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Cloning of a full-length cDNA of human testis-specific spermatogenic cell apoptosis inhibitor *TSARG2* as a candidate oncogene☆

Shang-Feng Liu,[a,b,c] Guang-Xiu Lu,[c] Gang Liu,[c] Xiao-Wei Xing,[c] Lu-Yun Li,[c] and Zhao Wang[a,b,*]

[a] *Institute of Biomedicine, Tsinghua University, Beijing 100084, People's Republic of China*
[b] *Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China*
[c] *Reproduction and Stem Cell Engineering Institute, Central South University, Changsha 410078, People's Republic of China*

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Abstract

A novel human gene full-length cDNA sequence-*TSARG2* was identified from a human testis cDNA library using the *SRG2* gene (GenBank Accession No. AF395083), which was significantly up-regulated in cryptorchidism, as an electronic probe. *TSARG2* was 1223 bp in length. The putative protein encoded by this gene was 305 amino acids with a theoretical molecular weight of 34,751 and isoelectric point of 9.85. The sequence shared no significant homology with any known protein in databases except *SRG2*. Northern blot analysis revealed that 1.7 kb *TSARG2* transcript was detected selectively in human testis. Furthermore, results of in situ hybridization assay confirmed that *TSARG2* was expressed in seminiferous tubules, more precisely in spermatogonia and spermatocyte. No mutation was found by PCR-SSCP in 122 cases of azoospermia, severe oligzoospermia, and cryptorchidism. The green fluorescence produced by pEGFP-C1/*TSARG2* was detected on the nucleus of COS7 cells after 24 h post-transfection. The pcDNA3.1(−)/*TSARG2* plasmid was constructed and introduced into MCF7 cells by liposome transfection. *TSARG2* can accelerate MCF7 cells to traverse the S-phase and enter the G2-phase compared with the control without transfection of *TSARG2*, which suggested that this gene plays an important role in the development of cryptorchid testis and is a testis-specific apoptosis candidate oncogene.

Keywords: TSARG2; Testis-specific expression; Spermatogenesis; Cryptorchidism

Cryptorchidism represents the most common disorder of sexual differentiation in males. The rate of cryptorchidism in newborn males varies from 1.5% to 3.4%, and increases to 30.4% in premature infants [1]. In addition to its potentially adverse effect on subsequent fertility, cryptorchidism is the major known risk factor for testicular cancer. About 8–15% of testicular cancer is converted from cryptorchidism [2–4]. In mammals, the scrotal temperature is slightly lower than the temperature of the abdomen. This cooler temperature in the scrotum is a requirement for normal spermatogenesis, since naturally occurring cryptorchidism in boys and experimental cryptorchidism in animals cause germcell loss and result in reduced fertility and sterility. It is not known whether cryptorchidism represents an independent risk factor for testicular cancer or whether both disorders have a common cause. Despite extensive histological and cytological characterization of cryptorchid testes, the nature of temperature-induced destruction of spermatogenesis is not well understood. It has been confirmed that the spermatogenic process is controlled by many testis spermatogenic cell apoptosis-related genes, such as *Mcl-1* [5], *p53* [6], *CREM* [7], *Fas* [8], *Hsp*

☆ The cDNA sequence reported in this paper has been submitted to GenBank and has Accession No. AY040204.

* Corresponding author. Fax: +86-10-62772240.
E-mail address: zwang@tsinghua.edu.cn (Z. Wang).

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[9,10], *TRAIL* [11], *c-myc* [12], and *TR2* [13]. Mouse cryptorchidism has been used as a model to investigate the molecular cues that guide the apoptosis, expansion, and differentiation of spermatogonial germ cells [14–16].

Testis spermatogenic cell apoptosis is a complicated polygene-related process. At present, the research on testis spermatogenic cell apoptosis is in the early stages. As far as we know, specific testis spermatogenic cell apoptosis-related genes have not been cloned. Cloning new apoptosis-related novel genes is a key to further understanding of the apoptosis mechanism and the biological process of germ cells, and it is of momentous significance in clarifying the physiological and pathological process of spermatogenesis.

In a previous study, Jiang [17] cloned 24 ESTs of mouse testis spermatogenic cell apoptosis-related genes by creating mouse cryptorchidism models and making use of suppression subtractive hybridization. The results are registered in GenBank. Beginning with the EST BE644542, one of the ESTs mentioned above, we cloned a novel mouse gene full-length cDNA sequence-*SRG*2 from a mouse testis cDNA library and successfully performed a primary functional study [18]. Based on the *SRG*2 sequence, we cloned a human testis-specific spermatogenic cell apoptosis-related gene *TSARG*2 as candidate oncogene.

Materials and methods

Isolation of the TSARG2 cDNA [19]. Based on the *SRG*2 sequence (AF395083) we used Blast algorithms through the National Center of Biotechnology Information and found a series of ESTs: BG150360, BE972693, AA429726, H53400, H53354, and AA382855. A 1175 bp fragment was obtained from these ESTs by splicing on an EST annotation machine at IFOM (http://bio.ifom-firc.it/EST_MACHINE/index.html). Gene-specific primers A1 (5′-AGTCTGTGTCAAATACCCTTTTTCC-3′) and A2 (5′-GGCGAAAGTGACGGTGACTTGTCTA-3′) from the 1175 bp fragment were designed for nest PCR to identify *TSARG*2 5′-end. PCR was performed on a GeneAmp PCR System 9600 (Perkin–Elmer) in 10 μl of 1× PCR buffer, containing 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM gene-specific primer A1, 0.4 μM of vector-specific primer AP1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′), 2 mM $MgCl_2$, 1 U advantage 2 *Taq* DNA polymerase (Clontech), and approximately 200 ng Marathon-Ready cDNA of human testis (Clontech) as template. We used the following touch-down PCR procedure: initial denaturation at 95 °C for 1 min 30 s, and 5 cycles of 94 °C for 10 s, and 72 °C for 3 min, then 5 cycles of 94 °C for 10 s, and 70 °C for 3 min, then 25 cycles of 94 °C for 10 s, and 68 °C for 3 min, hold at 4 °C. The second PCR was performed using gene-specific primer A2 and vector-pecific primer AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) with the above PCR mixture as template. This PCR fragment was cloned into pUCm-T vectors and sequenced. The full-length cDNA sequence was generated by merging the overlapping sequences obtained.

