Identification and Characteristics of a Novel Testis-Specific Gene, Tsc24, in Human and Mice

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Testis-specific genes are essential for the spermatogenesis in mammalian male reproduction. We have identified a novel gene, Tsc24, from the results of the Affymetrix Genechip analysis in the developmental stage of days 4, 9, 18, 35, 54 and 6 months of postnatal Balb/C mouse testis. The scaling signal intensities of Tsc24 in the six stages of mouse testis showed that the expression of Tsc24 was not detected on day 4, 9 or 18 but on day 35, 54 and 6 months. The full cDNA length of mouse Tsc24 was 899 bp, with a 624 bp open reading frame encoding a 207 amino acid protein with a predicted molecular weight of 23.997 kDa. The results of semi-quantitative RT-PCR showed that the expression of mouse Tsc24 can only be detected after the mouse was 35 d old and the expression level increased gradually from day 35 to 6 months. Of the eight tissues (liver, spleen, heart, lung, brain, kidney, epididymis, and testis) examined in mice, and of the 12 tissues (liver, kidney, muscle, brain, spleen, adipose, lung, heart, epididymis, testis, ovary and uterus) examined in human, Tsc24 was exclusively expressed in the testis, but in none of the other studied tissues. The result of subcellular localization of GFP-Tsc24 fusion protein in Cos-7 cells supports that Tsc24 protein is expressed in nuclear. Our study should be a basis for function characterization of the Tsc24 gene, leading to the elucidation of the molecular events underlying mammalian male reproduction.

Key words testis-specific gene; DNA microarray; spermatogenesis

Sperm development, termed spermatogenesis, is characterized by a mitotic (spermatogonia), a meiotic (spermatocytes) and a differentiative haploid (spermatids) phase. This complex process is orchestrated by the expression of thousands of genes encoding proteins that play essential roles during specific phases of germ cell development. The dissection of the mechanisms that regulate the mitotic and meiotic cell cycles in mammalian germ cells is useful for: Screening and characterizing key genes in sperm progress, and thus for better understanding of the molecular requirements for spermatogenesis to occur, which is often based on the treatment of male sterility due to abnormal sperm development; Protection of the withering of male sexual potency; Offering new contraceptive targets and health care drugs.①

The testes are part of a select set of organs necessary for the continued propagation of a species that reproduces sexually. Specifically, testes are the sites of spermatogenesis, a complex process in which the Sertoli cells of the seminiferous epithelium support the differentiation of germ cells into functionally competent spermatozoa. Compared to somatic cells, germ cells have some unique events including meiotic cell cycles, nuclear protein exchange and large morphology change. Identification of testis-specific or germ cell-specific genes involved in these unique events during sperm development will shed light on the mechanisms of spermatogenesis. These genes, for example, CERM,② Hh,③ tesmin,④ Cdc2,⑤ Dmc1,⑥ Cyt,⑦ Gsk3-beta⑧ and Ldhc⑨ have been proven to be the testis-specific genes that play important roles during spermatogenesis. Various approaches have been developed to obtain these tissue-exclusive expressed genes including suppression subtractive hybridization (SSH), differential display RT-PCR, and DNA microarray.⑩ Of these, the DNA microarray⑪ is a useful, high throughput method, providing a platform to evaluate the thousands of genes in parallel, and allowing the monitoring of changes in gene expression occurring during developmental events. Considerable progress has been made in screening testis-specific genes in mouse and human using the DNA microarray. Sha et al.⑫ have compared gene expression profiles between adult and fetal human testes using a self-made cDNA chip comprised of 9216 genes, and 731 differently expressing genes have been characterized. Beissbarth et al.⑬ analyzed gene expression in the mouse testis from day 1, 4, 8, 11, 14, 18, 21, 26, 29 to day 60 using the Affymetrix (Santa Clara, CA, U.S.A.) Mouse U74v2 chip, containing ca. 12500 known mouse genes or EST sequence, and thus spanning approximately 1/3 rd of the mouse genome; they estimated that >2300 genes (ca. 4% of the mouse genome) are dedicated to male germ cell-specific transcripts, >99% of which are first expressed during or after meiosis.

