TSEG-1, a novel member of histone H2A variants, participates in spermatogenesis via promoting apoptosis of spermatogenic cells

Chaohui Gu a,b,1, Qiangsong Tong a,b,1, Liduan Zheng b,c, Zhulin Liang a,b, Jiarui Pu a,b, Hong Mei a, Tao Hu a, Zhiyong Du a, Fengyan Tian a, Fuqing Zeng a,b

a Department of Surgery, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, P. R. China
b Human Disease Related Gene Research Group, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China
c Department of Pathology, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

A novel member of histone H2A, named as testis specific expressed gene 1 (TSEG-1, approved symbol: H2afb1), was identified from adult mouse testis. The TSEG-1 gene is 610-bp in length and consists of one exon. TSEG-1 transcript was robustly and exclusively expressed in adult mouse testis, mainly in spermatocytes. In developmental testis, the TSEG-1 transcript was robustly expressed since postnatal day (P) 21, peaked at P30, and gradually decreased in the testis of aging mouse. The surgical cryptorchidism mouse model showed an increase in the TSEG-1 expression, accompanied by enhanced apoptosis of spermatogenic cells. The EGFP-tagged TSEG-1 protein is located in the nuclei of cultured spermatocytes (GC-2spd cells). Transfection of TSEG-1 into GC-2spd cells resulted in suppressed cell viabilities, increased apoptosis, and decreased mitochondrial membrane potential. Intratesticular injection of TSEG-1 resulted in increased apoptosis of spermatogenic cells in vivo. These results suggest that TSEG-1 may participate in the spermatogenesis via regulating the apoptosis of spermatogenic cells.

Introduction

Genetic abnormalities are main causes of oligozoospermia and azoospermia in male infertility [1]. It has been indicated that a number of genes involve in male spermatogenesis. Mutations or polymorphisms of these genes result in the majority of idiopathic forms of spermatogenic disorders [2]. Meanwhile, the structural maintenance of chromosomes and recombination of germ cell DNA in different developmental stages are required for normal spermatogenesis [3]. The abnormal regulation of these steps will result in a severe spermatogenesis dysfunction. Thus, it is very important to reveal the function and regulation mechanisms of the genes involved in spermatogenesis. The screening of candidate spermatogenesis-related genes will help us reveal the mechanisms of spermatogenesis, enhance the knowledge of abnormal spermatogenesis, and provide the treatment methods for male infertility [4].

So far, gene cloning strategies are used to identify genes involved in spermatogenesis. These strategies include differential display polymerase chain reaction (DD-PCR), suppression subtractive hybridization (SSH), cDNA microarrays, and expressed sequence tag (EST)-based in silico cloning [5,6]. Previous studies have showed the successful application of these strategies in the identification of spermatogenesis-related novel genes. For example, carcinoembryonic antigen-related cell adhesion molecule 6 (CeaCam6) and Ran-binding protein 5 (IP05) were identified by DD-PCR [6]. Tumor protein translationally-controlled 1 (TPT1), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), and nuclear mitotic apparatus protein 1 (NUMA1) were identified by SSH [7]. The human sex hormone binding globulin (SHBG), testis-specific gene with ankyrin repeats and PEST domain (TSAP), and patched domain containing 2 (PTCHD2) were identified by in silico cloning [8]. In these strategies, in silico cloning integrates bio-information and mathematics to facilitate genomic mapping, gene identification, and SNP identification, and is an important strategy to significantly accelerate the identification of novel genes [9].

In the current study, we indentified the testis specific expressing gene 1 (TSEG-1, approved symbol: H2afb1), a novel member of histone H2A variants, via in silico cloning strategy. We observed the cellular and subcellular localization and the expression profiles of TSEG-1 in mouse multiple tissues, testis of different development stages, and spermatogenesis-impaired cyrtorchidism model. Through transfection of TSEG-1, we observed the influence of TSEG-1 on the apoptosis of spermatogenic cells in vitro and in vivo. We demonstrated for the first time that TSEG-1 may play a critical role in spermatogenesis through regulating the apoptosis of spermatogenic cells.
Results

Identification, cloning and characterization of TSEG-1

Using the mouse testis-specific EST BY076573.1 as a template, nine homologous ESTs (BY070589.1, BY070689.1, BY070747.1, BY070715.1, BY070245.1, BY715086.1, CB274190.1, BX634402.1, CN832581.1) were found in dbEST database and assembled into one EST cluster. A 610-bp novel gene was predicted in homologous genome. Reverse transcription polymerase chain reaction (RT-PCR) was undertaken to clone it from mouse testis. As shown in Fig. 1A, a 336-bp fragment was amplified from mouse testis cDNA and genomic DNA, and designated as testis specific expressing gene 1 (TSEG-1). To verify the transcript of TSEG-1, a 336-bp probe was generated by PCR amplification and labeled by digoxin (DIG).

