Developmental expression pattern of a novel gene, TSG23/Tsg23, suggests a role in spermatogenesis

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ABSTRACT: A novel gene, TSG23/Tsg23, was identified by comparing the expression profiles of human adult and fetal testis using Affymetrix Genechips. RT–PCR analysis from multiple human and mouse tissues indicated TSG23/Tsg23 mRNA was mainly expressed in the testis. In situ hybridization revealed that TSG23/Tsg23 mRNA was located in spermatocytes and round spermatids of the seminiferous tubules in human and mouse testis. To further confirm the result from RT–PCR, the antibody for human TSG23 was generated against the protein encoded by the gene. Western blot analysis demonstrated that TSG23 was mainly expressed in human testis, with a molecular weight of about 23 kDa. Immunohistochemistry showed that TSG23 was predominantly located in spermatocytes and round spermatids, consistent with the results from in situ hybridization. In order to explore the function of TSG23 in spermatogenesis, the study compared the expression of TSG23 in the testis from fertile persons and from patients with azoospermia. The results showed that there was less expression in patients with obstructive azoospermia compared with fertile persons, and no detectable TSG23 at mRNA and protein levels in patients with non-obstructive azoospermia. The expression of Tsg23 mRNA was considerably decreased in a time-dependent manner in the testis of an azoospermic mouse model induced by Busulfan. These data suggest that TSG23/Tsg23 is involved in human and mouse spermatogenesis.

Key words: spermatogenesis / azoospermia / TSG23/Tsg23 / testis

Introduction

Infertility has affected as many as 15% of couples worldwide, and males are known to be responsible for half of the instances (Nishimune and Tanaka, 2006). This condition is now considered to be a complex set of diseases involving genetic, physical and environmental factors, with genetic factors accounting for 15% of cases of male infertility (Cram et al., 2004; Carrell et al., 2006; Orstavik, 2008). Thus, genetic analysis is important for both prognosis and treatment of the infertile man. It is currently believed that idiopathic infertility is mainly of genetic origin. Thus, a more complete understanding of the physiological mechanisms in spermatogenesis is needed before solutions to the problem can be found.

Spermatogenesis is a form of development that continues throughout adult life in most mammals and can be divided into three phases: mitosis (self-renewal and multiplication of spermatogonia), meiosis (reduction of chromosomal number from diploid to haploid) and spermiogenesis (spermatid differentiation into spermatozoa). Defects in any one of these three processes could result in infertility. The highly ordered and complicated process of spermatogenesis in the testis requires a precise coordination of cell-cycle events as well as regulatory pathways, which depend on the expression of many genes. Many of these genes are expressed predominantly or exclusively in spermatogenic cells, and their regulation can involve control at the transcriptional or post-transcriptional level. The regulation of gene expression during spermatogenesis can be thought of as occurring in three levels: intrinsic, interactive and extrinsic (Eddy, 2002; Wolgemuth et al., 2002). The identification of testis-specific or germ cell-specific genes involved in these unique events provides excellent tools to dissect the differentiation program and to study the mechanisms by which spermatogenesis is controlled. To date, many testis-specific genes have been identified in human and mouse, e.g. SRG4 (Xing et al., 2004), SPERGEN-1 (Miyamoto et al., 2003), Tssk3 (Zuercher et al., 2000), Fank1 (Zheng et al., 2007), TSGA10...
(Modarressi et al., 2001), mtLR1 (Nie et al., 2005) and Tex24 (Fan et al., 2006). Schultz et al. (2003) analyzed gene expression in mouse testes from Day 1, 4, 8, 11, 14, 18, 21, 26 and 29 to Day 60 by using the Affymetrix Mouse U74Av2 chip, and estimated that about 4% of the mouse genome was dedicated to germ cell-specific transcripts, >99% of which are first expressed during or after meiosis. In addition to the genes mentioned above, other testis-specific genes may exist and serve unique functions in the testis.