Recently, we isolated testis from day 4, 9, 18, 35, 54 and 6 months of postnatal Balb/C mice and the cRNAs prepared from these six developmental testes have been hybridized with a commercially available GeneChip Mouse Genome 430 2.0 Array (Affymetrix Inc.) chip, containing ca. 34000 known mouse genes and 8000 unknown genes or ESTs (Expressed Sequence of Tags), and thus spanning the whole mouse genome. In mining the microarray data, we identified 2058 up-regulated transcripts with developmental stages from day four to six months postnatal. These transcripts including some unknown genes or EST may be related to testis development and spermatogenesis (detailed information will be published in other papers). Herein, a novel testis-specific conserved gene in human and mice with predicted protein weights of 24 kDa, which we called Tsc24 (Testis-Specific Conserved gene), is reported. Its characteristics and possible correlation with spermatogenesis are also discussed.

MATERIALS AND METHODS

Samples Cos-7 cell line was obtained from ATCC and maintained in a humidified atmosphere of 5% CO2 and in

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DMEM medium supplemented with 10% fetal calf serum (Life Technologies, Inc, Rockville, MD, U.S.A.), 3 mM 1-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. Male and female Balb/c mice (aged 4—6 weeks) were obtained from Laboratory Animals Center of South Medical University (Guangzhou, China) and maintained in a temperature- and humidity-controlled room. All the animals had free access to standard mouse chow and water. Male and female mice mated naturally, and the day of birth was designated as day 1. Testes were individually collected from these mice on days 4, 7, 9, 14, 18, 21, 35, 54, and 6 months. Other organs including brain, heart, lung, liver, kidney, spleen, epididymis, ovary and uterus from adult mice and human patients or volunteers were also collected, and all samples immediately frozen in RNA stabilization reagent RNAlater (QIAGEN, Valencia, CA, U.S.A.). Human tissues including lung, muscle, liver, adipose, kidney, spleen, epididymis, and testis were taken from biopsies or operation, and other human tissues including brain, heart, ovary and uterus were donated by the patient’s relative. The age range of participants was 24—40 years. The ethics committee of Shenzhen Hospital of Peking University granted research approval prior to sample collection. Animal experiments were approved by the Animal Test Center of China.

**RNA Extraction and Affymetrix Genechip Analysis**

Total RNA was extracted from mouse and human organs by using Trizol reagent (Invitrogen, San Diego, CA, U.S.A.) 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 (formaldehyde), and the purity of total RNA was confirmed using an RNeasy Mini Kit (QIAGEN, Valencia, CA, U.S.A.). Total RNA from the testis of day 4, 9, 18, 35, 54, and 6 months postnatal Balb/C mice 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 45000 pairs of probes including 39000 transcripts in which 34000 well expressed mouse genes, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, U.S.A.) as described by the manufacturer. The primer sequences were as follows: mouse Tsc24, Forward primer: gCAgCAgAAATTCTgggCAgCTAgAgAA; Reverse primer: gCAgCTCCAgACgACACAgAgggTgAA; human Tsc24, Forward primer: ATCATgTcggCCA-AgAgggCAGgCAAT; Reverse primer: CTACCCATAgATCgAggTCCACTCTgCA; β-actin, Forward primer: AgAgggAAATCgTcggTcAg, Reverse primer: CCAAgAgggACgCTggAAAA. All samples from various date testis and other organs were plated in triplicate PCR reactions.

**Construct of GFP-Tsc24 Fusion Vector and Subcellular Localization**

The coding sequence of mouse Tsc24 was amplified using the primers: CcTCTCgAgCTCCCATCATgTcggCCA (introducing an EcoR I site in 5’), ACTAgAAgTcAgCTACCATAgATCCAggAgTC (introducing a Xho I site in 5’), and the PCR products were double-digested with EcoR I/Xho I, then the digested products were inserted to the Xho I/EcoR I site of pEGFP-C1 to produce the fusion protein expressed vector, pEGFP-C1-Tsc24, and the coding sequence of EGFP-Tsc24 expressing cassette was confirmed by sequencing. The expressed plasmid, pEGFP-C1-Tsc24, was transferred to Cos-7 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Twenty-fourth after the transfer, the EGFP-expressing Cos-7 cells were stained with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and the expression of GFP-Tsc24 in these treated cells was detected by fluorescent microscopy LEICA DM4000B (Zeiss, German).

