Asian Journal of Andrology (2022) 24, 478–486
www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Male Reproduction

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

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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 azoospermia, 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.

Asian Journal of Andrology (2022) **24**, 478–486; doi: 10.4103/aja202190; published online: 17 December 2021

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[1] and are classified by size and specific biological sources,[2] such as exosomes (diameter between 30 nm and 200 nm),[3] microvesicles (approximately 200 nm in diameter), and apoptotic bodies (1–2 μm in diameter).[2,4] 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.[5] 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.[6–8] Epididymosomes, which are exosomes in the epididymis, can participate in the regulation of spermatogenesis and fertilization.[9–11] In addition, in the testes of infertile animal models, mesenchymal stem cell-derived exosomes also showed the ability to induce spermatogenesis.[12]

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)[13] 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 glial 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).[14] In addition, after busulfan-induced depletion of SSCs in mice, the transplantation of TECs has been proven to restore spermatogenesis.[14] 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.[13]

The BTB is considered as a "gatekeeper" to protect developing germ cells.[15] Moreover, the BTB has been shown to be closely related to spermatogenesis.[15] Interestingly, a large body of literature has focused on the relationship between Sertoli cells and spermatogenesis while only a few studies have examined TECs.[16,17] 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.

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Received: 21 July 2021; Accepted: 17 October 2021

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 with 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% CO_2, 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. In short, the culture medium was centrifuged at 2000*g* for 10 min and 10 000*g* for 30 min (SL4R Plus; Thermo Fisher Scientific). Then, the supernatant was transferred to an overspeed centrifuge tube and ultracentrifuged with an ultracentrifuge (SW 45 Ti rotor, Beckman Optima XPN-80 ultracentrifuge, 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**).

Nanoparticle tracking analysis

Exosomes from the sample were identified by grain size analysis, electron microscopy analysis, and western blotting according to the following methods. The sample of exosomes was diluted with 1× PBS and then was used for nanoparticle tracking analysis directly. The qNano Gold (Izon Science, Christchurch, New Zealand) and user guide were used to measure the grain size of isolated exosomes. Data were recorded and analyzed by Izon Control Suite (version 3.3.2.2001, Izon Science).

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 (Tecnai 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 chemiimager 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, NH_4HCO_3 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} NH_4HCO_3 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 miRNeasy 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

(version 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 HTEC-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-455-3p", "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 of 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 microRNA 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,[24,25] 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.[30] 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,[9,11,12] 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-

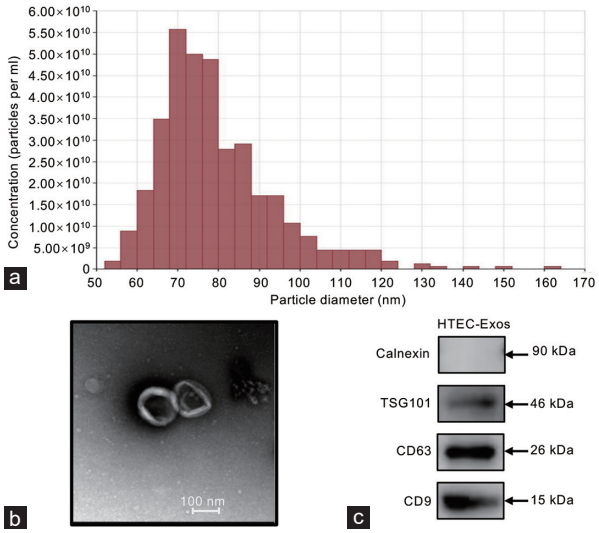

Figure 1: Characterization of testicular endothelial cell-derived exosomes. (**a**) Size distribution of HTEC-Exos by nanoparticle tracking analysis. (**b**) TEM image of HTEC-Exos. (**c**) The expression of calnexin, TSG101, CD9, and CD63 in HTEC-Exo lysates as detected by western blotting. HTEC-Exos: human testicular endothelial cell-derived exosomes; TEM: transmission electron microscopy; TSG101: tumor susceptibility 101.

