Sertoli cells from non-obstructive azoospermia and obstructive azoospermia patients show distinct morphology, Raman spectrum and biochemical phenotype

Meng Ma¹, Shi Yang¹, Zhenzhen Zhang², Peng Li¹, Yuehua Gong², Linhong Liu², Yong Zhu¹, Ruhui Tian¹, Yufei Liu¹, Xiaobo Wang¹, Feng Liu¹, Lin He³, Yang Liu², Hao Yang², Zheng Li¹,* and Zuping He²,4,5,*

¹Department of Urology, Renji Hospital, Shanghai Human Sperm Bank, Shanghai Jiao Tong University School of Medicine, Shanghai 200001, China ²Stem Cell Research Center, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, China ³Instrumental Analysis Center, Shanghai Jiao Tong University, 800 Dong Chuang Rd, Shanghai 200001, China ⁴Shanghai Key Laboratory of Reproductive Medicine, Shanghai 200025, China ⁵State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200032, China

*Correspondence address: Tel: +86-21-68383920; Fax: +86-21-68383916; E-mail: zupinghe@sjtu.edu.cn (Z.H.); lizhengboshi@163.com (Z.L.)

STUDY QUESTION: Are there differences in the morphology, spectrum and biochemical phenotype between Sertoli cells from non-obstructive azoospermia (NOA) patients and those from obstructive azoospermia (OA) patients with normal spermatogenesis?

SUMMARY ANSWER: Sertoli cells from NOA patients are distinct from those from OA patients in terms of morphological features, Raman spectrum and phenotype including the expression of genes and proteins (e.g. SCF, BMP4 and GDNF).

WHAT IS KNOWN ALREADY: NOA affects 10% of infertile men and has been diagnosed in 60% of azoospermic men. In contrast with OA patients who have normal spermatogenesis, NOA patients have an impaired spermatogenesis.

STUDY DESIGN, SIZE AND DURATION: This case–control study included 100 NOA patients (as cases) and 100 OA patients with normal spermatogenesis (as controls). The study was performed between January 2012 and January 2013.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: Karyotype analysis was performed to check the chromosome content and multiplex PCR was carried out to determine the expression of numerous Y chromosome genes in NOA patients. Human Sertoli cells were then isolated from the testes of NOA and OA patients by two-step enzymatic digestion and differential plating. Transmission electron microscopy was used to determine the ultrastructure of the Sertoli cells and real-time Raman microspectroscopy was used to assess their spectrum. We further compared the two groups of patients for expression of SCF, GDNF and BMP4 in Sertoli cells, using RT–PCR, microarray analysis, immunofluorescence, immunohistochemistry and Western blots.

MAIN RESULTS AND THE ROLE OF CHANCE: NOA patients had normal chromosome karyotypes and Y chromosome microdeletions were excluded. In morphology, Sertoli cells isolated from NOA patients had a series of abnormal ultrastructural features compared with the control Sertoli cells: (i) existence of small and spindle-shaped nuclei, (ii) smaller diameter, (iii) deficient nucleolus or endoplasmic reticulum and (iv) more vacuoles. Spectral intensities in Sertoli cells of NOA patients were distinct at four typical Raman peaks compared with the control Sertoli cells. In phenotype, SCF, BMP4 and GDNF transcripts and proteins were significantly lower in Sertoli cells of NOA patients than in the control Sertoli cells.

LIMITATIONS AND REASONS FOR CAUTION: The Sertoli cells of NOA patients were not compared with Sertoli cells of normal fertile men due to the fact that it is hard to obtain adult testes from normal donors.
**Introduction**