**Bioinformatics Analysis**

The complete coding sequence of novel gene, Tsc24, was derived by automated computational analysis using the gene prediction method: GNOMON. 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. The hydrophobicity of the amino acid sequences was analyzed by the protScale (http://cn.expasy.org/tools/protscale.html). Molecular weight and isoelectric point were also obtained from the Compute pi/Mw Program (http://cn.expasy.org/tools/pi_tool.html). Prediction of subcellular localization of Tsc24 was performed by PSORT II Prediction (http://psort.im.s.u-tokyo.ac.jp/form2.html).

**RESULTS**

**The Results of Affymetrix Analysis**

The results of the Affymetrix Genechip analysis indicated that a transcript (Accession number AK006825), that we termed mouse Tsc24, has gradually increased expression in developmental stages of mouse testis. The hybridization signal intensities on day 4, 9, 18, 35, 54, and 6 months postnatal were 8.8, 4.6, 5.7, 393.6, 531.6, and 559, respectively, as shown in Fig. 1a. The signal intensities of Tsc24 on day 4, 9 and 18 showed that expression of Tsc24 in these developmental stages of mice testis was not detected by our Affymetrix chip analysis. On the other hand, the signal intensities of β-actin were 6888.8, 7647.8, 8129.0, 6968.7, 6797.1 and 6571.2, respectively.
mRNA Expressed Profile of Tsc24 To authenticate the expression profile of Tsc24 in the developmental stages of mice testis supported by Affymetrix chip analysis, the expression styles were determined by RT-PCR assay. As shown in Fig. 1b, expression of mouse Tsc24 can only be detected after the mouse is 35 d old. The expression then increases gradually from day 35 to 6 months and remains stable after 54 d. The result of RT-PCR assay was consistent with that of Affymetrix chip analysis.

The expression profile of TSC24 in various tissues was also studied using multi-tissue RT-PCR. Of the eight tissues (liver, spleen, heart, lung, brain, kidney, epididymis, and testis), Tsc24 was exclusively expressed in the testis, but not expressed in the other seven tissues, as shown in Fig. 2a. Of the 12 tissues (liver, kidney, muscle, brain, spleen, adipose, lung, heart, epididymis, testis, ovary and uterus) examined in human, its expression was only detected in human testis, not in the other 11 tissues, as shown in Fig. 2b.

Features of cDNA and Deduced Protein The full cDNA length of mouse Tsc24 was 899 bp, with a 624 bp open reading frame with a predicted molecular weight of 23,997 kDa and isoelectric point of 8.474 and 5.218 charge at pH 7.0. A Blast search in the mouse genome database localized the Tsc24 gene to mouse chromosome 1. The gene is spliced to 9 exons and 8 introns, encompassing an 9503 bp genomic DNA (from 939908 to 930405 bp) in NT039185.6. The amino acid sequence analysis of the coding protein using the SignalP (V3.0) predicts no signal peptide, indicating the Tsc24 gene is a non-secretory protein, and the prediction of subcellular localization its protein showed that Tsc24 is located in nuclear (60.9%). Blast-pr showed the Tsc24 protein was highly homologous with the human CAI23536 sequence (C1orf49 gene, 56%), Bos taurus AAI10124 (57%), chimpanzee XP_514025 (60%), Canis familiaris sequence XP_853632 (48%). The homologous gene C1orf49 (accession no.: NM_032126) in human, we called human Tsc24, with a 702 bp open reading frame from nt 117—818, which encodes a 233 amino acids protein with a predicted molecular weight of 26,533 kDa, isoelectric point of 9.051 and 11.855 charge at pH 7.0.

Subcellular Localization of Tsc24 Protein According to the expression of fusion protein EGFP-Tsc24 in Cos-7 cells

Fig. 1a. Developmental Expression Pattern of Tsc24 during Spermatogenesis Detected by Affymetrix Chip Analysis

Mice testis was isolated from days 4, 9, 18, 35, 54, and the 6 months postnatal day of Balb/C mice and applied to whole genomic analysis by Affymetrix chip. The scaling signal intensities of Tsc24 in testis in days 4, 9, 18, 35, 54, and 6 months were 8.8, 4.6, 5.7, 393.6, 531.6, and 559, respectively. In contrast, the signal intensities of β-actin were 6888.8, 7647.8, 8129.0, 6968.7, 6797.1 and 6571.2, respectively.

