Exosome-Derived microRNAs in Sertoli Cells Inhibit Spermatogonial Apoptosis

Huihui Gao¹, Heran Cao¹, Tianqi Jin¹, Guofan Peng¹, Yining Chen¹, Wenxian Zeng¹, Chao Zhu¹, Jian Du², Wuzi Dong¹*

¹ College of Animal Science and Technology, Northwest Agriculture and Forestry University, Yangling, China
² Center for Stem Cell Biology and Regenerative Medicine, Tsinghua University, Beijing, China

The first author: Huihui Gao E-mail: BerylGao2@163.com
The second author: Heran Cao E-mail: 392124735@qq.com
The third author: Tianqi Jin E-mail: 1491863424@qq.com
The fourth author: Guofan Peng E-mail: 1054181732@qq.com
The fifth author: Yining Chen E-mail: 3060755420@qq.com
The sixth author: Wenxian Zeng Email: zengwenxian2015@126.com
The seventh author: Chao Zhu Email: 1412851691@qq.com
The eighth author: Jian Du Email: duj17@mails.tsinghua.edu.cn

*Corresponding Author: Wuzi Dong Email: dongwuzi@nwsuaf.edu.cn

College of animal science and technology, Northwest Agriculture and Forestry University, No. 22 Xinong Road, Yangling, Shaanxi 712100, China
Tel. 86-15202967336
Fax. 86-029-87092164
Abstract

Background: Spermatogenesis is a highly complicated biological process that occurs in the epithelium of the seminiferous tubules. It is regulated by a complex network of endocrine and paracrine factors and juxtacrine testicular cross-talk. Sertoli cells (SCs) play a key role in spermatogenesis due to their production of trophic, differentiation and immune-modulating factors. However, many of the molecular pathways of SCs action remain controversial and unclear. Recently, research has focused on exosomes as an important mechanism of intercellular communication.

Results: We found that the exosomes derived from SCs (SC-Exos) significantly inhibited the apoptosis of the primary spermatogonial stem cells (SSCs). Total of 1016 miRNAs in SCs and 556 miRNAs in SC-Exos were detected using microRNA (miRNA) high-throughput sequencing. Further, 294 miRNAs were differentially expressed between SCs and SC-Exos. Based on the GO and KEGG analyses, the target genes of 37 (high-expressed in exosomes and RPM>1000) selected miRNAs were involved in multiple biological pathways, including the MAPK signaling pathway and PI3K/AKT signaling pathway. And miR-10b is one of the top ten exosomes with relatively large enrichment of microRNA. In addition, the overexpression of miR-10b down-regulated expression of the target KLF4 to reduce spermatogonial apoptosis in SSCs or C18-4 cell line.

Conclusions: The study indicated a large number of small RNAs loaded in exosomes was secreted form the donor SCs to target spermatogonial regulated the apoptosis. And miR-10b inhibits the apoptosis of spermatogonia through the target gene KLF4.

Keywords: Sertoli cells; exosomes; miRNAs; spermatogonial; apoptosis
Introduction

Mammalian spermatogenesis is a complex cellular differentiation process through which male spermatogonial stem cells (SSCs) develop sequentially into spermatogonia, spermatocytes, spermatids, and eventually spermatozoa. The testicular niche is the basic architectures of normal development and differentiation of SSCs during spermatogenesis [1]. The efficient production of sperm from spermatogonia requires the coordinated interplay between germ cells and various somatic cells making up the spermatogenic niche. The Sertoli cell (SC) is arguably the most important component of the testis niche by physically supporting SSCs and providing them with these extrinsic molecules [2]. SCs directly regulate development and differentiation of germ cells (GCs) by transferring signaling molecules into GCs via the niche. In rodent and other mammals, it had been proved that the extracellular components derived from SCs were essential for the self-renewal of SSCs [3]. Extracellular vesicle (EVs) from SCs take part in maintaining homeostasis of the niche of SSCs and regulating and differentiation of SSCs [4].

Exosomes are a subset of EVs, characterised spherical structures limited by a lipid bilayer with a size ranging from 30 to 200 nm in diameter [5]. They contain proteins, lipids, sugar, DNA, mRNA, non-coding RNA and are secreted by normal or sick cells as a means of communication [6]. Exosomes capture bioactive molecules responsible for direct stimulation and increased survival of target cells, transmission of infectious agents, and horizontal transfer of membrane and/or cargo molecules, which are enriched in specific proteins and nucleic acids. By transferring their cargo to recipient cells, they can alter the behavior of these cells [7]. It has the potential to identify unknown cellular and molecular mechanisms in intercellular communication and in organ homeostasis and disease. Exosomes from neural stem cells transmit TFN-γ to activate STAT1 signals in target cells [8]. GLB1L4 loaded in exosomes from caput epididymis could be transported to the cauda epididymis to regulate the sperm function [9]. Active Wnt proteins can be sorted in exosomes
traveled to target cell to stimulate downstream signal pathway activation [10]. Exosomes contained DNA released by topotecan-treated cancer cells activated dendritic cell via STING signaling [11]. Recently, non-coding small RNAs loaded in exosomes have been shown to play multiple roles in intercellular communication in neighboring or more distant recipient cells [12], including miRNA.