The primers 5TSARG2 (5′-CCGAATTCATGGCTGCCGCCGG-3′) and 3TSARG2 (5′-TAAGCTTGGCATCACTTCTTCAAAG-3′) were designed according to the above full-length cDNA sequence and used in PCR assay with Advantage 2 DNA polymerase and Marathon-Ready cDNA of human testis as template to confirm the open reading frame. PCR amplification cycles involved, initial denaturation at 95 °C for 2 min and 35 cycles of 94 °C for 10 s, 58 °C for 40 s and 72 °C for 3 min, and then 72 °C for 7 min, hold at 4 °C. This PCR fragment was cloned into pUCm-T vectors and sequenced. We checked the nucleotide sequence by sequencing the clone in two directions and submitted to the GenBank.

Computer-aided analysis of human TSARG2. The Chambon rule was used to identify exon–intron junctions of *TSARG*2 gene. The deduced amino acid sequence of *TSARG*2 was aligned against the GenBank databases (nucleotide, EST, and protein) at the National Center for Biotechnology Information, using BLAST to search for sequence matches. Motif searches were performed with PROSITE programs. Using BLAST to search human genome (http://www.ncbi.nlm.nih.gov/humangenomeblast/) revealed the chromosome position of *TSARG*2. The CpG Island Searcher (http://ccnt.hsc.usc. edu/cpgislands) was used to search for the CpG island, and PromoterInspector (http://www.genomatix.de/software_services/software/PromoterInspector/PromoterInspector.html) was used to search for the promoter. All other bioinformatic tools used in this paper including the open reading frame identification are at the ExPASy molecular biology WWW server of the Swiss Institute of Bioinformatics (http://www.expasy.ch).

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gcgctgaggtctcgccccataagactccgcccgccccacgaggccgcgccgccagcaata
      *           *
aggcccagccccgccttctgctgttgccgcagcaacccagaaagcgtcatggctgccgcc
                                                  M  A  A  A
ggccaggaaaaagggtatttgacacagactgcggcagccctagacaagtcaccgtcactt
 G  Q  E  K  G  Y  L  T  Q  T  A  A  A  L  D  K  S  P  S  L
tcgccacagctagcagctcccatccgagggaggcctaagaagtgtctggtctatccgcat
 S  P  Q  L  A  A  P  I  R  G  R  P  K  K  C  L  V  Y  P  H
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 A  P  K  S  S  R  L  S  R  S  V  L  R  W  L  Q  G  L  D  L
agcttcttccccaggaacatcaacagagatttttcaaatggcttcctaattgcagaaata
 S  F  F  P  R  N  I  N  R  D  F  S  N  G  F  L  I  A  E  I
ttctgtatatattacccctgggaacttgaattatcatcctttgaaaacgggacctcttta
 F  C  I  Y  Y  P  W  E  L  E  L  S  S  F  E  N  G  T  S  L
aaagtcaagttggataactgggcacagttggagaagttcctggcaagaaaaaatttaaa
 K  V  K  L  D  N  W  A  Q  L  E  K  F  L  A  R  K  K  F  K
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 L  P  K  E  L  I  H  G  T  I  H  C  K  A  G  V  P  E  I  L
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 I  E  E  V  Y  T  L  L  T  H  R  E  I  K  S  I  Q  D  D  F
gtgaatttcacggactatagctaccagatgcgtttacccctggtttccaggtctacagtt
 V  N  F  T  D  Y  S  Y  Q  M  R  L  P  L  V  S  R  S  T  V
tcgaagtctattaaagataacattaggtttatcagaattactaagcaatcccaacatgctg
 S  K  S  I  K  D  N  I  R  L  S  E  L  L  S  N  P  N  M  L
accaatgaacttaaagcagagttcctcatccttttacatatgttgcaaagaaaattaggc
 T  N  E  L  K  A  E  F  L  I  L  L  H  M  L  Q  R  K  L  G
agaaaattgaatccagaatggtttgatgtgaaaccaacagtgggagaagttactctcaat
 R  K  L  N  P  E  W  F  D  V  K  P  T  V  G  E  V  T  L  N
caccttcctgcccaagcctctgggcgcagatataatttaaaagttaaaagaggaagagtt
 H  L  P  A  Q  A  S  G  R  R  Y  N  L  K  V  K  R  G  R  V
gtccctgttttaccaaatataggtagtggtggcagttcacatagagaaatacatgtgaag
 V  P  V  L  P  N  I  G  S  G  G  S  S  H  R  E  I  H  V  K
caagctggacaacattcttattactctgctatgaaacctatcagaaacatggacaagaaa
 Q  A  G  Q  H  S  Y  Y  S  A  M  K  P  I  R  N  M  D  K  K
ccttgaaaagcacctgtcaattggctttgaagaagtgatgccaccatctctagccataat
  P  *
aaagaaacagaatcacctagtattggatgtatttcaataataaatagagtggtgtctttt
ccataattgtgttaaagaacatttctttttccattcaatttgtttattaaactcctatgcc
aggcattgttgaaaatacaaaattaaataaat
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Fig. 1. Nucleotide and predicted amino acid sequences of *TSARG*2 (human testis spermatogenic cell apoptosis related gene 2). Polyadenylation signal is underlined. Stop codon is indicated by asterisk (*).