Non-obstructive azoospermia (NOA), which affects 10% of infertile men and has been diagnosed in 60% of azoospermic men, is a major health problem (Jarow et al., 1989; Matsumiya et al., 1994). Sertoli cell-only syndrome (SCOS), one of the most common types of NOA, can be diagnosed when testicular biopsy reveals that seminiferous tubules are lined by only Sertoli cells with a complete absence of male germ cells. SCOS is the most serious type of NOA. Spermatogenesis is a complex process by which spermatogonial stem cells (SSCs) divide and differentiate into haploid sperm (He et al., 2008). In rodents, SSCs divide into two types, A1-type spermatogonia (A1) and B spermatogonia that produce several types of spermatocytes, spermatids, and mature sperm, respectively (de Rooij and Groote-goed, 1998). The A1 spermatogonia, in turn, give rise to several generations of spermatogonia, including type A1–A4, intermediate and type B spermatogonia that produce several types of spermatocytes, spermatids, and mature sperm, respectively (de Rooij and Groote-goed, 1998). In humans, Clermont et al. suggested that A4 dark spermatogonia are the reserve stem cells while A4 bright spermatogonia are the renewing stem cells, and spermatogenesis consists of three major phases, i.e. mitosis of spermatogonia, meiosis of spermatocytes and spermigenesis of round spermatids to elongated spermatids (Clermont, 1963, 1966a,b, 1972). Both genetic and environmental factors can lead to abnormal spermatogenesis. NOA was first described by Devroey et al. in 1996 to distinguish these patients from those suffering from obstructive azoospermia (OA) (Devroey et al., 1996). In contrast to OA patients who have normal spermatogenesis, NOA patients have an impaired spermatogenesis due to several reasons, such as Klinefelter syndrome, genomic AZF deletions, cryptorchidism, testicular torsion, radiation and toxins (Palermo et al., 1999; Erez, 2000; Donoso et al., 2007). It has been suggested that diagnosis of NOA should be made according to pathological findings, since clinical and endocrine parameters cannot accurately distinguish between OA and NOA patients (Tournaye et al., 1995; Devroey, 1998; Schoor et al., 2002; McLachlan et al., 2007). However, the molecular mechanisms resulting in the azoospermia of NOA patients, especially SCOS, remain unknown.

Spermatogenesis takes place in the seminiferous tubules, where there exists a particular microenvironment or niche. Sertoli cells, which were described first by Enrico Sertoli in 1865, are the most important somatic cells contributing to the microenvironment due to their production of crucial growth factors and cytokines and their contribution to the blood–testis barrier (Griswold, 1998). During the process of spermatogenesis, Sertoli cells secrete numerous growth factors and cytokines in order to appropriately regulate the self-renewal and differentiation of SSCs, meiosis of spermatocytes and transformation of round spermatids into spermatozoa (de Rooij, 2009). For instance, stem cell factor (SCF, KIT ligand, steel factor) is a cytokine essential for hematopoiesis, melanogenesis and gametogenesis. In the testis, the SCF/c-kit signaling system displays a cell type-specific pattern of expression: the receptor c-kit is localized to spermatogonia and Leydig cells, whereas the ligand SCF is exclusively produced by Sertoli cells (Mauduit et al., 1999). SCF has been shown to induce mouse spermatogonia to differentiate into round spermatids (Feng et al., 2000) and it is also required for the proliferation of mouse differentiating spermatogonia, specifically type A1 to A4 spermatogonia (Tajima et al., 1994; Hasthorpe, 2003). Gfalpha 1-derived neurotrophic factor (GDNF) promotes the survival and differentiation of undifferentiated spermatogonia including SSCs. GDNF is secreted by Sertoli cells, and SSCs express its co-receptors, c-Ret and GFRA1 (Meng et al., 2000). Bone morphogenic protein 4 (BMP4), which is also secreted by Sertoli cells, can induce the differentiation of SSCs as well as A1 and A4 spermatogonia (Nagano et al., 2003; Pellegrini et al., 2003). These findings illustrate that Sertoli cells play an essential role in regulation of rodent spermatogenesis. Nevertheless, it remains unclear whether abnormal growth factors and cytokines secreted by Sertoli cells result in human azoospermia.

Given the vital role of Sertoli cells in regulating spermatogenesis, we have for the first time compared the characteristics of Sertoli cells from NOA patients and those of Sertoli cells from OA patients with normal spermatogenesis. We found that Sertoli cells of NOA patients were distinct from those of OA patients in their morphologic characteristics, Raman spectrum and biochemical phenotype, as demonstrated by the significantly lower expression of SCF, BMP4 and GDNF. Therefore, this study offers novel insights into understanding the underlying causes of abnormal spermatogenesis in NOA and may provide a basis for developing new approaches for treating patients with NOA.

**Materials and Methods**

**Procurement of testicular biopsies from NOA patients and OA patients with normal spermatogenesis**

Testicular biopsies were obtained from 100 Chinese NOA patients and 100 Chinese OA patients (age ranging in age from 22 to 35 years) who underwent microdissection TESE (MD-TESE) at Renji Hospital affiliated...
Distinct characterization of NOA and OA patients

1865
to Shanghai Jiao Tong University School of Medicine. Cases of OA were caused by inflammation and vasoligation but not by congenital absence of the vas deferens (CBAVD) or other diseases, including cancer. Patients with NOA were diagnosed with SCO5 by histological analysis, and patients with Klinefelter syndrome and genomic AZF deletions or other diseases, including cancer, were excluded from this study. The clinical data of both the OA and the NOA patients were shown in Supplementary data, Table S1. This study was approved by the Institutional Ethical Review Committee of Renji Hospital (license number of ethics statement: 2012-01), Shanghai Jiao Tong University School of Medicine, and informed consent for testis tissues for research only was obtained from the donors.

