Proteomic analysis and miRNA profiling of human testicular endothelial cell-derived exosomes: the potential effects on spermatogenesis

Wen-Peng Song, Sheng-Ji Gu, Xiao-Hui Tan, Yang-Yang Gu, Wei-Dong Song, Jian-Yu Zeng, Zhong-Cheng Xin, Rui-Li Guan

Testicular endothelial cells have been found to play an important role in spermatogenesis and fertility, but their mechanism is obscure. Exosomes released by various cells are recognized as cell–cell communication mediators during the initiation and progression of many diseases. Therefore, the current study aimed to investigate the protein and miRNA components of human testicular endothelial cell-derived exosomes (HTEC-Exos) and to explore their potential effects on spermatogenesis. In this study, HTEC-Exos were first isolated by the ultracentrifugation method, and then identified by nanoparticle tracking analysis, transmission electron microscopy (TEM), and western blotting. The characteristics of HTEC-Exos were examined by liquid chromatography–mass spectrometry and microRNA (miRNA) chip analysis. Bioinformatics analysis was performed to explore the potential role of the exosomal content on spermatogenesis. A total of 945 proteins were identified, 11 of which were closely related to spermatogenesis. A total of 2578 miRNAs were identified. Among them, 30 miRNAs demonstrated potential associations with male reproductive disorders, such as azoosperma, and spermatogenesis disorders. In particular, 11 out of these 30 miRNAs have been proven to be involved in spermatogenesis based on available evidence. This study provides a global view of the proteins and miRNAs from HTEC-Exos, suggesting that HTEC-Exos may function as potential effectors during the process of spermatogenesis.

Keywords: exosomes; microRNAs; proteomics; testicular endothelial cells

INTRODUCTION
Extracellular vesicles (EVs) are closed structures with squamous lipid bilayer membranes that are secreted by various cells and are classified by size and specific biological sources, such as exosomes (diameter between 30 nm and 200 nm), microvesicles (approximately 200 nm in diameter), and apoptotic bodies (1–2 μm in diameter). Exosomes, containing lipids, proteins, and nucleic acids, can be internalized by local or distant cells and transfer biological signals, which are essential for various physiological and pathological processes. At present, increasing evidence has shown that exosomes play an important role in the process of spermatogenesis, including sperm maturation, sperm motility, capacitation, acrosome reaction, fertilization, and differentiation of spermatogonial stem cells. Epididymosomes, which are exosomes in the epididymis, can participate in the regulation of spermatogenesis and fertilization. In addition, in the testes of infertile animal models, mesenchymal stem cell-derived exosomes also showed the ability to induce spermatogenesis.

Testicular endothelial cells (TECs) are the key population in the male germline stem cell niche and are situated close to the blood–testis barrier (BTB). TECs can contribute to the self-renewal and maintenance of spermatogonial stem cells (SSCs), which are considered to have a significant role in spermatogenesis, through upregulation of gial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor-binding protein 2 (IGFBP-2), macrophage inflammatory protein 2 (MIP-2), and stromal cell-derived factor-1 (SDF-1). In addition, after busulfan-induced depletion of SSCs in mice, the transplantation of TECs has been proven to restore spermatogenesis. Furthermore, TECs can serve as an effective feeder layer to enhance the proliferation and self-renewal ability of rat SSCs in vitro while maintaining the characteristics of stem cells.

The BTB is considered as a “gatekeeper” to protect developing germ cells. Moreover, the BTB has been shown to be closely related to spermatogenesis. Interestingly, a large body of literature has focused on the relationship between Sertoli cells and spermatogenesis while only a few studies have examined TECs. Therefore, based on the regulation of spermatogonia by TECs and the role of exosomes in spermatogenesis, we investigated the possible role of human testicular endothelial cell-derived exosomes (TEC-Exos) on sperm production through proteomic analysis and miRNA profiling.
MATERIALS AND METHODS

Cell culture of human testicular endothelial cells (HTECs)
HTECs were purchased from ScienCell (#4500, ScienCell, Carlsbad, CA, USA) and cultured with endothelial cell medium (ECM, #1001, ScienCell) containing 5% fetal bovine serum (FBS, #0025, ScienCell), 5 ml of endothelial cell growth supplement (ECGS, #1052, ScienCell), and 5 ml of antibiotic solution (penicillin–streptomycin solution, #0503, ScienCell). HTECs were characterized by immunofluorescence antibodies specific to von Willebrand factor (vWF)/Factor VIII according to the product description. The cells were incubated in a humidified incubator (Forma 3110; Thermo Fisher Scientific, Waltham, MA, USA) which was adjusted to 37°C with 5% CO₂, and the culture medium was changed every 3 days. Under the conditions provided by ScienCell, the HTECs could be guaranteed to further expand for 15 population doublings. Therefore, the cells have not been further examined to maintain their initial characteristics and passage 3–5 HTECs were used for the subsequent experiment.

Isolation of exosomes
To isolate exosomes from the conditioned medium, medium with 5% exosome-depleted FBS (H-Wayen, Shanghai, China) was applied to culture HTECs for 48 h, followed by differential ultracentrifugation. The supernatant was transferred to an overspeed centrifuge tube and ultracentrifuged with an ultracentrifuge (SW 45 Ti rotor, Beckman Coulter, Brea, CA, USA) at 110,000 g for 75 min. The precipitate was resuspended and diluted with 1× phosphate-buffered saline (PBS), followed by filtration with 0.22-µm membranes. Once again, samples were ultracentrifuged at 110,000 g for 75 min. The precipitate was resuspended in 1×PBS and stored at −80°C for subsequent experiments (Supplementary Figure 1).