Northern blot. Northern blot analysis was performed using the PCR-amplified 532 bp segment of the *TSARG2* cDNA as a probe, which was labeled by [α-^{32}P]dCTP by using oligonucleotide primers A6 (5′-CGCATCTGGTAGCTATAGTCCGTG-3′) and 5TSARG2. Hybridization to a multiple-tissue Northern blot containing 2 mg poly(A)$^+$ mRNA from 8 human adult tissues (Clontech) was performed according to the manufacturer's instructions. Equal loading was confirmed using the β-actin probe as a control.

In situ hybridization on paraffin sections of human testis [20]. The hybridization probes were labeled by digoxigenin under PCR method using the primers 5TSARG2 and 3TSARG2. The paraffin-embedded testis tissue slides were dried at 60 °C for 30 min. These slides were cleared of paraffin with xylene, re-hydrated by sequential washings with graded ethanol solution (70–100%) and then deproteined by incubation with proteinase K (100 μg/ml) in 10 mmol/L Tris–HCl, pH 7.5, containing 2 mmol/L $CaCl_2$ (TES buffer) for 10 min at room temperature, and then fixed in 4% fresh paraformaldehyde for 5 min. The probes were labeled with digoxigenin by PCR methods and then denatured at 80 °C for 10 min. The deproteined slides were hybridized with 40 μl hybridization buffer (50% formamide, 4× SSC, 2× Denhart, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA) containing approximately 20 ng digoxigenin-labeled probes. Slides were covered with parafilm and incubated at 37 °C for 16 h in a humid chamber.

Amplification of each exon of TSARG2 gene from genomic DNA and SSCP analysis. The reaction mixture contained 1 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 500 mM of each primer (Table 1), 200 mM dNTP, 80 ng template genomic DNA, and 2.5 U *Taq* DNA polymerase (Sangon) in a total volume of 10 μl. PCR amplification cycles involved, initial denaturation at 95 °C for 1 min 30 s and 35 cycles of 94 °C for 40 s, 58 °C for 30 s and 72 °C for 40 s, and then 72 °C for 5 min, hold at 4 °C. The six exons of the *TSARG2* gene were amplified separately using 80 ng of genomic DNA prepared from peripheral blood leukocytes. SSCP analysis was performed essentially as described by Liu et al. [21]. Briefly, 3 μl lis-denaturing buffer (0.05% bromophenol blue, 0.01% xylene cyanol, and 10% sucrose) was added to 3 μl of the PCR mixture. The mixed sample was incubated at 95 °C for 5 min, rapidly chilled on ice, and subjected to electrophoresis on a 6% or 8% polyacrylamide gel.

TSARG2–EGFP plasmid construction and transfection. An EGFP–TSARG2 expression plasmid was constructed by inserting *TSARG2* cDNA into pEGFP-N1 (Clontech) vector. PCR was performed using primers BD11 (5′-CGGAATTCTCATGGCTGCCGCCGG-3′) and BD12 (5′-CGGTCGACCAAGGTTTCTTGTCCATGTTTC-3′), and using *TSARG2* cDNA in the pUCm-T vector as template. The PCR mixture and pEGFP-N1 plasmid were digested using an *Eco*RI and *Sal*I restriction enzyme. The *TSARG2* cDNA was ligated into the pEGFP-N1 vector upstream of EGFP. Correct orientation was determined by restriction digestion and DNA sequencing. Cells were seeded at a density of 4×10^6 cells/100 mm culture dish and transfected with 2–6 μg plasmid using Lipofectamine (Promega) according to the manufacturer's standard protocol. Fluorescence of the EGFP–TSARG2 fusion protein was typically detected within 2 days and experiments were performed within 3 or 4 days after transfection. Prior to observation, COS7 cells were plated onto 35 mm glass coverslips and returned to the incubator until the cells had attached and begun to spread. As a precaution against potential artifact due to modification of the TSARG2 molecule by EGFP or the increased expression of *TSARG2* in cells transfected with the TSARG2–EGFP fusion protein, central experiments were duplicated for cells that had not been transfected and for cells that had been transfected with EGFP vector as control.

Establishment of stable expression TSARG2 in MCF7 cell. An eukaryotic expression vector pcDNA3.1(−)/*TSARG2* for expression of *TSARG2* was constructed. *TSARG2* cDNA in the pUCm-T vector was removed using *Eco*RI and *Hin*dIII restriction enzymes. Restriction endonuclease digestion and sequencing were used to check whether or not the gene was inserted correctly. MCF7 cells were maintained in minimal essential medium (MEM) with Earl's balanced salts (Gibco),

