Extracellular vesicles derived from hypoxia-preconditioned olfactory mucosa mesenchymal stem cells enhance angiogenesis via miR-612

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Abstract
Mesenchymal stem cells (MSCs) play important roles in tissue repair and regeneration, such as the induction of angiogenesis, particularly under hypoxic conditions. However, the molecular mechanisms underlying hypoxic MSC activation remain largely unknown. MSC-derived extracellular vesicles (EVs) are vital mediators of cell-to-cell communication and can be directly utilized as therapeutic agents for tissue repair and regeneration. Here, we explored the effects of EVs from human hypoxic olfactory mucosa MSCs (OM-MSCs) on angiogenesis and its underlying mechanism. EVs were isolated from normoxic (N) OM-MSCs (N-EVs) and hypoxic (H) OM-MSCs (H-EVs) using differential centrifugation and identified by transmission electron microscopy and flow cytometry. In vitro and in vivo, both types of OM-MSC-EVs promoted the proliferation, migration, and angiogenic activities of human brain microvascular endothelial cells (HBMECs). In addition, angiogenesis-stimulatory activity in the H-EV group was significantly enhanced compared to the N-EV group. MicroRNA profiling revealed a higher abundance of miR-612 in H-EVs than in N-EVs, while miR-612 inactivation abolished the N-EV treatment benefit. To explore the roles of miR-612, overexpression and knock-down experiments were performed using a mimic and inhibitor or agomir and antagomir of miR-612. The miR-612 target genes were confirmed using the luciferase reporter assay. Gain- and loss-of-function studies allowed the validation of miR-612 (enriched in hypoxic OM-MSC-EVs) as a functional messenger that stimulates angiogenesis and represses the expression of TP53 by targeting its 3’-untranslated region. Further functional assays showed that hypoxic OM-MSC-EVs promote paracrine Hypoxia-inducible factor 1-alpha (HIF-1α)-Vascular endothelial growth factor (VEGF) signaling in HBMECs via the exosomal miR-612-TP53-HIF-1α-VEGF axis. These findings suggest that hypoxic OM-MSC-EVs may represent a promising strategy for ischemic disease by promoting angiogenesis via miR-612 transfer.

Keywords: Olfactory mucosa, Mesenchymal stem cell, Angiogenesis, microRNA, EVs
Introduction
Over the past few years, the mesenchymal stem cell (MSC) therapy has attracted widespread attention for the treatment of ischemic disease, such as skin wound healing, peripheral and coronary vascular disease [1], cerebral infarction [2], and acute kidney ischemia injury [3]. MSCs are tissue-derived cells with unique characteristics that include a self-renewing ability, multilineage differentiation potential, and immunomodulatory properties [4]. Studies in both animal and human settings have demonstrated the therapeutic potential of MSCs in the treatment of a range of disorders, including ischemic disease. Recently, extracellular vesicles (EVs) have been reported to be essential paracrine components of MSCs, and they may offer a suitable alternative to cell-based therapies. Specifically, MSC-derived extracellular vesicles (MSC-EVs) possess an angiogenic function and are highly effective for treating ischemic diseases.

MSC-derived extracellular vesicles (MSC-EVs) may overcome the problems associated with MSC therapy. EVs are small 40–150 nm membrane particles of endosomal origin that play crucial roles in intercellular communication by delivering micro RNAs (miRNAs), mRNAs, and proteins to recipient cells [5, 6]. EVs exhibit stem cell-like pro-regenerative properties and direct treatment with them may avoid many adverse effects of stem cell transplantation therapy [7]. Because EVs are not live cells, the low efficacy of MSC therapy due to poor survival can be overcome by the MSC-EVs therapy. Many studies have reported that the local injection of EVs secreted by human MSCs from different sources can promote angiogenesis, suggesting that EV-based therapy is a promising treatment [8].

The olfactory mucosa (OM) is an attractive source of transplantable stem cells for central nervous system repair, which possesses several distinct advantageous attributes, including lifelong renewal, easy access, no risk to donors, no ethical problems, and autotransplantation potential to avoid immune rejection [9]. It is believed that olfactory mucosa mesenchymal stem cells
OM-MSCs), a novel type of resident stem cells in the olfactory lamina propria, have a high proliferation rate, self-renewal capacity, and ability to differentiate into multiple lineages. In addition to their potential application in tissue repair and regeneration, OM-MSCs have also been utilized as a convenient and effective method for the regeneration of both hippocampal neurons in mice and the spinal cord in humans [10]. Our previous work has identified 274 secreted proteins secreted by OM-MSCs using LC–MS/MS. It is well known that these molecules are important in neurotrophy, angiogenesis, cell growth, differentiation, apoptosis, and inflammation, which are all highly correlated with tissue repair [11]. Moreover, we conducted an in-depth study on the role of olfactory mucosa mesenchymal stem cells (OM-MSCs) in the treatment of ischemic stroke. It was found that OM-MSCs exert neuroprotective effects in cerebral ischemic/reperfusion (I/R) injury via the Golgi apparatus secretory pathway and alleviate mitochondrial dysfunction [12, 13]. However, the direct use of stem cells for therapeutic purposes remains limited by many risk factors, such as tumor formation, thrombosis, poor survival in inflammatory and hypoxic condition, and unwanted immune responses [14, 15]. Additionally OM-MSCs also contain abundant EVs [16]. Nevertheless, few studies to date have directly utilized OM-MSCs to harvest EVs for therapeutic uses.