MiRNA as a type of small non-coding RNA of 19–23 nucleotides can be loaded in exosomes to participated in the intercellular communication by a variety of ways, for example, facilitating the invasion capacity of breast cancer cells [13], inducing differentiation of recipient monocytes [14], altering the niche of the cancer cell [15]. miR-133b loaded exosomes from mesenchymal stromal cells can be transferred the neural cells to improve neurite growth [16]. Let-7 microRNAs from metastatic gastric cancer cell can via exosomes into the extracellular environment to maintain oncogenesis[17]. Exosomal miR-92a can be released by leukemia cells into endothelial cell to enhance migration [18]. Sharma et al. demonstrated that epididymosomes, as exosome-like structures, can protect RNA from degradation [19]. The researches displayed that individual microRNA loaded exosomes can also act on target cells. There are a lot of researches have reported that miRNAs packaged in exosomes are involved in reproduction. Exosomes of seminal plasma are related to sperm maturation [20]. These miRNAs are associated with the expression of interleukin (IL-10 and IL-13), raising the possibility that exosomes play a role in the immunity of genital residents [21]. Molecular analysis showed that microRNAs Let-7a, Let-7b, miR-148a, miR-375 and miR-99a were enriched in seminal plasma derived exosomes from multiple human donors [21]. exosome-derived miR-92b-3p and miR-17-5p might be used to differentiate the pregnancy status as early as several days after insemination in pigs [22].

In the present study, the mouse primary SCs were in vitro cultured and the exosomes form SCs (SC-Exos) was purified by gradient hyper-centrifugation. And then effect of SC-Exos on spermatogonial stem cells was examined. MicroRNAs profiles from SCs and SC-Exos were
sequenced via high-throughput sequencing and the differences between exosomal microRNAs and Sertoli cellular microRNAs were comparatively analyzed by bioinformatics. Further, the function of the specific high-abundance exosomal microRNA was revealed by examining the expression of target gene which was identified involve in regulating proliferation and apoptosis of spermatogonial stem cells. The findings of this study would provide a new insight about the SCs regulating the niche homeostasis of spermatogonial stem cells via exosomes or exosomal microRNAs.

Materials and methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Northwest A&F University in China. The mice were housed in the specific pathogen free facility in 50% humidity at 22°C. The care and use of experimental animals completely conformed to the university animal welfare laws, guidelines, and policies.

Isolation and culture of Sertoli cells

SCs were isolated from 5-7 day old ICR mice according to the protocol previously described [23]. Briefly, after tunica albuginea was removed from testes under stereoscope, seminiferous tubules were detached gently and then washed 3 times with DPBS. For remove Leydig cells, the tubules were digested with 1 mg/mL collagenase IV (Invitrogen) for 3 h, and washed 3 times with DPBS. To obtain the single cell, the samples were sequentially digested with 0.25% Trypsin-EDTA (Gibco) for 5 min at 37°C and then the dispersed testicular cells were filtered through a 40-μm mesh. The single cell suspension was pelleted by centrifugation at 350 g for 5 min and resuspended in DMEM/F12 supplemented with 5% (v/v) FBS (Gibco). 5×10⁶ cells/mL was seeded into dishes and cultured in a 5% CO₂ incubator at 34°C.
To remove peritubular myoid cells (PMCs), the above cells (including SCs, GCs and PMCs) were cultured for 20 min and the no-adhering cells (mainly content SCs and GCs) was transferred into a new dish. Further to remove GCs, the weakly adhering cells were removed after the cells were cultured for 6 h and the adhering cells were seeded into new dish. The proceed was repeated twice.

**Sertoli cells proliferation**

The purified SCs (5×10^5 cells/mL) were cultured in DMEM/F12 containing 10% FBS and incubated at 34 °C and 5% CO₂. The number of SCs was calculated from a dish of SCs after digested with 0.25% Trypsin-EDTA (Gibco) every 12 h.

**Immunocytochemistry**

SCs (0.5-1×10^4 cells) were fixed with 4% paraformaldehyde for 20 min in a 96-well plate. Cells were washed 3 times in PBS and permeabilized for 10 min with 0.5% Triton X-100/PBS. After washing, the cells were blocked with 4% BSA for 1h, then incubate with primary antibodies, including anti-SOX9 (1:1000, Cat: ab185966, Abcam), anti-GATA4 (1:50, Cat: ab84593, Abcam), overnight at 4℃. Thereafter, cells were washed in PBS, incubated with secondary antibody, including goat anti-rabbit IgG/Alexa Fluor 488 (Sangon Biotech), donkey Anti-Rabbit IgG/TRITC (Sangon Biotech) for staining 1 h at room temperature. After washing 3 times with PBS, DAPI was used to counterstain the nuclei, and images were captured using a fluorescence microscope (Olympus IX-71; Tokyo, Japan).

**Isolation of exosome from SCs**

Exosomes from SCs were isolated as described previously with minor modifications [24]. 1.5×10^5/mL SCs were cultured in DMEM/F12 supplemented with 10% FBS until cells reach 60% confluence (about 36 h), and then the medium was replaced with DMEM/F12 supplemented with 10% KnockOut SR-Multi-Species (Thermo) and continually cultured 48 h. The 200 mL medium was
collected and centrifuged serially at 300 \( \times g \) for 10 min, 2000 \( \times g \) for 20 min, and 12,000 \( \times g \) for 20 min, followed by filtration through a 0.22-\( \mu \)m pore filter (Millipore) to remove SCs, the apoptotic bodies, cell debris and shed vesicles. Subsequently, the supernatant was ultracentrifuged at 120,000 \( \times g \) for 2 h to collect exosomes. And the pallets washed once in PBS by ultracentrifugation. Part of exosomal pallets preserved in liquid nitrogen for small RNA sequencing and the other part of pallets were resuspended in 200 \( \mu \)L PBS.

**Western blotting**

Proteins were prepared in cold RIPA with 1mM phenylmethylsulfonyl fluorid (PMSF) for 30 min on ice. Productions were centrifuged at 12,000g for 15 min, and the concentrations of supernatant were measured by BCA protein assay kit. Lysates were separated by SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking with 5.0% skim milk in TBST for 1 h, membranes were processed for CD63 primary antibodies (1:1000, Cat: ab59479, Abacam), caspase3(1:1000, Cat: ab13847, Abacam), cleaved caspase-3(1:1000, Cat: ab32042, Abacam), bax(1:500, Cat: WL01637, Wanleibio), bcl-2 (1:500, Cat: WL01556, Wanleibio), klf4(1:1000, Cat: ab214666, Abacam), beta-ACTIN(1:200, Cat: CW0096M, CWBIO) incubation overnight at 4°C. Then HRP-conjugated antibody was used as secondary antibodies. The bound antibodies were detected using an ECL kit (Amersham, Bioscience).

