

MiR-150-5p-modified Bone Marrow Mesenchymal Stem Cells Derived Exosomes Ameliorate Osteonecrosis of Femoral Head by Promoting Endothelial Cell Angiogenesis

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Research

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Abstract

Background: Osteonecrosis of femoral head (ONFH) is a common ischemic disease that induces femoral head necrosis. The role of exosomes and miRNA in ONFH has been elucidated, however, whether miRNA-modified exosomes improve the therapy of ONFH is not clear.

Methods: We screened ONFH-related miRNAs by RNA sequencing in plasma exosomes of ONFH patients and healthy donors. The key miRNA was overexpressed in bone marrow mesenchymal stem cells (BMSC) exosomes. The regulatory functions of miRNA-modified BMSC exosomes in vascular endothelial cells were illustrated through angiogenesis assay and scratch assay.

Results: We identified 9 differently expressed miRNAs (DEmiRNAs) in plasma exosomes between ONFH and healthy groups, with 6 up-regulated and 3 down-regulated miRNAs. Function and pathway analysis revealed DEmiRNAs were primarily involved in angiogenesis, cell migration, focal adhesion. Moreover, miR-150-5p was declined in ONFH exosomes and regulated multiple angiogenesis-related pathways. The miR-150-5p-overexpressed BMSC exosomes were successfully obtained and transported miR-150-5p to endothelial cells. Moreover, the miR-150-5p-modified BMSC exosomes promoted the angiogenesis and migration of endothelial cells.

Conclusion: Our results elucidate the exosomal miRNA expression profiles in ONFH, and miR-150-5p-modified BMSC exosomes protect against ONFH by promoting angiogenesis, suggesting a new molecular knowledge for the clinical application of ONFH.

Introduction

Osteonecrosis of femoral head (ONFH), also known as avascular necrosis of the femoral head, is a complicated skeleton disorder that shows a high disability rate [1]. ONFH is characterized by a femoral head cells apoptosis and accompanied by diminished function of the hip joint [2]. ONFH is a progressive disease which occurs primarily in young adults and may lead to the deformation or even collapse of the femoral head [3]. Various macroscopic risks and causative factors are closely linked to the occurrence of ONFH, including trauma disrupting, blood dyscrasias, corticosteroids usage, hyperlipidemia, excessive alcoholism, and other miscellaneous [4]. Currently, treatments for ONFH is based on condition develops through different stages to perform operatively and non-operatively. Conservative therapy includes pharmacologic agents, biophysical treatments, with femoral head replacement procedures (FHRP) and femoral head sparing procedures (FHSP) for advanced deterioration [1,5]. However, ONFH is still a major problem for no complete cure possibilities. ONFH originates from the cellular level and carries a poor prognosis, a precise and effective therapy in clinical is needed.

Bone mesenchymal stem cells (BMSCs), nonhematopoietic pluripotent stromal cells, which can differentiate into a variety of cell types as well as can promote cell regeneration [6,7]. Previous studies have reported that the failure of bone repair and reconstruction during osteonecrosis is mainly caused by abnormal osteogenesis and adipogenesis of BMSCs [8]. At present, in the treatment of femoral head

necrosis, mesenchymal stem cells (MSCs) have received more applications. The autologous implantation or ex vivo expanded of BMSCs has emerged to effectively delay or avoid early-stage ONFH deterioration [9]. Betaine, which attenuates the ethanol-induced inhibition of mineralization of hBMSCs and osteogenesis, is potential pharmacotherapy for ONFH with alcohol induction [10]. Inherently, the therapeutic ability of BMSCs depends to a great extent on their secreted regulatory carriers, such as exosomes. Exosomes are a type of small bilayer membrane-bound nanovesicles (30 - 150 nm) that deliver proteins, lipids, and nucleic acids (miRNA, tsRNA, etc) between cells [11], and play essential roles in many biological processes. Exosomes derived from BMSCs can modulate cell survival and tissue repair [12]. However, the effect of MSCs-derived exosomes on ONFH remains unclear.

MicroRNAs (miRNAs) are endogenous small RNAs of less than 22 base pairs, which participate in the post-translational regulation of gene expression [13]. MiR-15b improves ONFH through inhibits the BMSCs-based osteogenic differentiation of via targets Smad7 [14]; MiR-144-3p inhibits the BMSCs cell activity and osteogenic differentiation by targeting FZD4, indicating that MiR-144-3p may mediate ONFH progression and may serve as a new target [15]. Previous findings showed that miRNAs involve in the mediation of various biological processes via exosomes. The decline of microRNA-224-3p in BMSC exosomes resulted in the upregulation of FIP200 and thereby potentiates angiogenesis and endothelial cell proliferation, invasion, and migration in traumatic ONFH [16]. Liao et al. found miR-122-5p over-expressed exosomes impaired the development of ONFH by inhibiting SPRY2 expression via the RTK/Ras/MAPK signaling pathway [17]. These findings testify that MSCs-derived exosomes can be conducive to the mend of ONFH via the transfer of specific miRNA.

Our study aimed to identify exosomal miRNAs beneficial for ONFH, and construct the miRNA-overexpressed BMSC exosomes to verify its therapeutic efficacy in ONFH. We identified differentially expressed miRNAs in exosomes from ONFH patients and healthy donors through RNA-seq. The functions of key miRNA-modified BMSC exosomes were illustrated by matrix-gel based in vitro angiogenesis assay and scratch assay. We look forward to exploring a novel molecular for the study basis and clinical application of ONFH.