Previously, we characterized the gene expression profiles of 2058 spermatogenesis-related genes in mice by performing large-scale cDNA analysis using GeneChip Mouse Genome 430 2.0 (Xiao et al., 2008), and a number of novel genes were identified and characterized (Tang et al., 2006, 2007; Yu et al., 2007). Here, as an ongoing study on testis-specific genes, we identified a novel gene, TSG23/Tsg23, which is mainly expressed in testis of human and mouse, and we analyzed its characteristics at the gene transcript level. Further, we characterized the protein encoded by the gene and predicted that it could play an important role in spermatogenesis. In an attempt to obtain greater insight into the function of TSG23/Tsg23, we compared the gene expression differences between fertile and azoospermic testes at the mRNA and protein levels. To the best of our knowledge, this is the first report on the mRNA and protein expression characteristics of the novel gene with a potential role in spermatogenesis.

Materials and Methods

Sources of tissues

Human tissues including lung, muscle, liver, kidney, esophagus, stomach and fetal tissues were collected during surgical operations, whereas fertile human testis specimens were obtained from post-mortem studies and orchiectomies. The age range of subjects was 24–40 years old. The testicular tissue samples from 20 azoospermic patients (9 obstructive azoospermia and 11 non-obstructive azoospermia) aged 22–36 years were obtained, after informed consent, from clinical biopsies for pathological diagnosis. Male and female Balb/c mice (aged 4–6 weeks) were obtained from the Laboratoral Animals Center of South Medical University (Guangzhou, People’s Republic of China) and maintained in a temperature- and humidity-controlled room. All animals had free access to standard mouse chow and water. Male and female mice were mated naturally, and the day of birth was designated as Day 1. Testes were individually collected from mice aged 4, 6, 9, 11, 15, 18, 24, 31, 35, 40 and 54 days to 6 months. Other organs including brain, heart, lung, liver, kidney, spleen, epididymis, colon and pancreas from adult mice were also collected, and all the samples were immediately frozen in RNAlater liquid (Qiagen, Valencia, CA, USA). The Ethics Committee of Peking University Shenzhen Hospital granted approval for the study prior to sample collection. Animal experiments were approved by the Animal Test Center of China.

Affymetrix Genechip analysis

Total RNA was extracted from human and mouse organs by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendations. The concentrations and the integrities of total RNA were assessed by measuring the 260:280 nm ratios and by fractionation in 1% denaturing agarose gel. Total RNA from the testis of adult and fetal human with a 260:280 nm ratio of 1.8 or higher was used to generate biotinylated cRNA target for the GeneChip Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA), containing almost 45,000 probe sets representing more than 39,000 transcripts derived from ~33,000 well-substantiated human genes. Total RNA from the testis of Day 4, 9, 18, 35 and 54 to 6 months post-natal Balb/c mouse, and with a 260:280 nm ratio of 1.8 or higher, was used to generate biotinylated cRNA target for the GeneChip Mouse Genome 430 2.0 (Affymetrix), which contained 45,000 pairs of probes including 39,000 transcripts for 34,000 well-characterized mouse genes and in which 8000 expressed sequence tags were located. All of these procedures were carried out as described by Affymetrix. After hybridization, the array was washed, stained with streptavidin phycoerythrin using the Affymetrix GeneChip Fluidics Workstation 400 and scanned on a Hewlett-Packard gene array scanner (Hewlett-Packard Co., Palo Alto, CA, USA). After the arrays were scanned, the signals generated were determined and analyzed by MAS 5.0 software. The absolute and comparison analyses were also performed using MAS 5.0. After normalization of these data, a comparison analysis was made of an experimental array and a baseline array so as to monitor changes in the expression of transcripts across the samples targeted to different arrays (refer to http://www.Affymetrix.com for details on the statistics of these analyses).

Semi-quantitative RT-PCR

Semi-quantitative RT–PCR was carried out to analyze and confirm the expression of candidate genes. Total RNA (1 μg) was reverse-transcribed into cDNA in a reaction primed by oligo deoxynucleotideT (dT)₉ primer using RevertAid™ M-Mulv Reverse Transcriptase (Fermentas, Glen Burnie, MD, USA) according to the manufacturer’s instructions. Reverse and forward oligonucleotide primers, specific to the chosen candidate genes, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. The primer sequences were as follows: human TSG23 (TSG23), forward primer: 5′-GGG ACA ATG GCT ACT CCA GAA GCA GA-3′; reverse primer: 5′-GGA TGG GCC ATC ATG AAA GCT TGA G-3′; mouse TSG23 (Tsg23), forward primer: 5′-CTG TTC TCC CAG AAG CAG AGC TCC CTA TCT-3′, reverse primer: 5′-GTA CCT CAC TAA AGG CAT CTG TCC AGA AG-3′; β-actin, forward primer: 5′-TCA TCA CTA TTG GCA ACG AGC-3′, reverse primer: 5′-AAC AGT CCG CCT AGA AGC AC-3′. The following PCR conditions were used: 94°C for 3 min; 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s; followed by 72°C for 5 min. All samples from various date testes and other organs were plated in triplicate PCRs.