Table 1
Oligonucleotide primers for the amplification of *TSARG2* gene

Exon	Primer	Expected DNA size (bp)
1	Sense: 5′-CAGGCGCTGAGGTCTCGC-3′ Antisense: 5′-GCGGGGGGGCCTAGGACTG-3′	363
2	Sense: 5′-CACTTTATTTCATTTCCTAATTGCC-3′ Antisense: 5′-TAACAGTTAAAAGTGTAAATGACTG-3′	198
3	Sense: 5′-CTTCATAGGTAAATAAAAGGGAGA-3′ Antisense: 5′-GTGTTACTAGGAAATTACAAAGAAC-3′	248
4	Sense: 5′-CAACAGTATTTTATTTTTTATTTCC-3′ Antisense: 5′-CATCTGTGCTCAAGAATAGCCTG-3′	313
5	Sense: 5′-GGCTTTTTAAGAAATAACTTTTGTG-3′ Antisense: 5′-CCAGTGTAATTGGCAGTGTAGG-3′	292
6	Sense: 5′-CGTTGTATGAATATTTAGTAACC-3′ Antisense: 5′-GAAAAAGTAGATTCACCATTCCT-3′	451

Table 2
Exon–intron junctions of *TSARG2* gene

Exon	Exon size (bp)	5′ splice donor	Intron size (bp)	3′ splice acceptor	Intron
1	327	CAACAG**gt**gctt	1606		1
2	130	GAGAAG**gt**acgt	376	gatc**ag**AGATTT	2
3	119	TCGAGA**gt**aagt	110	ttaa**ag**TTCCTG	3
4	221	ATCCAG**gt**aagt	4390	ttcc**ag**AATTAA	4
5	117	TTTTAC**gt**aagt	1,07,435	ttat**ag**AATGGT	5
6	319			tccc**ag**CAAATA	

Uppercase and lowercase letters indicate exon and intron sequences, respectively; conserved splice donor and acceptor dinucleotide sequences are indicated in bold.

supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine (Sigma), and 1% non-essential amino acids (Sigma). The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere and subcultured every 5 days. Cells were plated at 50% confluence in 100 mm dishes. Fifteen micrograms of purified DNA (Qiagen) was used for each transfection, and G418 was added to the cell media 48 h post-transfection at a final concentration of 0.5 mg/ml.

Replicate samples of 2×10^4 breast tumor cells MCF7 were plated in individual wells of a 24-well plate in standard culture medium. After allowing 24–48 h for cells to become adherent, the medium was changed to Opti-mem, and oligonucleotides at a final concentration of 1 mM in the presence of 10 mg/ml lipofectin were added for 4 h. The medium was then changed to standard medium lation of cell-sorted tumor cells that had received a containing serum, and the cells were cultured for 6 days. Cells were then taken up in trypsin–EDTA and viable cells were counted using a hemocytometer in the presence of trypan blue. Each treatment group was performed in triplicate. Control growth cultures were treated with 10 mg/ml lipofectin without the addition of plasmid or with the addition of pcDNA3.1(−) vector.

The effect of *TSARG2*/pcDNA3.1(−) vectors on cell cycle dynamics was examined using flow cytometry. The cells were washed with PBS, fixed with ice-cold 70% ethanol, and stored at 4 °C for 2 h. Subsequently, the cells were incubated with 50 μl of 1 mg/ml RNaseA for 60 min in a 37 °C water bath. After incubation, 400 μl of 50 μg/ml PI was added to the cells. This mixture was incubated for 60 min on ice in the dark. Cell cycle analysis was performed by flow cytometry. At least 1×10^4 cells were analyzed.

Results

Cloning and characterization of full-length TSARG2 cDNA

Primers were designed according to the 1175 bp sequence previously mentioned and used in PCR assay with advantage 2 DNA polymerase (Clontech) and Marathon-Ready cDNA of mouse testis (Clontech) as template. The PCR fragment was cloned into pUCm-T vectors and sequenced. The resulting 152 bp fragment was merged with the 1175 bp fragment, with overlapping sequences. This resulted in a 1233 bp fragment which was the full-length cDNA of *TSARG*2. The nucleotide and predicted amino acid sequences of *TSARG2* are shown in Fig. 1. Exon–intron junctions of *TSARG*2 gene are shown in Table 2 .

Analysis of the human *TSARG2* sequence revealed the existence of an ORF of 918 bp encoding a putative protein of 305 amino acids. The predicted protein has a theoretical molecular mass of 34,751 Da and a calculated isoelectric point of 9.85. There are 3 potential polyadenylation signals (AATAAA) at nucleotide positions 1079, 1120, and 1227. Homology searches using the Swiss-Prot program found no homology between TSARG2 and any known proteins. No signal peptide or transmembrane region was found in TSARG2 protein, and TSARG2 protein is most probably a nucleoprotein (60.9%). We searched for *TSARG*2 gene in the human genome (http://www.ncbi.nlm.nih.gov/humangenomeblast/), and the Blast result showed that the full-length of *TSARG*2 gene and the human genome BAC DNA sequence NT_006118.12 on 4q34.1–34.2 had high homology, so *TSARG*2 gene was mapped on 4q34.1–34.2 (Fig. 2). CpG island searches using CpG Island Searcher found a 441 bp CpG island with 61.6% GC value from −277 to +163. We also found a typical 208 bp promoter from −191 to +16 in this CpG island (Fig. 3).

