ORIGINAL ARTICLE: ANDROLOGY

Decreased expression of SAM68 in human testes with spermatogenic defects

Le-Jun Li, M.D.,[a] Feng-Bin Zhang, M.D.,[a] Shu-Yuan Liu, M.D.,[a] Yong-Hong Tian, M.D.,[a] Fang Le, M.D.,[a] Hang-Ying Lou, M.D.,[a] He-Feng Huang, M.D.,[a] and Fan Jin, M.D.[a,b]

[a] Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine; and [b] Key Laboratory of Reproductive Genetics, National Ministry of Education (Zhejiang University), Women's Reproductive Health Laboratory of Zhejiang Province, Zhejiang, People's Republic of China

Objective: To assess the expression patterns of SAM68 in the testes of azoospermic patients with normal and abnormal spermatogenesis.
Design: Retrospective study and in vitro study.
Setting: University hospital.
Patient(s): Testicular biopsies of azoospermic men with normal spermatogenesis (OAZ; n = 20), with maturation arrest at the spermatocyte stage (MA; n = 20), and with Sertoli cell–only syndrome (SCOS; n = 10).
Intervention(s): No interventions with patients. Knockdown of Sam68 was performed in the GC-2spd(ts) cell line.
Main Outcome Measure(s): SAM68 expression was analyzed using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR), Western blot, and immunohistochemistry analysis in tissues. Moreover, Sam68 was knocked down in GC-2spd(ts) cells. Cell viability was measured using the MTT assay, and the apoptosis rate was detected using flow cytometry with the Annexin V–FITC kit.
Result(s): Using qRT-PCR, the expression level of testicular SAM68 mRNA in MA and SCOS patients was statistically reduced compared with in OAZ patients. In addition, using qRT-PCR, Western blot, and immunohistochemistry analyses, mRNA and protein expressions of SAM68 were absent or barely detectable in testicular tissues in 45% (9 of 20) of patients with MA and in all patients with SCOS. Furthermore, decreased expression of Sam68 suppressed germ cell proliferation and induced apoptosis in transfected GC-2spd(ts) cells.
Conclusion(s): Deficient SAM68 expression was observed in the human testis with MA at the spermatocyte stage and SCOS. These results may offer new perspectives on the molecular basis of abnormal spermatogenesis. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: SAM68, spermatogenic defect, male infertility, RNA-binding proteins

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/lil-sam68-human-testes-spermatogenic-defects/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Approximately 15% of idiopathic male factor infertility is due to azoospermia, which is defined as the complete absence of spermatozoa in an ejaculate (1). Despite considerable advances in the diagnostic evaluation of azoospermia, such as Y-chromosomal microdeletions (2, 3), the cause of abnormal spermatogenesis in the majority of cases remains unclear.

Received October 22, 2013; revised March 2, 2014; accepted March 20, 2014.
L.-J.L. has nothing to disclose. F.-B.Z. has nothing to disclose. S.-Y.L. has nothing to disclose. Y.-H.T. has nothing to disclose. F.L. has nothing to disclose. H.-Y.L. has nothing to disclose. H.-F.H has nothing to disclose. F.J. has nothing to disclose.
This work was supported by the National Natural Science Foundation of China, National Basic Research Programs of China, Zhejiang Provincial Natural Science Foundation of China, and Zhejiang Provincial Department of Education Foundation of China (grant nos. 81370760, 2014CB943302, LZ13H040001, 2012CB944901, 2181200475, and 30901493).
Reprint requests: Fan Jin, M.D., Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine, Xueshi Road 2, Hangzhou, Zhejiang 310006, People's Republic of China (E-mail: jinfan@zju.edu.cn).

Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.03.036

During spermatogenesis, the transcriptional and translational control of gene expression is tightly regulated (4). De novo transcription is not a continuous process, and mRNAs are synthesized and stored at specific times with RNA-binding proteins (RBPs), which protect the mRNAs during the transcriptionally inactive stages of spermatogenesis (5). Many RBPs are highly or uniquely expressed in germ cells (4, 6, 7), and, accordingly, many knockout mouse models for genes encoding these RBPs are infertile (8–10), which suggests an important role of these proteins in male germ cells. In the human, the deleted in azoospermia

genes encode potential RNA-binding proteins that are strong candidates for human fertility factors (11). RNA-binding motif Y, encoding a male germ cell–specific RNA-binding protein, is frequently deleted in infertile men (12).