Materials And Methods

Human plasma specimens

In the current study, three paired plasma samples (ONFH patients and healthy individuals) were used for sequencing, an itional nine paired plasma samples were used for PCR validation. The blood specimens were placed in BD vacutainer K2 EDTA blood collection tubes and mixed lightly. Next, the anticoagulant-treated blood samples were performed to centrifuge separates at 1000 rpm for 15 min at 20 °C to removes the blood cell debris and impurities. The upper layer containing plasma was then obtained from each tube and transferred into sterile EP tubes by the pipette. Taking care not to touch the bottom sediment during this step. At last, each plasma was labeled and stored at -80 °C for subsequent experiments. None of the ONFH patients received any therapy before blood collection. Each participant

was signed written informed consent before the experiment. This study was performed with the approval of the Human Ethics Committee of the First Affiliated Hospital of Fujian Medical University

Isolation and identification of exosomes

The ExoQuick™ Plasma Prep and Exosome Precipitation Kit (SBI, EXOQ5TMA-1, Japan) were used to isolate exosomes from plasma. Briefly, plasma samples were incubated for 1 h at -20 °C and 4 °C, respectively. After ultra-centrifuged for 15 min (13,000 rpm), partial cells and their debris were removed from plasma samples. Add 5 µL SBI Thrombin Reagent to the supernatant and mixed. Then centrifuge isolation was conducted at 10,000 rpm for 5 min at 4 °C to help dissolve the fibrin. After refrigerated 30 min at 4°C, discarded the supernatant, the exosomes pellet were resuspended with 1X PBS (20 µg exosomes per 1 mL PBS) and stored at - 80 °C. The exosomes were negatively stained with the 3% (w/v) sodium phosphotungstate solution, and a photograph was captured using an LVEM5 TEM (DeLong America, Montreal, QC, Canada). NTA (NanoSight; Malvern Panalytical, Worcestershire, UK) was carried out to the diameter and size distribution of exosomes.

Small RNA sequencing and bioinformatics analysis

Illumina sequencing was performed for plasma exosomal small RNA sequencing according to the instructions of the Multiplex Small RNA Library Prep Kit (Illumina, USA). The concrete operations were briefly described as follows. Total RNA from plasma exosomes was firstly extracted using the Trizol method (Qiagen, Germany), measured and quantified by NanoDrop 2000 (Thermo, USA). For each sample, 3' adaptor was connected to RNA (200 ng), and reverse primer hybridization and 5' adaptor connection were disposed of orderly. Previous products were synthesized into cDNA and then enriched by PCR. The sequencing of screened DNA fragments was performed using the HiSeq platform (Illumina, USA). The original sequence data (ONFH patients and healthy participants) were filtered and mapped to reference genome, the internationally recognized algorithm DESeq2.0 was adopted to select the differentially expressed miRNAs (DEmiRNAs) with the threshold of p-value < 0.05 and |Log Fold Change| > 1. Subsequently, the hierarchical clustering of DEmiRNAs was analyzed by using MEV software and plotted the heatmap. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology terms (GO) annotation analysis were used to infer the functional roles of DEmiRNAs. Additionally, RNAHybrid and Miranda were used for the target gene prediction of miRNAs and the miRNA-mRNAs interaction network analysis was constructed using Cytoscape software 3.6.1 (<https://cytoscape.org/>).

Isolation and culture of cells

Bone marrow was respectively obtained from SD rat using a sterile syringe with heparin, followed by quick mixed with heparin. Next, the fat cells were removed from the BMSCs with a centrifuge separates (1000 rpm for 20 min), and then rinsed the sediment three times with Dulbecco s Modified Eagle Medium (DMEM), and cultured the BMSCs in RPMI-1640 media (GIBCO) with additional 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (hUVEC) (Procell Life Science&Technology Co., Ltd) were incubated in modified ECM medium (added 10% FBS, P/S, and 1% ECGS). All cells were incubated at 37

°C with 95% air and 5% CO₂. After 72 h of incubation, the whole medium was replaced, and the culture medium changed every 2-3 d. When cell confluence reached about 80%, cell subculture was performed.

Cell transfection

BMSCs were prepared in a 6-well plate for miRNA transfection. Briefly, 100 pmol of synthetic miR-150-5p mimics or negative control (NC) RNA were mixed with 250 µL serum-free Opti-MEM (Gibco, Grand Island, NY, USA) and incubated for 5 min at room temperature, respectively. Meanwhile, 5 µL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was diluted with 250 µL serum-free medium Opti-MEM at room temperature for 5 min. The above two reagents were mixed and incubated at room temperature for 20 min and added to the wells containing BMSCs. The 6-well plate was placed in a 37 °C incubators for 6 - 8 h and refreshed the medium. Subsequent tests were performed after an additional 24 - 48 h incubation.

Internalization of exosomes

The exosomes derived from BMSCs were extracted and ultra-centrifuged (120,000 x g) at 4 °C for 3 h after PBS dilutions using a Beckman tabletop ultracentrifuge. After washed with 100 ml medium, the exosomes' precipitation was resuspended in 700 ml diluent C. The exosomes were labeled by PKH67 Fluorescent with PKH67 Fluorescent Cell Connection Kit (Sigma-Aldrich) according to manufacturer protocol. Exosomes solution (250 ml) was mixed with diluted PKH67 dye and incubated for 4 min, and neutralize the excess PKH67 dye with bovine serum albumin (1%, 4.2 ml). The dye-labeled exosomes were centrifuged at 120,000 x g for 3 h (4 °C) and washed the precipitation with 1 x PBS. The HUVEC cells (1.3 x 10⁴ cells /well) were placed on 8-well slides and cultured for 24 h. After 1 x PBS washing, the medium containing PKH67-labeled exosomes was added and incubated for 48 hours. The cells were washed and incubated with a 4% paraformaldehyde solution at room temperature for 10 minutes. After 1 x PBS washing, the nuclear staining was employed by ProLong Gold Antifade Reagent containing DAPI (Thermo Fisher Scientific, USA). The internalization of exosomes in HUVEC cells was observed with a confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany).

Matrix-gel based in vitro angiogenesis assay

HUVECs were suspended in a serum-free ECM culture medium, seeded in a 24-well plate, and incubated with miR-150-5p-exosomes (miR-Exo), NC-exosomes (NC-Exo), and PBS for 24 h. The angiogenesis analysis was employed on ice, adding a 200 ul cooled Matrigel Matrix (10mg /ml; Becton Dickinson, USA) and incubated at 37 °C for 1 h. HUVECs (6 × 10⁴ per group) were seeded at coagulated Matrigel and incubated for 18 h at 37 °C. The blood vessels formation of each group was observed with an inverted microscope (NIKON, Japan).