Transmission electron microscopy (TEM)
The HTEC-Exos were dropped onto the copper grid and incubated for 5 min at room temperature. Then, the cells were incubated with a drop of 2% uranyl acetate for 1 min and dried for 20 min. TEM (Technai G2 Spirit BioTwin, FEI Company, Hillsboro, OR, USA) was used for observing the morphology of HTEC-Exos.

Western blotting
To collect all protein, the resuspending was added with isovolumetric radioimmunoprecipitation assay (RIPA) lysis buffer (strong). Bicinchoninic acid protein assay kit (Thermo Fisher Scientific) was used for measuring the concentration of protein. The protein lysate was separated on the 4%–20% Bis-Tris polyacrylamide gel and then transferred to the polyvinyl fluoride membrane. Then, the polyvinyl fluoride membrane was blocked with 5% (w/v) skim milk and incubated overnight with primary antibody against calnexin, tumor susceptibility 101 (TSG101), CD9, and CD63 (Abcam, Cambridge, MA, USA) at 4°C. After incubated with secondary antibodies, the image of membranes’ signals was obtained by chemilumager 4000 (Alpha Innotech Corporation, San Leandro, CA, USA) via ECL Plus Western Blotting Substrate (#2132, Thermo Fisher Scientific).

Peptide preparation and digestion
For peptide preparation and digestion, chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Solvents were received from Thermo Fisher Scientific. The EVs pellets were sonicated and lysed in 2% sodium dodecyl sulfate (SDS), 7 mol l⁻¹ urea, and 1× protease inhibitor solution, after which protein lysate was kept on ice for 2 h. Next, the lysate was centrifuged at 19,000 g at 4°C for 20 min and the supernatant was transferred to a new 1.5 ml EP tube. Then, the volume of 100% acetone was added and precipitated overnight at −20°C. Precipitation was dissolved in 6 mol l⁻¹ guanidine hydrochloride and 300 mmol l⁻¹ triethylammonium bicarbonate (TEAB) after two washes with ethanol–acetone–acetic acid (50: 50: 0.1) solution. The acquired exosomal proteins were digested with trypsin according to filter-aided sample preparation approach (FASP).¹⁸

The proteins were reduced with 20 mmol l⁻¹ dithiothreitol (DTT) for 1 h at 37°C and then alkylated with 90 mmol l⁻¹ iodoacetamide (IAA) for 40 min in the dark. Next, NH₂HCO₃ was added to the filter unit and centrifuged at 16,000 g for 15 min for four times. Then, 50 ml of 50 mmol l⁻¹ NH₂HCO₃ with trypsin (enzyme to protein, 1: 50) was added to the sample for digestion overnight at 37°C. The next day, peptides were eluted by centrifuging for 15 min at 13,000 g. The 100 µl water was used for a second elution. Samples were dried using a vacuum centrifuge. Then, the prepared samples were desalted by using ZipTip-C18 column and then used for liquid chromatography–mass spectrometry (LC-MS) analysis.

LC-MS analysis
LC-MS analysis was carried out by an Easy nLC 1200 (Thermo Fisher Scientific) coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Then, 1 µg samples were loaded onto a C18 reverse-phase analytical column (2 μm, 100 Å, 50 μm × 15 cm; nanoViper™, Thermo Fisher Scientific) in a data-dependent acquisition mode. Survey full-scan MS spectra were acquired over 300–2000 m/z at a resolution of 60,000. The MS equipment has a higher-energy collision dissociation for fragmentation with a normalized collision energy of 28%.

MS raw files were analyzed by using the MaxQuant software, version 1.5.8.3 (Max Planck Institute of Biochemistry, Martinsried, Germany). Two missed trypsin cleavage sites were allowed for protein identification. Carbamidomethylation of cysteine residues was set as a fixed modification, while methionine oxidation and N-terminal acetylation were set as variable modifications.

miRNA microarray profiling
Total miRNAs were isolated from HTEC-Exos using Qiagen miRNasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. FlashTag biotin HSR RNA labeling kit (Affymetrix, Santa Clara, CA, USA) was used to label the miRNAs, which was later loaded into the GeneChip miRNA 4.0 arrays (#902412, Applied Biosystems, Waltham, MA, USA). GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) was then used for hybridization, washing, and staining. Then, arrays were scanned by Scanner 3000 7G (Affymetrix) according to the user manual to obtain the original array images. Gene-specific probes were used to perform quality control of the gene expression data.

The raw images were processed by GeneChip Command Console software (version 4.5, Affymetrix), and Expression Console software
Asian Journal of Andrology

13, 14

1.4.1, Affymetrix) was used for robust multiarray analysis (RMA) normalization. Then, to study the expression profiles of miRNA, Transcriptome Analysis Console software (version 3.1) was used to assess the quality of microarray data. Differentially expressed miRNAs with statistical significance were selected based on the P < 0.05.

Statistical and bioinformatic analyses
The predicted target genes were annotated with Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by using the Database for Annotation, Visualization, and Integrated Discovery (version 6.8). Target genes of differentially expressed miRNAs were predicted by miRTarBase. The significance level of enrichment was evaluated by Fisher’s exact test, which was conducted using the R program package. Specifically, we searched for the database of GO term “spermatogenesis” (GO:0007283) to analyze the protein groups identified by LC-MS and the target genes of miRNAs with high signal values.