Multiplex PCR
Peripheral venous blood was obtained from 10 normal men and 10 NOA patients. Multiplex PCR was performed to check the completeness of the genomic DNA sequences of Y chromosome genes, including SRY, sY254, sY127, sY86, sY134, sY84 and sY255, according to a procedure described previously (Sun et al., 2012). The primers of the chosen genes have been shown previously (Sun et al., 2012).

Chromosomal karyotype analysis
Chromosomal karyotype analysis of peripheral blood lymphocytes from 10 NOA patients was performed, and the karyotypes were interpreted using the recommendation of the International System for Human Cytogenetic Nomenclature.

Isolation of Sertoli cells from NOA and OA patients
Testicular biopsies obtained from 20 NOA and 20 OA patients were washed three times aseptically in Dulbecco modified Eagle medium (DMEM) containing antibiotic with penicillin and streptomycin (Gibco). Sperm-free tubes were isolated from human testis biopsies using the first enzymatic digestion according to a procedure we have previously described (He et al., 2010). Sertoli cells were obtained using a second enzymatic digestion with 4 mg/ml collagenase IV, 2.5 mg/ml hyaluronidase (Sigma), 2 mg/ml trypsin (Sigma) and 1 µg/ml Dnase I and followed by differential plating according to a procedure previously described (He et al., 2010). For differential plating, cell suspensions were seeded into culture plates in DMEM/F-12 (Gibco) supplemented with 10% FBS and incubated at 34°C in 5% CO2 for 3 h. After incubation, the media containing male germ cells were removed, and Sertoli cells were attached to culture plates and cultured for 3 days. The viability of freshly isolated Sertoli cells was assessed by exclusion of trypan blue staining, and Sertoli cells were identified by immunostaining with anti-GATA4 and WT1 as described below.

Transmission electron microscopy
Freshly isolated human Sertoli cells from 10 patients with OA and NOA were fixed with 2.5% w/v glutaraldehyde in 0.1 M cacodylate buffer. After extensive washing in phosphate-buffered saline (PBS), the cells were post-fixed in 1% w/v OsO4 for 30 min, dehydrated in a graded solution of ethanol and embedded in Epon. Ultrathin sections were cut and examined under a Phillips EM 301 electron microscope after staining with uranyl acetate and lead citrate.

Real-time Raman microspectroscopy
Raman microspectroscopy is a laser-based noninvasive technique that has been shown to provide a detailed chemical ‘fingerprint’ of a number of types of cells. We have recently demonstrated that Raman microspectroscopy can be used for distinguishing human zona pellucida-bound sperm from unbound sperm (Lu et al., 2012). The spectra of human Sertoli cells from 10 OA and 10 NOA patients were scanned using the Senterra R200-L dispersive Raman microscope (Bruker Optik Gmbh, Ettlingen, Germany) with a 532-nm laser source with accumulation times of 2 × 5 s for single spectra. For each sample, 10 Sertoli cells were scanned. Each individual cell was detected with six scanning points, including cytoplasm and nucleus. Wave number was calibrated automatically using the OPUS 6 software, and the assignment of the peaks was based on the contributions of recent research. Standard principal component analysis (PCA) was performed on the data of the Raman peaks.

RNA extraction and RT–PCR
Total RNA was extracted from human Sertoli cells of 20 patients with OA and 20 patients with NOA, using Trizol reagent (Invitrogen). Reverse transcription (RT) was performed using a First Strand cDNA Synthesis Kit (Fermentas, Lithuania) and PCR was performed according to the protocol we described previously (He et al., 2007). The forward and reverse primers of the chosen genes, including SCF, GDNF, BMP4 and GAPDH, were designed and are listed in Supplementary data, Table SII. The PCR reaction started at 94°C for 2 min and was performed as follows: denaturation at 94°C for 30 s, annealing at the temperature (Tm) indicated in Supplementary data, Table SII for 45 s and elongation at 72°C for 45 s. After 35 cycles, the samples were incubated for an additional 5 min at 72°C. PCR products were separated by electrophoresis on 1.2% agarose gels, and the gels were exposed to chemiluminescence (Chemi-Doc XRS, Bio-Rad, Hercules, CA).