**Observe exosomes by electron microscopy**

For scanning electron microscopy (SEM), 5 \( \mu \)L of exosomes were added to a clean silicon slice and fixed with 2.5% glutaraldehyde in PBS for 15 min, washed twice with PBS. The exosomes were dehydrated in increasing concentrations of ethyl alcohol (35, 50, 70, 80, 90, and 100%) every 20 min. After air-drying under a ventilation hood, exosomes were post-fixed in 1% osmic acid for 1 h and then next, samples were subjected to a 10-min treatment with isoamyl acetate. Finally, the specimens
were sputter coated with palladium gold and viewed under a SEM. For transmission electron microscopy (TEM), samples of exosomes were observed using previously described methods [25].

Nanoparticle tracking analysis

Size distribution and quantification of exosome were analyzed by NanoSight NS3000 instrument (Malvern, England) as described previously [26]. Briefly, exosomes from SCs were diluted in 1mL of DPBS and disaggregated by using a syringe and needle. Then the sample was injected into the chamber and three individual samples were tested.

RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted from the testes, cells and exosomes using TRIzol reagent (Invitrogen) and reverse transcription was performed for cDNA using Takara Reverse Transcription kit according to the manufacturer’s instructions. sRNA-specific stem-loop RT primers were used to synthesize cDNA. The PCR mixture consisted of 1 μL of the cDNA sample, 10 μL primestar buffer, 4 μL dNTP mixture, 1μL of each primer (0.1mM), 1 μL primestar hs DNA polymerase and 33 μL water. The PCR reactions started at 98 °C for 3 min, and then denature at 98 °C for 30 s, anneal at 58-62 °C for 5 s and elongate at 72 °C for 30 s for 30 cycle.

Transcript level of klf4, cd63, sox9, α-sma, plzf and 3β-hsd were performed by PCR in a 20 μL volume, containing 10 μL 2×mix, 0.5 μL each of forward and reverse primers, 1 μL cDNA, and 8 μL ddH2O. The PCR protocol was as follows: initial denaturation for 5 min at 98 °C; followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 30 s at 72 °C and a final extension for 10 min at 72 °C, with subsequent cooling to 4 °C. The products were performed by agarose gel electrophoresis. gapdh gene was used as internal control gene for mRNA expression normalization.
TaqMan MicroRNA assays were used to quantify the level of mature sRNAs. The 20 µl volume included 1 µl RT product, 10 µl of 2 × TaqMan Universal PCR Master Mix, 0.4 µM TaqMan probe, 3 µM forward primer and 1.5 µM reverse primer. The reactions were started at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate. The results were performed via the $2^{-\Delta\Delta CT}$ method in terms of the protocol as described previously [27]. The CEL-miR-39 spike-in control (GenePharma, China) was used as additional internal reference for sRNA expression normalization in exosomes and SCs [28, 29]. For this purpose, 50 fmol of CEL-miR-39 was added to the RT reaction and was measured by qPCR using specific primers. Differences were considered statistically different at $p < 0.05$ and analysis were performed using $t$-test. All of the primers are presented in Sup: Table S1.

**Bioinformatics Analysis**

Total RNA was isolated from SCs and exosomes from the SCs, using the Trizol reagent (Takara, Japan), following the manufacturer's instructions. The quantity and quality of the total RNA were determined using a NanoDrop® 174 ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) at 260/280 nm (ratio $> 2.0$), and its integrity was tested using 2100 Bioanalyzer and RNA 6000 Nano LabChip® 176 Kit (Agilent, USA) with an RNA integrity number greater than 8.0. The sample sequenced in the Illumina HiSeq 2500 system (Illumina, USA) following the vendor's instructions. Three batches of RNA samples of SCs and exosome were prepared for sequencing. The original image data obtained by the Illumina HiSeq™ 2500 sequencing analyzer were automatically transformed into raw reads using base calling. After eliminating adaptor sequences, low-quality reads, sequences smaller than 17 bp and reads with no insertion, clean reads were obtained and used for further analysis. The sequences were mapped genome using BWA software [30], classified using unitas tool [31] by comparing them with the following non-coding RNAs that are deposited in Ensembl [32], miRbase [33], GtRNAdb [34] and SILVA rRNA database [35]. We also compared
small RNAs expression levels between the SCs and exosomes and the number of reads per million (RPM) clean tags was calculated. Target genes of selected miRNAs were predicted by miRanda [36], PITA [37] and RNAhybrid [38]. Then the predicted target genes were analyzed by DAVID [39] for Gene Ontology (GO) annotation and KOBAS software [40] for KEGG pathway analysis.

Cell transfection

Before transfection, 1.5×10^5 cells were seeded on plates (6-well) with fresh complete DMEM/F12 medium. The commercial Lipofectamine® 2000 reagent (Lipo2000) was used according to the manufacturer’s instruction. MiR-10b mimics were assembled in serum-free Opti-MEM medium at 37 °C for 30 min. The final concentration of the mimics was adjusted to 50 nM with complementing Opti-MEM medium. After 6h of incubation, the transfection mixture was replaced with the complete medium and maintained for further incubation up to 48 h. The free naked NC mimics was used as a negative control.