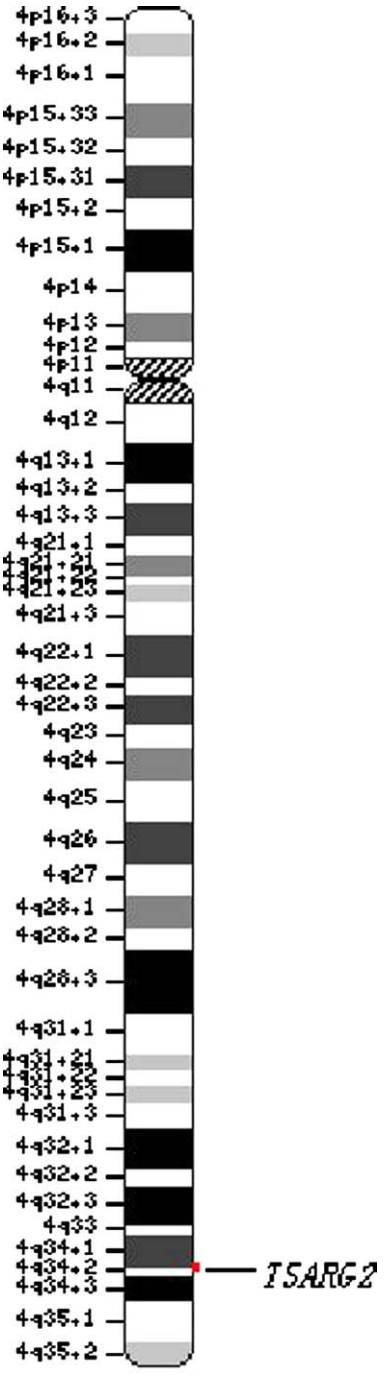

Fig. 2. Chromosome position of *TSARG*2. The chromosome position of *TSARG*2 was determined to be at 4q34.1–34.2 by searching the Drift Human Genome in NCBI.

tttgtttgtttgtttgttttgtagcgaaagtctctccaccttgtgttgcccaggctggtcttgaactcctgagctcaaacgatcctcccgcc
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cccgcctgctttccgccgcctgccaggcctcagattgcctaccctggcctggcttatgtttca

Fig. 3. CpG island of *TSARG*2 was searched by the CpG Island Searcher program and the promoter of *TSARG*2 was searched by the PromoterInspector program. The CpG island is shaded; the promoter is underlined; and the start codon is framed.

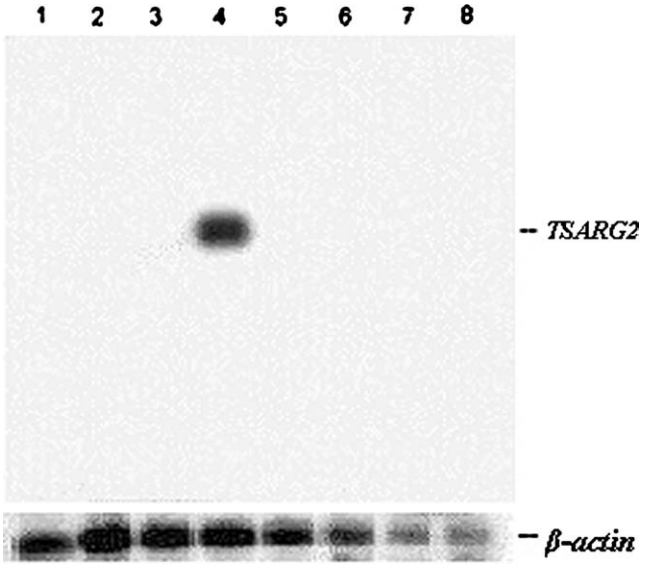

Fig. 4. Northern blot analysis of *TSARG*2 in 8 kinds of human tissues. A single strongly expressed, 1.7 kb mRNA species was observed exclusively in adult testis (overnight exposure); no hybridization signal was detected in other tissues even after 7 days exposure at −80 °C. 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon (no mucosa); and 8, peripheral blood leukocyte.

Northern blot analysis

The expression of the *TSARG*2 gene was examined in 8 different human tissues using Northern-blot analysis (see Materials and methods). A single strongly expressed, 1.7 kb mRNA species was observed exclusively in adult testis (overnight exposure); no hybridization signal was detected in other tissues even after 7 days exposure at −80 °C (Fig. 4).

In situ hybridization

PCR labeling was performed using the *TSARG*2/pUCm-T vector as template. The probe labeled with digoxigenin was hybridized with the tissue of human testis. The result indicated stronger hybridized signals observed in the seminiferous tubules, more precisely in spermatogonia and spermatocyte (Fig. 5). Thus, it was verified that the expression of the *TSARG*2 gene was high in adult testis.

PCR-SSCP analysis

One hundred twenty-two samples of peripheral blood specimens of azoospermia, severe oligzoospermia and cryptorchidism were collected. The DNA was extracted and used to establish a DNA sample pool. But PCR-SSCP did not detect the mutation of this gene in the samples, suggesting that this gene might carry out its function through up-regulation or down-regulation (Fig. 6).

TSARG2–EGFP fusion proteins localize to cell nucleus in vitro

To observe *TSARG*2 fusion protein expression in mammalian cells, a pEGFP-N1/*TSARG*2 fusion plasmid was constructed and transiently introduced into COS7 cells by liposome transfection. Under a fluorescence microscope, the green fluorescence produced by pEGFP-N1/*TSARG*2 was detected on the nucleus of COS7 cells after 24 h post-transfection, while the fluorescence produced by pEGFP-N1 was detected through the cells. Consistent with the prediction by bioinformatics, this result suggested that *TSARG*2-coding product is a nucleus protein (Fig. 7).

The effects of TSARG2 on cell growth

To determine whether *TSARG*2 gene resulted in changes in cell proliferation, the effects of such treatment on tumor cell growth were examined in vitro. As shown in Fig. 8, *TSARG*2 treatment results in significant acceleration of MCF7 cell growth compared with control cells.

