SPANXN2 in TGCTs, firstly. Secondly, the effect of SPANXN2 on TGCTs progression was conducted by assays *in vitro*. Finally, we explored the underlying mechanism by Western-Blotting analysis. Taken together, our findings may in favor of discover novel TGCTs biomarkers or targeted therapies.

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 TGCTs tissue samples were obtained from eleven testicular seminomas and three non-seminomas. Fresh tissues were collected and frozen in liquid nitrogen for storage at -180°C. And 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 1μg 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 β-actin was used as the housekeeping gene for normalization. Amplification was performed with the following thermos-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 and $2^{-\Delta\Delta CT}$ method were used to analyze the threshold cycle (CT) values. The gene-specific primers used were as follows:

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

Cells were cultured as mentioned above and divided into negative control (NC), and test group (SPANXN2 group). The sequence of SPANXN2 was cloned into the CMV-MCS-DsRed2-SV40-Neomycin-GV147 vector. The GV147 empty vector (NC) and the GV147 vectors expressing the SPANXN2 were transfected into cells. Cells were seeded in a 6-well culture plate (5×10^5 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 to perform 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 by 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 5×10^3 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

After transfected 48 h, firstly, cells were enzymatically dissociated into single cells by 0.05% trypsin and then fixed with 75% cold ethanol in PBS overnight at 4℃. Secondly, 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℃ 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 for analyzing the cell cycle distribution according to the guideline.

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 (2×10^4 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% CO_2 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 to harvest proteins. 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℃ with the appropriate primary detection antibodies: GAPDH (Sigma, 1:1000 dilution), α-Tubulin (Sigma, 1:600 dilution), Snail (Cell Signaling Technology, 1:1000 dilution), and Vimentin (Cell Signaling Technology, 1:1000 dilution); AKT (ProteinTech, 1:1000 dilution) and p-AKT (ProteinTech, 1:1000 dilution). After washing three times with TBST for 5 min, the membrane was incubated with secondary antibodies（Cell Signaling Technology, 1:1000 dilution） for 1 h at 37℃. 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 expressions of α-Tubulin and GAPDH were used as internal reference.

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 Software5.0 (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 TGCTs tissues (Fig. 1A).

Subsequent qRT-PCR analysis of TGCTs samples and normal samples also confirmed the differential expression of SPANXN2 (Fig. 1B). These results revealed that TGCTs tissues express a lower level of SPANXN2 relative to those detected in normal tissues.

Transfection of TGCT cell lines

As shown in Figure 2A, 2B, most cells (>75%) showed red fluorescence that means 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 TGCTs cell lines (TCAM-2, NCCIT), using β-actin as a reference (Fig. 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 TGCTs cells to form colonies was investigated in colony formation assays. The colony formation ability of TGCTs cells overexpressing SPANXN2 was significantly decreased compared with that of the cells in the NC group ($P < 0.05$, Fig. 3A and 3B).

To investigate the effect of SPANXN2 overexpression on TGCT cell proliferation. NCCIT and TCAM-2 cells were transfected with SPANXN2 plasmid and their negative control plasmid. The MTT assay results revealed that it had no significant difference in the cell proliferation rates of the NC and SPANXN2 groups ($P> 0.05$, Fig. 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 TGCTs cells overexpressing SPANXN2 compared with the cells in the NC group ($P> 0.05$, Fig. 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 TGCTs cells, but has no significant effect on the cell cycle distribution and apoptosis.

SPANXN2 negatively regulates TGCTs cell migration

The effect of SPANXN2 overexpression on the migration of TGCTs cells was investigated in Transwell assays. At 48 h after transfection, the cell number that migrated was significantly fewer in the SPANXN2 group compared with that in the NC group ($P < 0.05$, Fig. 5A and 5B). The results indicated that SPANXN2 overexpression inhibited the migration of TGCT cells.

SPANXN2 influences EMT-related proteins 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 TGCTs 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 proteins, Vimentin and Snail was decreased (Fig. 5C), suggesting that SPANXN2 regulates the EMT-related proteins. Similarly, the AKT related molecules had also detected. AKT and p-AKT protein expression levels were lower after SPANXN2 overexpression (Fig. 5D). The results above showed that SPANXN2 overexpression regulated EMT-related proteins 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 showed 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 for diagnosing and treating TGCTs, TGCT patients still have a high risk of relapse with poor prognosis *(Qin et al., 2020)*. The underlying mechanisms of TGCTs development have yet to be fully elucidated *(Kalavska et al., 2017)*.