Bioinformatics analysis

The complete coding sequence of novel gene, TSG23, was derived by automated computational analysis using the gene prediction method: DNA sequences of the differentially expressed genes were entered in the database for the search of homologous proteins using BLAST (http://www.ncbi.nlm.nih.gov/blast) and SMART (http://smart.embl-heidelberg.de/) programs. ClustalW program was used for multiple DNA sequence alignment (http://www.ebi.ac.uk/clustalw/). SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used for signal peptide prediction. Molecular weight and isoelectric point were also obtained from the Compute pl/Mw Program (http://cn.expasy.org/tools/pi_tool.html). Prediction of subcellular localization was performed by PSORT II Prediction (http://psort.im.s.u-tokyo.ac.jp/form2.html).

In situ hybridization

To synthesize the probes of TSG23 for in situ hybridization (ISH), the PCR products of TSG23 were purified and subcloned into pDrive Clone Vector according to the manufacturer’s protocol (Invitrogen), antisense and sense constructs were derived by sequencing and were linearized by Sall (human and mouse) and BamHI (mouse), HindIII (human) digestion, and DIG-antisense and sense riboprobes were synthesized with SP6
polymerase and T7 polymerase using DIG RNA labeling Kit (Roche, Mannheim, Germany), respectively. The probes were precipitated using ethanol at −20°C overnight and resuspended in DEPC-treated water for hybridization.

The testis samples of adult human and mouse were sliced and immersed in 4% paraformaldehyde for 1 h. The tissues were then placed in fresh 4% paraformaldehyde at 4°C overnight, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin. The embedded testes were sliced into 5 μm sections and placed on slides pretreated with polylysine. The sections were deparaffinized in xylene, rehydrated in ethanol and washed with DEPC-treated water and TBS. After being treated with proteinase K (20 μg/ml) for 10 min at 37°C, sections were post-fixed by 4% paraformaldehyde for 10 min and then washed twice with TBS. The sections were prehybridized in hybridization buffer without probe at 50°C for 2 h to block non-specific binding sites, and then overlaid with a hybridization mixture consisting of 50% formamide, 2 × standard saline citrate (SSC) buffer, 10 mM DTT, 1 mg/ml herring sperm DNA, 500 μg/ml yeast tRNA, 1 mg/ml BSA and 100 ng/ml probe for TSG23, covered with frame seals, and incubated overnight at 50°C. After hybridization, the hybridization solution was removed by thoroughly rinsing the slides in 4 × SSC. The slides were washed twice in 2 × SSC and 1 × SSC for 15 min at 50°C. To remove non-specifically bound DIG-labeled RNA probes, the slides were incubated with RNase A for 10 min at 37°C. The slides were then washed twice in 0.1 × SSC for 10 min at 50°C. Immunological detection was carried out with AP-conjugated anti-DIG antibodies and visualized with nitroblue tetrazolium (250 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (225 μg/ml) according to the manufacturer’s instructions (DIG Nucleic Acid Detection Kit; Roche).

Western blot analysis
A synthetic peptide (105DNLYSRSPDLGGP123 of TSG23) was used to immunize mice. After three immunizations, sera were collected, and polyclonal antibodies purified with the synthetic antigen peptide column using an AminoLink Immobilization kit (Pierce, Rockford, IL, USA).

All human tissue samples were lysed with lysis buffer for 1 h on ice in the presence of protease inhibitor cocktails (BIODEV, Beijing, China). Lysed proteins with non-ionic detergent were centrifuged for 10 min at 12,000g. The supernatant fractions from the lysate were mixed with 5 × SDS sample buffer and boiled for 5 min. Samples were then reduced with 5% β-mercaptoethanol and stored at −20°C until use.