Fig. 1b. Developmental Expression Pattern of Tsc24 during Spermatogenesis Detected by RT-PCR

The total RNA samples were prepared from the mouse testes harvested on different postnatal days. RT-PCR was then performed to detect the expression of Tsc24. The number above each lane indicates the postnatal day. β-actin was used as the RT-PCR control. M lane stands for marker used in the detection of the products. As shown, expression of mouse Tsc24 can only be detected after the mouse is 35 d old. The expression then increases gradually from day 35 to 6 months and remains stable after 54 d.

Fig. 2. RT-PCR Expression Analysis of the Expression of Tsc24 in Multiple Tissues of Mice (a) and Human (b)

M lane stands for marker used in the detection of the RT-PCR products. β-actin was used as internal control for concentration of the individual cDNA population. The exclusive expression of the Tsc24 was evident in testis of mice and human by RT-PCR assay.
cells, as shown in Fig. 3, the protein of Tsc24 was located in nuclear, which is consistent with the deduced results of Tsc24 in the subcellular localization predicted by PSORT II software.

DISCUSSION

In this study we have identified a novel gene, Tsc24, from the results of Affymetrix Genechip analysis in the developmental stages of the postnatal days 4, 9, 18, 35, 54 and 6 months of Balb/C mouse testis. Of six stages of testis isolated from different postnatal days, the expression of Tsc24 was detected in days 35, 54 and 6 months, while not detected in days of 4, 9 or 18.

To identify the expression profile of Tsc24, RT-PCR assay was also applied on mouse testes of eight different developmental stages. The results of RT-PCR showed that Tsc24 is not expressed in mice testis of days 4, 7, 9, and 18, but expressed in that of days 35, 54 and 6 months. The expression increased gradually from 35 to 54 d and remains stable after day 54. The results of multi-organs RT-PCR showed that this gene expresses exclusively in mouse and human testis. During mice spermatogenesis, round spermatids begin to elongate in testis of 35-d-old mouse. Comparing the different expression stages of Tsc24 and its distribution in multiple organs of mice and human, we propose that Tsc24 performs in the process from round spermatids to elongating spermatids. It might be a marker in the morphologic changes of spermatids during spermatogenesis in mammalian animals.

The group of genes exclusively expressed in the testis and encoding proteins with essential roles during spermatogenesis, has been called ‘chauvinist’ genes. These genes can be grouped into three categories: (i) homologous genes only in spermatogenic cells; (ii) unique genes without significant similarity in nucleotide sequence to those expressed in any other cells; and (iii) genes producing transcripts in both somatic and spermatogenic cells but germ cell-specific transcripts due to alternative transcription start site, transcript-splicing sites, or polyadenylation signals. A special feature of chauvinist genes is that expression of all of these genes is developmentally regulated during meiotic and post-meiotic phases. Consistently, the novel gene Tsc24 identified in this study, falling into the category of unique genes, is expressed in developmentally regulated patterns.

The mouse testis-specific gene, Tsc24, is a 23.997 kDa protein. The amino acid sequence analysis of the coding protein using the SignalP (V3.0) predicts no signal peptide indicating the Tsc24 gene is a non-secretory protein, and the prediction of subcellular localization of this protein shows that Tsc24 is located in nuclear. The results of subcellular localization of GFP-Tsc24 fusion protein in Cos-7 cells also supported that this protein is located in nuclear. Multi-alignment analysis of the protein of Tsc24 gene among mice, human, Bos Taurus, chimpanzee and Canis familiaris, indicates that Tsc24 is highly conserved protein in mammalian animal spermatogenesis.

In summary, we have isolated a novel gene, Tsc24, which is developmentally and exclusively expressed in the mice and human testis. The function of Tsc24 in mouse spermatogenesis is uncertain. However, to our knowledge, this is the first time that Tsc24 has been found specifically in these tissues. Further investigation of the molecular mechanisms in the distribution of Tsc24 protein in multiple tissues in mice and human, its interaction with other proteins, and the downregulation of Tsc24 by RNAi15) or knockout techniques is needed to provide adequate information to address its biological functions in the mouse spermatogenesis. Our study should be a basis for the functional characterization of the gene, leading to the elucidation of molecular events underlying mammalian male reproduction.
REFERENCES