In recent years, researchers have become committed to using exogenous means to enhance the ability of MSCs to promote angiogenesis and improve ischemia and injury. It has been reported that MSCs cultured in hypoxia (3% O2) significantly increases in vitro cell survival, proliferation, and angiogenesis-related growth factors. Furthermore, hypoxia preconditioning of MSCs is a beneficial approach to promote cell survival and treat several diseases, such as spinal cord injury [17] and cerebral ischemia [18]. We have previously demonstrated that hypoxia preconditioning of OM-MSCs can regulate their production of paracrine mediators, conferring neuroprotection against cerebral I/R injury [18]. Therefore, it was hypothesized that the angiogenesis-promoting effect of OM-MSC-EVs might be enhanced by culture in hypoxic conditions. In the present study, we compared pro-angiogenic effect between hypoxia OM-MSC derived EVs (H-EVs) and normoxia-cultured OM-MSC derived EVs (N-EVs) both in vitro and in vivo. Furthermore, advanced studies have shown that miR-612 in OM-MSCs can be transfected into endothelial cells through EVs and promote endothelial cell specification via direct suppression of its target TP53. To the best of our knowledge, this is the first study to show that hypoxic OM-MSC-EVs have the ability to promote angiogenesis and to elucidate the underlying mechanism.

**Materials and methods**

All experiments were carried out in accordance with the approved guidelines and were approved by Hunan Normal University and the institutional ethical and animal care committees. Human nasal mucosa biopsies were obtained with informed consent and all experiments were approved by the Ethics Committee at the Second Affiliated Hospital of Hunan Normal University (Ethical Approval Document No. 2018-30), and all clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

**Isolation, culture and identification of human OM-MSCs**

OM-MSCs were obtained from healthy male volunteers for scientific purposes (20–50 years old) at the Second Affiliated Hospital of Hunan Normal University. The isolation and culture of OM-MSCs were carried out using a protocol from our previous studies. Cell surface markers (CD34, CD45, CD44, CD73, CD90, CD105, CD133, CD146) were used to characterize OM-MSCs by flow cytometric analysis.

**Isolation and identification of normoxia and hypoxia OM-MSC-EVs**

Normoxia and hypoxia OM-MSC-EVs were purified by differential centrifugation, as described previously [19]. Before collecting the supernatant, the flow apoptosis of hypoxic and normoxic OM-MSCs was determined, and the supernatant could be collected only if the apoptosis rate was less than 3%. Hypoxia OM-MSCs was cultured at 3% oxygen concentration for 48 h. Briefly, the OM-MSCs were cultured to 90% confluence in complete DMEM; then, the complete medium was replaced with DMEM supplemented with 10% sEV-depleted FBS. The sEV-depleted FBS was prepared by centrifuging FBS at 120,000 g for 24 h and then passing it through a 0.22-μm filter (Millipore, SLGP033RB). 48 h the conditioned medium was collected and centrifuged at 300 g for 10 min, at 2000 g for 10 min, and at 10,000 g for 30 min to remove cells and cell debris. The clarified supernatant was then concentrated with a 0.22 μm syringe filter before EVs preparations. Firstly, the supernatant was then transferred to Ultra-Clear tubes and centrifuged at 100,000 g for 70 min at 4 °C with a SW32Ti rotor (Beckman Coulter, Netherlands). The exosome-containing pellet was washed with phosphate-buffered saline (PBS) and centrifuged at 100,000 g for 70 min. Finally, the pellet was then carefully re-suspended in 100 μL PBS. The protein concentration was determined by BCA protein assay kit (Beyotime, China). The samples were used immediately or stored at − 80 °C.

The identification of OM-MSC-EVs were carried out using a protocol from our previous study.

**Proteome analysis**

The protein
content of the concentrated EVs was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China). The OM-MSC-EVs size and concentration was assessed by nanoparticle tracking analysis (NTA) using a Nanosight NS300 (Malvern, UK). EVs markers CD63, CD81 and TSG101 (1:1000, ProteinTech, China) and OM-MSCs marker Nestin (1:1000, ProteinTech, China) were determined using Western blot.

**miRNA arrays**

Total RNA extracted from the normoxia and hypoxia OM-MSC-EVs was used for miRNA arrays. miRNA profiling was performed with OE Biotech’s (Shanghai, China) miRNA microarray service based on Affymetrix miRNA 3.0 Array.

**Culture of human brain microvascular endothelial cell and transfection**

Human brain microvascular endothelial cells (HBMECs; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle's medium: nutrient mixture F12 (DMEM/F12; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen, United States) at 37°C in 5% CO₂ atmosphere. Cells were incubated at 37 °C, 5% CO₂.

According to the manufacturer’s instructions, when HBMECs at 80% confluency, they were transfected with 50 nM miRNAs using Lipofectamine 2000 in Opti-MEM (Invitrogen). The synthetic miR-612 mimic, miR-612 inhibitor, mimic negative control (NC), inhibitor negative control (NC), were purchased from RiboBio (Guangzhou, China). After transfection for 5 h, the culture medium was replaced with complete medium. Other cells were cultured in 6-well culture plate and transfected with miR-612 inhibitor or the negative control inhibitor using Lipofectamine 2000 (Invitrogen), and cultured in complete medium containing 100 μg/mL normoxia or hypoxia OM-MSC-EVs (200 μg/well) or an equal volume of PBS. After 24 h of incubation, the downstream experiments were performed.