Microarray analysis
Total RNA was obtained from Sertoli cells of 10 patients with OA and 10 patients with NOA, using Trizol reagent as mentioned above. The concentration and purity of total RNA were evaluated by measuring the 260/280 nm ratios, and the integrity of total RNA was assessed by 1.0% denaturing agarose gel electrophoresis. Total RNA samples (5 μg) were reverse-transcribed into single-strand cDNA that was subsequently converted into double-strand cDNA. The purified double-strand cDNA served as a template to synthesize biotin-labeled cRNA using the GeneChip2 WT Terminal Labeling Kit (Affymetrix), and the biotin-labeled cRNA was purified using the GeneChip Sample Cleanup Module kit (Affymetrix) and fragmented to 35~200 bases. The fragmented cRNA was hybridized to Affymetrix GeneChip Human Gene 1.0 ST Arrays and stained using the GeneChip2 Hybridization, Wash and Stain Kit (Affymetrix). The arrays were scanned with a GeneChip2 Scanner 3000 7G (Affymetrix), and raw data were analyzed using the GeneSpringGX software.

Immunohistochemistry
To prepare sections, testes from 10 OA and 10 NOA patients were fixed in 4% paraformaldehyde (PFA), washed with PBS and dehydrated through a series of graded alcohols. The testes were then embedded in paraffin at 60°C overnight and sections were cut for immunohistochemistry. The testis sections were hydrated with series of graded alcohols. After endogenous peroxidase activity was blocked with 3% H2O2, non-specific binding was blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 10% normal goat serum. The sections were incubated with primary antibody against SCF (Abcam, catalogue no: ab52603) and the biotinylated cRNA was hybridized to Affymetrix GeneChip Human Gene 1.0 ST Arrays and stained using the GeneChip2 Hybridization, Wash and Stain Kit (Affymetrix). The arrays were scanned with a GeneChip2 Scanner 3000 7G (Affymetrix), and raw data were analyzed using the GeneSpringGX software.
Histological examination
Testicular biopsies from 10 OA and 10 NOA patients were fixed in Bouin’s fixative overnight, embedded in paraffin and sectioned at 5 μm thickness. The sections were stained with hematoxylin and eosin (H&E) and observed under a microscope.

Immunofluorescence
For immunofluorescence, the freshly isolated human Sertoli cells from 10 OA and 10 NOA patients were fixed with 4% PFA and permeabilized in 0.4% triton-X 100 (Sigma-Aldrich) for 45 min. After washing with PBS, the cells were blocked in 1% BSA for 30 min and this was followed by incubation with primary antibodies at a dilution of 1:100 overnight at 4°C. Primary antibodies used here included anti-WT1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, catalogue no: sc-192), anti-GATA4 (Santa Cruz, catalogue no: sc-1237), anti-SCF (Abcam, catalogue no: ab52603), anti-GDNF (Abcam, catalogue no: ab18956), anti-BMP4 (Santa Cruz, catalogue no: sc-6896) and anti-Vimentin (Santa Cruz, catalogue no: sc-5565). After three washes with PBS, the cells were incubated with the secondary antibody, including FITC-conjugated or rhodamine-conjugated IgG (Jackson Immuno Research Laboratories) at a 1:200 dilution for 1 h at room temperature. DAPI (4′-6-diamidino-2-phenylindole) was used to stain cellular nuclei and the cells were observed for epifluorescence, using a confocal fluorescence microscope (Leica).

Western blots
Freshly isolated human Sertoli cells from 10 patients with OA and 10 patients with NOA were lysed with RIPA buffer (Santa Cruz) for 30 min on ice. After 30 min lysis on ice, cell lysates were cleared by centrifugation at 12,000 g for 10 min at 4°C. The supernatants were dialyzed against PBS prior to loading on SDS-PAGE (Bio-Rad Laboratories, Richmond, CA) and Western blots were performed according to a protocol described previously (He et al., 2008). The chosen antibodies were anti-SCF (Abcam, catalogue no: ab52603), anti-GDNF (Abcam, catalogue no: ab18956), anti-BMP4 (Santa Cruz, catalogue no: sc-6896) and anti-GAPDH (Santa Cruz, catalogue no: sc-5565). After extensive washes in PBS, the blots were visualized by chemiluminescence (Chemi-Doc XRS, Bio-Rad, Hercules, CA).

Statistical analysis
All the values were presented as mean ± SEM, and statistically significant differences (P < 0.05) between NOA and OA patients were determined using analysis of variance (ANOVA) and a two-tailed t-test.

Results
NOA patients had normal karyotypes and no Y chromosome microdeletions
We first checked the chromosome karyotype and the expression of numerous Y chromosome genes of NOA patients. Karyotype analysis showed that NOA patients had normal chromosome karyotypes (Fig. 1A). Multiplex PCR on genomic DNA was used to check whether NOA patients had Y chromosome microdeletions. Numerous Y chromosome genes, including SRY, sY254, sY127, sY186, sY134, sY84 and sY255, were present in NOA patients, thus excluding Y chromosome microdeletion of these genes in NOA patients (Fig. 1C). As shown in Supplementary data, Table S1, there is no obvious difference in body mass index (BMI), luteinizing hormone (LH), testosterone (T), prolactin (PRL) or estradiol (E2) between NOA and OA patients, whereas testicular volumes of NOA patients were significantly reduced compared with those of OA patients and follicle-stimulating hormone (FSH) levels of NOA patients were statistically higher than those of OA patients.