Scratch assay

The cells were seeded in a 6-well plate and formed adherent cells. The monolayer cell was scraped in a straight line to create a "scratch" by a p200 pipette tip when cell density reached 90–100% confluent.

Then the cells incubated with BMSCs-derived exosomes (10 mg/mL). Images of the scratch were acquired at 0 h and 48 h. The migration distance of the scratched area and the node numbers were observed, and the multiple visual fields of cell migrated were randomly selected and photographed and performed a comparison between groups.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted as the previous method. After the detection of RNA quality, purity, and content, 1ug RNA was reverse-transcribed into cDNA with a PrimeScript™ RT reagent kit with gDNA Eraser kit (TaKaRa, Tokyo, Japan). The Reverse transcription-quantitative polymerase chain reaction (qRT-PCR) reaction was performed with the SYBR Green PCR kit (Toyobo, Osaka, Japan) on Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The reaction conditions were: 10 min of pre-denaturation at 95 °C, 45 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 60 s. The internal references in qRT-PCR were β -actin and U6. Three independent experiments were conducted. The relative expression of each factor was evaluated using the $2^{-\Delta\Delta Ct}$ method. The RT primer and special PCR primers were listed in Additional file 1.

Statistical analysis

All of these experiments were repeated three times. Statistical analyses were assessed with the SPSS v.21.0 software (IBM, USA). The comparisons of means among the two groups were evaluated by Student's t-test. One-way ANOVAs were performed for multiple comparisons. For all tests, $p < 0.05$ was considered to be statistically significant.

Results

Characterization of plasma exosomes derived from ONFH patients

To comprehensively characterize the morphologies and structures of exosomes derived from plasma of ONFH patients and healthy donors, we performed TEM and NTA methods. Both TEM and NTA showed that there was no significant difference in the plasma exosomes between ONFH patients and healthy donors (Fig 1A and B). TEM image indicated plasma particles were identified as two-layer membrane structures, mainly presented with a morphology of round or cup-shaped (Fig 1A). NTA revealed that the mean size of particles was 121.9 and 125.0 nm, with an average concentration of 5.4×10^{11} and 5.2×10^{11} particles/mL in ONFH patients and healthy donors, respectively (Fig 1B). All these results unequivocally confirmed that exosomes were successfully isolated from human plasma.

Exosomal DE miRNAs between ONFH and healthy controls

To identify the ONFH-related exosomal miRNAs, we performed small RNA sequencing in plasma exosomes from ONFH patients and healthy donors. From the six sequencing libraries, an average of 19.85 million clean reads per library was obtained. Among the clean reads, approximately 9.57 million

reads per library were mapped to miRNA, with the mapped rate ranged from 40.7% to 52.9% (Additional file 2). In this study, a total of 1,353 exosomal miRNAs were identified in the plasma of ONFH patients and healthy donors. Especially, 1014 exosomal miRNAs were commonly expressed in ONFH and healthy individuals, while 158 were uniquely expressed in ONFH, and 181 miRNAs were uniquely identified in healthy exosomes (Fig 2A). Based on $|\log_2 \text{fold change}| > 1$ and FDR value < 0.05 we identified 9 differently expressed miRNAs in exosomes between ONFH and healthy control groups (Fig 2B). Among these differently expressed miRNAs, 6 exosomal miRNAs (including miR-4800-3p, miR-150-5p, hsa-miR-6717-5p, hsa-miR-3192-3p, hsa-miR-4497, hsa-miR-3178) were upregulated, and 3 exosomal miRNAs (including hsa-miR-122b-5p, hsa-miR-122-3p, hsa-miR-452-5p) were downregulated in ONFH compared to those in healthy control.

Function and pathway analysis of exosomal DEmiRNAs

To further investigate the roles of DEmiRNAs, we predicted target genes for these miRNAs, and the function and pathway of the target genes were analyzed by GO and KEGG analysis. Using RNAhybrid and Miranda, we identified 1,156 target genes for the DEmiRNAs (Additional file 3). GO enrichment analysis indicated 914 targeted genes were significantly enriched in the GO terms. Biological process analysis revealed DEmiRNAs mainly involved in the function of angiogenesis, phosphorylation, transcription regulation, signal transduction, and cell migration. For instance, numerous targeted genes enriched in the GO terms of “neuron migration”, “phosphorylation”, “regulation of transcription, DNA-templated”, “sprouting angiogenesis”, “intracellular signal transduction” (Fig 3A, Additional file 4). Cell composition analysis showed DEmiRNAs were mainly associated with neuronal cell body, cytoplasm, terminal bouton, growth cone (Additional file 5). Molecular function analysis showed DEmiRNAs were related to double-stranded DNA binding, protein binding, kinase activity, nucleotide binding (Additional file 6). A total of 365 targeted genes were mapped to terms in KEGG database. We found that targeted genes were primarily involved in the signaling pathway of “Focal adhesion”, “ErbB signaling pathway”, “mTOR signaling pathway”, “Rap1 signaling pathway”, and “Ras signaling pathway” (Fig 3B).

Exosomal miR-150-5p is down-regulated in ONFH and involved in angiogenic signals

To identify the key exosomal miRNAs related to ONFH, two DEmiRNAs were selected for qRT-PCR validation in 9 ONFH patients and healthy controls, based on their large abundance and high fold changes. In accordant with the RNA-seq results, qRT-PCR results exhibited that miR-150-5p was significantly decreased in the ONFH exosomes compared to the healthy control exosomes (Fig 4A). The expression of miR-452-5p was upregulated in the ONFH exosomes, but the difference was not significant. Therefore, miR-150-5p was screened for further study. Molecular regulatory (Fig 4B) network demonstrated that miR-150-5p regulates multiple angiogenesis-related pathways by targeting the mRNAs. For instance, miR-150-5p was involved in the vital signaling of angiogenesis, VEGF signaling pathway, by targeting PRKCB and AKT2. Moreover, the target genes of miR-150-5p participate in the TGF- β signaling pathway, MAPK signaling pathway, HIF-1 signaling pathway, PI3K-Akt signaling pathway, and mTOR signaling pathway, which been proved to regulate angiogenesis [18,21]. The interruption of

blood supply to the proximal femur is the main cause of osteonecrosis, indicating that exosomal miR-150-5p might be involved in the treatment of ONFH by regulating angiogenesis.