To evaluate the relationship between knocking down or up-regulation of TP53 expression and miR-612 in endothelial angiogenesis, TP53 small interfering RNA (siRNAs) (TP53 siRNA#1, TP53 siRNA#2, TP53 siRNA#3) and pcDNA3.1-CMV-TP53 obtained from RiboBio (Guangzhou, China) were respectively used in HBMECs. Briefly, cells were transfected with siTP53, pcDNA3.1-CMV-TP53 or the universal negative control siRNA (Con siRNA) using Lipofectamine 3000 (Invitrogen) according to the instructions of the manufacturers. 24 h later, the efficiency of these siRNAs and pcDNA3.1-CMV-TP53 were verified by qRT-PCR. The siRNA sequences used in this study were the following:

- **TP53 siRNA #1:** 5′-GTACCACCATCCTACAA-3′;
- **TP53 siRNA #2:** 5′-AGAGAGATCTCCGAAGAA-3′;
- **TP53 siRNA #3:** 5′-GGAGATTTGGGATGACAG-3′.

**EVs uptake assay**

OM-MSC-EVs were labeled with a green fluorescent dye PKH67 (Sigma Alderich, USA) according to the manufacturer’s instructions. Then the cell-labeled suspension was centrifuged at 300g for 15 min and the supernatant was discarded. Cells were washed twice with PBS and seeded into culture flasks for 48 h of incubation. Next, EVs were isolated from the conditioned media of MSCs and incubated with HBMECs at 37 °C for 3 h. Cells were then washed with PBS and fixed with 4% paraformaldehyde for 15 min. After washing with PBS, nuclei were stained with DAPI (Invitrogen, USA). The signals were analyzed with a fluorescence microscope (Leica DMI6000B, Germany).

**In vitro on HBMECs**

**Proliferation assay**

In brief, cells (5 × 10³ cells per well; five replicates per group) were seeded into 96-well culture plates and treated with EVs (100 μg/mL) from different groups or VEGF (30 ng/mL) or PBS. A group without cells served as the control group.

### Table 1: Primer sequence for qRT-PCR

| miRNA/mRNA | Sequence (5′-3′) |
|------------|-----------------|
| GAPDH F    | ACAGGCTCAAGATCATCAGC | R → GGTCATGAGTCCTCCACGAG |
| U6 F       | CTGGCTTCGAGCACA   | R → AACGCTCAGAATTTGGGT |
| hsa-miR-612| GCT GGG CAG GGC TTC TGA G | R → GCTGGGCAGGGCTTCTGAG |
| TP53 F     | ACATCTCCATTTGTTCCC | R → CTCCCCACAAAAACCCAGT |
| HIF-1α F   | TGAGCTACTCGAGAGAAG | R → GCCAGCAGAAGTTAAGCAC |
| VEGF F     | AGGGCAGAGATCATCAGGAAGT | R → AGGGTCTCGATTGGATGAC |


Identification of N-EVs and H-EVs. A Flow cytometric analysis showed the levels of apoptosis in N-OM-MSCs and H-OM-MSCs. Both apoptosis rates are less than 0.5% and there are no significant differences. (n = 5). (ns: no significance). B Western blot detected the expression of positive markers (TSG101, CD63, and CD81) and negative markers (Calnexin) in H-EVs and N-EVs. C Morphological characteristics of the H-EVs and N-EVs were analyzed using TEM images, Bar = 100 nm. D Size distribution analysis of the H-EVs and N-EVs, analyzed by nanoparticle tracking assay (NTA). E The BCA assay quantified the protein concentration of H-EVs and N-EVs secreted by equal cells. (n = 3). *P < 0.05. F, G The schematic representation of EVs uptake experimental design and fluorescence image of PKH67-labeled H-EVs and N-EVs uptake by HBMECs, Bar: upper, 200 μm; lower, 50 μm.
blank. On 6 and 12 h, cell counting kit-8 reagent (CCK-8; 10 μL per well; DOJINDO, Japan) was added to the culture medium (100 μL per well). After incubation at 37 °C for 2 h, the absorbance of each well was measured at 450 nm by a microplate reader (Bio-Rad 680, Hercules, USA) and cell proliferation was represented through the mean absorbance of each individual well minus the blank value.

**Scratch wound healing assay**
Cells (5 × 10⁵ cells per well; three replicates per group) were seeded into a 6-well plate and incubated at 37 °C. After the cells had attached, the monolayer was scratched with a p200 pipette tip, washed with PBS to remove floating cells and then exposed to EVs (100 μg/mL) from different groups or an equal volume of PBS. Mitomycin-C (5 μg/mL; Sigma) was present throughout the migration assays to exclude the influence of cell proliferation on wound closure. HBMECs were photographed at 6 and 12 h after wounding. The rate of migration area was calculated as the ratio of closure area to initial wound as described previously [20]:

\[
\text{Migration area (\%) = } \frac{A_0 - A_n}{A_0} \times 100,
\]

where \(A_0\) represents the area of initial wound area and \(A_n\) represents the remaining area of wound at the metering point.