Isolation and identification of human Sertoli cells from NOA and OA patients
Histological analyses were performed to evaluate the spermatogenesis status of testicular tissues of OA and NOA patients. Histological examination showed that there was normal spermatogenesis in the seminiferous tubules of OA patients (Supplementary data, Fig. S1A), whereas only Sertoli cells or rare male germ cells remained in the seminiferous tubules of NOA patients (Supplementary data, Fig. S1B).

Human Sertoli cells from patients with OA and patients with NOA were isolated by a 2-step enzymatic digestion followed by differential plating. Seminiferous tubules were isolated from the testis biopsies of OA and NOA patients, using the first enzymatic digestion (Fig. 2A). Cell mixtures containing male germ cells and Sertoli cells were obtained using a second enzymatic digestion (Fig. 2B) and cultured with DMEM/F12 and 10% FBS for 3 h (Fig. 2C). After differential plating, only Sertoli cells remained in the culture dish (Fig. 2D). Cell viability was over 98% as assayed by trypan blue exclusion (data not shown).

We verified the identity of freshly isolated human Sertoli cells from patients with OA and patients with NOA, using two markers for Sertoli cells, GATA4 (He et al., 2007) and WT1 (Sharpe et al., 2003; Sheng et al., 2012). Immunofluorescence revealed that freshly isolated human Sertoli cells from OA and NOA patients were positive for both GATA4 (Fig. 2E) and WT1 (Fig. 2F). After differential plating, the purity of isolated Sertoli cells was ~100% as assessed by their expression of GATA4 and WT1.

Distinct ultrastructural features of human Sertoli cells in NOA and OA patients
We further compared the ultrastructure of Sertoli cells from patients with OA and patients with NOA, using transmission electron microscopy (TEM). Sertoli cells from patients with OA had intact organelles, including mitochondria and endoplasmic reticulum in the cytoplasm, a well-developed nucleolus and steady nuclear chromatin (Fig. 3A and B). As shown in Fig. 3C and D, Sertoli cells from patients with OA and patients with NOA, using two markers for Sertoli cells, GATA4 (He et al., 2007) and WT1 (Sharpe et al., 2003; Sheng et al., 2012). Immunofluorescence revealed that freshly isolated human Sertoli cells from OA and NOA patients were positive for both GATA4 (Fig. 2E) and WT1 (Fig. 2F). After differential plating, the purity of isolated Sertoli cells was ~100% as assessed by their expression of GATA4 and WT1.

Distinct ultrastructural features of human Sertoli cells in NOA and OA patients
We further compared the ultrastructure of Sertoli cells from patients with OA and patients with NOA, using transmission electron microscopy (TEM). Sertoli cells from patients with OA had intact organelles, including mitochondria and endoplasmic reticulum in the cytoplasm, a well-developed nucleolus and steady nuclear chromatin (Fig. 3A and B). As shown in Fig. 3C and D, Sertoli cells from patients with OA and patients with NOA, using two markers for Sertoli cells, GATA4 (He et al., 2007) and WT1 (Sharpe et al., 2003; Sheng et al., 2012). Immunofluorescence revealed that freshly isolated human Sertoli cells from OA and NOA patients were positive for both GATA4 (Fig. 2E) and WT1 (Fig. 2F). After differential plating, the purity of isolated Sertoli cells was ~100% as assessed by their expression of GATA4 and WT1.

Distinct Raman spectrum of human Sertoli cells from NOA and OA patients
We next determined Raman spectrum of human Sertoli cells from patients with NOA and patients with OA. Real-time Raman
Microspectroscopy showed that the shape of spectrum was similar between Sertoli cells from patients with OA (Fig. 4A) and patients with NOA (Fig. 4B). PCA analysis revealed that the dataset of 80% typical spectral intensity was included (Fig. 4C). However, statistical analysis indicated that the mean spectral intensity was distinct at four typical Raman peaks in Sertoli cells obtained from NOA patients compared with that in Sertoli cells of OA patients (Fig. 4D).

**Figure 1** Karyotype and the completeness of genomic DNA sequence of various Y chromosome genes in NOA patients. (A) Karyotype analysis of a representative NOA patient. (B and C) Multiplex PCR showed the expression of various Y chromosome genes SRY, sY254, sY127, sY86, sY134, sY84 and sY255 in a normal man (B) and a patient with NOA (C).