The results of our analysis of clinical TGCTs specimens were consistent with those of the GEPIA database, in which SPANXN2 expression was lower in TGCTs at the mRNA level. Until now, some researches that explored the role of the 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 TGCTs samples, Siska et al. demonstrated that activated T-cell infiltration and PD-1/PD-L1 interaction are closely correlated with seminoma histology and good prognosis *(Siska et al., 2017)*. To our knowledge, this is no report that SPANXN2 plays a role in TGCTs and based on assays *in vitro*. So far it is not yet clear if SPANXN2 acts as tumor suppressor genes or oncogenes in TGCTs. 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)*. But 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 might play a role in the progression of TGCTs.

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 maybe in some other ways.

Metastasis is an important aspect of cancer progression and may cause a poor prognosis in tumor patients. In addition to SPANXN2, other SPANX gene family members also play important roles in tumors metastasis. A 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 migration in TCGTs cells. It suggested that SPANXN2 might be a tumor suppressor gene in TGCTs 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 dysregulated in TGCTs is still need further research, although our results showed that SPANXN2 inhibited the colony formation and migration abilities of TGCTs cell lines. To our knowledge, our study is the first study 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 et al., 2019)*. Epithelial cells convert to interstitial phenotypes, characterized by the up-regulation of interstitial proteins, such as Vimentin *(Mitschke et al., 2019; Jolly et al., 2019)*. EMT transcriptional drivers like Snail *(Thiery et al., 2009)*. In our study, we found that SPANXN2 suppressed the metastasis of TGCTs cells and made changes in EMT-related proteins, Vimentin, Snail. This result is similar to the study of Hsiao et al, who reported that SPANXA inhibits the invasion and metastasis of lung cancer cells *in vitro* and *in vivo* by inhibiting the EMT pathway of lung adenocarcinoma *(Hsiao et al., 2016)*. Previous studies have also been suggested that CTAs, such as the SPANX protein family, induced the EMT signaling pathway in cancer stem cell-like cells, and led 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 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 et al., 2016)*. In our study, the AKT proteins were downregulated in the TGCT cells of SPANXN2 overexpression. Above all, these results suggested that SPANXN2 might suppress tumor cell migration and colony formation and regulated by the EMT-related proteins and AKT-related proteins.

Certain limitations of qRT-PCR analysis should be noted. Due to clinical specimens are hard to come by, we included biological tissues from only thirteen 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 regulating by the EMT signaling pathways and the AKT/p-AKT signaling pathways. On the other hand, the protein level of SPANXN2 is failed detected due to its sufficient homology among SPANX gene family members. The available antibody is the non-specific binding of SPANXN2, which is a limitation of our current study. Finally, the functions of these molecules in the progression of TGCTs remain future works to be elucidated. According to large-scale sequencing studies of testicular germ cell tumors, there is no major high-penetrance TGCTs predisposition gene *(Crockford et al., 2006; Litchfield et al., 2018)*. It is likely to be due to the involvement of many genes in the occurrence and development of TGCTs.

Conclusions

In summary, this is the first report about the role of SPANXN2 on TGCTs. Assays *in vitro* demonstrated that SPANXN2 significantly inhibited the migration and colony formation of TGCTs cells. SPANXN2 regulated the expression of EMT-related proteins and AKT-related proteins. It needs further experiments to prove that SPANXN2 may suppress tumor cell migration and colony formation regulating by EMT signaling pathway and AKT signaling

pathways. Our results indicated that SPANXN2 has the potential to become therapeutic targets for TGCTs. It will facilitate accurate and effective treatment for TGCTs.