TUNEL assay

To detect cell apoptosis, the cells were seeded on 96-well plates. After transfection, cell apoptosis was detected using a TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Jiangsu, China, A113-01). The TUNEL assay was performed 48h after transfection in spermatogonial, following the manufacturer’s instructions. In brief, cells were fixed in 4% paraformaldehyde and permeabilized for 10 min using 0.1% Triton-X100 solution at room temperature. Then, the cells were incubated with 50 μl TdT incubation buffer at 37 °C for 1 h in darkness. Finally, cells were stained with DAPI and evaluated under a fluorescent microscope (Olympus, Tokyo, Japan). The ratio of the TUNEL-positive cells (red fluorescence) indicated the spermatogonial apoptosis. Experiments were repeated three times, and each time we randomly selected 5 fields for statistics.
Spermatogonial cells were plated in 96-well plates and cultured for 24 hours. After this, CCK8 solution (Beyotime, Shanghai, China) was added to each plate, and the absorbance at 490 nm was measured using a microplate reader.

**Annexin V-FITC/PI Flow Cytometry Assay**

For Annexin V/PI analysis, Dead Cell Apoptosis Kit with Annexin V FITC and PI, for flow cytometry (40305ES20, YEASEN, China) was used. Cells were collected by centrifuged and washed with PBS. The supernatant was removed and stained with Annexin V-Alexa 488 and PI in binding buffer for 20 min. All experiments were tested in Flow cytometer.

**Results**

1. **Culture of the Sertoli cells and identification of its’ exosomes**

The purified SCs from testes of 5-7 dpn mouse exhibited typical morphology of epithelial cells and strong growth activity by a serial of different plating (Figure 1A). RT-PCR results displayed that the purified cells expressed *sox9* (a marker for SCs), *cd63* (a marker for exosomes), while not *plzf* (a marker for spermatogonia) and *3β-hsd* (a marker for Leydig cells) (Figure 1B). And the immunofluorescence experimental showed that the SOX9 and GATA4 positive cells were more than 95% (Figure 1C). The SCs were cultured for following experiments.

The EVs were isolated from the medium by a serial of gradient ultracentrifugation after SCs were cultured in DMEM/F12 medium supplemented with 10% KnockOut SR-Multi-Species for 48 h (Figure 1A). The EVs were circular double-layer membrane vesicles by SEM and TEM (Figure 2A and 2B). the diameter size of EVs is about 122.5 ± 4.1 nm, the size of most EVs was at 100 nm by the Nanosight assay and the concentration of EVs was about $10^{12}$ particles/mL. (Figure 2C).
Meanwhile, CD63 and HSP70 were detected in the EVs by Western blot (Figure 2D). The results showed that the EVs from *in vitro* primary SCs were a typical class of exosomes.

**2. Exosomes from Sertoli cells maintain spermatogonial cells survival**

To investigate the possibility communication between SCs and SSCs via exosomes, we performed an *in vitro* transwell-membrane model to culture donor cells (primary SCs), and receptor cells (primary spermatogonial cells or C18-4 cell line). In the context of transwell system, both of the Dil-labeled SCs on 0.4 μm transwell membrane and the spermatogonial cells or C18-4 cells on the dish could be grown well. The red fluorescence on spermatogonial cells or C18-4 cells was observed after the cells were culture for 48 h in saturation humidity incubator at 35 °C and 5% CO₂. The experiments showed that the DiI-labeled substances from Sertoli cells can penetrate the 0.4 μm transwell membrane and be transferred to recipient cells through culture medium (Sup: Figure 1). The final concentration 100 ng/μL DiI-labeled exosomes from SCs were added into serum-free DME/F-12 medium for culturing primary spermatogonial cells or C18-4 cells. The fluorescence (DiI-labeled exosomes) was detected as a granular pattern surrounding cells (Figure 3A). These experiments indicated the exosomes secreted by mouse SCs can target the spermatogonial cells.

Comparing with control (the medium without exosomes), spermatogonial cells or C18-4 cells added exosomes are in better state that the cells are plump and smooth after 4 d of culture, the cell viability increased by CCK8 analysis (Figure 3B). The rates of apoptosis of primary spermatogonial cells and C18-4 cells treated with exosomes were significantly down-regulated by Annexin V/FITC staining (Figure 3C). However, effect of exosomes on proliferation of C18-4 cell intimated no significant by the quantitative analysis of EdU staining (Sup: Figure 2). In conclusion, exosomes secreted from SCs can target spermatogonia to inhibit apoptosis, but no effect on proliferation.

**3. Small RNA profiles of Sertoli cells and exosomes**
Exosomes from specific cells are involved in regulating the function of receptor cells through sorting of specific miRNAs. Small RNA transcriptomes from SCs and the exosomes were sequenced by means of Illumina HiSeq™ 2500 platform and the data were analyzed by bioinformatics. The number of raw reads of the SCs were 14,271,751 and the exosomes were 15,549,820, respectively. After removing low-quality sequences, smaller sequences, contaminants which were formed by adapter-adapter ligation, the clean reads obtained from the SCs were 13,046,249 and from exosomes libraries were 14,348,194 (Table 1). The mapped of reads were 12,586,353 (96.47% of clean reads) and 11,286,869 (78.66% of clean reads) for samples of SCs and exosomes, respectively.

Pearson correlation analysis showed a moderate correlation between the SCs and exosomes (correlation coefficient, 0.733) (Figure 4A). The sequences common by SCs and exosomes were 3.59% (32,630), cell-specific were 65.26% (592,652), and exosomes-specific were 31.15% (282,834) (Figure 4B). Analyzed the size distribution between 17 and 40 nt of reads, showed that 3 peaks at 21–23 nt for both SCs and exosomes. In addition, exosomes had a peak at 33 nt (Figure 4C). After mapping to mouse genome, the proportion of annotated miRNAs was 15.8% in SCs, and 6.3% in exosomes. The annotated tRNAs accounted for 2.5% in SCs, 1% in exosomes. The rRNA in exosomes was 4.5% more than in SCs (8.4% in exosomes, 3.9% in SCs) (Figure 4D).