**Figure 2** Isolation and identification of human Sertoli cells from patients with OA and patients with NOA. (A) Seminiferous tubules were isolated from human testicular specimens, using a first enzymatic digestion with collagenase IV. (B) The cell suspension, including Sertoli cells and male germ cells, was obtained using a second enzymatic digestion. (C) Sertoli cells were adhered to culture dishes after incubation for 3 h. (D) Sertoli cells remained in the culture dishes. (E and F) Immunofluorescence revealed the expression of GATA4 (E) and WT1 (F) in human Sertoli cells from patients with OA and patients with NOA. Scale bars in A–D = 100 μm; scale bars in E and F = 20 μm.

**Differential gene expression in Sertoli cells from NOA and OA patients**

We used semi-quantitative RT–PCR to determine the expression of SCF, BMP4 and GDNF mRNA in Sertoli cells from patients with NOA and patients with OA. As shown in Fig. 5, the transcripts of SCF, GDNF and BMP4 were detected in Sertoli cells of OA and NOA patients; however, the mRNA expression of these genes was
significantly lower in Sertoli cells of NOA patients compared with that in Sertoli cells of OA patients. Microarray analysis was further used to compare gene expression in human Sertoli cells from patients with NOA and patients with OA. The integrity of total RNA used for microarray was assessed by denaturing agarose gel electrophoresis (Fig. 6A). In total, 33,297 expressed sequence tags (EST) were present in the Sertoli cells of both OA and NOA patients. There were 1,120 gene transcripts that were differentially expressed (up-regulated or down-regulated with 1.5 folds or more) in Sertoli cells from patients with OA and patients with NOA. Among them, SCF was expressed at a lower level in the Sertoli cells of NOA patients compared with that in Sertoli cells of OA patients (Fig. 6B), which was consistent with our RT–PCR data (Fig. 5). Additionally, in Sertoli cells of NOA patients compared with Sertoli cells of OA patients, there were lower levels of expression of numerous genes, including ACAN (aggrecan; fold change: 0.5493), AIM1 (absent in melanoma 1; fold change: 0.2713), ANO4 (anoctamin 4; fold change: 0.4516), ATRNL1 (attractin-like 1; fold change: 0.4901), BDKRB1 (bradykinin receptor B1; fold change: 0.3058), CLIC2 (chloride intracellular channel 2; fold change: 0.3714), EMB (embign; fold change: 0.3522), EPB41L3 (erythrocyte membrane protein band 4.1-like 3; fold change: 0.2908), FGL2 (fibrinogen-like 2; fold change: 0.1362), FLJ16171 (FLJ16171 protein; fold change: 0.3065), HSPB8 (heat shock 22 kDa protein 8; fold change: 0.4579), IL7 (interleukin 7; fold change: 0.2635), MAP2 (microtubule-associated protein 2; fold change: 0.4377), PAPPA2 (pappalyisin 2; fold change: 0.2679) and SMC2 (structural maintenance of chromosomes 2; fold change: 0.3508).

Distinct protein expression of SCF, BMP4 and GDNF in human Sertoli cells from NOA and OA patients

We further compared protein expression of SCF, BMP4 and GDNF in human Sertoli cells from patients with NOA and patients with OA. Immunofluorescence revealed that SCF, BMP4 and GDNF proteins were expressed at lower levels in Sertoli cells of NOA patients compared with that in Sertoli cells of OA patients (Fig. 7A–C), whereas there was no obvious difference in the expression of GATA4 in Sertoli cells of OA patients and NOA patients (Fig. 7D). Immunohistochemistry showed that SCF was present at Sertoli cells along the basement membrane of OA patients (Fig. 8A and B), whereas SCF was weakly expressed in Sertoli cells of NOA patients (Fig. 8C and D). Furthermore, Western blots demonstrated that SCF, BMP4 and GDNF proteins were expressed at significantly lower levels in Sertoli cells of NOA patients compared with that in Sertoli cells of OA patients (n = 6, Fig. 8E and F).

Discussion

SSCs and their progeny are located along the basement membrane in the seminiferous tubules. Normal spermatogenesis requires a micro-environment or niche that consists of somatic cells and growth factors and cytokines secreted by somatic cells. The Sertoli cell, the only somatic cell within the seminiferous tubules, plays an essential role in the regulation of spermatogenesis and in altering the rates of spermatogenesis. Sertoli cells can provide structural support and nutrition to developing male germ cells and produce a number of growth factors and cytokines that regulate and/or respond to pituitary hormone releases and influence the mitotic activity of spermatogonia (de Rooij, 2009). In this study, we have for the first time compared the characteristics of morphology, Raman spectrum and biochemical phenotype of Sertoli cells from NOA patients and OA patients with normal spermatogenesis in order to provide novel insights into the causes of NOA.