References:

Albers, P., Albrecht, W., Algaba, F., Bokemeyer, C., Cohn-Cedermark, G., Fizazi, K., Horwich, A., Laguna, M.P., Nicolai, N., and Oldenburg, J. 2015. Guidelines on Testicular Cancer: 2015 Update. *EUROPEAN UROLOGY* 68:1054-1068. DOI:10.1016/j.eururo.2015.07.044

Chen, Y., Lu, J., Xia, L., Xue, D., Yu, X., Shen, D., Xu, L., and 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., and 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, M.L., Sauer, R., Weinknecht, S., Kohrmann, K.U., and 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, G.P., Linger, R., Hockley, S., Dudakia, D., Johnson, L., Huddart, R., Tucker, K., Friedlander, M., Phillips, K.A., Hogg, D., Jewett, M.A., Lohynska, R., Daugaard, G., Richard, S., Chompret, A., Bonaiti-Pellie, C., Heidenreich, A., Albers, P., Olah, E., Geczi, L., Bodrogi, I., Ormiston, W.J., Daly, P.A., Guilford, P., Fossa, S.D., Heimdal, K., Tjulandin, S.A., Liubchenko, L., Stoll, H., Weber, W., Forman, D., Oliver, T., Einhorn, L., McMaster, M., Kramer, J., Greene, M.H., Weber, B.L., Nathanson, K.L., Cortessis, V., Easton, D.F., Bishop, D.T., Stratton, M.R., and Rapley, E.A. 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, P.C., Pesce, L., Facchini, S., Imbimbo, C., and Pisconti, S. 2019. Prognostic and predictive factors in testicular cancer. *Eur Rev Med Pharmacol Sci* 23:3885-3891. DOI:10.26355/eurrev_201905_17816

Gan, Y., Wang, Y., Tan, Z., Zhou, J., Kitazawa, R., Jiang, X., Tang, Y., and 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, M.H., Kratz, C.P., Mai, P.L., Mueller, C., Peters, J.A., Bratslavsky, G., Ling, A., Choyke, P.M., Premkumar, A., Bracci, J., Watkins, R.J., McMaster, M.L., and Korde, L.A. 2010. Familial testicular germ cell tumors in adults: 2010 summary of genetic risk factors and clinical phenotype. *Endocr Relat Cancer* 17:R109-R121. DOI:10.1677/ERC-09-0254

Hsiao, Y.J., Su, K.Y., Hsu, Y.C., Chang, G.C., Chen, J.S., Chen, H.Y., Hong, Q.S., Hsu, S.C., Kang, P.H., Hsu, C.Y., Ho, B.C., Yang, T.H., Wang, C.Y., Jou, Y.S., Yang, P.C., and Yu, S.L. 2016. SPANXA suppresses EMT by inhibiting c-JUN/SNAI2 signaling in lung adenocarcinoma. *Oncotarget* 7:44417-44429. DOI:10.18632/oncotarget.10088

Jolly, M.K., Somarelli, J.A., Sheth, M., Biddle, A., Tripathi, S.C., Armstrong, A.J., Hanash, S.M., Bapat, S.A., Rangarajan, A., and Levine, H. 2019. Hybrid epithelial/mesenchymal phenotypes promote metastasis and therapy resistance across carcinomas. *Pharmacol Ther* 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., and Mego, M. 2017. Prognostic value of intratumoral carbonic anhydrase IX expression in testicular germ cell tumors. *Oncology Letters* 13:2177-DOI:2185. 10.3892/ol.2017.5745

Kouprina, N., Mullokandov, M., Rogozin, I.B., Collins, N.K., Solomon, G., Otstot, J., Risinger, J.I., Koonin, E.V., Barrett, J.C., and Larionov, V. 2004. The SPANX gene family of cancer/testis-specific antigens: rapid evolution and amplification in African great apes and hominids. *Proc Natl Acad Sci U S A* 101:3077-3082. DOI:10.1073/pnas.0308532100

Kouprina, N., Noskov, V.N., Pavlicek, A., Collins, N.K., Schoppee, B.P., Ottolenghi, C., Loukinov, D., Goldsmith, P., Risinger, J.I., Kim, J.H., Westbrook, V.A., Solomon, G., Sounders, H., Herr, J.C., Jurka, J., Lobanenkov, V., Schlessinger, D., and Larionov, V. 2007. Evolutionary diversification of SPANX-N sperm protein gene structure and expression. *PLoS One* 2:e359. DOI:10.1371/journal.pone.0000359