4. Comparing analysis of miRNAs between the Sertoli cells and its’ exosomes

The sequencing results displayed that there were total 1016 miRNAs in SCs and total 556 miRNAs in exosomes. 524 miRNAs of them were shared in SCs and exosomes (Figure 5A). Besides, we found that 32 miRNAs were only detected in exosomes and 492 miRNAs in SCs were not sorted into exosome (Figure 5A, Table 2).

Comparing the expression of miRNAs between the SCs and exosomes ($p$ value < 0.05 and fold change > 2), a total 294 miRNAs were differentially expression between the SCs and exosomes.
of which were more highly expressed in SCs and 91 of which were more highly expressed in the
exosomes (Figure 5B and Sup: Table S2).

Furthermore, analyzing reads per million (RPM) of miRNAs, the expression abundance of the
top 10 miRNAs reached 82.33% and 89.23% of total miRNAs RPM in SCs and in exosome,
respectively. Comparing the top 10 miRNAs between in SCs and in exosome, five of them in
exosomes did not appear in the top 10 miRNAs of SCs (such as miR-10b-5p), although other five
miRNAs in SCs occupied in the top 10 miRNAs of exosomes (Figure 5C and 5D).

5. GO annotation and KEGG pathway analysis of the target genes of the high-expression
expressed miRNAs in exosomes

To understand the function of miRNAs in exosomes, the target genes of the high-expressed
miRNAs (RPM>1000) were predicted 3,809 (Sup: Table S3). The target genes of the high-expression
expressed miRNAs in exosomes were analyzed by GO annotation and KEGG pathway analysis (Sup:
Table S4 and S5). The results identified the more target genes of the candidate miRNAs were
associated with cellular component and biological process by GO annotation (Figure 6A). In KEGG
pathway annotation, the number of miRNAs-target genes were mainly involved in the axon guidance,
pathways in cancer, FoxO signaling pathway, cell cycle, proteoglycans in cancer, Hepatitis B,
metabolic pathways, PI3K-Akt signaling pathway, endocytosis and MAPK signaling pathway
(Figure 6B).

6. Experimental validation of miRNAs expression

In this study, the expression levels of the differentially expressed miRNAs showed similar
trends between the RNA-seq and RT-qPCR results (Figure 7). A total of 8 uniquely expressed and
differently expressed miRNAs that identified from miRNA-seq were selected for validation using
stem-loop RT-qPCR (Figure 7). Four DE miRNAs showed similar trend as detected by RNA-seq. Specially, the expressions of miR-26a-5p and let-7i-5p were highly expressed in the SCs (Figure 7A and 7B), while miR-148a-3p and miR-10b-5p were highly expressed in the exosomes ($p<0.05$) (Figure 7C and 7D). miR-10b-5p seemed to be more readily enriched by exosomes. Meanwhile, miR-135b-5p, miR-221-5p and miR-30b-3p only detected in SCs (the results not shown). However, the expression of miR-6538 could not be detected in both exosomes and SCs by RT-qPCR (CT value $<35$), which may be due to their low abundance and the relative low content of miRNAs in SCs and exosomes (Table 2).

7. Exosomal miR-10b derived from SCs inhibited SSCs apoptosis and targeted KLF4

The expression of miR-10b was significantly higher in exosomes than in SCs (Sup: Figure 2). Interestingly, there are reports miRNA-10b inhibits apoptosis of SSC through Kruppel-like factor 4 (KLF4) [41]. To corroborate the biological function of miR-10b in spermatogonial, we transfected miRNA-10b mimics to C18-4 cells for 48h, the transfection efficiency was up to 50 times higher (Figure 8A). To determine whether exosomal miR-10b inhibits SSCs apoptosis, we performed CCK-8 and TUNEL assays, and showed that the survival of SSCs transfected with miR-10b mimics was significantly increased ($p<0.01$) compared with those transfected with NC (Figure 8B and 8C). The pro-apoptotic protein BAX was downregulated while the anti-apoptotic protein BCL2 was upregulated. Meanwhile, the cleavage of caspase-3 was decreased after miR-10b treatment 48h (Figure 8D). These data confirm that miR-10b inhibits SSCs apoptosis. According to the previous reports [41], the qPCR and western blot were executed to analysis the expression of Klf4 in miR-10b overexpression cells, the result showed miR-10b overexpression significantly reduced the expression of Klf4 (Figure 8E-8F).
Discussion

The mouse testicular seminiferous tubule mainly contains SCs, PMCs and GCs. The SCs is the only somatic cells in the spermatogenic epithelium. Sertoli cells secrete cytokines that acts on SSCs participating in the composition of niche to regulated the physiological function of SSCs. The successful isolation and purification of the SCs in vitro is very important for the study of the effect of SCs on spermatogonia. Whereas, in order to obtain SCs, the PMCs and GCs must be removed. It has been reported the methods of isolating and purifying SCs including fluorescence-activated cell sorting (FACS) [42-44], velocity sedimentation separation using STA-PUT chambers [45], serial of different plating [46]. In our study, the GATA+/SOX9+ SCs was enriched up to >95% by the improved method of different plating.