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.[31] Sodium/potassium-transporting ATPase subunit alpha-4 (Na, K-ATPase α4) 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.[23] 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

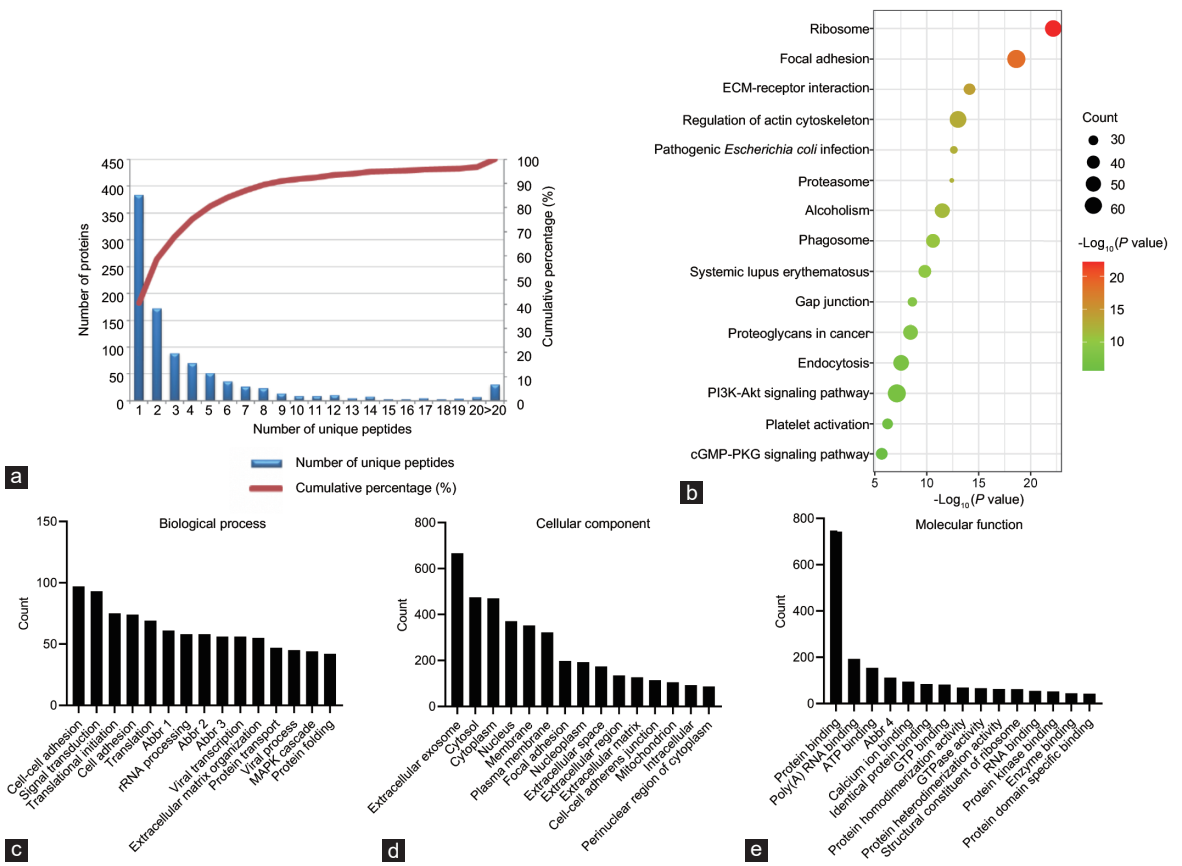

Figure 2: Proteomic analyses of the exosomes from testicular endothelial cells. (**a**) Bicoordinate distribution diagram of the number of unique peptides contained in all proteins identified in this experiment. The proteins with high confidence were those containing ≥2 unique peptides. (**b**) KEGG enriched signaling pathways. GO enrichment analysis of (**c**) biological processes, (**d**) cellular components, and (**e**) molecular function. Abbr 1: nuclear-transcribed mRNA catabolic process, nonsense-mediated decay; Abbr 2: negative regulation of apoptotic process; Abbr 3: SRP-dependent cotranslational protein targeting to membrane; Abbr 4: cadherin binding involved in cell–cell adhesion; GO: gene ontology; ECM: extracellular matrix; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; cGMP: cyclic guanosine monophosphate; PKG: protein kinase G; MAPK: mitogen-activated protein kinase; KEGG: Kyoto Encyclopedia of Genes and Genomes.