![Fig. 1. Identification of TSEG-1 from mouse testis.](image-url)

Using the mouse testis-specific expressed sequence tag (EST) BY076573.1 as a template, the homologous testis-specific ESTs were searched and assembled into one contig of the EST clusters. The GenScan procedure predicted a 610-bp novel gene. (A) Reverse transcription polymerase chain reaction (RT-PCR) was performed to clone the gene from mouse testis tissue. A 336-bp fragment was amplified from mouse testis cDNA and genomic DNA, and named as testis specific expressing gene 1 (TSEG-1). (B) To verify the transcript of TSEG-1, a 336-bp probe was generated by PCR amplification and labeled by digoxin. (C) Northern blot indicated the existence of TSEG-1 transcript in mouse testis. (D) Two-way sequencing results indicated that its sequence was consistent with the in silico cloning.
Fig. 2. Bioinformatics analyses of TSEG-1 gene. Homologous nucleic acid and amino acid sequences in GenBank were searched. (A) The amino acid sequence of TSEG-1 was highly homologous to histone H2A variants of different species, such as humans and rat. (B) TSEG-1 gene located in chromosome 12A (17,010K-18,830K), and was consisted of one exon. (C) Phylogenetic tree and (D) homology tree analyses indicated that TSEG-1 had a closest genetic relationship with the rat histone H2A.
TSEG-1 suppressed the cell viability of spermatocyte GC-2spd cells (Fig. 1B). Northern blot indicated the existence of TSEG-1 transcript in mouse testis (Fig. 1C). Two-way sequencing results indicated that its sequence was consistent with in silico cloning, and encoded a protein of 111 amino acids (Fig. 1D). Its sequence was not identical to any known genes, and the GenBank accession number EU079024 was assigned.

The amino acid sequence of TSEG-1 was highly homologous to histone H2A variants of different species, such as humans and rat (Fig. 2A). This gene is located in chromosome 2A3 (17,010K-18,830K) and consists of one exon (Fig. 2B). A H2A superfamily domain was detected between 18 residues and 96 residues of TSEG-1 (Fig. 2B). The phylogenetic and homology trees (Figs. 2C and D) indicated that TSEG-1 had a closest genetic relationship with rat histone H2A. The theoretical molecular weight of TSEG-1 protein was 12842.58 Dalton, and its theoretical isoelectric point was 11.4. The prediction by the Gene Ontology database, ProtFun 2.2 Server and PROSITE motif search program indicated that TSEG-1 may participate in spermatogenesis, regulation of Rho protein signal transduction, cell cycle, Rho GTPase activator activity, and damaged DNA binding.

Expression pattern of TSEG-1 in multiple mouse tissues and testsis of developmental stages

To further investigate the expression pattern of TSEG-1, Northern blot and real-time quantitative PCR were undertaken to measure its transcriptional levels in multiple mouse tissues. As shown in Fig. 3A, the TSEG-1 transcripts were detected exclusively in mouse testis, but not in brain, heart, lung, liver, spleen, stomach, intestine, kidney, bladder, prostate and skeletal muscle via Northern blot (Fig. 3A). These results were further verified by real-time quantitative PCR (Fig. 3B). The TSEG-1 transcript levels in mouse testis of different developmental stages were further determined by RT-PCR and real-time quantitative PCR. The results showed a robust TSEG-1 expression from postnatal day (P) 21 through P60, peaking at P30, but not at P4, P7, P14, P18, and P120 (Figs. 3C and D).

Localization of TSEG-1 mRNA in adult mouse testis

The cellular localization of TSEG-1 mRNA in mouse testis was determined by in situ hybridization. The results showed that the hybridization signal was associated with the seminiferous tubules and was limited to certain populations of germ cells in mouse testis. Strong positive signals were observed in the primary and secondary spermatocytes in adult mouse testis (Fig. 4A). The specificity of hybridization was validated by unadenatured probe as a negative control (Fig. 4A).

Expression pattern of TSEG-1 in spermatogenesis-impaired model

To further investigate whether TSEG-1 was a novel spermatogenesis-related gene, the expression profile of TSEG-1 was detected in established surgical cryptorchidism model. Hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) indicated the impaired spermatogenesis and increased apoptosis of spermatogenic cells in cryptorchid and contralateral testes (Fig. 4B). The apoptosis rates of spermatogenic cells in cryptorchid and contralateral testes were higher than those of sham group (Fig. 4C). Real-time quantitative PCR indicated robust TSEG-1 mRNA levels in testes of sham group (Fig. 4D). However, the expression of TSEG-1 was significantly upregulated in the cryptorchid and contralateral testes (Fig. 4D).