Kouprina, N., Noskov, V.N., Solomon, G., Otstot, J., Isaacs, W., Xu, J., Schleutker, J., and

Larionov, V. 2007. 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., and 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, D.T., Reid, A., Huddart, R., Broderick, P., Houlston, R.S., and 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, A.L., Vilela-Salgueiro, B., Rodrigues, A., Guimaraes, R., Cantante, M., Lopes, P., Antunes, L., Jeronimo, C., and 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, H.M., Hoekstra, H.J., Sijmons, R.H., Sonneveld, D.J., van der Steege, G., Sleijfer, D.T., and Nolte, I.M. 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, E.A., Westcott, J.M., Prechtl, A.M., Dang, T.T., Whitehurst, A.W., and Pearson, G.W. 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, L., Todoerti, K., Pilotti, S., Perrone, F., Seregni, E., Agnelli, L., Neri, A., Greco, A., and Borrello, M.G. 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, U.C., and Reinheckel, T. 2019. The role of proteases in epithelial-to-mesenchymal cell transitions in cancer. *Cancer Metastasis Rev*. DOI:10.1007/s10555-019-09808-2

Peng, D., Wei, J., Gan, Y., Yang, J., Jiang, X., Kitazawa, R., Xiang, Y., Dai, Y., and 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, C.M., and Zhao, Z. 2020. MicroRNA and transcription factor co-regulatory networks and subtype classification of seminoma and non-seminoma in testicular germ cell tumors. *Sci Rep* 10:852. DOI:10.1038/s41598-020-57834-w

Rapley, E.A., Crockford, G.P., Teare, D., Biggs, P., Seal, S., Barfoot, R., Edwards, S., Hamoudi, R., Heimdal, K., Fossa, S.D., Tucker, K., Donald, J., Collins, F., Friedlander, M., Hogg, D., Goss, P., Heidenreich, A., Ormiston, W., Daly, P.A., Forman, D., Oliver, T.D., Leahy, M., Huddart, R., Cooper, C.S., Bodmer, J.G., Easton, D.F., Stratton, M.R., and Bishop, D.T. 2000. Localization to Xq27 of a susceptibility gene for testicular germ-cell tumours. *NATURE GENETICS* 24:197-200. DOI:10.1038/72877

Rijlaarsdam, M.A., and Looijenga, L.H. 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, T.B., Furu, K., Skotheim, R.I., Haugen, T.B., Grotmol, T., and 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

Salemi, M., Calogero, A.E., Vicari, E., Migliore, E., Zaccarello, G., Cosentino, A., Amore, M., Tricoli, D., Castiglione, R., Bosco, P., and Rappazzo, G. 2009. A high percentage of skin melanoma cells expresses SPANX proteins. *Am J Dermatopathol* 31:182-186. DOI:10.1097/DAD.0b013e3181978d6f

Schmidberger, H., Bamberg, M., Meisner, C., Classen, J., Winkler, C., Hartmann, M., Templin, R., Wiegel, T., Dornoff, W., Ross, D., Thiel, H.J., Martini, C., and Haase, W. 1997. Radiotherapy in stage IIA and IIB testicular seminoma with reduced portals: a prospective multicenter study. *Int J Radiat Oncol Biol Phys* 39:321-326. DOI:10.1016/s0360-3016(97)00155-7

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

Siska, P.J., Johnpulle, R., Zhou, A., Bordeaux, J., Kim, J.Y., Dabbas, B., Dakappagari, N., Rathmell, J.C., Rathmell, W.K., Morgans, A.K., Balko, J.M., and Johnson, D.B. 2017. Deep exploration of the immune infiltrate and outcome prediction in testicular cancer by quantitative multiplexed immunohistochemistry and gene expression profiling. *OncoImmunology* 6:e1305535. DOI:10.1080/2162402X.2017.1305535

Tang, Z., Li, C., Kang, B., Gao, G., Li, C., and 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, J.P., Acloque, H., Huang, R.Y., and Nieto, M.A. 2009. Epithelial-mesenchymal transitions in development and disease. *CELL* 139:871-890. DOI:10.1016/j.cell.2009.11.007

van den Belt-Dusebout, A.W., de Wit, R., Gietema, J.A., Horenblas, S., Louwman, M.W., Ribot, J.G., Hoekstra, H.J., Ouwens, G.M., Aleman, B.M., and van Leeuwen, F.E. 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, A.W. 2014. Cause and consequence of cancer/testis antigen activation in cancer. *Annu Rev Pharmacol Toxicol* 54:251-272. DOI:10.1146/annurev-pharmtox-011112-140326

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

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

Yilmaz-Ozcan, S., Sade, A., Kucukkaraduman, B., Kaygusuz, Y., Senses, K.M., Banerjee, S., and Gure, A.O. 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., and 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., and 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.