BMSC exosomes promote endothelial cell angiogenesis

Next, we investigated whether exosomes overexpressed miR-150-5p could regulate angiogenesis. BMSC exosomes have been demonstrated to display therapeutic effect on various diseases including osteonecrosis[22], so we chose BMSC exosomes as miR-150-5p transporter. We first evaluated the action of BMSC exosomes in angiogenesis. As expected, the fluorescent tracer technique revealed BMSC exosomes derived from health rats could be internalized by HUVECs (Fig 5A). Moreover, we explored the angiogenesis potential by a matrix-gel based in vitro angiogenesis assay. The results displayed that BMSC exosomes derived from the healthy rats remarkably increased the tube formation of HUVECs, compared to PBS (Fig 5B and C). Collectively, BMSC exosomes were internalized by endothelial cells and promoted angiogenesis.

miR-150-5p modified BMSC exosomes enhance endothelial cell angiogenesis

To obtain the miR-150-5p overexpressed exosomes, we transfected the healthy BMSCs with miR-150-5p mimics and NC RNA. qRT-PCR verified that the BMSC exosomes with highly expressed miR-150-5p were successfully obtained (Additional file 7). Interestingly, the fluorescent tracer technique showed BMSC exosomes successfully transferred the miR-150-5p to the cytoplasm of HUVECs (Fig 6A). BMSC exosomes with miR-150-5p overexpression dramatically increased the tube forming capacity of HUVECs, compared to the control exosomes (Fig 6B). Furthermore, miR-150-5p overexpression promoted the enhanced effect of BMSC exosomes on the migration capability of HUVECs (Fig 6C). Taken together, these results indicated that miR-150-5p modified BMSC exosomes mediated the ONFH progression via promoted the angiogenesis of HUVECs.

Discussion

ONFH is a progressive pathologic disease characterized by dysfunction of endothelial cells, lipid metabolic disorders, and bone mass loss [23,24]. Our previous study has revealed BMSC-derived exosomes were involved in osteonecrosis by regulating osteogenesis [25], however, whether miRNA-modified exosomes can enhance the therapeutic effect remain unknown. Moreover, the expression profile of plasma exosomes from ONFH has not been reported. In this study, we first revealed the exosomal miRNA expression profiles of ONFH and identified 9 exosomal DEmiRNAs between ONFH and healthy donors. Exosomal miR-150-5p was significantly decreased in the ONFH plasma and was predicted to be involved in the angiogenesis-related signaling pathways. Moreover, BMSC exosomes carrying miR-150-5p induced the migration and tube formation of endothelial cells.

Exosomes themselves or exosomes carrying target molecules are being actively investigated as therapeutic agents [26]. Compared with other drug carriers, exosomes can effectively enter other cells and deliver functional molecules with minimal immunological clearance [27]. Especially, exosomes derived

from stem cells have exerted a therapeutic effect on various diseases, including cancer, cardiovascular disease, graft-versus-host disease, and osteonecrosis [28,30]. Emerging evidence showed stem cell-derived exosomes involved in angiogenesis. For instance, human urine-derived stem cell exosomes alleviate ONFH by reversing the GC-induced inhibition of endothelial angiogenesis [31]. HIF-1 α modified rabbit BMSC exosomes induced neovascularization by promoting the HUVEC cell ability, migration, and tube formation [32]. BMSCs have been shown to transfer miR-29b-3p into brain microvascular endothelial cells and enhance angiogenesis, resulting in amelioration of ischemic brain injury [33]. In accordant with previous studies, our results indicated miR-150-5p modified BMSC exosomes promoted the migration and tube formation of HUVECs.

Previous reports revealed that miR-150-5p promotes angiogenesis in extravillous trophoblast cells and synovial fibroblasts [34,35]. However, the mechanism by which miR-150-5p regulates angiogenesis remains unclear. In this study, we showed that the target genes of miR-150-5p were involved in VEGF, PI3K-Akt, MAPK, and HIF-1 signaling pathway, which can regulate angiogenesis and were related to ONFH. VEGF is a key regulator of angiogenesis, VEGF overexpressed adipose stem cells can provide rapid angiogenesis and osteogenesis in inhospitable avascular environments of ONFH [36]. Desferrioxamine ameliorates glucocorticoid-induced ONFH by inducing angiogenesis via HIF-1 α /VEGF pathway [37]. Exosomes secreted by induced pluripotent stem cell-derived mesenchymal stem cells mediate a protective effect in ONFH by inducing local angiogenesis via the activation of the PI3K/Akt signaling pathway [38]. Induction of ONFH caused increased expression of angiogenic responses-related genes, which were implicated in HIF-1, PI3K-Akt, and MAPK signaling pathways [39]. Mesenchymal stem cells-derived exosomal miR-122-5p protects against ONFH progression by triggering the RTK/Ras/ MAPK signaling pathway [17]. Here, our findings indicated that miR-150-5p-overexpressed BMSC exosomes may ameliorate ONFH by promoting angiogenesis via these signaling pathways.

Conclusions

Our results first provided the miRNAs expression profiles of ONFH plasma exosomes, and identified miR-150-5p was significantly decreased in the plasma exosomes of ONFH patients. The molecular regulatory network suggested that miR-150-5p was involved in the angiogenesis-related signaling pathways. Moreover, the overexpression of miR-150-5p in BMSC exosomes attenuated the angiogenesis of endothelial cells. This study expands the knowledge of BMSC exosomes and provides a new therapeutic target for ONFH.

Declarations

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Conflict of interest

The authors declare that there is no conflict of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the local Ethics Committee. Written informed consent for research purposes were obtained from all patients for study participation.

Authors' contributions

Conceptualization and Funding acquisition, P.C.; Data curation, S.F. and T.H.; Formal analysis, J.J., Y.L. and H.H.; Investigation, S.F., T.H., J.J., Y.L. and H.H.; Methodology, S.F. and T.H.; Writing - original draft, S.F., T.H., J.J., Y.L. and H.H.; Writing - review & editing, S.F. and T.H.; All authors read and approved the final manuscript.