RESULTS
Characterizations of TECs-Exos
Nanoparticle tracking analysis, TEM, and western blotting were used to explore the characterization of TECs-Exos. TEC-Exos morphology showed the structure of circular or elliptical, most of which were with a diameter of 70 nm–200 nm (Figure 1a and 1b). Then, western blotting was performed to examine calnexin, TSG101, CD63, and CD9 of these exosomes. The marker of exosomes (TSG, CD63, and CD9) could be detected while the marker of endoplasmic reticulum (calnexin) was absent (Figure 1c).

Proteomic analysis of TECs-Exos
LC-MS analysis was used to determine the protein composition of exosomes (Supplementary Table 1). A total of 945 proteins and 5470 peptides were identified from TEC-Exos. A unique peptide found only in one protein can be used as an important parameter to clarify the existence of the corresponding proteins. The proteins present with high confidence were those containing ≥2 unique peptides. In short, a total of 562 out of 945 identified proteins (59.5%) contained greater than or equal to two unique peptides (Figure 2a).

Based on KEGG annotations, the top 20 enriched signaling pathways were determined, among which “Ribosome”, “Focal adhesion”, and “ECM-receptor interaction” were more significantly enriched (Figure 2b). GO enrichment analysis was performed to identify the proteins in TEC-Exos and selected the top fifteen terms for drawing in terms of molecular function, cellular component, and biological process. The biological process term revealed an enrichment of “cell-cell adhesion” followed by “signal transduction” and “translational initiation” (Figure 2c). For the cellular component term, proteins isolated from HTEC-Exos were enriched in “extracellular exosome”, “cytosol”, and “cytoplasm” (Figure 2d). Molecular function term showed that “protein binding”, “poly(A) RNA binding”, and “ATP binding” were assigned the top three number of genes (Figure 2e).

miRNA profiling of TECs-Exos
A total of 2578 miRNAs were identified. Thirty miRNAs with signal values greater than five were screened, among which, “hsa-miR-3613-5p”, “hsa-miR-455-5p”, “hsa-miR-6732-5p”, and “hsa-miR-4487” were most abundantly expressed (Supplementary Table 2). The target genes of miRNAs were predicted via the miTarBase database, and then, GO enrichment analysis and KEGG analysis were performed.

The biological process analysis revealed enrichment of HTEC-Exos related to “endomembrane system organization”, “protein autophosphorylation”, and “Ras protein signal transduction” (Figure 3a). For the cellular component, these target genes were enriched in “focal adhesion”, “cell-substrate adherens junction”, and “cell-substrate junction” (Figure 3b). The molecular functions were mainly enriched in “transforming growth factor beta receptor signaling pathway”, “ubiquitin-like protein ligase binding”, and “ubiquitin protein ligase binding” (Figure 3c). The pathways enriched from the KEGG analysis are shown in Figure 3d, which included “Herpes simplex virus 1 infection”, “p53 signaling pathway”, and “Shigellosis”.

Proteins and miRNAs related to spermatogenesis
QuickGO (https://www.ebi.ac.uk/QuickGO/) is a tool for browsing the gene ontology and associated electronic and manual gene ontology annotations, containing a large number of GO terms such as spermatogenesis (GO: 0007283), male gamete generation, and sexual reproduction.19 Obtained target genes from the most abundant miRNA, and the screened proteins, existed in the database of GO term “spermatogenesis” (GO: 0007283) and its child terms. The existence of repeated proteins and target genes is shown in Table 1 and 2, respectively, such as the disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), sodium/potassium-transporting ATPase subunit alpha-4 (ATP1A4), hsa-miR-3613-5p, and hsa-miR-455-3p. Some miRNAs were proven to be related to spermatogenesis similar with other publications (Table 2).20–22 We screened the miRNAs that were duplicated in the systematic review and displayed them in Table 3, such as hsa-miR-638 and hsa-miR-149-3p.

DISCUSSION
In our study, a total of 945 proteins and 2578 miRNAs were identified from HTEC-Exos via LC-MS and miRNA chip microarray, respectively, and some of them were reported to be related to spermatogenesis. By bioinformatics analysis, the potential role of exosomal content in spermatogenesis was primarily explored.

Testicular stem cells, Leydig cells, peritubular myoid cells, and Sertoli cells are often considered as constituent cells of the testis, while testicular endothelial cells, the important component of the testis vasculature, were usually ignored as the constituent of the testis. The role of Leydig cells, peritubular myoid cells, and Sertoli cells during the process of sperm production has been confirmed by a large number of studies,16,17,26 but only a few studies have focused on the testicular endothelial cells.13,14 Evidence that endothelial cells can organ-specifically regulate developmental processes and maintain normal organ homeostasis by generating tissue-specific secretomes has been mounting.14,27,28 Soluble factors released by cerebral endothelial cells could promote the self-renewal and inhibit the differentiation of neural stem cells while promoting the production of neurons.29 In the testis, it has been reported that the disruption of testicular vascular development blocks testis cord formation and then prevents proper structural development of the testis.10 TECs, as a niche of germline stem cells, have also been proven to enhance the proliferation and self-renewal of spermatogonial stem cells.13,14

Due to the regulatory effects on spermatogenesis by exosomes and TECs,21,11,12 we investigated HTEC-Exos via proteomic analysis and miRNA profiling for the first time, through which we found several proteins and miRNAs related to spermatogenesis, which had not been previously reported in HTECs. For instance, the ADAM10 in HTEC-
Proteomics and miRNA profiling of HTEC-Exos