TSEG-1 suppressed the cell viability of spermatocyte GC-2spd cells

To further investigate the subcellular localization of TSEG-1 protein, the enhanced green fluorescent protein (EGFP)-tagged TSEG-1 eukaryotic expression vector (pEGFP-TSEG-1) was constructed, validated by nucleic acid sequencing, and transfected into cultured spermatocytes (GC-2spd cells). Seventy-two hours post-transfection, the transfection efficiencies were 72.5% and 69.2% in vector (pEGFP-N1) and TSEG-1 (pEGFP-TSEG-1) transfection groups, respectively. As shown in Fig. 5A, the subcellular localization of EGFP-tagged TSEG-1 protein was observed within nuclei of GC-2spd cells under a fluorescence microscope. Meanwhile, the EGFP localized at the cytoplasm of GC-2spd cells in the vector transfection group. The cell viabilities of GC-2spd cells were suppressed by TSEG-1 transfection (Fig. 5B). Since previous prediction indicated that histone H2A variants may participate in the regulation of cell cycle [10,11], we measured the influence of TSEG-1 on cultured spermatocytes. The cell cycle assay showed that transfection of TSEG-1 induced a slight G2/M arrest in cultured GC-2spd cells, without statistical difference when compared with vector control (Figs. 5C and D).

TSEG-1 induced apoptosis of spermatocyte GC-2spd cells

Since testis-specific gene plays a critical role in the regulation of apoptosis during spermatogenesis, we hypothesized that TSEG-1 might influence the apoptosis of spermatogenic cells. As shown in Fig. 6A, acridine orange (AO)/ethidium bromide (EB) and Hoechst 33258 staining indicated that transfection of TSEG-1, rather than vector, into GC-2spd cells resulted in characteristic changes of apoptosis. The annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining flow cytometry further confirmed the apoptosis of GC-2spd cells induced by transfection of TSEG-1 (Fig. 6B). In addition, the mitochondrial membrane potential was reduced in TSEG-1-transfected GC-2spd cells as revealed by 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethyl- benzimidazolyl-carbocyanine iodide (JC-1) staining flow cytometry (Fig. 6C).

TSEG-1 induced apoptosis of spermatogenic cells in vivo

To further investigate the effects of TSEG-1 on the apoptosis of spermatogenic cells, the in vivo TSEG-1 transfection was performed via polyethyleneimine (PEI)-mediated intratesticular injection. As shown in Fig. 7A, 72 hrs post-transfection, the EGFP and EGFP-tagged TSEG-1 expression was observed within seminiferous tubules under a fluorescence microscope. The in vivo transfection efficiencies were determined by calculating the percentage of green fluorescence-positive seminiferous tubules. The results showed that the transfection efficiencies were 75.4% and 73.2% in vector (pEGFP-N1) and TSEG-1 (pEGFP-TSEG-1) groups, respectively (Fig. 7B). One week post-transfection, impaired spermatogenesis and extensive apoptosis of spermatogenic cells were observed in testes transfected with TSEG-1, but not in those transfected with vector (Fig. 7A). The TUNEL-labeled apoptotic cells were counted, and the apoptosis rates were determined by calculating the average percentage of the total cells per seminiferous tubule. The results indicated that in vivo transfection of TSEG-1 resulted in enhanced apoptosis of spermatogenic cells (Fig. 7C).