Each extract containing ~50 μg protein was subjected to 10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (MilliPore, Bedford, MA, USA). Membranes were blocked in TBST containing 5% non-fat dry milk or 1% BSA overnight at 4°C, and hybridized for 2 h with TSG23 antibody (2 μg/ml dilution) at 37°C, followed by three washes for 10 min with TBST (5 mmol/l Tris–HCl, pH 7.4, 136 mmol/l NaCl, 0.1% Tween 20). Membranes were then incubated with HRP-conjugated goat anti-IgG mouse (dilution 1:2000) (Covalab, France) for 1 h at room temperature and washed three times with TBST. Bound antibodies were detected using the chemiluminescence western blotting detection kit (Pierce) according to the manufacturer’s recommendations and visualized under chemiluminescence detection equipment (ChemiDoc XR, BIO-RAD, Hercules, CA, USA).

Immunohistochemistry
Paraffin sections of human testis were deparaffinized using xylene, rehydrated through a graded series of 100, 95, 80 and 70% ethanol and incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Tissue sections were heated in a microwave oven for 15 min in 10 mM citrate solution (pH 6.0) for antigen retrieval and washed three times in PBS. After blocking with 5% normal goat serum for 30 min, sections were incubated with the primary antibodies overnight at 4°C. After incubation with the TSG23 antibody, the sections were rinsed, washed (3 × 5 min) in PBS and then incubated for 30 min at room temperature with biotinylated-conjugated anti-mouse secondary antibody. After incubation with the secondary antibody, the sections were rinsed, washed (3 × 5 min) in PBS and incubated for 10 min at room temperature with streptavidin conjugated to HRP and with DAB which generated brown color at the site of peroxidase activity. Sections were then rinsed and washed (3 × 5 min) in deionized water, and the nuclei were counterstained with Mayer’s hematoxylin. Finally, the sections were mounted. In negative control slides, the primary antibody was replaced by mouse serum without peptide immunization. The results were observed under microscope (DMLB, Leica Microsystems, Germany).

Establishment of azoospermic model
Seven to eight weeks male mice were given a single i.p. injection of Busulfan (Sigma Chemical Co., St Louis, MO, USA) at 40 mg/kg in 50% DMSO/PBS to establish azoospermic model (Brinster and Zimmermann, 1994; Nayernia et al., 2004). The control mice were injected with a comparable volume of 50% DMSO/PBS. At 1, 2 and 3 months after injection of Busulfan, the mice were killed, then the testes were collected, immediately placed in TRIzol reagent and subjected to RT-PCR.

Results
Identification of the novel gene TSG23/Tsg23 in human and mouse
By hybridizing human adult or fetal testis cDNA samples with a human Affymetrix GeneChip, we identified a novel gene, TSG23 (GenBank accession number NM_080608). This gene was highly expressed in the adult testis. The relative hybridization signal intensities of the gene in adult and fetal testes were 124.7 and 2.2, respectively, 60-fold stronger in adult than in fetal testes. Similar age-dependent expression of its mouse homologous gene, Tsg23 (GenBank accession number XM_130672), was observed in post-natal developmental stages of mouse testes. The hybridization signal intensities of Tsg23 on Day 4, 9, 18, 35, 54 and 6 months post-natal were 10.2, 1.1, 10.3, 284.1, 220.3 and 233.3, respectively, and the signals on Day 4, 9 and 18 were not detected by Affymetrix chip analysis. On the other hand, the signal intensities of β-actin were 3688.8, 3764.78, 3812.9, 3696.87, 3679.71 and 3757.12, respectively.

Features of cDNA and deduced protein
TSG23 gene was mapped to chromosome 20q13.12. The full cDNA length of TSG23 is 752 bp, with a 684 bp open reading frame from nt10-693, which encodes a 227 amino acids protein with a predicted molecular weight of 23.83 kDa and isoelectric point of 8.59. The amino acid sequence analysis of the coding protein using the SignalP (V3.0) predicted that the probability of a signal peptide was 98.4%, with the cleavage site located between positions 62 and 63, indicating the TSG23 was a secretory protein (Fig. 1A). With TMPred search server, two transmembrane helices were found at the 19–40 and 152–172 positions in the TSG23 gene, with the N-terminal lying outside the membrane (Fig. 1A). Searching for conserved function domain by Blast-p, we identified a putative polynucleotide phosphorylase (Pnp) domain (amino acids 132–172) (Fig. 1A), which is homologous with the RNase_PH domain belonging to polynucleotide
nucleotidytransferase. A BLAST search revealed a high homology of TSG23 to a mouse cDNA XM_130672. The two genes were 87% identical in nucleotide sequence. Blast-pr showed that the encoded protein by TSG23 gene in amino acid sequences was 82.7% identical to Tsg23 gene (Fig. 2). These data indicated a high level of homology between TSG23 and the mouse homologue at both the nucleotide and protein level; therefore, TSG23 is a human–mouse homologous gene.