Consent for publication

All the authors agree to publish the research.

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Figures

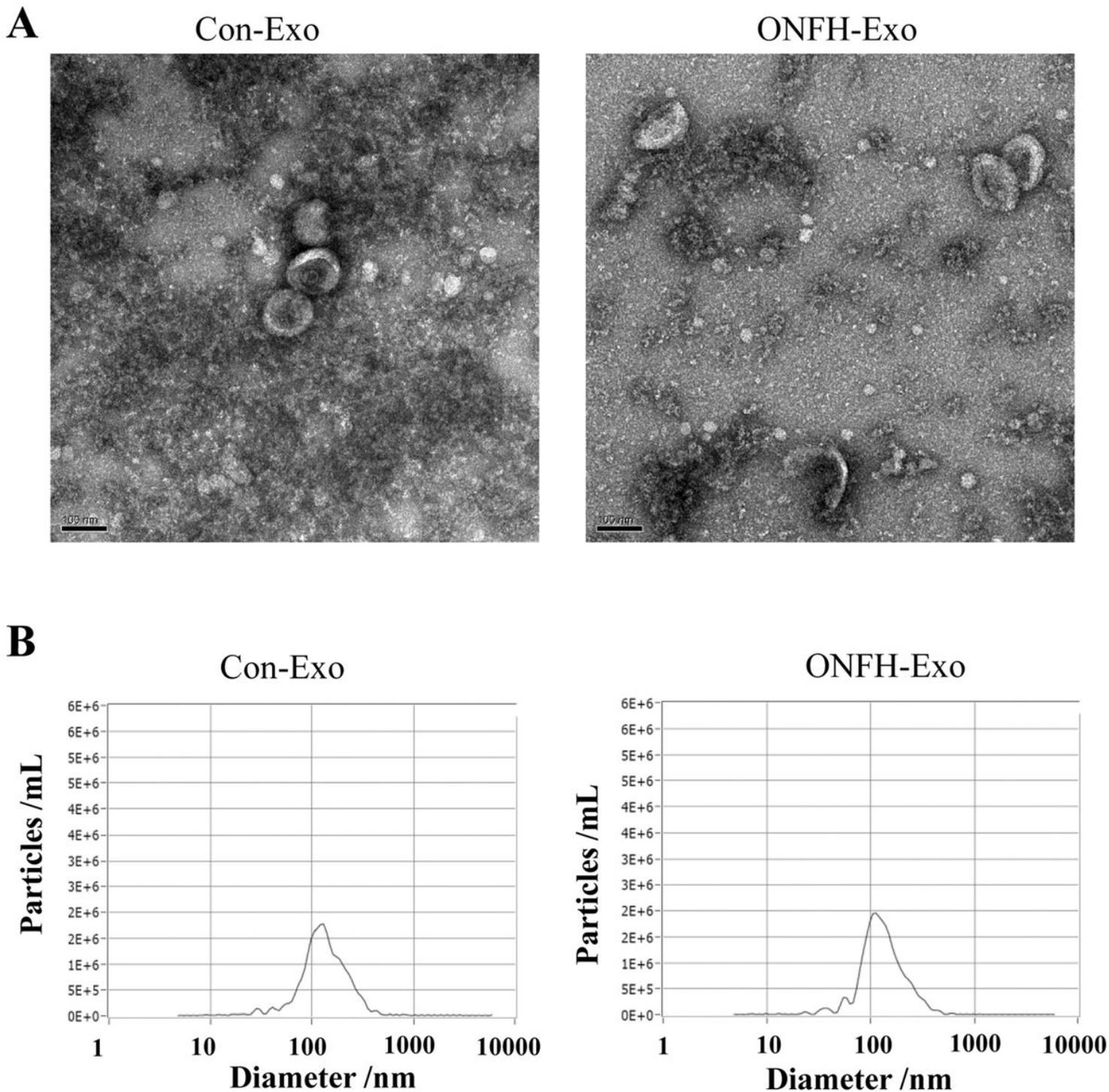