Figure legends

Figure 1. SPANXN2 is downregulated in TGCTs relative to normal tissues. (A) The GEPIA database shows the mRNA levels of SPANXN2 expression was significantly downregulated in TGCTs tissues compared to normal tissues. TPM=Transcription per million. (B) Independent quantitative RT-PCR validation of TGCTs tissue samples and normal samples. "T" indicates TGCTs specimens, "N" indicates normal specimens.

Figure 2. Transfection of TGCT cell lines. (A, B) Plasmid transfection efficiency evaluated by fluorescence microscopy. (C, D) Relative expression of SPANXN2 in TCAM-2 and NCCIT cells was measured by qRT-PCR in the NC and SPANXN2 groups using β-actin as a reference. Data represent the mean ± SEM and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bar in A and B, 100μm.

Figure 3. SPANXN2 upregulation demised the colony formation ability but had no effect on cell proliferation. (A) Colony formation ability was evaluated in colony formation assays. (B) Relative cell colony formation rate; $*P < 0.05$, $**P < 0.01$ compared with the NC group. (C, D) Cell proliferation was evaluated in MTT assays.

Figure 4. The cell cycle distribution. (A, B，D，E)The cell cycle distribution was analyzed by flow cytometric analysis and FlowJo7.6.2. (C, F) The bar graphs of each cell phases.

Figure 5. Effects of SPANXN2 on cell migration and the related proteins of EMT and AKT. (A) Image showing migration of TGCTs cells overexpressing SPANXN2. (B) The relative number of migrated cells in the NC and SPANXN2 groups; $*P < 0.05$ compared with the NC group. (C) Expression of Vimentin and Snail proteins detected by western blot analysis. (D) Expression of AKT and p-AKT proteins detected by western blot analysis. Scale bar in A, 100μm.

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SPANXN2 is downregulated in TGCTs relative to normal tissues

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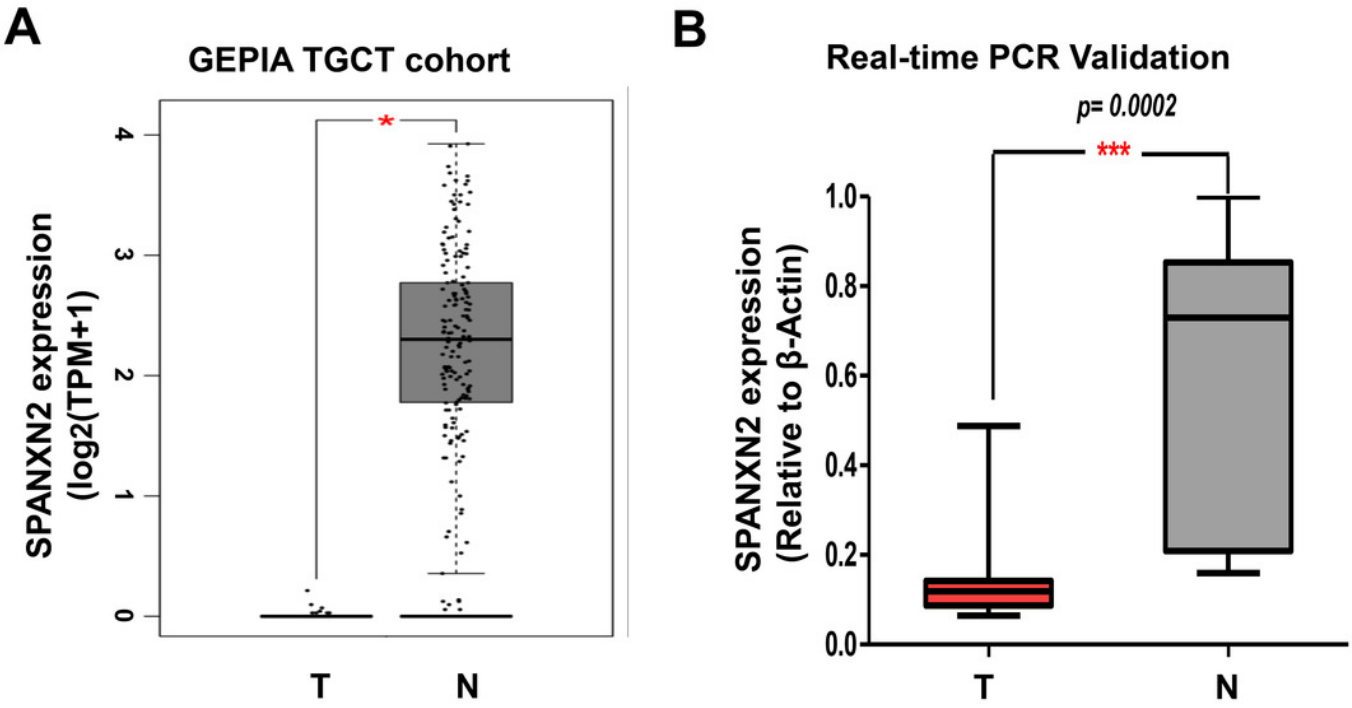