The percentage of cells resident in each cell-cycle phase is indicated (Fig. 9). In control MCF7 cells, the cell-cycle distribution is 72.0% of cells in G1, 21.7% of

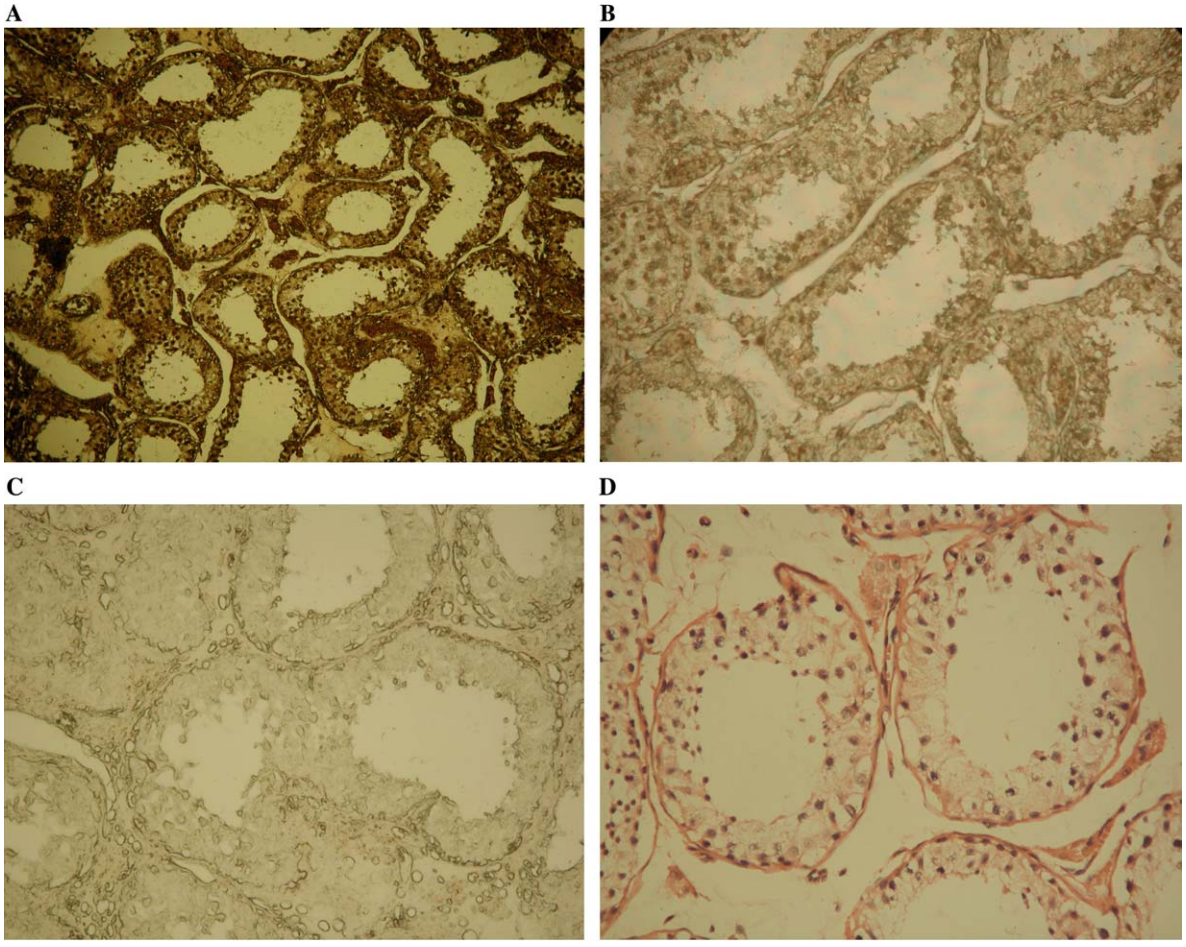

Fig. 5. In situ hybridization analysis of *TSARG2* in human testis paraffin sections. *TSARG2* transcript was detected in testis cryostat sections with digoxigenin-labeled RNA probe. (A) 100× and (B) 400×, using labeled probe; (C) 400×, using undenatured probe as a control. (D) 500× using unlabeled probe as a control (HE dye). The results indicated stronger hybridized signals observed in the seminiferous tubules, more precisely in spermatogonia and spermatocyte (A,B). Unlabeled or undenatured probe detected no positive signals (C,D).