**Transwell migration assay**
Boyden chamber assays were performed using 24-well transwell inserts (Corning, NY, USA) with 8 μm pore-sized filters and 24-well culture plates as described previously [20]. Cells (4 × 10⁴ cells per well; three replicates per group) were suspended in low serum (5% FBS) medium and plated into the upper chamber. 500 μL complete medium (containing 10% FBS) supplemented with EVs (100 μg/mL) from different groups or an equal volume of PBS was added to the lower chamber. After incubation for 16 h, cells attached to the upper surface of the filter membranes were removed by cotton swabs and cells on the bottom side of the filter (the migrated cells) were stained with 0.5% crystal violet for several minutes. The number of migrated cells was counted under an optical microscope at a 100× magnification (Leica). The absorbance of each well was measured at 550 nm by a microplate reader (Bio-Rad 680, Hercules, USA) and cell migration was represented through the mean absorbance of each individual well.

**Tube formation assay**
130 μL cold Matrigel per well was transferred into each well of a 24-well plate and incubated at 37 °C for 30 min. Then, HBMECs (2 × 10⁴ cells per well; three replicates per group) were plated into the Matrigel-coated 24-well plates and treated with EVs (100 μg/mL) from different groups or PBS. 6–8 h after seeding, tube formation was detected under an inverted microscope (Leica). The indicators (total number of branches length, junctions, nodes and meshes) revealing the abilities to form tubes were measured by using Image-J software.

**In vivo matrigel plug assay**
Athymic-nude (nu/nu) mice (female, 7–8 weeks old) were purchased from Slac Laboratory Animal Co., Ltd. (Shanghai, China) and were used for in vivo Matrigel plug studies. At the end of the experiment mice were euthanized by CO₂ inhalation. Mice were randomly allocated to 12 groups (n=6 mice per group for the groups): (1) Matrigel only, (2) Matrigel + N-EVs, (3) Matrigel + H-EVs, (4) Matrigel + HBMECs, (5) Matrigel + HBMECs + N-EVs, (6) Matrigel + HBMECs + H-EVs, (7) Matrigel containing HBMECs transfected with an miR-612 agomir negative control (agomir NC group), (8) miR-612 agomir (miR-612 agomir group), (9) miR-612 antagonir negative control (antagomir NC group), (10) miR-612 antagonir (miR-612 antagonir group), (11) miR-612 antagonir + H-EVs (miR-612 antagonir + H-EVs group), and (12) miR-612 antagonir + N-EVs (miR-612 antagonir + N-EVs group).

To test whether normoxia or hypoxia OM-MSC-EVs affected angiogenesis, normoxia and hypoxia OM-MSC-EVs (100 μg/mL) were resuspended in 500 μL of ice-cold Matrigel respectively, and implanted subcutaneously on the back of nude mice, while an equal volume of Matrigel without EVs was implanted as negative control. Matrigel plugs were removed for analysis 14 days later.

To test whether normoxia or hypoxia OM-MSC-EVs affected HBMECs angiogenesis, 5 × 10⁶ HBMECs were mixed with 500 μL of Matrigel (BD, USA) at a ratio of 1:1, while normoxia and hypoxia OM-MSC-EVs (100 μg/mL) were resuspended in 500 μL of ice-cold Matrigel respectively, and implanted subcutaneously on the back of nude mice, while an equal volume of Matrigel with 5 × 10⁶ HBMECs was implanted as negative control. Matrigel plugs were removed for analysis 14 days later.

To determine whether exosomal miR-612 is responsible for the proangiogenic effect of normoxia and hypoxia OM-MSC-EVs, HBMECs were loaded with miR-612 agomir NC/miR-612 agomir (1.5 nmol/mouse) and miR-612 antagonir NC/miR-612 antagonir (3 nmol/mouse), respectively. Next, 5 × 10⁶ HBMECs were mixed with 500 μL of Matrigel (BD, USA) at a ratio of 1:1, while an equal volume of Matrigel with normoxia and hypoxia OM-MSC-EVs (100 μg/mL) were implanted as negative control. Then, the cell suspensions were injected subcutaneously in the dorsal region of nude mice. Finally, the Matrigel plugs were removed for analysis on Day 14.
Fig. 2 H-EVs enhance the angiogenic activities of HBMECs in vitro. A The schematic representation of in vitro experimental design. B CCK-8 analysis was applied to measure the effect of H-EVs and N-EVs on proliferation of HBMECs, (n = 6), ***P < 0.001. C Representative images of tubule formation for each treatment group, Scale bar: 200 μm. D Quantitative data of tube formation using ImageJ, (n = 9), ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001. E Representative images of wound healing assay for each treatment group, Scale bar: 200 μm. F Quantitative data of migration rate using ImageJ, (n = 3), ns: no significance, ***P < 0.001. G Representative images of transwell assay for each treatment group, Scale bar: 100 μm. H Quantitative data of migrating cells OD value using ImageJ, (n = 9), ***P < 0.001.
Histology and immunofluorescence
Matrigel plugs were collected, fixed with 4% PFA, embedded in paraffin, and sectioned. For immunohistochemical analyses, Matrigel plug sections were stained with primary antibodies against CD31 (1:100, ab222783, abcam) and DAPI (Invitrogen, USA) as previously described [21].

Bioinformatics analysis and luciferase reporter assay
Putative targets of miR-612 were searched using TargetScan (http://targetscan.org/) and miRDB (http://www.mirdb.org/). TP53, which was predicted as a target of miR-612, was then assessed by luciferase reporter assay. The 3′-UTR of TP53 containing either wild-type (WT) or mutant-type (MT) binding sites of miR-612 were synthesized by GenePharma Co., Ltd. and inserted into the pmirGLO vector (Promega), with the resultant constructs denoted as WT-TP53 and MT-TP53, respectively. HEK293 cells were cotransfected with miR-612 mimics or miR-NC and reporter plasmids WT-TP53 or MT-TP53 using Lipofectamine 2000. The activities of Renilla and firefly luciferase were examined using a Dual-Luciferase® Reporter assay kit from Promega based on the manufacturer’s protocols. The activity of firefly luciferase was normalized to that of Renilla luciferase.