Exosomes are the nanosized membrane-bound delivery vesicles, which play a critical role in regulating cellular biofunction by mediating cell-to-cell communication. Mesenchymal stem cell-derived exosomes protect skin from oxidative stress-induced skin damage through adaptive regulation of Nrf2 defense system [47]. Exosomes extracted from mesenchymal stem cell ameliorate cardiomyocyte apoptosis through by miR-144 targeting the PTEN/AKT pathway [48]. In contrast, vascular endothelial cell-secreted exosomes accelerate the development of osteoarthritis by promoting chondrocyte apoptosis [49]. Additionally, The role of exosomes in reproduction has been reported in many literatures, seminal plasma exosomes combined with sperm maintain sperm function by infiltrating into the sperm membrane [50], GLB1L4 from rat caput epididymis could be transported to the cauda epididymis to regulate the sperm function by exosomes [9]. Seminal plasma-derived exosomes restrict infection by limiting viral replication in the reproductive tract [51]. In our research, after spermatogonia treated with exosomes secreted by SCs, proportion of apoptotic cells was significantly reduced, but the quantity of proliferation did not.
Many observations have been reported small RNAs could be transferred between cells via exosome [52]. The small RNAs in cell-released exosomes can circulate with the associated vehicles to reach neighboring cells and distant cells. After being delivered into acceptor cells, exosomal small RNAs play functional roles. Although it is difficult to completely exclude the effects of other exosomal cargos on recipient cells, small RNAs are considered the key functional elements. In our study, the results showed the presence of some highly expressed sRNAs in exosomes, i.e., miR-148a, let-7 miRNA family, miR-99a, which may be involved in the regulation of target cell function. Similarly, Ohshima et al. found that members of the let-7 miRNA family are abundant in exosomes derived from the gastric cancer cell line AZ-P7a [53], same with the exosomes derived from SCs. MiR-148a was expressed in exosomes dataset with high proportion. The number of target genes for miRNA-148a were 116 including Hbs1l, Atg12. There have been reports that miR-148a promotes apoptosis and cell proliferation by targeting Bcl-2 in colorectal cancer [52] and p27 in gastric cancer cells [53], respectively. Besides, miR-99a, the abundant miRNA in SCs and exosomes, modulate TGF-β induced epithelial to mesenchymal plasticity in normal murine mammary gland cells [54]. Therefore, the exosomes secreted from SCs could have important implications for regulate recipient cells. Some of top abundance miRNAs detected in exosomes were not abundant ones in SCs, especially the high-expression small RNA in exosomes, the inconsistence in small RNA spectrums might participate in important spermatogenesis, reflect different aspects of physiological and pathological conditions in testes. Furthermore, GO and KEGG based target prediction showed small RNAs potential roles in biological process, cellular component, human diseases and organismal systems. However, these predictions need further functional confirmation. Clearly, once transferred into target cells via exosomes, the highly enriched small RNAs may participate directly regulation of mRNA translation and influence cell functions. In our prediction, miRNAs from SCs might participate in the different processes and they could regulate the genes expression targeting to testicular cells by exosomes.
Recent researches have indicated that miR-10b involved in regulating cell proliferation and apoptosis. MiR-10b promote myoblasts proliferation [55], antagonizes hypoxia-induced cardiomyocyte apoptosis[56], meantime, exosomal miR-10b in early-stage hepatocellular carcinoma promote cancer cell proliferation [57]. Another study had shown microRNA-10b regulates the renewal of SSCs through KLF4 [41]. Our results demonstrated that miR-10b is relatively more highly expressed in exosomes than in SCs. We also validated the functionality of the miR-10b in SSCs, the results displayed miR-10b inhibit spermatogonial apoptosis. Conversely, it has been reported that miR-10b can promote the proliferation of SSC [41], but in our experimental the proliferation of SSC was not regulated by exosomes, which could be because the composition of exosomes is more complex, and there are other miRNAs masking the effect of miR-10b. The results also showed that miR-10b inhibits the expression of KLF4 gene mRNA and protein levels. It indicated that miR-10b may exert its biological function through targeting KLF4 after being delivered to spermatogonia by exosomes. Study has shown that KLF4 as a transcription factor can induce the totipotency of spermatogonial stem cells, implying the important role of KLF4 in germ cells [58]. In addition, miR-9-5p downregulates KLF4 to inhibit the apoptosis of liver cancer cells through the AKT signaling pathway [59]. KLF4 could also act on the MAPK signaling pathway through ectopic expression to promote the apoptosis of melanoma cells [60]. In this experiment, the enriched significantly different signaling pathways include PI3K/AKT and MAPK signaling pathways. This indicates that KLF4 may affect spermatogonia apoptosis through PI3K/AKT and MAPK signaling pathways.

Conclusion

In conclusion, mouse SCs delivered exosomes protected the spermatogonia by decreasing cell apoptosis, and this protective effect was partly generated through the exosome miRNAs by RNA sequencing. GO and KEGG analyses showed that the significant difference signal pathways include
PI3K/AKT and MAPK signal pathways which involved in regulating the apoptosis of spermatogonia. The results also suggest that miR-10b inhibits the apoptosis of spermatogonia through the target gene KLF4 (Figure 9).

Abbreviations

SCs: Sertoli cells; SSCs: spermatogonial stem cells; GCs: germ cells; EVs: extracellular vesicles; tsRNA: transfer-RNA-derived small RNA; PMCs: peritubular myoid cells; PMSF: phenylmethylsulfonyl fluorid; SEM: scanning electron microscopy; TEM: transmission electron microscopy; RPM: reads per million; FACS: fluorescence-activated cell sorting; MVs: microvesicles; KLF4: Kruppel-like factor 4

Ethics approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Northwest A&F University in China. The care and use of experimental animals completely conformed to the university animal welfare laws, guidelines, and policies.

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study can be obtained from the corresponding author upon request.
Competing interests

The authors have no conflicts of interest to declare.

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Authors’ contributions

WD designed this study. HG wrote the manuscript and analyze the data. HC performed the experiments on cell cultures and the isolated of exosomes. TJ performed the RT-qPCR to analysis the expression of genes and sRNAs, Western blot to authenticate of the exosomes. GP and YC tested the collected exosomes including SEM, TEM and nanosight assay. WZ provided guidance for the program. CZ performed the immunofluorescence analysis. The animals were feeding by JD. All authors contributed to the report and approved the final version of the manuscript.