WP Song et al

481

Exos was a member of the ADAM family, which existed in germ and Sertoli cells during all the stages of spermatogenesis and was activated at particular events of rat spermatogenesis. Sodium/potassium-transporting ATPase subunit alpha-4 (Na, K-ATPase a4) was also screened from HTEC-Exos; in its absence, spermatozoa are unable to fertilize eggs in vitro, resulting in a severe reduction in sperm motility and hyperactivation typical of sperm capacitation. The remaining 15 proteins screened from HTEC-Exos were shown to be closely related to spermatogenesis, spermatid development, spermatid nucleus differentiation, and acrosome assembly (Table 1). It has been reported that the deregulation of the expression of miRNA in sperm cells, epididymis, seminal plasma, and extracellular vesicles (i.e., exosomes and microvesicles) may lead to the alterations in spermatogenesis and embryogenesis, thereby resulting in various forms of infertility. By searching the GO term "spermatogenesis" (GO: 0007283) and its child terms, we identified thirty miRNAs involved in spermatogenesis from the results of miRNA profiling (Table 2), 11 of which were proven by this study (Table 3).

Given the importance of proteins and miRNAs in sperm production, our results may reveal the potential effect of HTEC-Exos on spermatogenesis, which may provide an explanation for certain unclear problems in the sperm formation process. In addition, SSCs are particularly sensitive to cytotoxic treatments; therefore, many patients...
Figure 3: miRNA profiling of the exosomes from testicular endothelial cells. GO enrichment analysis of (a) biological processes, (b) cellular components, and (c) molecular functions. (d) KEGG enriched signaling pathways. Abbr 1: transforming growth factor-beta receptor signaling pathway; Abbr 2: positive regulation of proteasomal ubiquitin-dependent protein catabolic process; Abbr 3: transcriptional activator activity, RNA polymerase II transcription regulatory; Abbr 4: transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; miRNA: microRNA.

Table 1: The list of proteins related to spermatogenesis expressed in human testicular endothelial cell-derived exosomes

| Protein                                      | Protein ID | Gene    | Function                   | Reference                      |
|----------------------------------------------|------------|---------|---------------------------|--------------------------------|
| Disintegrin and metalloproteinase domain-containing protein 10 | O14672     | ADAM10  | Spermatogenesis            | Urriola-Munaz et al.31         |
| Sodium/potassium-transporting ATPase subunit alpha-4 | Q13733     | ATP1A4  | Spermatogenesis            | Jimenez et al.54,55            |
| PMCA4                                        | P23634     | ATP2B4  | Spermatogenesis            | Okunade et al.54               |
| Tyrosine-protein kinase receptor UFO         | P30530     | AXL     | Spermatogenesis            | Chen et al.57                  |
| Caireticulin                                  | P27797     | CALR    | Spermatogenesis            | Nakamura et al.38,55           |
| Cartilage-associated protein                 | O75718     | CRTAP   | Spermatogenesis            | Zimmerman et al.60            |
| Eukaryotic translation initiation factor 5A-2| Q9QZ4      | EIF5A2  | Spermatogenesis            | Carelli et al.51               |
| Gap junction alpha-1 protein                 | P17302     | GJA1    | Spermatogenesis            | Sridharan et al.62             |
| Histone H2AX                                  | P16104     | H2AFX   | Spermatogenesis            | Lewis et al.63                 |
| Histone H3.3                                  | P84243     | H3F3A   | Spermatogenesis, Spermatid development | Yuen et al.64                  |
| Septin-2                                     | Q15019     | SEPTIN2 | Spermatogenesis            | Ronfani et al.65               |
| Septin-7                                     | Q16181     | SEPTIN7 | Spermatogenesis            | Wang et al.71                  |
| Platelet-activating factor acetylhydrolase IB subunit alpha | P43034     | PAFAH1B1| Acrosome assembly          | Yao et al.66                   |
| Phosphoglycerate mutase 2                    | P15259     | PGAM2   | Spermatogenesis            | Fundele et al.67               |
| Peroxiredoxin-4                              | Q13162     | PRDX4   | Spermatogenesis            | Shii et al.56                  |
| 60S ribosomal protein L10                    | Q96L21     | RPL10L  | Spermatogenesis            | Jiang et al.58                 |
| PMCA4: plasma membrane calcium-transporting ATPase 4 |           |         |                           |                                 |
become permanently infertile after completing cancer treatment.\textsuperscript{12} Harvesting SSCs before chemotherapy and reinjecting them into the testes after treatment can avoid the cytotoxic effects of chemotherapeutic drugs on SSCs.\textsuperscript{33,34} However, there are only a small number of SSCs in the testes of boys before adolescence, and it is necessary to expand the SSCs before injection into the testes.\textsuperscript{14} TEGs can provide necessary supports to SSCs.