Sam68 (Src-associated substrate in mitosis of 68 kD, also known as KH-DRBS1) belongs to the STAR (signal transduction and activation of RNA) family of RNA-binding proteins, which regulate a range of processes, including RNA stability, export, splicing, and mRNA translation. Analysis of the reproductive phenotype of *Sam68* knockout mice revealed that males are completely infertile (13), indicating the requirement of Sam68 expression in fertility. Mice lacking Sam68 failed to produce mature spermatozoa, which did not dramatically affect the size of the testes. Because this phenotype resembles certain cases of human infertility (14), we hypothesized that an aberrant expression of human SAM68 protein underlies the sterility observed in some idiopathic patients.

Thus, in light of these findings, we examined specimens obtained from testicular biopsies of azoospermic males and investigated the expression profile of SAM68. In vitro analyses of Sam68 were also performed in the GC-2spd(ts) cell line to determine the role of Sam68 in spermatogenesis.

MATERIALS AND METHODS

Patients and Samples

This study was approved by the Ethics Committee of the women's hospital. All participants provided their informed consent. Patients (n = 50) with azoospermia were recruited between May 2012 and April 2013, and patients with abnormal karyotypes or Y-chromosome microdeletions, as well as those undergoing hormone treatments or who had been exposed to alcohol or drugs were excluded from the study. Testicular tissue specimens were obtained from patients who underwent either a diagnostic testicular biopsy or a sperm retrieval procedure of the testicular tissues. Histological classification was determined by the most advanced spermatogenetic cell identified in the combined histological and cytological examination. Males with obstructive azoospermia (OAZ) who were found to have normal sperm upon wet preparation and histological examination of the testicular tissue specimen with the presence of late spermatids as the predominant feature were categorized as having normal spermatogenesis (15).

TESE samples were divided into three groups: OAZ with normal spermatogenesis (n = 20), maturation arrest at the spermatocyte stage (MA; n = 20), and Sertoli cell–only syndrome (SCOS; n = 10).

Serum concentrations of FSH, LH, T, and E_2 were measured using chemiluminescence assays. Testicular volumes were evaluated using ultrasound examination.

RNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Owing to the small size of the testicular tissue specimen, the samples were mechanically homogenized in TRIZOL or RIPA buffer using 5-mm stainless steel beads (Qiagen) with a Qiagen Tissue Lyser II set at 30 Hz for three sessions of 30 seconds' duration before RNA isolation or protein extraction. RNA was extracted using a commercially available monophasic solution of phenol and guanidine isothiocyanate (Life Technologies). RNA concentration was spectrophotometrically measured at 260 nm, and the purity was confirmed using the A260/A280 ratio. Reverse transcription was performed using the PrimeScript RT reagent/gDNA eraser kit (catalogue no. RR047A, Takara).

Quantitative Real-time PCR Analysis

Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-time PCR system as described by the manufacturer's protocol (Takara). The PCR mixture for each reaction contained 10 μL SYBR premix Ex Taq (perfect real time; Takara), 0.4 μL of each primer (10 μmol/L), and 50 ng cDNA adjusted to a final volume of 20 μL using dH_2O. All reactions were performed in duplicate. Real-time PCR was performed using the following protocol: 15 seconds at 94°C followed by 40 repetitive cycles at 94°C for 5 seconds, and 60°C for 15 seconds. The primer sequences are provided in Supplemental Table 1. The mRNA expression level of SAM68 was normalized against the expression of the housekeeping gene GAPDH. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

SDS-PAGE and Immunoblot Analysis

Tissue was homogenized as previously described and kept on ice for 60 minutes. The homogenate was then centrifuged at 10,000 rpm for 20 minutes at 4°C, and the supernatant was removed. Before storage at −80°C, a small aliquot of supernatant was removed and the protein concentration was measured using the BCA Protein Assay Kit (Applygen) according to the manufacturer's instructions. Approximately 30 μg of protein was loaded onto each gel and electrotransferred onto polyvinylidene fluoride membranes using standard procedures. SAM68 was detected using a rabbit monoclonal antibody (1:1,000; Epitomics) followed by a mouse anti-rabbit secondary antibody (MultiSciences Biotech). Horseradish peroxidase–coupled secondary antibodies were detected using the luminol chemiluminescent substrate (Biological Industries). Quantification of the band intensity with respect to GAPDH (MultiSciences Biotech) expression was assessed using the Image Quant TL7.0 software.

Immunohistochemistry

Briefly, Bouin's solution-fixed paraffin-embedded sections of testicular tissue were cut into 5-mm sections. After quenching the endogenous peroxidase activity, the sections were blocked using a blocking serum and then incubated overnight at 4°C with primary antibodies against SAM68 (1:300; Epitomics). The sections were then incubated with an HRP-conjugated secondary antibody (ZhongShan Biotechnology). To confirm the specificity of the SAM68 antibody, negative controls were processed in an identical manner by replacing the primary antibody with a normal rabbit IgG antibody.