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Transfection of TGCT cell lines

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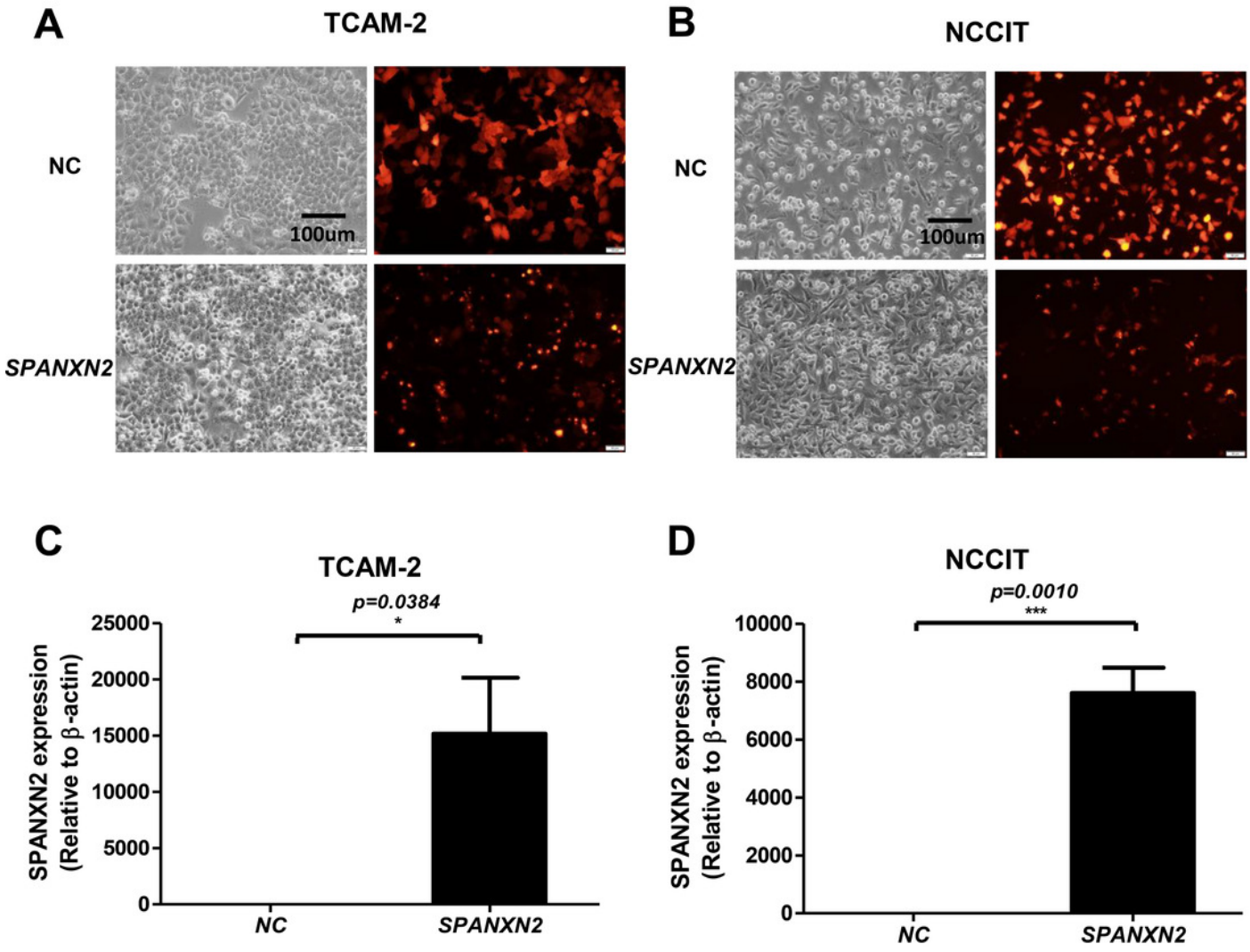