Discussion

Abnormal spermatogenesis is one of the main causes of male fertility. Based on the developmental process, spermatogenesis includes mitotic, meiotic and postmeiotic phases [12]. Correspondingly, the related cell types are spermatogonia, spermatocytes and spermatids, respectively [12]. Mitosis and meiosis are the key procedure of the differentiation of spermatogonia, and are involved in the structural maintenance of chromosomes and chromatin [13,14]. Meanwhile, disequilibrium of germ cell is the important factor for the regulation of spermatogenesis [15]. If the mechanisms involved in spermatogenesis are exactly elucidated, male infertility would be completely treated. Thus, many researches were carried out in order to reveal the association of promoters, transcription factors and the balance of germ cell apoptosis during spermatogenesis.
In this study, we identified a novel member of histone H2A variant family, named as TSEG-1, by in silico cloning. We found that TSEG-1 was highly homologous with H2afb1 (also known as H2LA2) via Basic Local Alignment Search Tool (BLAST) analysis. Previous studies suggested histone H2A variants play critical roles in the regulation of chromatin structure and function. Moreover, H2A variants are associated with activation of transcription, DNA repair and heterochromatin formation [10,16]. Especially, histone variants H2AL1 and H2AL2 are associated with the pericentric heterochromatin regions of male genome. H2AL1 and H2AL2 specifically mark the pericentric regions in condensing spermatids, and participate in the formation of new nucleoprotein structures [10]. H2AL1 and H2AL2 quickly disappear from the paternal pericentric heterochromatin regions after sperm-egg fusion [10]. Thus, histone variant TSEG-1 possibly participates in the modification of chromatin structure and plays a critical role in spermatogenesis. However, the relationship between TSEG-1 and germ cell apoptosis still remains largely exclusive. In this study, we explored the expression patterns of TSEG-1 in development of testes, spermatogenesis-impaired model, and the influence of TSEG-1 on the apoptosis of spermatogenic cells.
Fig. 4. Cellular localization of TSEG-1 and expression profile of TSEG-1 in spermatogenesis-impaired animal model. Thirty male BALB/C mice (6 weeks old) were divided into cryptorchidism group (n = 20) and sham group (n = 10). In cryptorchidism group, the left testis was anchored to the inner lateral abdominal wall. The right testis was not operated in the scrotum as a contralateral control. In sham group, the animals were treated identically except that the testes were not anchored to the lateral abdominal wall. (A) In situ hybridization was applied to determine the cellular localization of TSEG-1 mRNA in mouse testis. Strong positive signals were observed in the primary and secondary spermatocytes in adult mouse testis. The specificity of hybridization was validated by undenatured probe as a negative control. (B) In established surgical cryptorchidism model, hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining indicated the reduction of seminiferous tubules, decrease of lumen, and apoptosis of spermatogenic cells in mouse cryptorchid and contralateral testes (arrowhead). Original magnification, ×400. (C) The apoptosis rates of spermatogenic cells in cryptorchid and contralateral testes were higher than those of sham group. (D) Real-time quantitative PCR indicated that the expression of TSEG-1 was significantly enhanced in the cryptorchid and contralateral testes than that in sham group. The symbol (*) indicated a significant increase from sham (P<0.05). Triplicate experiments were undertaken with identical results.
Firstly, we investigated the characteristics and expression profiles of TSEG-1. The prediction program Genescan and our results from the amplification of genomic DNA and cDNA showed that TSEG-1 is consists of only one exon, which was consistent with previous evidences that most of histone variants are consisted of one exon [17]. The multiple tissue profiles showed the unique expression of TSEG-1 in testis as confirmed by Northern blot and real-time quantitative PCR, which was consistent with previous study [11]. The detection of TSEG-1 mRNA in the testes of different development stages revealed the highest levels of TSEG-1 transcripts in one-month testis. It has been demonstrated that elongating spermatids apparently emerge at postnatal days 24–25 and elongated spermatids are apparently produced at postnatal days 27–28 [18]. Combined with the function of histone H2A variants, these results suggest that TSEG-1 may participate in the process of spermatogenesis.

Secondly, we analyzed the cellular localization of TSEG-1 in mouse testis via in situ hybridization, and detected the TSEG-1 transcripts in surgical cryptorchidism model. Our evidences show that the TSEG-1 mainly localizes in spermatocytes, and TSEG-1 protein localizes within the nuclei of cultured cells. The TSEG-1 mRNA was significantly up-regulated in cryptorchid and contralateral testes contrast to sham controls. In addition, the upregulation of TSEG-1 transcripts was accompanied by increased apoptosis of spermatogenic cells in cryptorchid and contralateral testes. However, the correlation between TSEG-1 expression and apoptosis of spermatogenic cells in cryptorchidism still remains exclusive. Further investigation, such as in vivo knock-in or knock-out study, is needed to elucidate it clearly.

Apoptosis of testicular germ cells is a critical factor for normal spermatogenesis under physiological conditions, and the unbalance of testicular germ cell apoptosis is associated with various diseases such as cryptorchidism. We further observed the influence of TSEG-1 on the apoptosis of cultured spermatocytes (GC-2spd cells). We found that the cell viabilities of GC-2spd were suppressed by TSEG-1 transfection. In addition, the TSEG-1-transfected GC-2spd cells were slightly arrested at G2/M phase, without statistically significant difference when compared with vector group. Using fluorescent staining and flow cytometry methods, we demonstrated the TSEG-1-induced apoptosis in GC-2spd cells in vitro. The PEI-mediated in vivo gene transfection offers a convenient and efficient method for the functional research of genes related to the intrinsic regulation of spermatogenesis [19]. It has been reported that PEI-mediated gene transfection in the seminiferous tubules occurred in a restricted population of spermatogenic cells, mainly the spermatocytes [19]. In this study, we demonstrated that PEI was efficient for in vivo gene transfection of testes. PEI-mediated TSEG-1 transfection resulted in increased apoptosis of spermatogenic cells. These findings suggest that TSEG-1 may participate in spermatogenesis through regulating the apoptosis of spermatogenic cells.