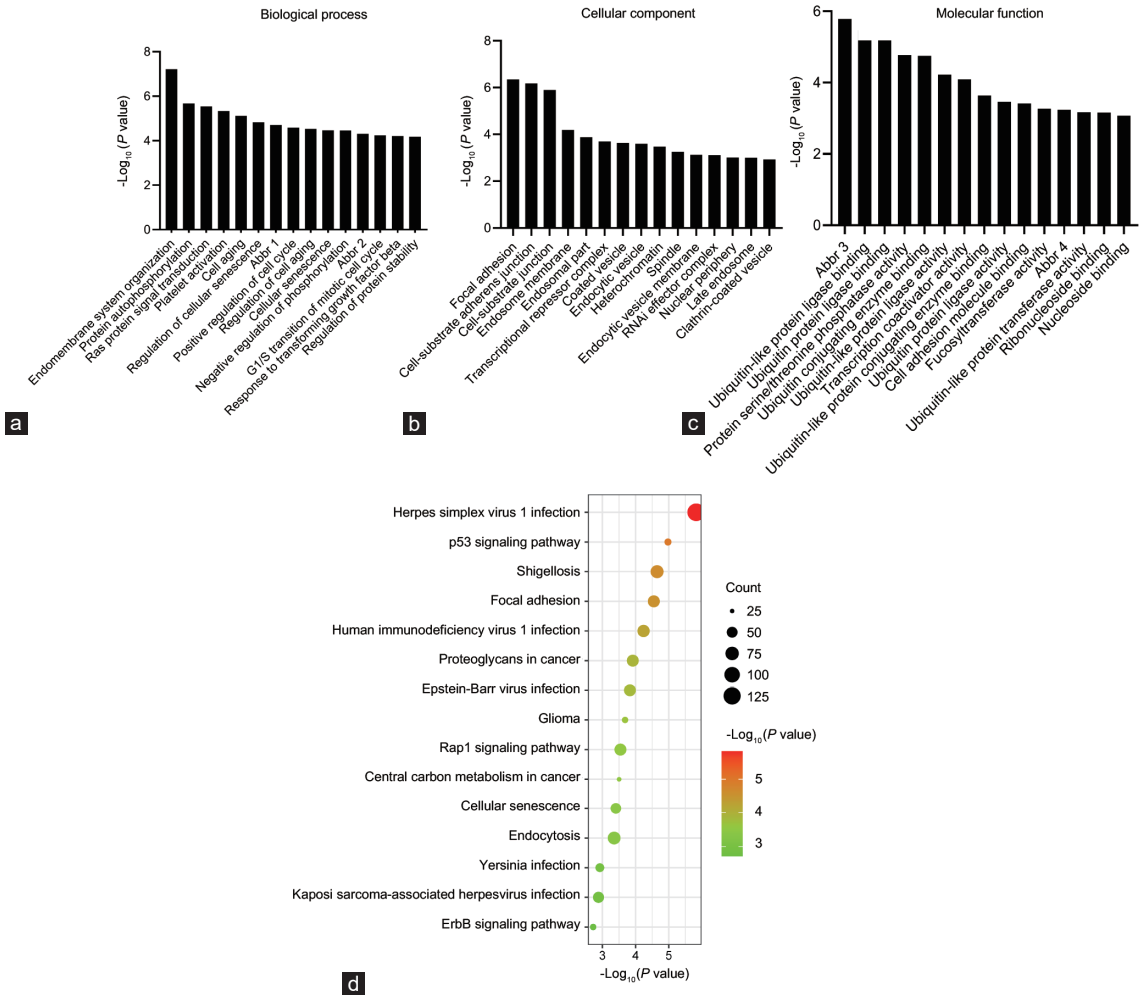

Figure 3: miRNA profiling of the exosomes from testicular endothelial cells. GO enrichment analysis of (**a**) biological processes, (**b**) cellular components, and (**c**) molecular functions. (**d**) KEGG enriched signaling pathways. Abbr 1: transforming growth factor-beta receptor signaling pathway; 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.*[56]
Tyrosine-protein kinase receptor UFO	P30530	*AXL*	Spermatogenesis	Chen *et al.*[57]
Calreticulin	P27797	*CALR*	Spermatogenesis	Nakamura *et al.*[58,59]
Cartilage-associated protein	O75718	*CRTAP*	Spermatogenesis	Zimmerman *et al.*[60]
Eukaryotic translation initiation factor 5A-2	Q9GZV4	*EIF5A2*	Spermatogenesis	Carrell *et al.*[61]
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]
High mobility group protein B2	P26583	*HMGB2*	Spermatogenesis Spermatid nucleus differentiation	Ronfani *et al.*[65]
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	Shi *et al.*[68]
60S ribosomal protein L10	Q96L21	*RPL10L*	Spermatogenesis	Jiang *et al.*[69]
Septin-2	Q15019	*SEPTIN2*	Spermatogenesis	Lin *et al.*[70]
Septin-7	Q16181	*SEPTIN7*	Spermatogenesis	Wang *et al.*[71]