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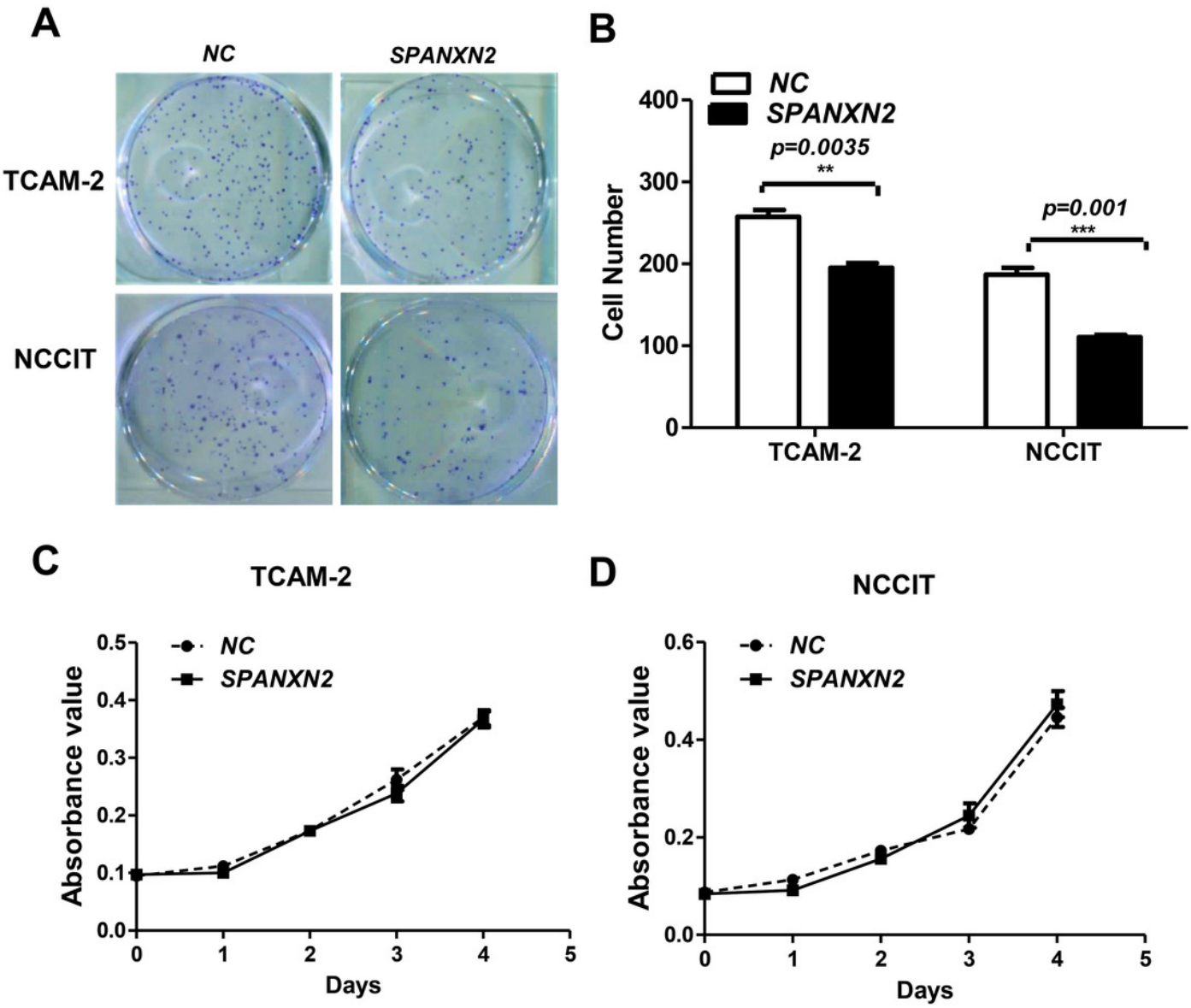