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References

1. Oatley, J.M. and R.L. Brinster, The germline stem cell niche unit in mammalian testes. Physiol Rev, 2012. 92(2): p. 577-95.
2. Griswold, M.D., The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol, 1998. 9(4): p. 411-6.
3. Chen, S.R. and Y.X. Liu, Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli...
cell signaling. Reproduction, 2015. 149(4): p. R159-67.
458
4. Mancuso, F., et al., Testosterone and FSH modulate Sertoli cell extracellular secretion: Proteomic analysis. Mol Cell Endocrinol, 2018. 476: p. 1-7.
459
5. Taylor, D.D., S. Akyo, and C. Gercel-Taylor, Pregnancy-Associated Exosomes and Their Modulation of T Cell Signaling (Retraction of vol 176, pg 1534, 2006). Journal of Immunology, 2015. 194(12): p. 6190-6190.
461
6. Thery, C., L. Zitvogel, and S. Amigorena, Exosomes: composition, biogenesis and function. Nat Rev Immunol, 2002. 2(8): p. 569-79.
463
7. Kalluri, R. and V.S. LeBleu, The biology, function, and biomedical applications of exosomes. Science, 2020. 367(6478).
465
8. Cossetti, C., et al., Extracellular vesicles from neural stem cells transfer IFN-gamma via IFngr1 to activate Stat1 signaling in target cells. Mol Cell, 2014. 56(2): p. 193-204.
467
9. Dong, D., et al., Palmitoylated GLB1L4 transfers via exosomes to maintain sperm function in rat epididymis. Reproduction, 2021. 161(2): p. 159-172.
469
10. Gross, J.C., et al., Active Wnt proteins are secreted on exosomes. Nat Cell Biol, 2012. 14(10): p. 1036-45.
471
11. Kitai, Y., et al., DNA-Containing Exosomes Derived from Cancer Cells Treated with Topotecan Activate a STING-Dependent Pathway and Reinforce Antitumor Immunity. J Immunol, 2017. 198(4): p. 1649-1659.
473
12. Janas, T., et al., Mechanisms of RNA loading into exosomes. FBS Lett, 2015. 589(13): p. 1391-8.
475
13. Yang, M., et al., Microvesicles secreted by macrophages shuttle invasion-potentiating miRNA into breast cancer cells. Mol Cancer, 2011. 10: p. 117.
477
14. Ismail, N., et al., Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. Blood, 2013. 121(6): p. 984-95.
479
15. Kosaka, N., et al., Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. J Biol Chem, 2013. 288(15): p. 10849-59.
481
16. Xin, H., et al., Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. Stem Cells, 2012. 30(7): p. 1556-64.
483
17. Ohshima, K., et al., Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS One, 2010. 5(10): p. e13247.
485
18. Umez, T., et al., Leukemia cell to endothelial cell communication via exosomal miRNAs. Oncogene, 2013. 32(22): p. 2747-55.
487
19. Sharma, U., et al., Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science, 2016. 351(6271): p. 391-396.
489
20. Sullivan, R., et al., Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis, 2005. 35(1): p. 1-10.
491
21. Vojtech, L., et al., Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. Nucleic Acids Res, 2014. 42(11): p. 7290-304.
493
22. Zhou, C., et al., Deep-sequencing Identification of MicroRNA Biomarkers in Serum Exosomes for Early Pig Pregnancy. Front Genet, 2020. 11: p. 536.
495
23. Chang, Y.F., et al., Isolation of Sertoli, Leydig, and spermatogenic cells from the mouse testis. Biotechniques, 2011. 51(5): p. 341-2, 344.
497
24. Greening, D.W., et al., A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods Mol Biol, 2015. 1295: p. 179-209.
499
25. Jung, M.K. and J.Y. Mun, Sample Preparation and Imaging of Exosomes by Transmission Electron Microscopy. J Vis Exp, 2018(131).
501
26. Mazzeo, C., et al., Exosome secretion by eosinophils: A possible role in asthma pathogenesis. J Allergy Clin Immunol, 2015. 135(6): p. 1603-13.
503
27. He, Z., et al., Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod, 2007. 77(4): p. 723-33.
505
28. Kosaka, N., et al., Secretary mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem, 2010. 285(23): p. 17442-52.
507
29. Foss, K.M., et al., mir-1254 and miR-574-5p: serum-based microRNA biomarkers for early-stage non-small cell lung cancer. J Thorac Oncol, 2011. 6(3): p. 482-8.
509
30. Li, H. and R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics, 2009. 25(14): p. 1754-60.
511
31. Gebert, D., C. Hewel, and D. Rosenkranz, unitas: the universal tool for annotation of small RNAs. BMC Genomics, 2017. 18(1): p. 644.
Acidic Microenvironment Up-Regulates Exosomal miR-21 and miR-10b in Early-Stage

miR-10b-5p Regulates C2C12 Myoblasts Proliferation and Differentiation.

MIR-99a and MIR-99b modulate TGF-beta induced epithelial to mesenchymal plasticity in gastric cancer cells.

Boar seminal plasma exosomes maintain sperm function by infiltrating into the sperm membrane.

Vascular endothelial cell-secreted exosomes facilitate osteoarthritis pathogenesis by promoting cell proliferation by targeting p27 in gastric cancer cells.

Fast and effective prediction of microRNA/target duplexes.

Nat Genet, 2007.

via the AKT signaling pathway.

pluripotency by the transcription factors Oct3/4, c-Myc, Sox2 and Klf4.

1965-1979.

Hepatocellular Carcinoma to Promote Cancer Cell Proliferation and Metastasis.