The Tsg23, which was located on chromosome 2H3, was found to encode a 226 amino acids protein with a predicted molecular weight of 23.55 kDa, isoelectric point of 7.67. The amino acid of Tsg23 was predicted no signal peptide, indicating that Tsg23 was a non-secretory protein. Similarly, two transmembrane helices were found in Tsg23 (Fig. 1B).

**Tissue distribution of TSG23/Tsg23 mRNA in human and mouse**

The tissue expression pattern of TSG23 was examined by RT–PCR in seven different human tissues including testis, lung, muscle, liver, kidney, esophagus and stomach. The gene was mainly expressed in testis (Fig. 3A). In the 10 tissues (testis, brain, heart, lung, liver, spleen, epididymis, colon and pancreas) examined in mice, its expression was high in testis, weak in epididymis and non-existent in the other tissues (Fig. 3B). The expression of TSG23/Tsg23 mRNA was evident in testis of human and mice (as well as in mouse epididymis), indicating that TSG23/Tsg23 gene was mainly expressed in the testis in both human and mouse.

**Development-dependent expression of Tsg23 mRNA in mouse testis**

To authenticate the expression pattern of Tsg23 in the developmental stages of mouse testis supported by Affymetrix chip analysis, we performed RT–PCR using mouse testes obtained at different days after birth. As shown in Fig. 3C, expression of Tsg23 was detected after Day 15 and gradually increased from Day 15 to 5 months. The result from the RT–PCR assay was consistent with that from the Affymetrix chip analysis, which demonstrated that Tsg23 mRNA was developmentally regulated.

**Localization of TSG23/Tsg23 mRNA in human and mouse testis**

The cellular localization of TSG23/Tsg23 mRNA in human and mouse testis was determined by ISH. The results showed the hybridization signal was associated with the seminiferous tubules and was limited to certain populations of germ cells both in human and mouse. Strong positive signals were detected in the spermatocytes and round spermatids of the seminiferous tubules in adult human testis. In some areas of the seminiferous tubule, moderate-to-weak signals were generated, whereas other areas within the same tubule were stained intensely, indicating that the expression of TSG23 mRNA in the spermatogenic cells could be stage specific (Fig. 4A). The hybridization signal with antisense Tsg23 probe was limited to certain populations of germ cells, particularly in round spermatids. Weak or no signal was detected in Sertoli cells, Leydig cells and spermatogonia (Fig. 4C). No hybridization signal was observed when a sense TSG23/Tsg23 probe was used as a control (Fig. 4B and D).

**Generation of Tsg23 antibody and its tissue expression pattern**

We generated an antibody against the protein encoded by TSG23 using a synthetic peptide. To test the specificity of the antibody to TSG23 protein and confirm the results of RT–PCR, western blot analysis was carried out in the same tissue samples. As shown in Fig. 3D, the antibody recognized a distinct band at 23 kDa comparable to the predicted molecular weight. The band was only detected in the

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**Figure 1** Nucleotide and deduced amino acid sequences of TSG23/Tsg23 in human (A) and mouse (B). Underlined sequences show the transmembrane helices in amino acid sequences. The boxed represents the cleavage site in human amino acid sequences. TSG23 contains a polynucleotide phosphorylase (Pnp) domain (shaded).
human testis. This result suggested that TSG23 protein is mainly expressed in human testis.

Abnormal expression of TSG23/Tsg23 mRNA in azoospermic testis

To investigate the contribution of TSG23 to spermatogenesis, we compared its expression in the testis between patients with azoospermia and fertile persons. RT–PCR studies found no detectable TSG23 mRNA in patients with non-obstructive azoospermia, and less expression of TSG23 in patients with obstructive azoospermia compared with fertile persons (Fig. 5A). In the mouse model of azoospermia induced by Busulfan, which caused structural damage in testis by depletion of spermatogenic cells, we observed that the expression of Tsg23 mRNA was weak at 1 month, and little or no expression was detectable at 2 and 3 months after treatment (Fig. 5B).