### Table 2: The miRNAs related to spermatogenesis expressed in human testicular endothelial cell-derived exosomes

| miRNA     | Signal | Target gene | Possible function                                                                 |
|-----------|--------|-------------|-----------------------------------------------------------------------------------|
| hsa-miR-3613-5p | 8.78792 | KatNAL1, STRBP, YY1, PSMC3, STRBP_0, STRBP_2, STRBP_1, B92Y1 | Spermatogenesis, spermatid development                                             |
| hsa-miR-455-3p* | 8.51647 | NDRG3, SLC4A2, FSTL3, PHF7, METTL14, TLL1, TYRO3 | Spermatogenesis, spermatid development, spermatogonial cell division, spermatid differentiation, sperm individualization, sperm axoneme assembly, male germline cyst formation |
| hsa-miR-6732-5p | 8.1828 | CALR, CDK16, AMH, SMARC1, ZFX, HSF2, TMBIM6, HMBG2 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4487 | 7.9967 | CCNB1, GSR, AGO3, GOLGA3, JAM2, METTL14, PHF7, PLD6, RHBDD1 | Acrosome assembly, sperm capacitation, spermatid development, spermatid differentiation, sperm axoneme assembly |
| hsa-miR-6087 | 7.88397 | KN1, TLL1, TYRO3, GOLGA3, ZNF35, JAM2, METTL14, PHF7 | Acrosome assembly, male germline cyst formation, spermatogenesis, spermatid development, spermatid differentiation, sperm axoneme assembly |
| hsa-miR-3960 | 7.83086 | WSCD1, TCP1 | Spermatogenesis |
| hsa-miR-8075 | 7.50747 | DAZAP1, CATSPER4, KIFC1, STK11 | Spermatogenesis, spermatid development |
| hsa-miR-6089 | 7.3477 | CCNB1, GSR, CRLAP, Dlx3, ERCC1, IGF2R, PATZ1, TPT1, UBE2B | Spermatogenesis, spermatid development, spermatid nucleus differentiation, sperm axoneme assembly |
| hsa-miR-6090 | 7.2333 | H2AFX, HMGAI, RIMBP3C | Spermatogenesis, spermatid development |
| hsa-miR-3665 | 7.07463 | ALKBH5, DDX4, S100A11, SHS4A | Spermatogenesis, spermatid development, sperm axoneme assembly, primary spermatocyte growth, spermatocyte division |
| hsa-miR-5787 | 6.88067 | ADAM17, CELF1, CEPI35, CLU, NEURL1, PEX2 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-6125 | 6.72017 | H2AFX | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4530 | 6.66846 | ATAT1, CATSPER4, KIFC1, STK11 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4508 | 6.51407 | AMH | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-6088 | 6.26322 | BRD2, CCR6, MYCBP, PLEKHA1, SGL1, SLC2A14, TERT, USP42 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-2861 | 6.18234 | CLU, MEI1 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4466 | 6.06271 | AGO3 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-2115-5p | 5.9418 | AGO3, PYG02, SLC4A1, YY1 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4516 | 5.90823 | ALKBH5, CCND2, PHC2, SLC4A2, SLC2A6, TP53 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-638* | 5.89988 | TP53 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-1237-5p | 5.71595 | CLU, H2AFX, ERCC1, WSCD1, SBF1, SIX5, SPATA2 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-6729-5p | 5.70038 | ATAT1, CCNB1, HERPUD2, SF3A | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-3196 | 5.698 | H2AFX, WSCD1, SBF1, SPATA2, AMH | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-8069 | 5.61643 | DICER1, PLEKHA1, STK11, CRAP, TERT, USP42 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-149-3p* | 5.5248 | SPATA2, PHC2, YY1, PLEKHA1, STK11, CRAP, TERT, USP42 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-6126 | 5.50762 | CLDN11, NRE6A1, PIAS2 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-8072 | 5.45165 | WSCD1, POF8 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-6869-5p | 5.44576 | HMGB2, APOB, HMGAI, OCA2, POP1A, ZFP37 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |
| hsa-miR-4459 | 5.4443 | METTL14, TLL1, TYRO3, CRKL, P2R2B, ERCC1, ALKBH5, SGPL1, ActRA1A, BCL2L11, CCDC36, DNM2, EL3, MAK, MYBL1, NPAP1, PSME3 | Spermatogenesis, spermatid development, spermatid assembly, male germline cyst formation |
| hsa-miR-4484 | 5.39124 | CADMI, PEBP1 | Spermatogenesis, spermatid development, spermatid nucleus differentiation |

*The miRNA had been proved by other articles to be related to spermatogenesis. miRNA: micro ribonucleic acid
growth factors for the self-renewal and expansion of SSCs, and provide more options for treatment.3,5,6 The results of this study suggested that TEC-Exos might be related to azoospermia and spermatogenesis disorders based on proteomic analysis and miRNA profiling. Further studies are needed to focus on the mechanism of TECs-Exos in regulating spermatogenesis, which may provide new insights into the treatment and diagnosis of spermatogenesis disorders.

The pathways via KEGG analysis showed the potential impact on the regulation of spermatogenesis. Cytoskeletal dynamics underlie many key spermatogenic processes, in which the actin cytoskeleton is involved deeply.38 The migration of germ cells, maintenance of cell–cell interactions, and niche of SSCs are based on integrin- and actin-based adhesion junctions and the regulation of the actin cytoskeleton.39-41 In addition, the cytoskeleton based on actin and microtubule is necessary for the transport of both sperm and phagocytes across the spermatogenic epithelium in testes.42-44 It was reported that gap junction is essential during spermatogenesis through the maintenance and differentiation of stem cells in the testis.45-47 The pathways of "pathogenic Escherichia coli infection" and "herpes simplex virus 1 infection" were detected via KEGG analysis, while infection with E. coli and herpes simplex virus 1 has been reported to play an important role in male factor infertility.48-49 Spermatogenesis is tightly regulated by ubiquitination, acetylation, and proteosomal degradation,46,47 which might be related to the pathway "proteasome". Besides, "p53 signaling pathway", "Rap1 signaling pathway", "endocytosis", and "PI3K-Akt signaling pathway" were all proven to be the key pathways of spermatogenesis.48-51