RNA isolation and quantitative real-time PCR
Total RNA from cells and tissues, and exosome-derived RNA were extracted using TRIZOL (Tiangen, Beijing, China). The primers used in this study were synthesized by Sangon Biotech Company (shanghai, China) (Table 1). Using GAPDH and U6 as internal reference primers, the relative mRNA expression of target genes was calculated by the 2 − △△Ct method [22].

Western blotting
EVs, cells and matrigel tissue were processed for Western blot as described [12, 13]. Immunoblot analyses were performed using the following primary antibodies against Calnexin (1:1000, ProteinTech, China), TSG101 (1:1000, ProteinTech, China), CD81 (1:1000, ProteinTech, China), HIF-1α (5 µg/mL, abcam, UK), VEGF (5 µg/mL, abcam, UK), TP53(1:1200, ProteinTech, China), GAPDH (1:3000, ProteinTech, China). The anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Protein-tech. The proteins were visualized using an enhanced chemiluminescent (ECL) detection kit (Advanta Inc., United States).

Statistical analysis
All experiments were performed in at least three replicates. Data are expressed as mean ± SEM. Differences between groups were estimated using two-side dunpaired Student’s t-test or two-sided ANOVA with the Bonferroni correction for the post hoc t-test as appropriate. Statistical analysis was conducted with GraphPad Prism 6 Software (La Jolla, CA, United States). Differences with the probability of P < 0.05 were considered significant.

Results
Identification of normoxic and hypoxic OM-MSCs and OM-MSC-EVs
To study the roles of EVs in endothelial cell angiogenesis, normoxic and hypoxic OM-MSCs were first isolated and characterized as previously described [23]. Flow cytometry analysis revealed that both normoxic and hypoxic OM-MSCs were highly positive for MSC surface markers, including CD44, CD73, CD90, CD105, CD133, CD146, and CD29, but negative for CD34 and CD45 (Additional file 1: Fig. S1A). All of these results were consistent with the findings of previous studies [23].

In accordance with the requirements of minimal information for studies of extracellular vesicles 2018 (MISEV 2018) [24], hypoxic and normoxic OM-MSC apoptosis rates were analyzed by flow cytometry before extracting the supernatant. The apoptosis rates of hypoxic OM-MSCs (0.124 ± 0.018, n = 5) and normoxic OM-MSCs (0.118 ± 0.010, n = 5) were < 0.5% to eliminate the influence of apoptosis on the acquisition of EVs (Fig. 1A). H-EVs and N-EVs were isolated from hypoxic and normoxic OM-MSCs and characterized using western
Fig. 3 (See legend on previous page.)
blotting, electron microscopy, and NTA assays. Western blotting demonstrated that the exosomal marker proteins TSG101, CD63, and CD81 were present in these EVs as expected, and negative exosome marker calnexin was not detected in both OM-MSC-derived EV types (Fig. 1B). Transmission electron microscopy analysis showed that both types of EVs were round-shaped, with a diameter range of 30–100 nm (Fig. 1C). NTA results revealed a size distribution range of 30–150 nm for normoxic and hypoxic OM-MSC-EVs (Fig. 1D). Bicinchoninic acid protein assay was used to quantify the EV protein concentration as previously described [25]. The total protein amount was 0.620 ± 0.070 μg/μL for N-EVs and 0.832 ± 0.059 μg/μL for H-EVs (P < 0.01, n = 3; Fig. 1E). Therefore, there was a difference between the total protein concentrations in the two types of EVs. Collectively, the exosomal characteristics observed in the present study were consistent with those previously reported [16].

As a type of natural nanoparticle, EVs regulate the function of neighboring and distant cells by delivering diverse factors. To evaluate exosome internalization, HBMECs were incubated with the green fluorescent dye (PKH67)-labelled EVs for 6 h and observed using fluorescence microscopy (Fig. 1F). Green fluorescence was detected in the HBMECs, suggesting the internalization of both labeled H-EVs and N-EVs by HBMECs (Fig. 1F, G). Reassuringly, no fluorescent signal was observed in the cells receiving the dyes alone (Additional file 1: Fig. S1B) or unlabeled MSC-EV controls (Fig. 1F, G). Therefore, no fluorescent signal was observed in the cells receiving the dyes alone (Additional file 1: Fig. S1B) or unlabeled MSC-EV controls (Fig. 1F, G), showing that the signal was specific to the MSC-EVs being studied.