We found that the NOA patients had normal chromosome karyotypes and we excluded Y chromosome microdeletions. Notably, we discovered that Sertoli cells of NOA patients assumed an abnormal ultrastructure compared with Sertoli cells of OA patients with normal spermatogenesis. Under TEM, Sertoli cells of NOA patients had a small and spindle-shaped nuclei and a deficient nucleolus. Although we observed that the expression of vimentin is lower in Sertoli cells of OA patients compared with that of NOA patients, the nucleolus of NOA Sertoli cells may still have function, as evidenced
by our finding of normal intensity of RNA bands in NOA Sertoli cells. It is worth noting that more vacuoles, which represents lipid droplets (Chui et al., 2011), were detected in Sertoli cells from patients with NOA, suggesting that phagocytosis in Sertoli cells of NOA patients is increased since lipid droplet formation can be found in Sertoli cells after phagocytosis of apoptotic spermatogenic cells (Wang et al., 2006). Although the shape of the Raman spectrum was similar between Sertoli cells from patients with OA and patients with NOA, statistical analysis showed that spectral intensity of NOA and OA Sertoli cells was distinct at four typical Raman peaks and one of them, namely $1086\text{ cm}^{-1}$, represents phosphate, CC skeletal and COC string from glycosidic link (Maquelin et al., 2002). Collectively, these results suggest that the abnormalities in ultrastructure and Raman spectrum in Sertoli cells of NOA could contribute to their aberrant functions in regulating spermatogenesis.

Notably, we found that there were distinct levels of transcripts and proteins of SCF, BMP4 and GDNF in Sertoli cells from NOA patients compared with Sertoli cells from OA patients. SCF seems to play dual roles in regulating the proliferation and differentiation of mouse type A1 to A4 spermatogonia (Yoshinaga et al., 1991; Rossi et al., 1993). It has been demonstrated that SCF induces a mouse spermatogonial cell line to differentiate into meiotic spermatocytes and haploid round spermatids, as demonstrated by the formation of a synaptonemal complex and acrosome-like structure, respectively (Feng et al., 2002). These studies implicate that SCF is a key factor for full differentiation of spermatogonia and gametogenesis in vitro. In humans, SCF can be detected in Sertoli cells at mRNA level (Chui et al., 2011). In this study, we discovered that SCF mRNA was expressed at a significantly lower level in NOA Sertoli cells compared with OA Sertoli cells as shown by RT–PCR and microarray analysis. Our immunofluorescence, immunohistochemistry and Western blots further revealed that the SCF protein was significantly lower in NOA Sertoli cells compared with OA Sertoli cells. Our results are consistent with the finding that SCF mRNA was significantly lower in testicular biopsies of patients with spermatogenic failure (Plotton et al., 2006).

**Figure 4** Raman spectrum of human Sertoli cells from patients with OA and patients with NOA. (A and B) Real-time Raman microspectroscopy showed the spectrum of human Sertoli cells from patients with OA (A) and patients with NOA (B). (C) PCA analysis displayed the dataset of 80% typical spectral intensity in Sertoli cells from patients with OA and patients with NOA. (D) Statistical analysis showed different spectral intensities between NOA and OA Sertoli cells. *Statistically significant differences ($P < 0.05$) between OA and NOA patients.
GDNF is produced by Sertoli cells, and importantly it was the first identified growth factor to regulate the self-renewal and differentiation of mouse SSCs in vivo. Over-expression of GDNF can result in an accumulation of undifferentiated spermatogonia, and conversely GDNF knockout leads to the depletion of spermatogonia (Meng et al., 2000), indicating that GDNF is required for the self-renewal of SSCs. It has also been reported that GDNF is a crucial factor for self-renewal and maintenance of mouse SSCs in vitro (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004; Hofmann et al., 2005). We have recently demonstrated that GDNF promotes mouse SSC proliferation through c-Fos transcription by the Ras/Erk1/2 pathway (He et al., 2008). GDNF is detected in human Sertoli cells at mRNA level (Chui et al., 2011). It has been shown that FSH induces the expression of GDNF in mouse testis tissue and human fetal Sertoli cells to

Figure 5 Differential gene expression between NOA and OA Sertoli cells. RT–PCR analysis showed the expression of SCF, BMP4 and GDNF in Sertoli cells from patients with OA (lane 1) and NOA (lane 2). RT–PCR without primers was used as controls (lane 3), and GAPDH served as loading control of total RNA. PCR was also performed for each gene no reverse transcriptase as a negative control and no PCR product was observed (data not shown).

Figure 6 The expression of rRNA and SCF between NOA and OA Sertoli cells. (A) Agarose gel electrophoresis showed the integrity of total RNA used for microarray analysis. (B) Microarray analysis revealed SCF mRNA expression in human Sertoli cells from patients with OA and patients with NOA. *Statistically significant difference \((P < 0.05)\) between OA and NOA patients.