Expression characterization of TSG23 protein in azoospermic patients’ testis

To further elucidate whether the expression of TSG23 protein was associated with spermatogenesis, immunohistochemistry was performed in the testes from fertile and azoospermic patients. In the normal testis, all stages of spermatogenic cells were present in the seminiferous epithelia. In the obstructive azoospermic testis, reduced layers of spermatogenic cell, disordered cellular arrangement and considerable numbers of vacuoles were observed in the seminiferous tubules. In the non-obstructive azoospermic testis (spermatogenic...
arrest at spermatocyte level), the basal membrane of seminiferous tubules was thickened, and only spermatogonial cells and spermatocytes were found in the seminiferous epithelia. According to Clermonts’ study and Amann’s review (Clermont, 1963; Amann, 2008), we determined the spermatogenic stages in the human testis. As shown in Fig. 6A, TSG23 protein was predominantly located in the spermatocytes and round spermatids. In the obstructive azoospermic testis, the layers of spermatogenesis cells decreased, a number of vacuoles were observed in the lumen. Less signal of the TSG23 protein was detected in spermatocytes and round spermatids (C). In the non-obstructive azoospermic testis, the basal membrane of seminiferous tubules was thickened; only spermatogonial cells and spermatocytes were found in the seminiferous epithelia, defined as spermatogenic arrest. No signal of the TSG23 protein was detected (D). No staining was observed in tissue sections when TSG23 antibody was replaced by buffer containing 1% BSA (B). Bars = 50 μm.

Discussion

Thousands of genes are involved in human spermatogenesis, and only a small proportion of them has been identified and screened in infertile men so far (Krausz and Giachini, 2007). Identifying key genes that regulate this process will undoubtedly lay a solid base for our further understanding and better manipulation of spermatogenesis, and can be useful for the development of new contraceptive targets and health care drugs. Several studies have identified some genes...
expressed at a specific stage or in a particular cell types during spermatogenesis using differential display, cDNA/oligonucleotide array or subtractive hybridization (Sha et al., 2002; Schultz et al., 2003; Guo et al., 2004; Hong et al., 2005; Bonilla and Xu, 2008). Feig et al. (2007) filtered 1181 genes that exhibited exceptional statistical significance in testicular expression and identified gene expression patterns of the testis that correlated with the appearance of distinct stages of male germ cells. These studies provided information about the expression profile of a large number of germ cell genes with known or unknown function and revealed that the function of most of the testis-specific genes was unknown.

In the present study, a novel gene, TSG23/Tsg23, was identified from the results of Affymetrix human and mouse Genechip analysis. The chip results indicated that TSG23/Tsg23 was highly expressed in adult human testis and only detected in mouse testis after Day 35. Further characterization of TSG23/Tsg23 provided information about its nucleotide sequences and protein-encoded, tissue distribution and age-dependent expression of mRNA, and the abnormal expression of TSG23/Tsg23 mRNA and its encoded protein in patients and mice with male infertility. These results strongly suggested that TSG23/Tsg23 is involved in spermatogenesis.

The relationship between TSG23 and spermatogenesis was indicated by several lines of evidence. First, it was identified by a close correlation between the expression level of TSG23 and the testicular development. Hybridization of the human chip with adult and fetal testis indicated TSG23 was expressed more highly in human adult testis than in fetal testis. Second, multiple tissue distribution of TSG23 showed that it was predominantly expressed in testis; no expression was detectable in other tissues examined. The tissue specificity of TSG23 expression pattern implied its involvement in the functions of the testis, i.e. spermatogenesis. Although the function of TSG23 remains unknown at present, we predict that it may play a potential role in human spermatogenesis. Thirdly, in situ hybridization analysis demonstrated that the expression of TSG23 mRNA in adult testis was localized in certain populations of germ cells. Strong positive signals were detected in the spermatocytes and round spermatids of the seminiferous tubules in adult human testis. No signal was detectable in Sertoli cells, Leydig cells and spermatogonia. Fourthly, the involvement of TSG23 in spermatogenesis was further suggested by a close correlation between the expression of TSG23 and azoospermia. The expression of TSG23 in the testis from patients with non-obstructive azoospermia was absent in both mRNA and protein levels. These results provided compelling evidence for the involvement of TSG23 in human spermatogenesis.