In summary, we identified the TSEG-1, a novel member of histone H2A variant family. We found that TSEG-1 is consisted of one exon, and possesses the unique expression profile in mouse testis. Highest transcription levels of TSEG-1 were observed in testis at one-month postnatal. The expression pattern of TSEG-1 in testis is age-dependent and stage-specific. The results of crytorchidism model indicate that TSEG-1 may be involved in the pathogenesis of cryptorchidism. Cell culture and animal studies demonstrated that TSEG-1 promotes apoptosis of spermatogenic cells in vitro and in vivo. Further knock-in and knock-out studies are needed to better elucidate the function of TSEG-1 in spermatogenesis, and therapeutic strategies of male infertility.

Fig. 5. TSEG-1 suppressed the cell viability of spermatocyte GC-2spd cells. (A) The enhanced green fluorescent protein (EGFP)-tagged TSEG-1 eukaryotic expression vector was constructed and transfected into cultured spermatocytes (GC-2spd cells), Seventy-two hours post-transfection, the transfection efficiencies were 72.5% and 69.2% in vector (pEGFP-N1) and TSEG-1 (pEGFP-TSEG-1) transfection groups, respectively. The subcellular localization of TSEG-1 protein was observed within nuclei of GC-2spd cells under a fluorescence microscope. Meanwhile, the EGFP localized at the cytoplasm of GC-2spd cells in the vector transfection group. (B) Seventy-two hours post-transfection of TSEG-1, the cell viabilities of GC-2spd cells were reduced. (C) and (D) Cell cycle assay showed that 72 hrs post-transfection, TSEG-1 induced a slight G2/M arrest in cultured GC-2spd cells, without statistical difference when compared with vector control. The symbol (△) indicated a significant decrease from untransfected control (P<0.05), respectively. Triplicate experiments were undertaken with identical results.
Materials and methods

Animals

Adult BALB/c mice, 25 to 35 grams in weight, were bought from Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All experiments followed the protocols approved by the Animal Care and Use Committee of Tongji Medical College. The mice were housed and bred at 25 °C in a 12 l: 12 d cycle and given food and water ad libitum. Adult mouse tissues, including brain, heart, lung, liver, spleen, stomach, small intestine, kidney, bladder, prostate, testis, and skeletal muscle, were harvested and homogenized. Pregnant BABL/c mice were housed to deliver neonatal mice. The mouse testis specimens at developmental stages (Postnatal day 4, 7, 14, 18, 21, 30, 60, and 120) were collected and stored at -80 °C freezer.

Fig. 6. TSEG-1 induced apoptosis of spermatocyte GC-2spd cells. (A) Acridine orange (AO)/ethidium bromide (EB) and Hoechst 33258 staining was carried out to detect the characteristic changes of apoptosis. The results indicated that 72 hrs post-transfection of TSEG-1, rather than vector, resulted in morphological changes of apoptosis in GC-2spd cells. (B) The Annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining flow cytometry indicated that 72 hrs post-transfection, TSEG-1 exerted strong apoptosis-inducing effects on GC-2spd cells. (C) Seventy-two hours post-transfection, the mitochondrial membrane potential was reduced in TSEG-1-transfected GC-2spd cells as revealed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrachloro-benzimidazolyl-carbocyanine iodide (JC-1) staining flow cytometry. Triplicate experiments were undertaken with identical results.
Surgical cryptorchidism model

Thirty male BALB/C mice (6 weeks old) were divided into cryptorchidism group (n=20) and sham group (n=10). The mice were anesthetized by pentobarbital sodium (0.01 mg/g body weight, i.p.). All operations were performed under sterile conditions [20]. Briefly, the testes were observed through a midline abdominal incision. The left testis was anchored to the inner lateral abdominal wall. The right testis was not operated in the scrotum as a contralateral control. The sham operated animals were treated identically except that the testes were not anchored to the lateral abdominal wall. The anchored testes were found to reside within the abdominal cavity until sample collection. Mice were sacrificed under ether anesthesia at one week post-operation. The testes were dissected, and fixed in 4% formalin or stored at −80 °C freezer for morphological and molecular detection, respectively.