PMCA4: plasma membrane calcium-transporting ATPase 4

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*	*B9ZYY1*	Spermatogenesis, spermatid development
hsa-miR-455-3p*	8.51647	*YY1*	*NDRG3*	*SLC4A2*	*FSTL3*	*PHF7*	*METTL14*	*TTLL1*	*TYRO3*	Spermatogenesis, spermatid development, spermatogonial cell division, spermatid differentiation, sperm individualization, sperm axoneme assembly, male germline cyst formation
		JAM2	*ZNF35*	*ACOX1*	*ACTR1A*	*CCDC36*	*NPAP1*	*SKIL*	*SPEM1*	
		H2AFX	*CCNB1*	*AGO3*	*BRD2*	*IGF2R*	*KNL1*	*ZFX*	*BMP8A*	
		BRIP1	*DYNC1H1*	*HIST1H1E*	*KATNAL1*	*PDK1*	*PGM3*	*STYX*	*TMEM119*	
		ZFP42	*ZNF805*							
hsa-miR-6732-5p	8.1828	*CALR*	*CDK16*	*CD55*	*BCL2L1*					Spermatogenesis
hsa-miR-4487	7.99967	*CCNB1*	*KNL1*	*AMH*	*SMARCE1*	*ZFX*	*HSF2*	*TMBIM6*	*HMGB2*	Acrosome assembly, sperm capacitation, spermatid development, spermatid nucleus differentiation
		GSR								
hsa-miR-6087	7.88397	*KNL1*	*AGO3*	*GOLGA3*	*JAM2*	*METTL14*	*PHF7*	*PLD6*	*RHBDD1*	Acrosome assembly, male germline cyst formation, spermatogenesis, spermatid development, spermatid differentiation, sperm axoneme assembly
		TTLL1	*TYRO3*	*ZNF35*						
hsa-miR-3960	7.83086	*WSCD1*								Spermatogenesis
hsa-miR-8075	7.50747	*DAZAP1*	*TCP1*							Spermatogenesis, spermatid development
hsa-miR-6089	7.34772	*CCNB1*	*CRTAP*	*DHX36*	*ERCC1*	*IGF2R*	*PATZ1*	*TPT1*	*UBE2B*	Spermatogenesis, sperm axoneme assembly, regulation of transcription from RNA polymerase II promoter involved in spermatogenesis
hsa-miR-6090	7.23333	*H2AFX*	*HMGA1*	*RIMBP3C*						Spermatogenesis, spermatid development
hsa-miR-3665	7.07463	*CRTAP*	*ALKBH5*	*DDX4*	*S100A11*	*SHISA6*				Spermatogenesis
hsa-miR-5787	6.88067	*ADAM17*	*CELF1*	*CEP135*	*CLU*	*NEURL1*	*PEX2*			Spermatogenesis, spermatid development, sperm axoneme assembly, primary spermatocyte growth, spermatocyte division
hsa-miR-6125	6.72017	*H2AFX*								Spermatogenesis
hsa-miR-4530	6.66846	*ATAT1*	*CATSPER4*	*KIFC1*	*STK11*					Spermatogenesis, spermatid development, sperm capacitation
hsa-miR-4508	6.51407	*AMH*								Sperm capacitation
hsa-miR-6088	6.26322	*BRD2*	*CCR6*	*MYCBP*	*PLEKHA1*	*SGPL1*	*SLC2A14*	*TERT*	*USP42*	Spermatogenesis, positive regulation of flagellated sperm motility involved in capacitation
hsa-miR-2861	6.18234	*CLU*	*MEI1*							Spermatogenesis, spermatid development
hsa-miR-4466	6.06271	*AGO3*								Spermatogenesis, spermatid development
hsa-miR-2115-5p	5.9418	*AGO3*	*PYGO2*	*SLCO4C1*	*YY1*					Spermatogenesis, spermatid development, spermatid nucleus differentiation
hsa-miR-4516	5.90823	*ALKBH5*	*CCND2*	*PHC2*	*SLC4A2*	*SPATA6*	*TP53*			Spermatogenesis
hsa-miR-638*	5.89988	*TP53*								Spermatogenesis
hsa-miR-1237-5p	5.71559	*CLU*	*H2AFX*	*ERCC1*	*WSCD1*	*SBF1*	*SIX5*	*SPATA2*		Spermatogenesis, spermatid development
hsa-miR-6729-5p	5.70038	*ATAT1*	*CCNB1*	*HERPUD2*	*SF3A1*					Spermatogenesis
hsa-miR-3196	5.698	*H2AFX*	*WSCD1*	*SBF1*	*SPATA2*	*AMH*				Spermatogenesis, sperm capacitation
hsa-miR-8069	5.61643	*DICER1*								Spermatogonial cell division
hsa-miR-149-3p*	5.5248	*SPATA2*	*PHC2*	*TP53*	*YY1*	*PLEKHA1*	*STK11*	*CRTAP*	*UBE2B*	Spermatogenesis, spermatid development, Sperm axoneme assembly, sperm individualization
		GOLGA3	*METTL14*	*PLD6*	*TTLL1*	*TYRO3*	*ADRM1*	*CRKL*	*E2F1*	
		PARP11	*PPP2R2B*	*SPATA5*	*SPEM1*	*SREBF2*	*SSTR1*	*TESMIN*		
hsa-miR-6126	5.50762	*CLDN11*	*NR6A1*	*PIAS2*						Spermatogenesis
hsa-miR-8072	5.46156	*WSCD1*								Spermatogenesis
hsa-miR-6869-5p	5.44576	*HMGB2*	*APOB*	*HMGA2*	*OCA2*	*POC1A*	*ZFP37*			Spermatogenesis, spermatid development, spermatid nucleus differentiation
hsa-miR-4459	5.44443	*METTL14*	*TTLL1*	*TYRO3*	*CRKL*	*PPP2R2B*	*ERCC1*	*ALKBH5*	*SGPL1*	Spermatogenesis, spermatid development, sperm axoneme assembly, male germline cyst formation
		SHISA6	*HMGA1*	*JAM2*	*PHF7*	*ZNF35*	*GSR*	*ACOX1*	*ACTR1A*	
		BCL2L11	*CCDC36*	*DNM2*	*ELP3*	*MAK*	*MYBL1*	*NPAP1*	*PSME3*	
		SKIL								
hsa-miR-4484	5.39124	*CADM1*	*PEBP1*							Spermatogenesis, spermatid development, sperm capacitation