The TSG23 homologous gene in mouse, Tsg23, was also linked to mouse spermatogenesis. This gene had properties similar to those of TSG23. As shown in the mouse chip results, the expression of Tsg23 was detected at Day 35 and 54, and at 6 months, but not detected at Day 4, 9 and 18, which was further authenticated by both RT–PCR and in situ hybridization studies. RT–PCR analysis revealed that Tsg23 was weakly expressed in mice testis from Day 15, and the expression was obviously increased after Day 24. It has been reported that the seminiferous epithelium in mouse testis at Day 6 contains only primitive type A spermatagonia and Sertoli cells; type A and type B spermatogonia are present by Day 8; meiotic prophase is initiated by Day 10, with the germ cells reaching the early and late pachytene stages by days 14 and 18, respectively; and pachytene spermatocytes account for about one-third of the total cell population in the seminiferous epithelium by Day 15. Secondary spermatocytes and haploid spermatids appear in increasing numbers between days 18 and 20 (Belville et al., 1977; Hong et al., 2005). According to the expression characteristics of Tsg23 in testis, we proposed that Tsg23 may participate in the generation of late pachytene spermatocytes and round spermatids. In addition, the results of multi-tissue RT–PCR showed that this gene was highly expressed in testis and weakly in epididymis. The possible reason for the weak expression of Tsg23 in epididymis of mice was that some immature and mature sperm were stagnated in the epididymis. The results from ISH also showed that Tsg23 mRNA was expressed only in spermatogenic cells, mainly in spermatocytes and round spermatids in adult mouse testes. In the mouse azoospermic models induced by Busulfan, the expression of Tsg23 mRNA was decreased in a time-dependent manner. All of the data from the mouse implied that Tsg23 may play a role in spermatogenesis.

Although there is a close relationship between TSG23 and spermatogenesis, the exact function of TSG23 protein remains elusive. To gain further insight into the characteristics of the protein, we generated a specific antibody against the novel protein by using a synthetic peptide corresponding to amino acids 105–123 of the putative protein. The antibody explicitly recognized distinct bands, with the specific expected size of the protein in the testis. The western blot analysis of lysates from multiple human tissues showed the expression of this protein was restricted to testis. This result corroborated and extended the previous RT–PCR data that TSG23 mRNA was mainly expressed in testis.

To determine the localization of TSG23 protein, immunohistochemistry was performed on testicular sections with normal spermatogenesis. TSG23 protein was immunolocalized predominantly in the spermatocytes and round spermatids. The expression of TSG23 protein in the spermatogonial cells was stage specific. Immunoreactive TSG23 levels were low at stages I and II of spermatogenesis. However, the levels of immunoreactive TSG23 protein were higher in the cells at stages III and V. This result, together with the localization of TSG23 mRNA of ISH, indicated that the expression of TSG23 mRNA and its protein in the spermatogonial cells was stage specific. A stage-specific pattern of TSG23 expression was observed in spermatogenic cells, suggesting a temporal regulation of intracellular TSG23 expression.

To further validate the function and role of TSG23 protein in spermatogenesis, we compared the difference of TSG23 expression in the testis between fertile men and azoospermic patients. In the testis from fertile men, TSG23 protein was predominantly located in the spermatocytes and round spermatids, and the expression of TSG23 protein was significantly decreased in spermatocytes and round spermatids of the testis from patients with obstructive azoospermia and no signal was detected in testis of spermatogenic arrest. This result was in agreement with previous observation that TSG23 mRNA was not detected in patients with non-obstructive azoospermia and significantly decreased in patients with obstructive azoospermia. It was revealed that decreased expression of TSG23 were associated with spermatogenic failure in infertile men.

In summary, we have identified a novel gene, TSG23/Tsg23, which is developmentally and highly expressed in human and mouse testis. Most importantly, the expression of the gene is decreased in the
testis from patients with azoospermia. The results have provided evidence implicating the involvement of TSG23 in spermatogenesis. Further investigation is required to determine the molecular mechanisms of TSG23 in the spermatogenesis. A Tsg23 knockout mouse could provide further insight into the precise function of Tsg23. This study is currently under way. On the other hand, the screening of TSG23 gene mutations in patients with azoospermia by direct sequencing may help us to understand the role of TSG23 in human infertility.

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