Intratesticular injection

Thirty male BALB/C mice (8 weeks old) were divided into phosphate buffer saline (PBS) group (n=10), vector group (n=10) and TSEG-1 group (n=10). The mice were anesthetized by pentobarbital sodium (0.01 mg/g body weight, i.p.), and testes were exteriorized through a midline abdominal incision of approximately 5 mm. The intratesticular injection procedure was previously described [19]. In PBS group, the testes were injected with 10 μl of PBS. In vector and TSEG-1 groups, the plasmid DNA (pEGFP-N1 or pEGFP-TSEG-1) was mixed with polyethylenimine (PEI) at a PEI nitrogen: DNA phosphate ratio of 6, and injected into each testis. The total volume was 10 μl, containing 3 μg plasmid DNA. After injection, the testes were put back into the abdomen and the incisions were sutured. The testes were collected for

Fig. 7. TSEG-1 induced apoptosis of spermatogenic cells in vivo. Thirty male BALB/C mice (8 weeks old) were divided into PBS group (n=10), vector group (n=10) and TSEG-1 group (n=10). In PBS group, the testes were injected with 10 μl of PBS. In vector and TSEG-1 groups, the plasmid DNA (pEGFP-N1 or pEGFP-TSEG-1) was mixed with polyethylenimine (PEI) at a PEI nitrogen: DNA phosphate ratio of 6, and injected into each testis. The total volume was 10 μl, containing 3 μg plasmid DNA. (A) Seventy-two hours post-transfection, the EGFP-tagged TSEG-1 expression was observed within seminiferous tubules under a fluorescence microscope. One week post-transfection, HE and TUNEL staining indicated the impaired spermatogenesis and extensive apoptosis of spermatogenic cells in testes transfected with TSEG-1 (arrowhead), but not in those transfected with vector. Original magnification, ×200. (B) The PEI-mediated in vivo transfection efficiencies were determined by calculating the percentage of green fluorescence-positive seminiferous tubules under a fluorescence microscope. The results showed that the transfection efficiencies were 75.4% and 73.2% in vector and TSEG-1 groups, respectively. (C) The TUNEL-labeled apoptotic cells were counted, and the apoptosis rates were determined by calculating the average percentage of the total cells per seminiferous tubule. The results indicated that in vivo transfection of TSEG-1 resulted in enhanced apoptosis of spermatogenic cells. The symbol (*) indicated a significant increase from PBS (P<0.05).
analysis as indicated. The PEI-mediated in vivo transfection efficiencies were determined by calculating the percentage of green fluorescence-positive seminiferous tubules under a fluorescence microscope.

Full-length cDNA prediction and cloning

Mouse testis-specific EST BY076573.1 was obtained from online EST database ZooIDD (http://bio301.iis.sinica.edu.tw/~zooIDD/new/main.php) [21]. Homologous ESTs were obtained via search in EST database (http://www.ncbi.nlm.nih.gov/dbEST/). The Biolign program (Tom Hall, Ibis Therapeutics, Carlsbad, CA) was applied to splice the EST cluster, and the homologous genome sequence was obtained through genome BLAST (www.ncbi.nlm.nih.gov/mouse genome blast/) program in the National Biological Information Center (NCBI). The GenScan procedure (http://genes.mit.edu/GENSCAN.html) was applied to predict the exons and introns of the sequence [22]. The open reading frame (ORF) was applied to recognize the open reading frame.

RT-PCR was undertaken to clone the novel gene. Total RNA was isolated with RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The reverse transcription reactions were conducted with RevertAidTM (QIAGEN, Valencia, CA). The reverse transcription reaction was carried out at 50 °C for 1 hr, and extension at 72 °C for 7 min. The PCR product was separated on a 0.8% agarose gel, purified by PCR product purification kit (Omega Biotech Inc., Norcross, GA), and cloned into pEASY-T3 (TransGen Biotech Co., China). The sequence of cloned gene was validated by DNA sequencing.

Bioinformatics analysis

Homologous nucleic acid and amino acid sequences in GenBank were searched through BioEdit sequence alignment editor (version 7.0.8.0, North Carolina State University, USA). The isoelectric point and molecular weight of the encoded protein were predicted by ExPASy database (http://www.expasy.ch/tools/). The phylogenetic and homology trees were analyzed by DNAMAN software (Version 6, Lynnon Biosoft Corporation, QC, Canada). The gene function was predicted by Gene Ontology database (http://www.geneontology.org/), ProtFun2.2 Server (http://www.cbs.dtu.dk/services/ProtFun/), and PROSITE motif search program (http://www.expasy.ch/prosite/).