It is worth noting that our study has several limitations. First of all, the exosomes verified in our experiments were extracted from infant HTECs, which cannot fully reflect the condition of the exosomes derived from HTECs in men of all ages. Age is an important factor for spermatogenesis and affects multiple aspects including sperm morphology, sperm concentration, and sperm motility.52,53 In follow-up experiments, the data on the exosomes derived from HTECs in men of different ages should be supplemented and compared with the data from infant HTECs to study the influence of age on the composition of proteins and miRNAs in exosomes. Second, our experiment only studied the miRNA and protein composition of HTEC-Exos, and screened the miRNAs and proteins that may affect spermatogenesis. However, we did not explore the specific role and underlying mechanism of these screened miRNAs and proteins in the process of spermatogenesis. More studies are warranted to address these issues.

### Table 3: The miRNAs expressed in human testicular endothelial cell-derived exosomes which were proved to be related to spermatogenesis by other articles

| miRNA      | Signal | Variation | Positions | Comparison | Study              |
|------------|--------|-----------|-----------|------------|--------------------|
| hsa-miR-455-3p | 8.51647| Upregulated| Testis    | SCO vs Oaz| Muñoz et al.20     |
| hsa-miR-638  | 5.89988| Upregulated| Testis    | NOA vs N  | Li et al.21        |
| hsa-miR-149-3p| 5.2548 | Upregulated| Spermatozoa| NI vs NF  | Salas-Huetos et al.22|
| hsa-miR-320a | 2.38864| Downregulated| Spermatozoa| O vs N    | Muñoz et al.20     |
| hsa-miR-1224-5p| 1.84448| Upregulated| Testis    | vs N      | Zhou et al.22      |
| hsa-miR-1246 | 1.82329| Upregulated| Epididymis| Cryptorchid tissue vs N| Tang et al.23       |
| hsa-let-7b-5p | 1.79294| Downregulated| Seminal plasma| A vs N    | Belleannée et al.24|
| hsa-miR-511  | 1.23289| Downregulated| Seminal plasma| SpF (NOA or severe O) vs Oaz| Zhou et al.22      |
| hsa-miR-222-3p| 1.14998| Downregulated| Seminal plasma| V vs N    | Hu et al.75        |
| hsa-miR-1973 | 1.08043| Downregulated| Spermatozoa| A vs N    | Abu-Halima et al.76|
| hsa-miR-26a-5p| 1.00665| Upregulated| Testis    | Pachytene spermatocyte NOA vs Oaz| Yao et al.77       |

A: asthenozoospermia; N: normal spermatogenesis; NF: normozoospermic fertile; NI: normozoospermia infertile; NOA: nonobstructive azoospermia; O: oligozoospermia; Oaz: obstructive azoospermia; SCO: sertoli-cell-only syndrome; SpF: spermatogenic failure; miRNA: micro ribonucleic acid

### CONCLUSION

Exosomal cargos in HTECs including proteins and miRNAs were comprehensively characterized. Analysis of their composition provides insight into the potential role of HTEC-Exos during spermatogenesis and might represent new and potential diagnostic and prognostic tools for male infertility.

### AUTHOR CONTRIBUTIONS

RLG, ZCX, and JYZ were involved in conception design, manuscript writing, and administrative support. WPS carried out the collection and assembly of data, and manuscript writing. SJG participated in cell culture, collection and assembly of data, and manuscript writing. XHT performed the data analysis and interpretation together with YYG. WDS was involved in collection and assembly of data. All authors read and approved the final manuscript.

### COMPETING INTERESTS

All authors declare no competing interests.

### ACKNOWLEDGMENTS

The study was funded by the Horizontal Subject and the National Natural Science Foundation of China (Grant No. 81401194).

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

### REFERENCES

1. Gandham S, Su X, Wood J, Nocens AL, Ali SC, et al. Technologies and standardization in research on extracellular vesicles. Trends Biotechnol 2020; 38: 1066–98.
2. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 2009; 9: 581–93.
3. Rayamajhi S, Nguyen TD, Marasini R, Aryal S. Macrophage-derived exosome-mimetic hybrid vesicles for tumor targeted drug delivery. Acta Biomater 2019; 94: 482–94.
4. Harding CV, Heuser JE, Stahl PD. Exosomes: looking back three decades and into the future. J Cell Biol 2013; 200: 367–71.
5. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. Theranostics 2018; 8: 237–55.
6. Vickram AS, Srikumar PS, Srinivasan S, Jeyanthi P, Anbarasu K, et al. Seminal exosomes – an important biological marker for various disorders and syndrome in human reproduction. Saudi J Biol Sci 2021; 28: 3607–15.
7. Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles – roles in gamete maturation, fertilization and embryo implantation. Hum Reprod Update 2016; 22: 182–93.
8. Li Q, Li H, Liang J, Mei J, Cao Z, et al. Sertoli cell-derived exosomal MicroRNA-486-5p regulates differentiation of spermatogonial stem cell through PTEN in mice. J Cell Mol Med 2021; 25: 3950–62.
9. Xiao J, Wang X, Loo Y, Li X, Li XW. Research progress in sRNAs and functional
proteins in epididymosomes. *Yi Chuan* 2018; 40: 197–206.