8 Assoc, 2019.

83 Biochem, 2019.

7 Oncotarget, 2016.

18 Virology, 2015.

257 Biomaterials, 2020.

13 Aging (Albany NY), 2021.

144 BMC Genomics, 2018 (Database issue): p. 1-12.

394 J Vis Exp, 2016 (1): p. e53389.

365 Cell Regul, 2013 (5): p. 120264.

3156 J Cell Physiol, 2007 (18): p. 1140-6.

3527 PLoS One, 2012 (7): p. e31032.

3528 Mol Cell Biol, 2012 (32): p. 5767-76.

3529 J Biol Chem, 2004 (279): p. 33536-43.

3530 Cell Death Differ, 2011 (18): p. 1417-1429.

3531 Mol Cell, 2002 (9): p. 464-75.

3532 Mol Cell Biol, 2001 (21): p. 8920-32.

3533 Nat Immunol, 2002 (3): p. 350-6.

3534 Cell Death Differ, 2007 (14): p. 1927-36.
KLF4 is regulated by RAS/RAF/MEK/ERK signaling through E2F1 and promotes melanoma cell growth. Oncogene, 2017. 36(23): p. 3322-3333.

Figure legends

**Figure 1. Separation and purification of primary Sertoli cells** (A) The morphology of cultured primary SCs and the proliferation curve of SCs in DMEM/F12 supplemented with 10% (v/v) FBS. Verified the cell purity by PCR(B), immunocytochemical (C), C: Sertoli cells, T: testes, M: marker. Data are presented as Mean ± Standard deviation. Scale bar, 100 μm.

**Figure 2. SC-exosomes characterization** TEM (A) and SEM(B) images of SC-exosomes showing a homogeneous population of exosomes; (C) Western blot analysis of common exosome markers, where 20 μg of total protein was loaded in each lane; (D) Hydrodynamic size distribution of SC-exosomes measured by nanosight. Scale bar, 100 nm.

**Figure 3. SC-exosomes target spermatogonia to inhibit apoptosis.** (A) Dil-exosomes incubation spermatogonia after 12h, the positive signal was detected. Control: Dil incubation spermatogonia. (B) The viability of spermatogonia after exosome processing. (C) Annexin V/FITC staining of apoptotic
cells after exosomes treatment. Data are presented as Mean ± Standard deviation. ** p <0.01; Scale bar, 100 μm.

**Figure 4. Profiling of small RNAs in mouse Sertoli cells and exosomes samples.** (A) Correlation analysis of the small RNAs in SCs and exosomes; (B) Venn diagram showing the profile of kinds of small RNAs expressed in SCs and exosomes; (C) Size and frequency distribution of detected small RNAs (19-40nt); (D) The relative abundance of different classes of small RNAs.

**Figure 5. Profiling of miRNAs in mouse Sertoli cells and exosomes samples.** (A) Venn diagram showing the profile of miRNAs expressed in SCs and exosomes; (B) Volcano plot generated by clustering of the variable miRNAs in SCs and exosomes; (C-D) The pie chart of ten most abundant miRNAs. C: Sertoli cells. D: Exosomes. MiRNAs differed in two samples were colored and marked.

**Figure 6. Gene ontology and KEGG analysis of the predicted targets for high expressed miRNAs in exosomes.** The targeted genes of high expressed miRNAs (A) is analyzed in this figure. The right-hand-side scale is the targeted gene numbers corresponding to the GO terms. The left-hand-side scale is the -log10 (P-Value) of GO terms. Ten GO terms for each process are listed. (B) KEGG analysis of the 30 most enriched pathways. The right-hand-side scale is the targeted gene numbers corresponding to the KEGG pathway. The left-hand-side scale is the -log10 (P-Value) of KEGG pathway.

**Figure 7. Expression of selected different expression miRNAs between Sertoli cells and exosomes detected by RT-qPCR and miRNA-seq.** (A) Expression of miR-26a-5p; (B) Expression of let-7i-5p; (C) Expression of miR-148a-3p; (D) Expression of miR-10b-5p; Small RNAs expression from RT-qPCR represented by lines on the top and values are shown on the left vertical axis as relative abundance. Small RNA expression from miRNA-seq represented by bars on the bottom and
values are shown on the right vertical axis as RPM (normalized reads number). Data are presented as Mean ± Standard deviation. *, p<0.05, ** p<0.01.

Figure 8. miR-10b inhibits spermatogonia inhibit by targeting KLF4. (A) The expression of miR-10b after transfected with mimics (B) CCK-8 was used to detect the effect of miR-10b on spermatogonia. (C) The statistical results and representative figures of the effect of miR-10b on the apoptosis of spermatogonia as detected by TUNEL. (D) Western blot showing the apoptotic markers after miR-10b treatment. (E-F) The expression KLF4 detected by RT-PCR and Western blot. Data are presented as Mean ± Standard deviation. ** p<0.01; Scale bar, 100 μm.

Figure 9. Exosome-derived miR-10b in sertoli cells inhibit spermatogonial apoptosis by targeting KLF4. Sertoli cells deliver miR-10b to spermatogonia via exosomes. miR-10b inhibits spermatogonia apoptosis by targeting KLF4, leading to the down-regulation of the apoptosis-related gene BAX and Caspase-3.

Additional files

Supplement Figure 1: DiI-exosomes incubation spermatogonia after 12h, the positive signal was detected. Control: DiI incubation spermatogonia.

Supplement Figure 2: C18-4 cells were incubated with SC-exosomes, and the control group (NC) was cultured for the same medium without addition exosomes. Representative figures from 12 hours after cultivation. Scale bar, 100 μm.

Supplement file 1: Table S1. Stem-loop RT-PCR and qPCR primers of miRNAs (XLS 26KB).
Supplement file 2: Table S2. The basic expression data of miRNAs in Sertoli cells and exosomes (XLS 227KB).

Supplement file 3: Table S3. Predicted targets of exosomes miRNAs (XLS 56KB).

Supplement file 6: Table S4. The GO annotation of exosomes miRNAs (XLS 3010KB).

Supplement file 7: Table S5. The KEGG pathway analysis of exosomes miRNAs (XLS 103KB).