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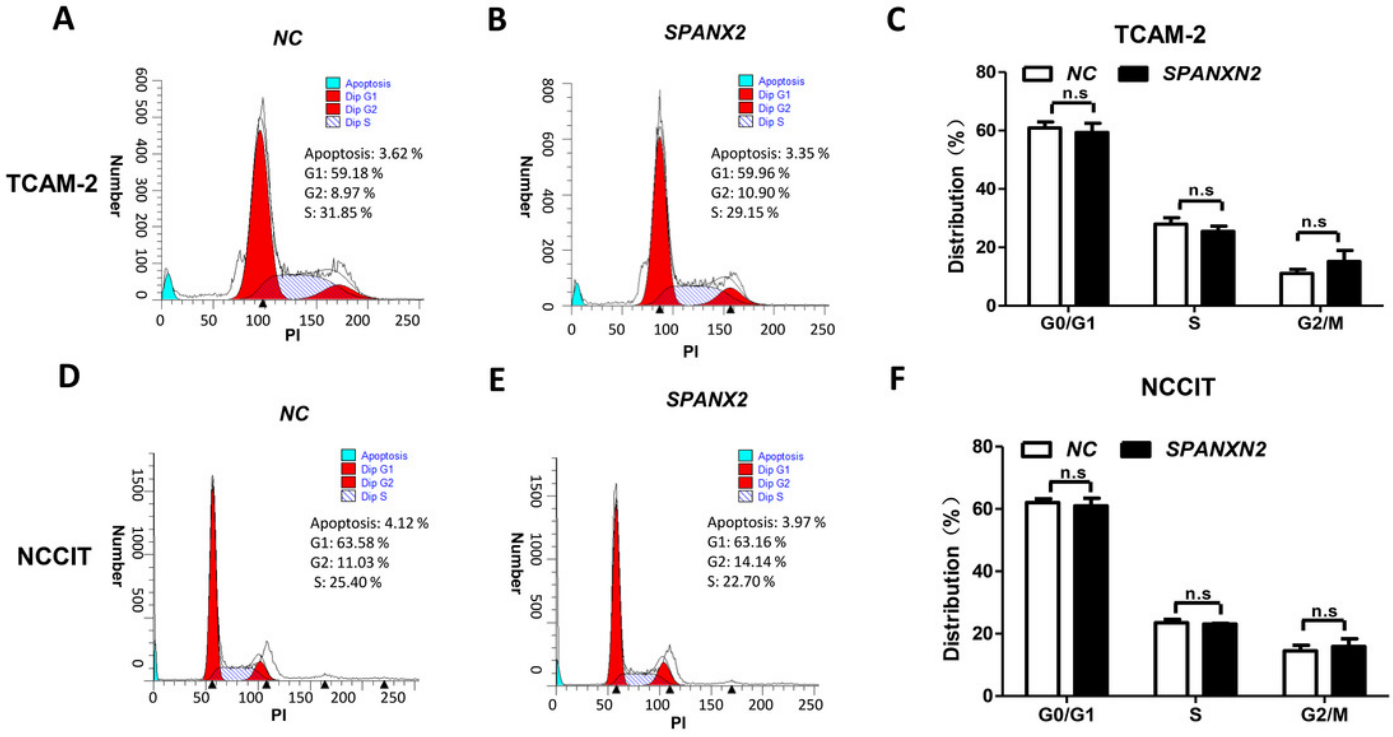

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