*The miRNA had been proved by other articles to be related to spermatogenesis. miRNA: micro ribonucleic acid

become permanently infertile after completing cancer treatment.[32] Harvesting SSCs before chemotherapy and reinjecting them into the testes after treatment can avoid the cytotoxic effects of chemotherapeutic drugs on SSCs.[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 *in vitro* before injection into the testes.[14] TECs can provide necessary

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	Lian *et al*.[21]
hsa-miR-149-3p	5.5248	Upregulated	Spermatozoa	NI *vs* NF	Salas-Huetos *et al*.[22]
hsa-miR-320a	2.38864	Upregulated	Spermatozoa	O *vs* N	Muñoz *et al*.[20]
		Downregulated	Seminal plasma	A *vs* N	Zhou *et al*.[72]
hsa-miR-1224-5p	1.84448	Upregulated	Testis	Cryptorchid tissue *vs* N	Tang *et al*.[73]
hsa-miR-1246	1.82239	Upregulated	Epididymis	Epididymis vasectomy *vs* no vasectomy	Belleannée *et al*.[74]
hsa-let-7b-5p	1.79294	Downregulated	Seminal plasma	A *vs* N	Zhou *et al*.[72]
hsa-miR-511	1.23289	Downregulated	Testis	SpF (NOA or severe O) *vs* Oaz	Muñoz *et al*.[20]
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: normozoopspermia infertile; NOA: nonobstructive azoospermia; O: oligozoospermia; Oaz: obstructive azoospermia; SCO: sertoli-cell-only syndrome; SpF: spermatogenic failure; miRNA: micro ribonucleic acid

growth factors for the self-renewal and expansion of SSCs, and provide more options for treatment.[13,14] 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 be focusing 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.[35] 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.[35–37] In addition, the cytoskeleton based on actin and microtube is necessary for the transport of both sperm and phagocytes across the spermatogenic epithelium in testes.[38–40] It was reported that gap junction is essential during spermatogenesis through the maintenance and differentiation of stem cells in the testis.[41,42] 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.[43–45] Spermatogenesis is tightly regulated by ubiquitination, acetylation, and proteasomal 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.

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.

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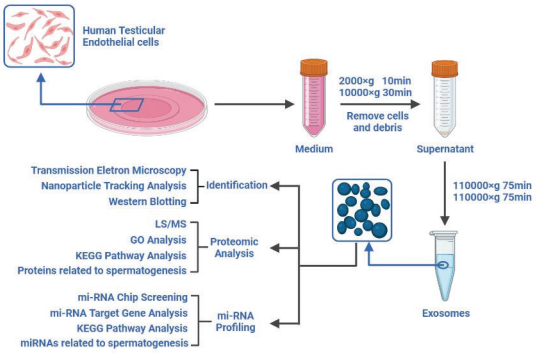

Supplementary Figure 1: HTEC-Exos isolation, identification, proteomic analysis, and miRNA profiling. HTEC-Exos: human testicular endothelial cell-derived exosomes; miRNA: microRNA.