**H-EVs enhance the angiogenic activities of HBMECs in vitro**

The impact of H-EVs on the angiogenic activities of endothelial cells was then assessed (Fig. 2A). CCK-8 analysis was used to measure the effect of normoxic and hypoxic OM-MSC-EVs on the proliferation of HBMECs. Both N-EVs and H-EVs exhibit similar properties to hypoxic OM-MSC-EVs on the proliferation of HBMECs (Fig. 2B). Nicotinamide adenine dinucleotide (NADH) is essential for the promotion of angiogenesis, and NADH can be used as a positive control and is able to stimulate the proliferation of HBMECs (Fig. 2B). Both N-EVs and H-EVs exhibit similar properties to hypoxic OM-MSC-EVs on the proliferation of HBMECs. Nicotinamide adenine dinucleotide (NADH) is essential for the promotion of angiogenesis, and NADH can be used as a positive control and is able to stimulate the proliferation of HBMECs (Fig. 2B). In addition, compared to N-EVs, H-EVs can better promote the proliferation of HBMECs. The tube formation assay on Matrigel was used as an in vitro model of angiogenesis. HBMECs treated with H-EVs and N-EVs showed a higher number of capillary-like structures compared to the control group (Fig. 2C). Quantitative measurements revealed that the branch length, junctions, nodes, meshes, length, and branch number were all significantly increased after normoxic and hypoxic OM-MSC-EV stimulation. Moreover, compared with N-EVs, HBMEC tube formation was significantly enhanced in the H-EV group as determined by the increase in the branch length and total number of junctions, nodes, and meshes (Fig. 2D). Similar results were found in scratch wound healing assay (Fig. 2E, F) and transwell assay (Fig. 2G, H). These findings indicate that OM-MSC-EVs augment the angiogenic activities of endothelial cells, and H-EVs significantly promote angiogenesis compared with the normoxic group.

**miRNA expression profile of H-EVs**

EVs play a vital role in intercellular communication, and their functions mainly depend on their internal contents. Therefore, deep sequencing of small RNAs from H-EVs and N-EVs was conducted. After trimming low-quality reads, contaminants, adaptors, and reads smaller than 17 nt, the remaining reads were mapped to noncoding RNA databases. Additional file 1: Fig. S2A shows the reads identified for categories of small RNA [ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclearRNA (snRNA), cis-regulatory element (Cis-reg), other-Rfam-RNA, gene, repeat, known-miRNA, and unannotation]. The percentage of miRNAs in the total RNA isolated from normoxic and hypoxic EVs corresponded to 22.20% ± 3.10% (n = 3) and 20.75% ± 5.87% (n = 3), respectively. There was no significant difference (P > 0.05) between the normoxic and hypoxic conditions (Additional file 1: Fig. S2B). Over 715 and 716 miRNAs were identified in H-EVs and N-EVs, respectively (Additional file 1: Fig. S2C; Additional file 2: Table S1). A total of 286 miRNAs were simultaneously identified both in H-EVs and N-EVs. The number of overlapping and unique proteins between the two groups is shown in Additional file 1: Fig. S2C. A set of miRNAs that were differentially expressed in H-EVs vs. N-EVs was also identified. Among these differentially expressed miRNAs, Fig. 3A shows the 19 miRNAs exhibit the greatest difference in abundance between normoxia and hypoxia.
Fig. 4 (See legend on previous page.)
Hypoxic OM-MSC-EV-enriched miR-612 enhances the angiogenic activities of HBMECs

To confirm whether miR-612 has a functional role in the EV-induced regulation of angiogenesis, EVs were obtained from OM-MSCs that were pretreated with an anti-miR-612 oligonucleotide (OM-MSC-EVanti-miR-612) or with a scrambled construct as the control (OM-MSC-EVNC). The proliferation of HBMECs was quantified by CCK-8 analysis. The OM-MSC-EVanti-miR-612 stimulation resulted in a significant decrease in HBMEC proliferation when compared with controls (Fig. 3G). Similar results were also found in the transwell assay (Fig. 3H) and tube formation assay (Fig. 3I). The pro-migratory effect of OM-MSC-EVs was attenuated, but not totally abolished in the OM-MSC-EVanti-miR-612 group when compared with the controls (Fig. 3I). Tube formation assay showed fewer capillary-like structures on Matrigel with HBMECs treated with OM-MSC-EVs with and without miR-612 compared with the OM-MSC-EVsNC (Fig. 3I). Quantitative analysis of the total junctions, meshes, and nodes further confirmed that down-regulation of miR-612 in OM-MSC-EVs blocked their positive effects on tube formation (Fig. 3I).

Thereafter, the effects of miR-612 on the regulation of angiogenesis were further analyzed using miR-612 mimics and its inhibitor in HBMECs. HBMECs transfected with the miR-612 mimics recapitulated the positive effects on tube formation (Fig. 4A, B), migration abilities (Fig. 4C, D), and cell viability (Fig. 4E) when compared with the scrambled group. These assays demonstrated that HBMECs exhibited a much stronger angiogenic ability, proliferative ability, and motility when transfected with miR-612 mimics. These findings indicate that miR-612 augments the angiogenic activities of endothelial cells. Conversely, transfection of HBMECs with the miR-612 inhibitor yielded the opposite results (Fig. 4F–J). The angiogenic ability, proliferative ability, and motility were decreased in response to the treatment with the miR-612 inhibitor (Fig. 4F–J). These results were again reversed when the HBMECs transfected with the inhibitor were incubated with H-EVs and N-EVs (Fig. 4F–J). Collectively, these findings suggest that miR-612 is required for OM-MSC-EV-induced promotion of endothelial angiogenesis.