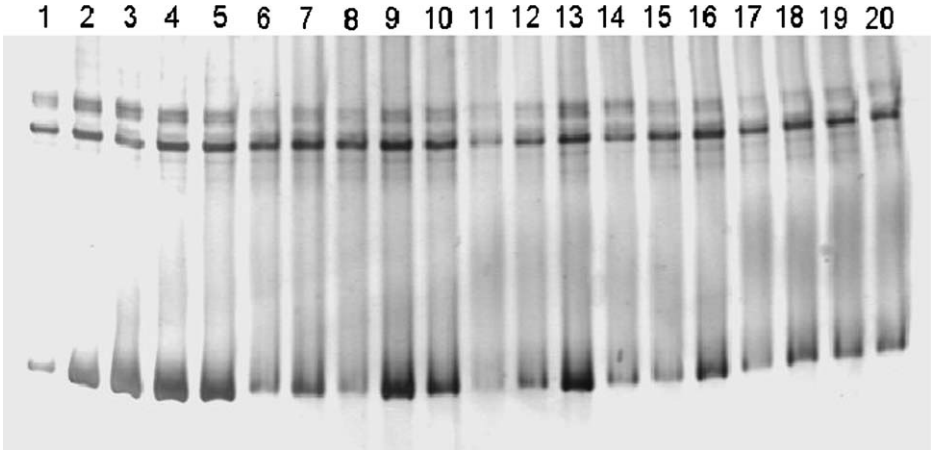

Fig. 6. PCR-SSCP analysis of exon 3 of the human *TSARG2* gene. An aliquot of the PCR mixture of exon 3 was denatured and separated on 6% polyacrylamide gel containing 5% glycerol. After electrophoresis at 4 °C for 12 h, DNA bands were visualized by silver staining. The PCR mixtures used were: lanes 1, sample from healthy volunteer; lanes 2–20, samples from volunteers with azoospermia, severe oligzoospermia or cryptorchidism.

cells in S-phase, and 6.3% in G2, whereas in transfection MCF7 cells, 55.6% of cells remain in G1, 29.3% of cells in S-phase, and 15.1% in G2. So *TSARG2* can accelerate MCF7 cells to traverse the S-phase and enter the G2-phase compared with the control without transfection of *TSARG2*.

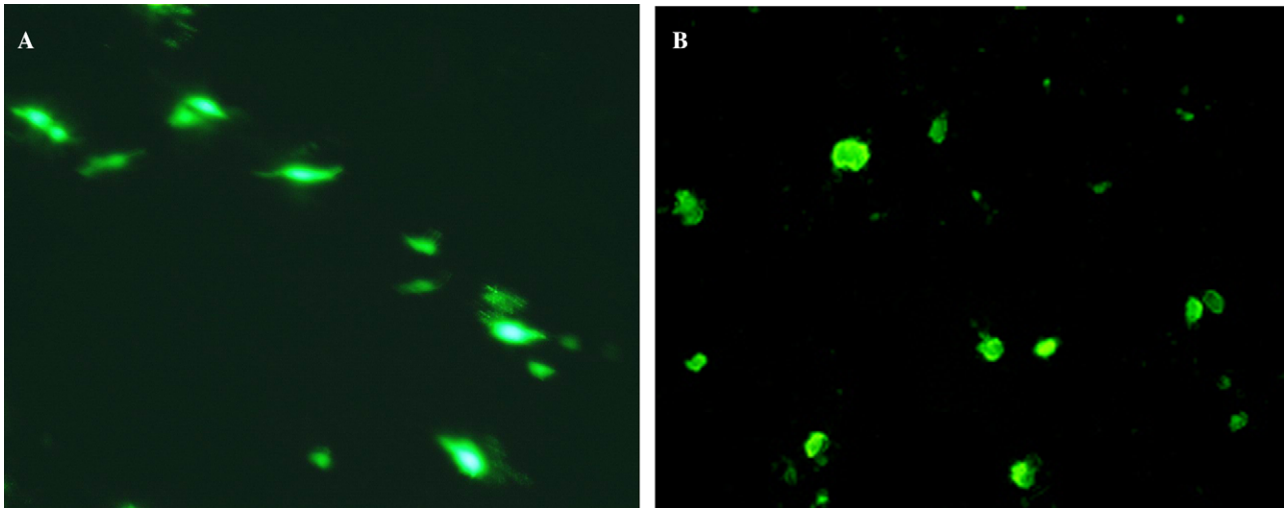

Fig. 7. (A) Expression of EGFP protein in COS7 cell line (400×); (B) expression of EGFP/TSARG2 fusion protein in COS7 cell line (400×). The results indicated that TSARG2–EGFP fusion proteins localized to cell nuclei in vitro.

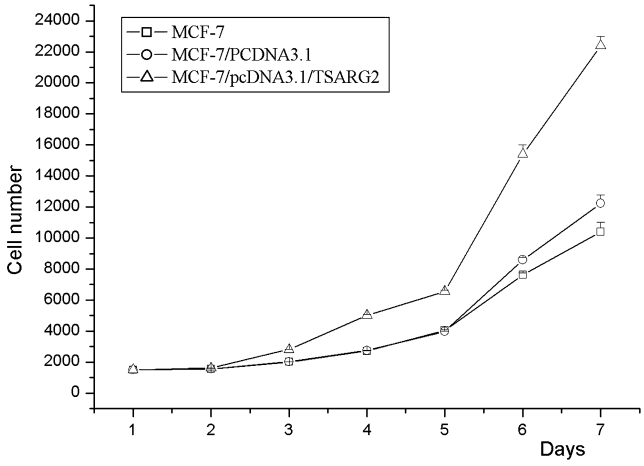

Fig. 8. The acceleration of monolayer growth of human MCF-7 cells by transfection of *TSARG2*.

Discussion

Northern blots indicated that *TSARG2* gene only had high expression in human testis; no signal was found in seven other tissues. Mouse *SRG2* gene, the homologous gene of *TSARG2*, also demonstrated the same results as *TSARG2* [18]. Many genes could be expressed in human testis, but only a few testis-specific genes were found, and most of them were structural genes [22–25]. Otherwise, TSARG2 protein was mostly found in the cell nucleus and could accelerate cell growth which is very different from other testis-specific genes. Bioinformation results showed *TSARG2* only coded 305 amino acids, so we think *TSARG2* may be a trans-acting factor.