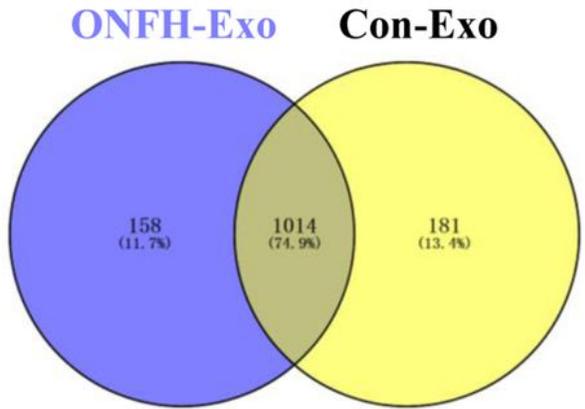
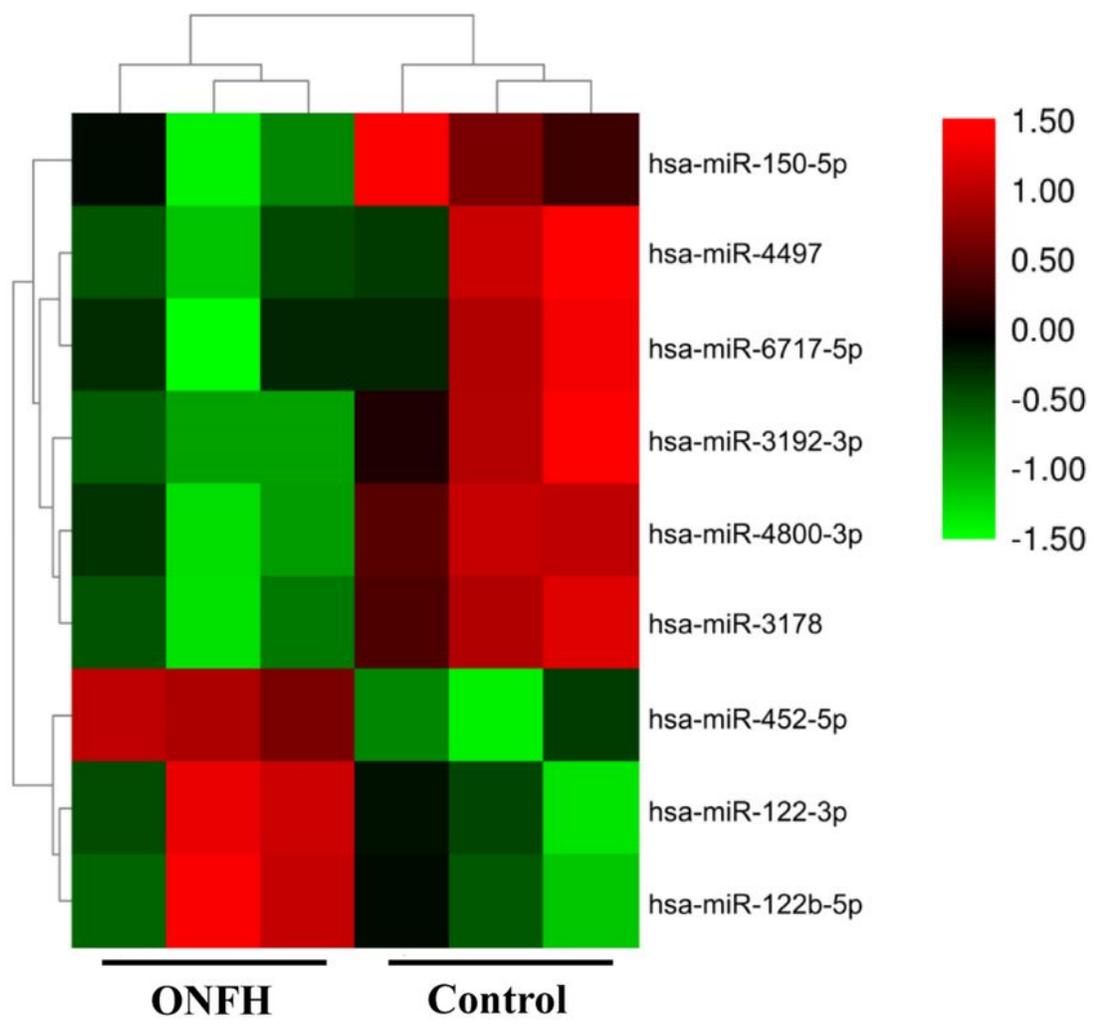
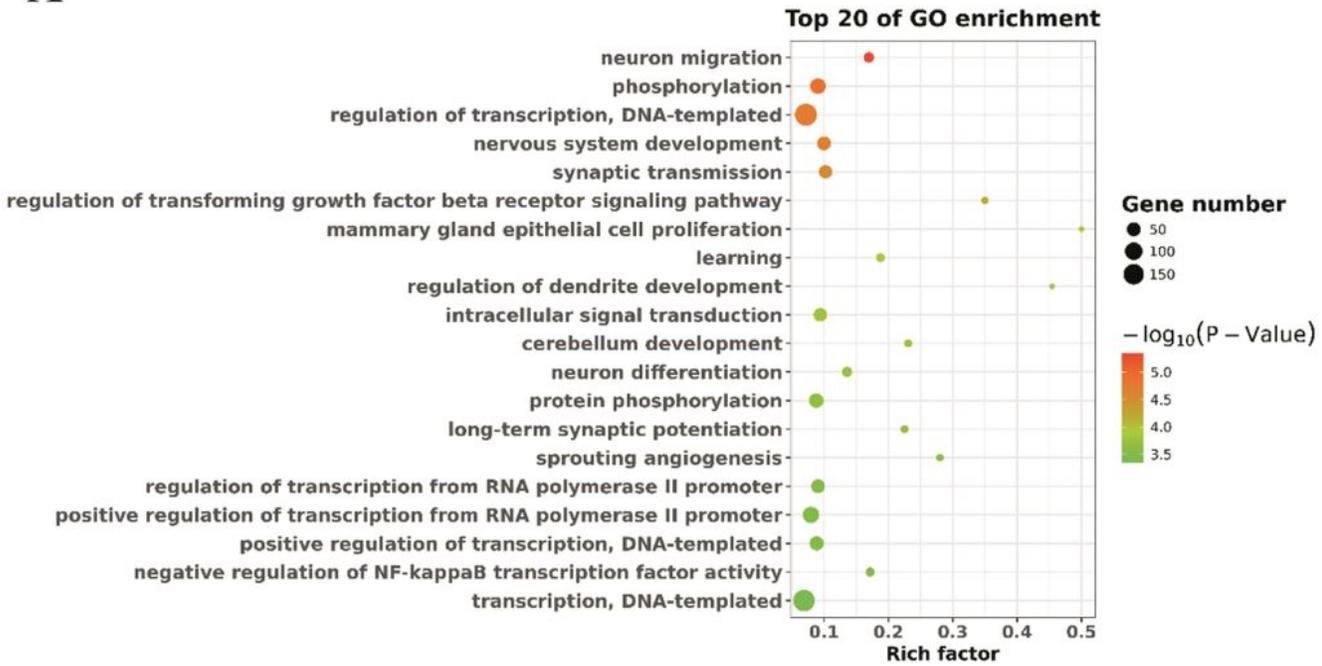
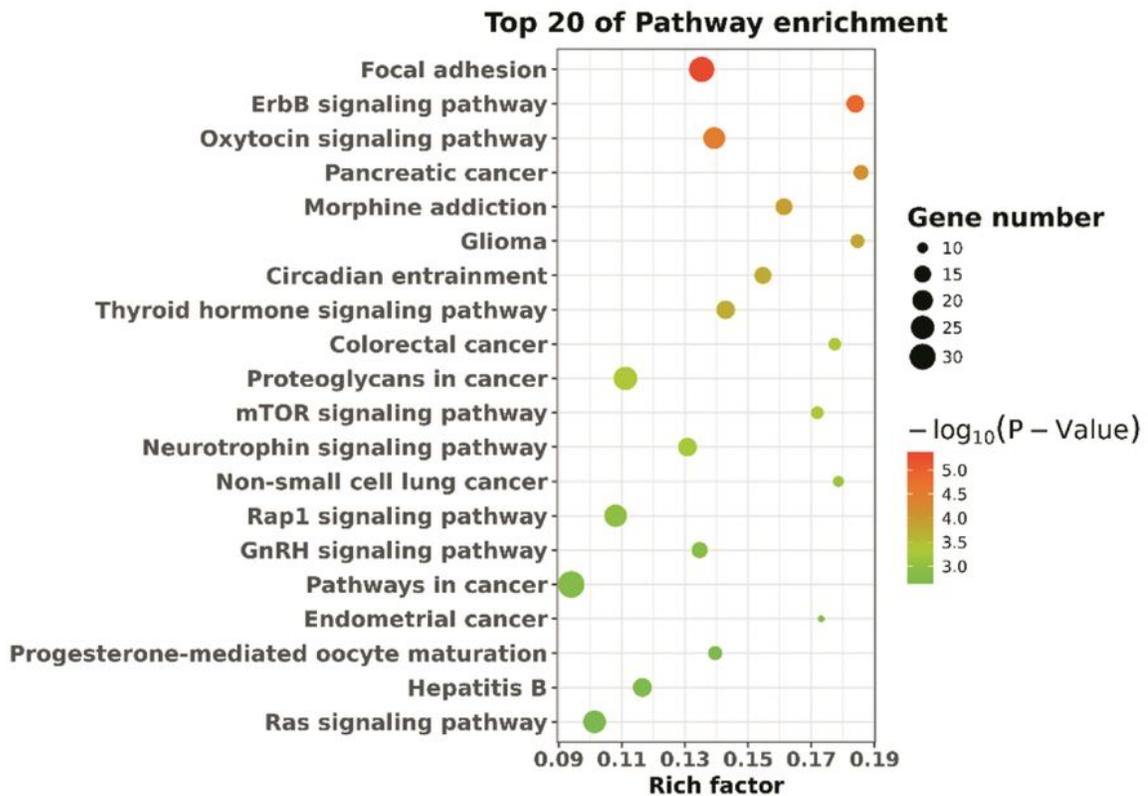