Northern blot

According to the ORF, the PCR primers were designed by Premier Primer 5.0 software as the following: forward: 5′-ATGGCCAGGAAAAGGCAAAG-3′; reverse: 5′-TCAGTTGTCATCAGG TTC-3′. Gradient PCR amplification was performed on a G-storm thermal cycler (Gene Technologies Limited, West Chester, PA). The PCR was performed using PCR Master Mix (Fermentas), according to the manufacturer's instruction. Thermal cycling consisted of preliminary denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1.5 min, with a final product extension at 72 °C for 7 min. The PCR product was separated on electrophoresis in 1.5% agarose gel, purified by PCR product purification kit (Omega Biotech Inc., Norcross, GA), and cloned into pEASY-T3 (TransGen Biotech Co., China). The sequence of cloned gene was validated by DNA sequencing.

RNA was transferred to Hybond-N+ membrane (Pall Corp., Port Washington, NY). Prehybridization was carried out at 65 °C for 30 min in DIG Easy Hyb solution (Roche). Hybridizations were performed at 65 °C for 16-18 hrs. Blots were triplicately washed with 2 × saline sodium citrate (SSC) and 0.05% sodium dodecyl sulfate (SDS) for 15 min, and washed with 0.1 × SSC and 0.1% SDS at 50 °C for 15 min. Then the blot was detected by anti-digoxin (DIG) antibody staining, and recorded on X-ray films with the chemiluminescence substrate (Roche) [23].

In situ hybridization

The probe was generated as described above and denatured at 80 °C for 10 min. The testes were harvested from 4 weeks old mice and fixed in 10% neutral-buffered formaldehyde. Testes were embedded in paraffin, and 4-μm-thick sections were prepared. Hybridization was performed according to published report [24]. The sections were treated with H2O2 mix (H2O2 : methanol = 1:50) for 30 min, and washed triplicately in distilled water for 5 min. Prehybridization was performed at 58 °C for 2 hrs. Hybridizations were carried out at 58 °C for 16 hrs. The sections were firstly washed in 2 × SSC at room temperature for 30 min, incubated in RNase A solution at 37 °C for 30 min, washed in 2 × SSC at 65 °C for 1 hr, and washed in 0.1 × SSC at 65 °C for 1 hr. Then, the sections were blocked with 3% bovine serum albumin blocking buffer at room temperature for 1 hr, and incubated with anti-Dig Fab-antibody conjugated to peroxidase (Sigma, St. Louis, MO) at room temperature for 2 hrs. After washing with PBS, the slides were incubated with peroxidase-conjugated anti-Dig antibody (DAKO, Glostrup, Denmark). The slides were incubated with 3, 3′-diaminobenzidine tetrahydrochloride (DAB, DAKO) and counterstained with haematoxylin, dehydrated with alcohol, cleared with xylene and covered with a cover slip for observation.

RT-PCR and real-time quantitative PCR

RT-PCR was undertaken to observe the gene expression characteristics as described above. The PCR primers for mouse house-keeping gene GAPDH were 5′-GCCAGGTCATCATCAGAACATTTGGA-3′ and 5′-GCTGTCGTTACACCCGTCGTC-3′, amplifying a 314-bp fragment. PCR bands were separated on ethidium bromide-stained agarose gels. GAPDH was used to normalize the initial variations in sample concentration and served as a control for reaction efficiency. Real-time quantitative PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was performed using ABI Prism 7900 Sequence Detector (Applied Biosystems) with the same sets of primers. Thermal cycling consisted of preliminary denaturation for 2 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 35 s. Fluorescent signals were collected during extension phase, Ct values of the sample were calculated, and mRNA expression level was analyzed by 2△△Ct method [25,26].

Cell cultures and transfections

The GC-2spd (CRL-2196) cells, an immortalized spermatocyte cell line established by stable transfection of the SV40 large T antigen gene [27,28], were purchased from American Type Culture Collection (ATCC, Manassas, VA), and grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies Inc.), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2. The novel gene fragment containing restriction enzyme sites Xho I and Kpn I was amplified from mouse testis cDNA with the following primers: forward 5′-CGCCCAAGCTTATGGCCAGGAAAAGGCAAAG-3′; reverse 5′-
CCCGGATCCTGGTTGTCA TCAGGTTCTGGT-3′. The fusion expression vector pEGFP-TSEG-1 was generated by inserting the gene fragment into pEGFP-N1 (Clontech, Mountain View, CA), validated by sequencing, and transfected into cultured GC-2spd cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection efficiencies were determined by calculating the percentage of EGFP-positive cells under a fluorescence microscope.