James ER, Cartelle DT, Aston KI, Jenkins TG, Yeast M, et al. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. *Int J Mol Sci* 2020; 21: 5377.

Sullivan R, Saez F, Grouard J, Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells Mol Dis* 2005; 35: 1–10.

Zhankina R, Baghban N, Askarov M, Sapiyeva D, Ibragimov A, et al. Mesenchymal stem cells and their exosomes for restoration of spermatogenesis in non-obstructive azoospermia: a systemic review. *Stem Cell Res Ther* 2021; 12: 229.

Kim YH, Oh MG, Bhang DH, Kim BJ, Jung SE, et al. Testicular endothelial cells promote self-renewal of spermatogenic stem cells in rats. *Biol Reprod* 2019; 101: 360–7.

Bhang DH, Kim BJ, Kim BG, Schadler K, Baek KH, et al. Testicular endothelial cells maintain a critical population in the germ cell stem cell niche. *Nat Commun* 2018; 9: 4379.

Su L, Mruk DD, Cheng CY. Drug transporters, the blood-testis barrier, and spermatogenesis. *J Endocrinol* 2011; 208: 207–23.

Ni FD, Hao SL, Yang WX. Multiple signaling pathways in Sertoli cells: recent findings in spermatogenesis. *Cell Death Dis* 2019; 10: 541.

Griswold MD. 50 years of spermatogenesis: Sertoli cells and their interactions with germ cells. *Biol Reprod* 2018; 99: 87–100.

Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation protocol for proteome analysis. *Nat Methods* 2009; 6: 359–62.

Binnis D, Dimmer E, Huntley R, Barrett D, Donovan C, et al. QuickGO: a web-based tool for Gene Ontology searching. *Bioinformatics* 2009; 25: 3046–7.

Muñoz X, Mata A, Bassas L, Larría S. Altered miRNA signature of developing germ-cells in fertile infants relates to the severity of spermatogenic failure and persists in spermatozoa. *Sci Rep* 2015; 5: 17991.

Lian J, Zhang X, Tian H, Liang N, Wang Y, et al. Altered microRNA expression in patients with non-obstructive azoospermia. *Biol Reprod Endocrinol* 2009; 7: 13.

Salas-Huetos A, Blanco J, Vidal F, Grossmann M, Pons MC, et al. Spermatogenesis from normozoospermic fertile and infertile individuals convey a distinct miRNA cargo. *PLoS One* 2015; 11: 32985.

Aivaliadou E, Riplone M, Brunetti F, Bertulli G. cAMP-Epac2-mediated activation of Rap1 in developing male germ cells: RA-RhoGAP as a possible direct down-stream effector. *Mol Reprod Dev* 2009; 76: 407–16.

Almeida S, Rato L, Sousa M, Alves MG, Oliveira PF. Fertility and sperm quality in the ageing male. *Curr Pharm Des* 2017; 23: 4429–37.

Jimenez T, Casamonti E, Krausz C. Age-dependent de novo mutations during spermatogenesis and their consequences. *Adv Exp Med Biol* 2011; 696: 24–49.

Jimenez T, Sanchez G, Wetheimer E, Blanco G. Activity of the Na,K-ATPase alpha4 isoform is important for mitochondrial potential, intracellular Ca2+, and pH to maintain motility in rat spermatozoa. *Reproduction* 2010; 139: 839–45.

Salas-Huetos A, Blanco J, Vidal F, Grossmann M, Pons MC, et al. Increased expression of the Na,K-ATPase alpha4 isoform enhances sperm motility in transgenic mice. *Biol Reprod* 2011; 84: 153–61.

Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, et al. Targeted ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a novel role for PMCA4. *Biol Chem* 2004; 279: 33742–50.

Chen Y, Wang H, Qi N, Wu H, Xiong W, et al. Expression characterization and functional implication of the collagen-modifying leucine-rich proteins in mouse gonadal tissue and male sperm. *Aims Genet* 2018; 1: 4–20.

Carrell DT, De Jonge C, Lamb DJ. The genetics of male infertility: a field of study whose time is now. *Arch Androl* 2014; 60(4): 310–5.

Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, et al. Male germ cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 2004; 304: 1338–40.

Combes AN, Wilhelm D, Davidson T, Dejana E, Harley V, et al. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. *Cell Mol Life Sci* 2019; 76: 2661–91.

Chioppi F, Casamonti E, Krausz C. Age-dependent de novo mutations during spermatogenesis and their consequences. *Adv Exp Med Biol* 2011; 696: 24–49.

Jimenez T, Sanchez G, Wetheimer E, Blanco G. Activity of the Na,K-ATPase alpha4 isoform is important for mitochondrial potential, intracellular Ca2+, and pH to maintain motility in rat spermatozoa. *Reproduction* 2010; 139: 839–45.

Nakamura M, Moriya M, Baba T, Michikawa Y, Yamano T, et al. An endoplasmic reticulum protein, calreticulin, is transported into the acrosome of rat sperm. *Exp Cell Res* 1993; 205: 101–10.

Nakamura M, Michikawa Y, Baba T, Okinaga S, Ari K. Calreticulin is present in the acrosome of spermatids of rat testes. *Biochem Biophys Res Commun* 1992; 186: 668–73.

Zimmerman SM, Besio R, Heid-Lipsmeyer ME, Dimorl M, Castagnola P, et al. Expression characterization and functional implication of the collagen-modifying leucine-rich proteins in mouse gonadal tissue and male sperm. *Aims Genet* 2018; 1: 4–20.