OM-MSC-EV-transferred miR-612 modulates HBMEC specification via TP53

It has been reported that TP53 inhibition can upregulate HIF-1α and VEGF expression. Interestingly, the levels of TP53 mRNA and protein were reduced in HBMECs treated with H-EVs and N-EVs, and the decrease in TP53 in the hypoxic group was greater than that in the normoxic group (Fig. 5A–C). However, the mRNA and protein levels of HIF-1α and VEGF were increased in both groups, while the expression of HIF-1α and VEGF in the hypoxic group was higher than that in the normoxic group (Fig. 5A–C). To investigate the mechanism by which miR-612 promotes angiogenesis, the bioinformatics tool TargetScan was used to identify putative targets of miR-612. TP53 was one of the predicted targets of
Fig. 5 (See legend on previous page.)
Fig. 6 TP53 induced angiogenesis in vitro. A Representative images of tubule formation for each treatment group. Scale bar: 200 μm. B Quantitative data of tube formation using ImageJ, (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001. C Representative images of wound healing assay for each treatment group, and quantitative data of migration rate using ImageJ, (n = 3), ***P < 0.001; Scale bar: 200 μm. D Representative images of transwell assay for each treatment group and quantitative data of migrating cells OD value using ImageJ, (n = 9), ***P < 0.001, Scale bar: 100 μm.
miR-612 (Fig. 5D). Therefore, luciferase vectors containing the wild-type or mutant 3′-untranslated region (UTR) sequence of TP53 were constructed. The miR-612 mimic significantly decreased the relative luciferase activity of the wild-type vectors, while the luciferase activity of mutant vectors was not altered (Fig. 5D). These findings suggest that miR-612 specifically binds to the 3′-UTR of TP53 mRNA. Considering the specific regulatory effect of miR-612 on TP53 expression and the close relationship between TP53, HIF-1α, and VEGF, it was speculated that the miR-612-TP53-HIF-1α-VEGF axis regulates the behavior of HBMECs after OM-MSC-EVs.

Transfection of mimics and inhibitor were verified by qRT-PCR, and the results showed that miR-612 expression was significantly upregulated in HBMECs transfected with miR-612 mimic and downregulated in HBMECs transfected with miR-612 inhibitor. Using qRT-PCR and western blotting, it was found that miR-612 overexpression significantly reduced both TP53 mRNA and protein levels (Fig. 5E, F). The mRNA and protein levels of TP53 were suppressed by miR-612 inhibitor, further supporting the hypothesis that miR-612 blocked the activation of the TP53 signaling pathway (Fig. 5E, G). In parallel, transfection of miR-612 mimics into HBMECs resulted in increased mRNA and protein levels of HIF-1α and VEGF (Fig. 5E, F). Conversely, transfection with miR-612 inhibitor resulted in decreased expression of HIF-1α and VEGF (Fig. 5E, G). Taken together, these results demonstrate that HIF-1α and VEGF expression is upregulated by miR-612, and that one miR-612 target, TP53, may function as a mediator in the miR-612-HIF-1α-VEGF axis.

Furthermore, the effect of TP53 on angiogenesis was investigated. HBMECs were transfected with pcDNA3.1-TP53 and TP53 siRNA (siTP53#1, siTP53#2, and siTP53#3) to upregulate and downregulate TP53, respectively. First, the angiogenic activities of HBMECs were assessed after TP53 interference using specific siRNAs (siTP53#1, siTP53#2, and siTP53#3). Downregulation of TP53 was verified by qRT-PCR and western blotting (Additional file 1: Fig. S3A, B). As evidenced by the tube formation assay (Fig. 6A, B), scratch wound assay (Fig. 6C), transwell assay (Fig. 6D), and CCK-8 assay (Additional file 1: Fig. S3E), the migration, angiogenic behavior of HBMECs after OM-MSC-EVs.

miR-612 is abundant in H-EVs and promotes endothelial cell angiogenesis in vivo

To assess the pro-angiogenic potential of H-EVs in vivo, a Matrigel plug assay was performed in athymic nude mice (Fig. 8A). Test groups included the following: Matrigel only, Matrigel+HBMECs, Matrigel+N-EVs, Matrigel+H-EVs, Matrigel+HBMECs+N-EVs, and Matrigel+HBMECs+H-EVs. These mixtures were subcutaneously injected into mice and the Matrigel plugs were removed after 14 days. Visual examination of the Matrigel plugs showed no new blood vessel formation in the Matrigel only and Matrigel+HBMECs groups, whereas blood (red blood cells shown in red color) and blood vessels were observed in the other four groups (Fig. 8B, C). Matrigel plugs in both H-EV groups had many more vessels than both of the N-EV groups (Fig. 8B, C). Results were validated by immunostaining for the endothelial biomarker CD31 (Fig. 8D, E). Immunostaining quantification for CD31-positive signals and meshes gave similar results (Fig. 8F, G). In the Matrigel+HBMECs+H-EVs group, the numbers of CD31-positive vessels were greater, and the vessel walls were thicker. These results indicated that all OM-MSC-EV groups induced new vessel formation in vivo. Moreover, EVs derived from hypoxic OM-MSCs induced greater vessel formation than those derived from normoxic OM-MSCs. Western blotting analysis showed that the expression of TP53 was reduced in the Matrigel+HBMECs+N-EVs and Matrigel+HBMECs+H-EVs groups. The
Fig. 7 (See legend on previous page.)
decrease of TP53 in the Matrigel + HBMECs + H-EVs group was greater than that in the Matrigel + HBMECs + N-EVs group (Fig. 8H). However, the expression of HIF-1α and VEGF were increased in both Matrigel + HBMECs + N-EVs and Matrigel + HBMECs + H-EVs groups, the expression of HIF-1α and VEGF in the hypoxic group was higher than that in the normoxic group (Fig. 8H). These results are consistent with what we found in vitro experiments.