**Measurement of cell viability**

Seventy-two hours post-transfection, cell viability was monitored by the 2-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) colorimetric assay [29]. Briefly, 20 μl of MTT (5 mg/ml) was added to each well. After 4 hrs of incubation at 37 °C, the cell supernatants were discarded, MTT crystals were dissolved with DMSO and the absorbance measured at 570 nm. Percent viability was defined as the relative absorbance of treated versus untreated control cells. All experiments were done with 6–8 wells per experiment and repeated at least three times.

**Cell cycle assay**

Seventy-two hours post-transfection, cell cycles were examined by flow cytometry [30]. Briefly, 2 × 10^6 of cells were collected, washed twice with 0.01 mol/L PBS and fixed in 70% ethanol overnight at 4 °C. Then, cells were washed once with PBS, digested by 200 μl of RNase (1 mg/ml) at 37 °C for 30 min, and stained with 800 μl of PI (50 μg/ml Sigma) at room temperature for 15 min. The DNA histograms were generated with a flow cytometer (Becton Dickson Co., San Jose, CA), using the CELLQUEST software.

**Cellular morphological observation**

To observe the changes in cellular morphology, the AO (Sigma) and EB (Sigma) staining method was performed [31]. Briefly, 72 hours post-transfection, cells were harvested with 0.125% trypsin and 0.01% ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), resuspended in 95 μl of DMEM medium, and incubated with 5 μl of AO/EB staining solution (100 mg/L of each dye in PBS) at room temperature for 15 min. Cells were examined using fluorescence microscopy and photographed (Olympus, Tokyo, Japan).

Morphological evidence of apoptosis was further obtained using Hoechst 33258 staining method [32]. Briefly, 72 hours post-transfection, cells were fixed with 4% formaldehyde in PBS solution at 4 °C for 10 min. After three washes with PBS, the cells were stained with 10 mg/L of Hoechst 33258 in PBS solution at 4 °C in dark for 10 min, and morphologic changes including cell shrinkage and nuclear condensation were observed under a fluorescence microscope (Olympus AX80, Olympus).

**TUNEL assay**

The detection of cells with fragmented DNA in testicular tissue sections was performed using the DeadEnd™ Colorimetric TUNEL System (Promega Corporation, Madison, WI) according to manufacturer's instruction [33,34]. Briefly, for permeabilization, sections were incubated in 10 μg/ml proteinase K in PBS (10 min, room temperature), and the Streptavidin horseradish peroxidase solution was diluted to 1: 1000 with PBS. Three independent samples in each group were assayed by TUNEL, with three sections of every sample. For each section, ten high power field (×200) images were captured, and the apoptosis rates were determined by calculating the average percentage of total TUNEL-positive cells per high power field under a microscope (Fluovert, Leitz, Germany) linked with a Sony DX500 digital camera (Sony, Tokyo, Japan).

**Apoptosis rates detection**

Apoptosis ratios of cells were determined by annexin V-FITC and PI staining flow cytometry [35]. Briefly, 72 hours post-transfection, cells were collected, washed twice with cold PBS, resuspended with 100 μl of binding buffer (10 mmol/L L-2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, 140 mmol/L NaCl, 2.5 mmol/L CaCl, pH 7.4) into 2–5× 10^5 cells/ml density, and incubated with annexin V-FITC (BD Pharmingen, San Diego, CA) at room temperature for 10 min. After washing with binding buffer, the cells were resuspended with 400 μl of binding buffer containing 10 μl of PI (20 μg/ml), and incubated on ice for 15 min. Apoptosis was analyzed by a flow cytometer (Becton Dickson Co.) at a wavelength of 488 nm.

**Mitochondrial membrane potential detection**

The mitochondrial potential of cultured cells was quantified by JC-1 (Biotium, Inc., Hayward, CA) staining flow cytometry. Briefly, 72 hrs post-transfection, 2×10^3 GC-2spd cells were collected, resuspended in 1× JC-1 reagent solution, and incubated at 37 °C for 15 min. After washing twice with 1× assay buffer, the cells were resuspended in 0.5 ml of 1× assay buffer, and analyzed immediately by flow cytometry (Becton Dickson Co.). Mitochondria containing red JC-1 aggregates in healthy cells were detectable in FL2 channel, and green JC-1 monomers in apoptotic cells were detectable in FL1 channel.

**Statistical Analysis**

Unless otherwise stated, all data were shown as mean± standard error of the mean (SEM). Statistical significance (P<0.05) was determined by t test or analysis of variance (ANOVA) followed by assessment of differences using SPSS 13.0 software (SPSS Inc., Chicago, IL).

**Acknowledgment**

This work was supported by National Natural Science Foundation of China (No. 30200284, No. 30600278, No. 30772359), Program for New Century Excellent Talents in University of China (NCET-06-0641), and Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (2008-889).

**References**