Carrell DT, De Jonge C, Lamb DJ. The genetics of male infertility: a field of study whose time is now. *Arch Androl* 2006; 52: 269–74.

Sridharan S, Brehm R, Bergmann M, Cooke PS. Role of connexin 43 in Sertoli cells and f-actin organization to support spermatid transport during spermatogenesis in the rat testis. *Endocrinology* 2016; 157: 2894–908.

Smedzliu CM, Mennsberg A, Vogl AW, Tanentzapf G. Bi-directional gap junction-mediated somato-germline communication is essential for spermatogenesis. *Development* 2015; 142: 2598–609.

Boyer A, Girard M, Thimmaiahalli DS, Levassor A, Cestele C, et al. mTOR regulates gap junction alpha1 protein trafficking in Sertoli cells and is required for the maintenance of spermatogenesis in mice. *Biol Reprod* 2016; 95: 13.

Chen M, Cai LY, Kanno N, Kato T, Lu J, et al. Detection of human herpesviruses (HHVs) in semen of human male infertile patients. *J Reprod Dev* 2013; 59: 457–62.

Malolina EA, Kuliubin A, Tulenev Iu A, Kushtch AA. (Destructive changes in the mouse testes in retrograde infection with herpes simplex virus). *Urologia* 2015; 55–5. (Article in Russian)

Bhushan S, Hossain H, Lu Y, Geisler A, Tchalilabachev S, et al. Uropathogenic E. coli induce different immune response in testicular and peritoneal macrophages: implications for testicular immune privilege. *PLoS One* 2011; 6: e28542.

Zhang Q, Ji SY, Busayavalka K, Shao J, Yu C. Meiosis I progression in spermatogenesis requires a type of testis-specific 20S core proteasome. *Nat Commun* 2019; 10: 3387.

Qian MX, Pang Y, Liu CH, Hanatake K, Du BY, et al. Acetylation-mediated proapoptotic degradation of core histones during DNA repair and spermatogenesis. *Cell* 2013; 153: 1012–24.

Wang D, Zhao W, Liu J, Wang Y, Yuan C, et al. Effects of HFG-1 on Spermatogenesis of varicocoele rats by regulating VEGF/FPI3K/Akt signaling pathway. *Reprod Sci* 2021; 28: 1161–74.

Fang Y, Wang L, Dong X, Wang H, He L, et al. Downregulation of vdac2 inhibits spermatozoa via JNK and p53 signalling in mice exposed to cadmium. *Toxicol Lett* 2020; 326: 114–22.

Xia W, Wong EW, Mruk DD, Cheng CY. TGF-beta3 and TNFalpha perturb blood-testis barrier (BTB) dynamics by accelerating the clathrin-mediated endocytosis of integral membrane proteins: a new concept of BTB regulation during spermatogenesis. *Dev Biol* 2009; 327: 48–61.

Proteomics and miRNA profiling of HTEC-Exos

WP Song et al
phosphoglycerate mutase-2 in the testis of the mouse. *Dev Biol* 1987; 124: 562–6.

68 Shi H, Liu J, Zhu P, Wang H, Zhao Z et al. Expression of peroxiredoxins in the human testis, epididymis and spermatozoa and their role in preventing H$_2$O$_2$-induced damage to spermatozoa. *Folia Histochem Cytobiol* 2018; 56: 141–50.

69 Jiang L, Li T, Zhang X, Zhang B, Yu C, et al. RPL10L Is required for male meiotic division by compensating for RPL10 during meiotic sex chromosome inactivation in mice. *Curr Biol* 2017; 27: 1498–505.e6.

70 Lin YH, Kuo YC, Chiang HS, Kuo PL. The role of the septin family in spermiogenesis. *Spermatogenesis* 2011; 1: 298–302.

71 Wang X, Fei F, Qu J, Li C, Li Y, et al. The role of septin 7 in physiology and pathological disease: a systematic review of current status. *J Cell Mol Med* 2018; 22: 3298–307.

72 Zhou R, Zhang Y, Du G, Han L, Zheng S, et al. Down-regulated let-7b-5p represses glycolysis metabolism by targeting AURKB in asthenozoospermia. *Gene* 2018; 663: 83–7.

73 Tang D, Huang Z, He X, Wu H, Peng D, et al. Altered miRNA profile in testis of post-cryptorchidopexy patients with non-obstructive azoospermia. *Reprod Biol Endocrinol* 2018; 16: 78.

74 Belleannée C, Légaré C, Calvo E, Thimon V, Sullivan R. microRNA signature is altered in both human epididymis and seminal microvesicles following vasectomy. *Hum Reprod* 2013; 28: 1455–67.

75 Hu L, Wu C, Guo C, Li H, Xiong C. Identification of microRNAs predominately derived from testis and epididymis in human seminal plasma. *Clin Biochem* 2014; 47: 967–72.

76 Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, et al. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. *Fertil Steril* 2013; 99: 1249–55.e16.

77 Yao C, Yuan Q, Niu M, Fu H, Zhou F, et al. Distinct expression profiles and novel targets of microRNAs in human spermatogonia, pachytene spermatocytes, and round spermatids between OA patients and NOA patients. *Mol Ther Nucleic Acids* 2017; 9: 182–94.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

©The Author(s)(2021)
Supplementary Figure 1: HTEC-Exos isolation, identification, proteomic analysis, and miRNA profiling. HTEC-Exos: human testicular endothelial cell-derived exosomes; miRNA: microRNA.