We found a CpG island in *TSARG2* gene's 5′-end by CpG Island Searcher. With this clue we found a 208 bp promoter in this sequence, which should be the

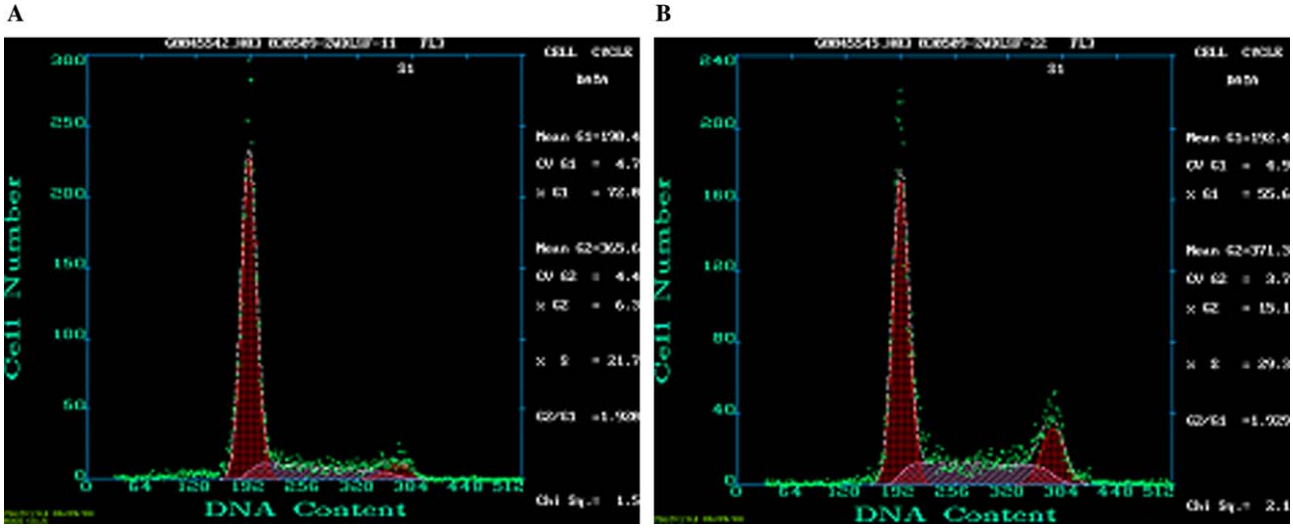

Fig. 9. The cell-cycle distribution of MCF7 cells by transfection of *TSARG2*. Transfection of *TSARG2* can accelerate MCF7 cells (B) to traverse the S-phase and enter the G2-phase compared with the control without transfection of *TSARG2* (A).

testis-specific gene's promoter which was mentioned above. We have identified the promoter by PCR assay, and the functions of this promoter will be examined in future studies. Another promising characteristic of the promoter of *TSARG*2 is its temperature sensitivity. The promoter would be very important for gene therapy passing through the blood–testis barrier and targeting the testis.

The mutation of this gene was not found in the samples by PCR-SSCP, suggesting that this gene might carry out its function to up-regulate or down-regulate through promoter methylation.

The open reading frame of *TSARG*2 was obtained from a human testis cDNA library by PCR. Using in situ hybridization on a tissue section of human testis, we conducted a preliminary study of the expression and function of *TSARG*2. The purple brown granules of hybridized signals were found in different stages of spermatogenesis cells. The results showed that this gene performs its function by different expression in different stages of spermatogenesis cells.

To observe *TSARG*2 protein function in mammalian cells, pcDNA3.1(−)/*TSARG*2 plasmid was constructed and introduced into MCF7 cells by liposome transfection. The results showed that *TSARG*2 gene could accelerate MCF7 cell growth and suppress cell apoptosis, leading to the supposition that when the hot signal started up the apoptosis in cryptorchid testis, *TSARG*2 gene received the apoptosis results and improved its expression to hasten spermatogenesis and suppress the excess apoptosis.

Northern blots showed that *TSARG*2 gene had a very high expression in normal testis and PCR results indicated that the expression abundance of *TSARG*2 was still very high even with varied testis cDNA libraries as template. Was this related to maintenance of normal spermatogenesis and a high rate of apoptosis? Confirming this supposition would provide a new target of medical treatment for azoospermia, severe oligzoospermia and cryptorchidism. For this purpose, *TSARG*2 gene was fused in expression vectors pGEX-KG and pGEX-4T-2. Then it was transformed into *Escherichia coli* BL21(DE_3). With IPTG induction, the gene was efficiently expressed. The fusion product of *TSARG*2 was purified according to the purification procedures of inclusion bodies and glutathione–Sepharose 4B affinity chromatography. SDS–PAGE analysis of the purified recombinant protein showed only one band (data not shown). Research of the physical and chemical characteristics and physiological function of this protein is ongoing.

TSARG2 is a testis-specific gene that may play an essential role in testis function. Northern blot showed that the mouse homologous *SRG*2 transcript was easily detected first in the testes of 21-day-old mice. At this time, second meiosis occurred and produced the first group of spermatids. After that, *SRG*2 gene expressed steadily in adult mouse testis (data not shown), indicating that these genes play an important role in the maturation of sperm. However, the mechanism and the significance of enhanced expression of *TSARG2* in cryptorchidism still remains to be defined.

According to the cloning of human testis spermatogenesis related genes, the function and the reciprocity of these genes is an aspect for further research. We are slowly gaining an understanding of when testis cell apoptosis begins, how signals are transducted among those cells and what roles they play in male infertility.

Altogether, this research represents the first functional characterization of *TSARG*2, a gene which may perform important functions in the development of the adult testis as well as in testicular germ cell tumorigenesis.

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