Figure 7 Differential protein expression of SCF, BMP4 and GDNF in Sertoli cells from patients with NOA and patients with OA. Immunofluorescence showed the expression of SCF (A), BMP4 (B), GDNF (C) and GATA4 (D) in the Sertoli cells from patients with NOA and patients with OA. Scale bars in A–D ¼ 10 μm.
Figure 8 Differential protein expression of SCF, GDNF and BMP4 in Sertoli cells from patients with OA and patients with NOA. (A–D) Immunohistochemistry revealed SCF expression in testis sections of OA patients (A) and NOA patients (C). Higher magnifications of SCF expression in testis sections of OA patients (B) and NOA patients (D) were also shown. Scale bars in A and B = 50 μm; scale bars in C and D = 25 μm. (E and F) Western blots showed the expression of SCF, BMP4 and GDNF in the Sertoli cells from representative patients with OA and representative patients with NOA. *Statistically significant differences (P < 0.05) between OA and NOA patients.
stimulate the proliferation of type A spermatogonia in vitro (Ding et al., 2011). In the present study, the expression of GDNF mRNA and protein in Sertoli cells from patients with NOA was obviously lower than that in Sertoli cells from patients with OA, whereas the serum FSH concentration of patients with NOA is over five times higher in NOA patients than in patients with OA (Supplementary data, Table S1). Our data indicate that the Sertoli cells from patients with NOA could not respond to increased serum FSH concentrations.

BMP4 is secreted by Sertoli cells and it has been shown to induce the maintenance of mouse SSCs, reflecting that it promotes SSC differentiation in vitro (Nagano et al., 2003). BMP4 appears to be important for both proliferation and differentiation of SSCs due to the fact that SSCs can self-renew and progress to differentiate at the same time (He et al., 2009). In addition, BMP4 has been found to induce the differentiation of mouse SSCs via the SMAD1/5/8 pathway (Pellegri et al., 2003; Itman and Loveland, 2008). In humans, BMP4 is detected in Sertoli cells at mRNA level (Chui et al., 2011). In this study, similar to SCF and GDNF, the mRNA and protein expression of BMP4 in Sertoli cells from patients with NOA was remarkably lower than that in Sertoli cells from patients with OA.

Considered together, the significantly lower or loss of transcripts and proteins of SCF, GDNF and BMP4 may account for the abnormal SSC self-renewal and differentiation and eventually contribute to the deficient niche of SSCs in NOA patients. It is difficult to obtain adult testes from normal donors in China to serve as a normal biological control for our data of OA and NOA. Nevertheless, the OA in patients we employed in this study was caused by inflammation or vasoligation, not by congenital or other diseases, and thus they could be used as a good control for NOA patients.

Conclusions

In summary, we have for the first time compared the morphology and ultrastructure, Raman spectrum and biochemical phenotype of Sertoli cells from NOA patients and OA patients. We highlight that Sertoli cells from NOA patients have distinct morphological features, Raman spectrum and phenotype (in terms of SCF, BMP4 and GDNF) compared with the control normal Sertoli cells. Therefore, this study can offer new insights into better understanding the underlying causes for NOA of patients and might provide a basic ground for developing new therapies for the patients with NOA.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

M.M. and S. Y. conducted laboratory experiments, generated and analyzed data and prepared the manuscript. Z. Z., P. L., Y. G. and L. L. performed laboratory research and collected data. Y. L., X. W., F. L. and L. H. were involved in patient recruitment and clinical data collection. Y. Z., R.T., Y. L. and H.Y. provided materials and analyzed data. Z.L. was responsible for the study conception and design, data analysis and interpretation, manuscript writing and final approval of manuscript. Z.H. was responsible for the study conception and design, supervision of all aspects of the laboratory experiments, data analysis and the preparation and final approval of the manuscript.

Funding

This study was supported by China National Key Project (2010CB945200), a key grant from National Nature Science Foundation of China (31230048), grants from National Science Foundation of China (31171422 and 31201109), the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, Shanghai Pujiang Program (11P1406400) and a key grant from the Science and Technology Commission of Shanghai Municipal (12JC1405900).

Conflict of interest

None declared.

References


Distinct characterization of NOA and OA patients


He Z, Jiang J, Hofmann MC, Dym M. Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod 2007;77:723–733.


Ploton I, Sanchez P, Durand P, Lejeune H. Decrease of both stem cell factor and clusterin mRNA levels in testicular biopsies of azoospermic patients with constitutive or idiopathic but not acquired spermatogenic failure. Hum Reprod 2006;21:2340–2345.