Figure 1

Characterization of plasma exosomes derived from ONFH patients. (A) Morphological characteristics of plasma exosomes detected by TEM. Representative TEM images of exosomes (Scale bars: 100 μ m). ONFH-Exo, the exosomes isolated from ONFH patient plasma. Con-Exo, the exosomes isolated from healthy donor plasma. (B) The size distribution and content plasma exosomes detected by NTA. X-axis: diameter of exosomes (nm); Y-axis: the content of exosomes/ml.

A**B****Figure 2**

Exosomal DE miRNAs between ONFH and healthy controls. (A) Venn diagram of identified miRNAs in ONFH exosomes and control exosomes. The violet and yellow circle show the miRNAs in ONFH exosomes and control exosomes, respectively. (B) Heatmap of DE miRNAs in plasma exosomes of ONFH patients and healthy donors. The high to low expression levels were represented as red to green.

A**B****Figure 3**

Function and pathway analysis of exosomal DEmiRNAs. (A) GO enrichment analysis based on the target genes of exosomal DEmiRNAs. (B) The top 20 enriched pathway terms of target genes of exosomal DEmiRNAs. X-axis: rich factor; Y-axis: GO or pathway terms; the size of each bubble: the number of genes; color: $-\log_{10}(P\text{-value})$.

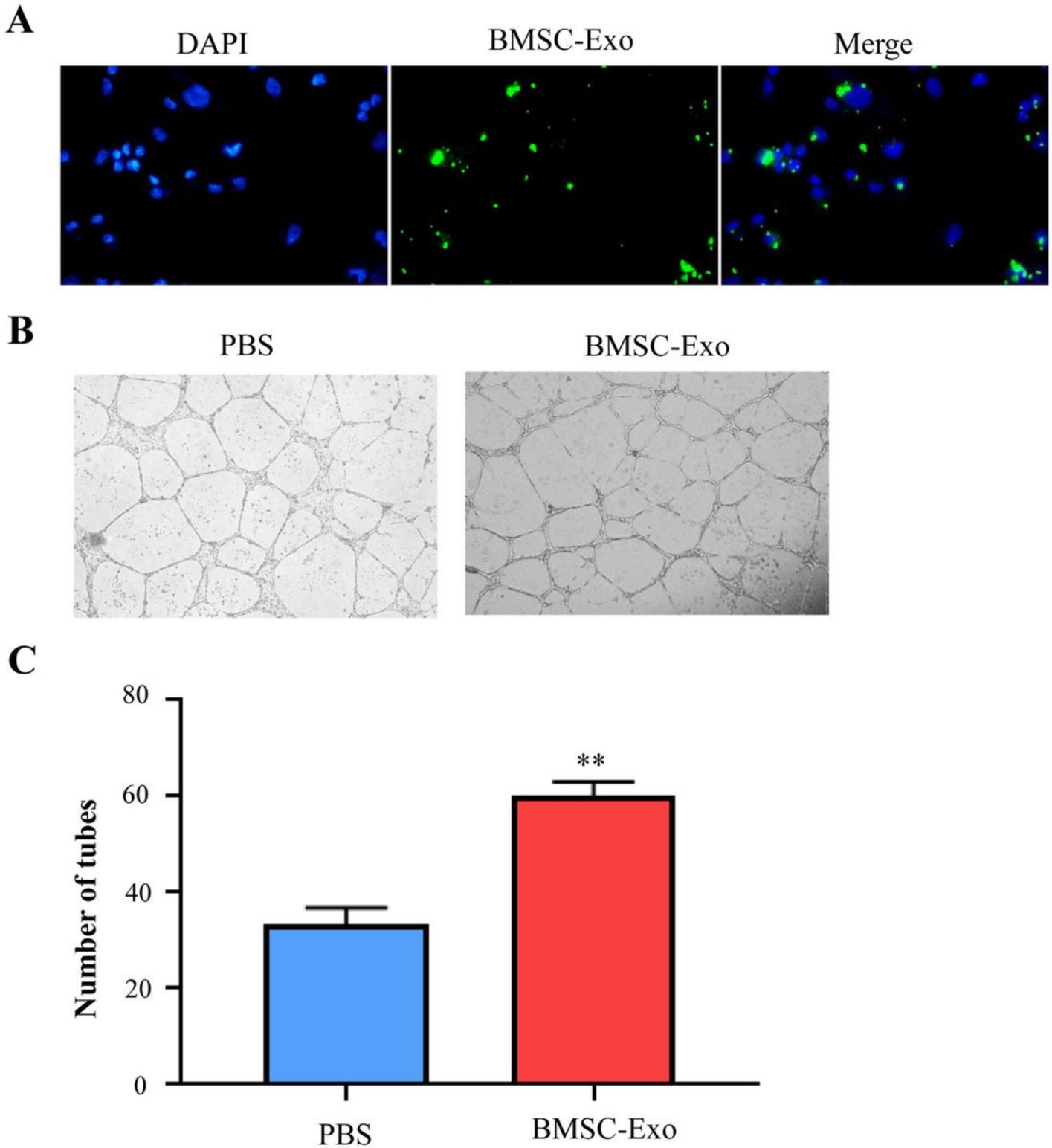


Figure 5

BMSC exosomes promote endothelial cell angiogenesis. (A) Internalization of the green fluorescence dye BMSC-Exos in HUVECs. BMSC-Exo, exosomes derived from BMSCs. (B) Tube forming capacity of HUVECs with or without BMSC-Exos was measured using matrix-gel based in vitro angiogenesis assay. (C) Histogram for the quantification of tube formation. Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$ in Student's t-test.

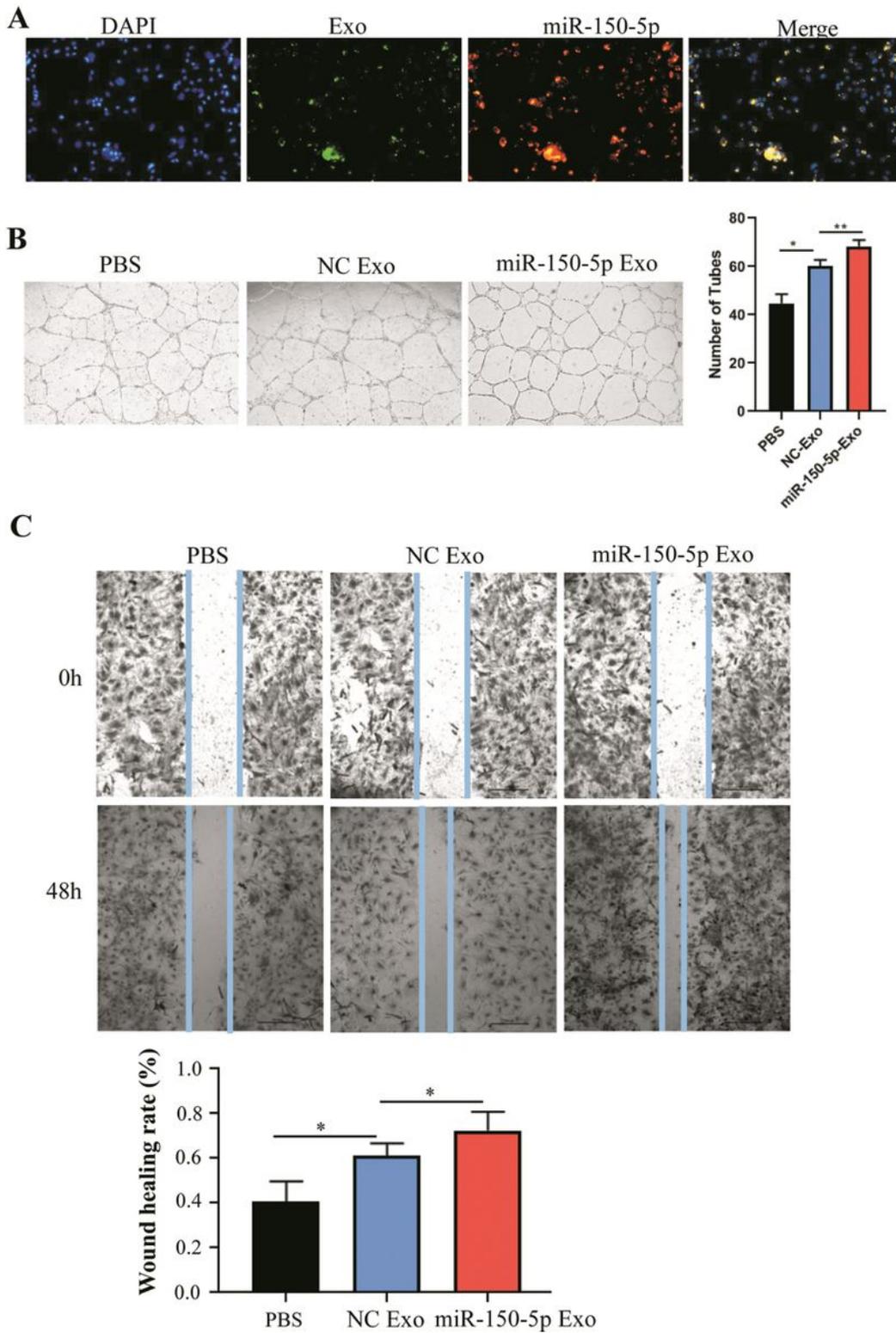


Figure 6

miR-150-5p modified BMSC exosomes enhance endothelial cell angiogenesis. (A) The transfer of miR-150-5 in BMSC exosomes and the cytoplasm of HUVECs was detected with a fluorescent tracer technique. (B) Tube forming capacity of HUVECs with or without miR-150-5p modified BMSC exosomes was measured using matrix-gel based in vitro angiogenesis assay. Right, the quantification of tube

formation. (C) Cell migration was measured by a scratch test. Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$ in One-way ANOVA.

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