Thus, H-EV-enriched miR-612 might modulate TP53 signaling in vivo. Six groups, including Matrigel containing HBMECs transfected with an miR-612 agonist negative control (agonist NC group), miR-612 agonist (miR-612 agonist group), miR-612 antagonist negative control (agonist NC group), miR-612 antagonist (miR-612 antagonist group), miR-612 antagonist + H-EVs (miR-612 antagonist + H-EVs group), or miR-612 antagonist + N-EVs (miR-612 antagonist + N-EVs group) were injected into athymic nude mice. Matrigel was excised after 14 days. The presence of blood vessels was subsequently assessed by immunofluorescence staining for CD31 (green). Consistent with the in vitro data, the number of vessels in the miR-612 agonist group was significantly increased compared with that in the agonist NC group, antagonist NC group, and miR-612 antagonist groups. Matrigel plug in the miR-612 agonist group demonstrated many more vessels than other groups (Fig. 9A). Moreover, the morphology and number of vessels in Matrigel plugs were directly visualized by immunofluorescence staining (Fig. 9B). Quantification of immunostaining for CD31-positive signals gave similar results (Fig. 9C). In addition, miR-612 overexpression by agonist transfection significantly decreased the expression of TP53 and increased the expression of HIF-1α and VEGF (Fig. 9D–G). Conversely, antagonist-612 significantly attenuated the pro-angiogenic effect of HBMECs (Fig. 9A) as revealed by quantification of CD31-positive signals (Fig. 9B, C). Western blotting analysis indicated that transfection of antagonist-612 can significantly increase the expression of TP53 and decrease the expression of HIF-1α and VEGF (Fig. 9D–G). Finally, these results were again reversed when the HBMECs transfected with antagonim-612 were incubated with H-EVs and N-EVs (Fig. 9A–G). Taken together, these results demonstrate that H-EV-enriched miR-612 regulates TP53 signaling in vivo.

**Discussion**

The present study demonstrates that OM-MSC-EVs might enhance angiogenesis in vitro and in vivo, especially after hypoxic pretreatment. It was also revealed that OM-MSC-EVs promote HIF-1α-VEGF signaling in HBMECs through the miR-612-TP53-HIF-1α-VEGF axis, therefore suggesting that H-EVs serve as a promising alternative treatment for ischemic disease.

Angiogenesis is a key process in tissue repair after ischemia that involves various cell types, such as endothelial progenitors and inflammatory cells [26]. MSCs have already been shown to promote angiogenesis after ischemia through their differentiation and paracrine signaling activity [27, 28]. Accumulating evidence has suggested that transplanted MSCs promote angiogenesis mainly through paracrine mechanisms, such as EVs, which have been described as the most important effective ingredients that play a significant role in cell-to-cell communication. EVs have been widely used as a natural nanocarrier for the delivery of therapeutic agents into cells due to their advantages in size, structure, stability, and biocompatibility [29–33]. MSCs and their EVs are found in many human organs and tissues, including adipose tissue [34], bone marrow [35], umbilical cord [36], umbilical cord blood [37], placenta [38], and urine [20]. OM-MSCs are localized in the nasal lamina propria and are a novel source of MSCs identified in recent research [10]. A population of OM-MSCs was identified that originate from the olfactory lamina propria, possessing the typical characteristics of stem cells. Due to MSCs’ pro-angiogenesis abilities, they have been widely utilized in the treatment of various ischemic diseases. There has already been evidence that OM-MSCs possess...
Fig. 8 (See legend on previous page.)
several advantages over BM-MSCs. An autologous transplant can be performed using OM-MSCs derived from the nasal lamina propria. In addition, OM-MSCs demonstrated a higher proliferation profile and greater suppressive capacity compared to BM-MSCs [39, 40]. OM-MSCs have been demonstrated to exert protective effects in various disease states, including Parkinson’s disease [41], global cerebral ischemia [42], cerebral I/R injury [12, 13], hippocampal lesions [10], and autoimmune arthritis [43]. Moreover, it has been reported that OM-MSC-derived EVs ameliorate murine Sjögren’s syndrome by modulating the function of myeloid-derived suppressor cells [40]. However, no study has investigated the angiogenesis-promoting effects of OM-MSC-EVs and H-EVs. To further explore the effect of EVs derived under hypoxic conditions, a series of experiments were conducted to verify that EVs secreted by OM-MSCs under hypoxic conditions have a strong pro-angiogenic effect. Endothelial cells are the major effector cells in tissue repair after ischemia. Their proliferation, migration, and tube formation are essential for angiogenesis. In the present study, the effects of H-EVs on the behavior of endothelial cells (HBMECs) in vitro were evaluated. The results revealed that these nanoparticles can be internalized by HBMECs, and can significantly enhance their proliferation, migration, and angiogenic tubule formation, which confirmed the pro-angiogenic property of H-EVs. In the experiment, we found that H-EVs seemed to be internalized by HBMECs more effectively than N-EVs, although there is no statistical analysis to support this. Additionally, there has been evidence that MSCs derived from other sources can be more efficiently internalized by endothelial cells following hypoxic preconditioning [34, 44]. Hence, we will verify whether H-EVs are internalized more by HBMECs in the future. Further studies have shown that H-EVs increase vascularization of implanted Matrigel plugs in vivo. These results predicted that H-EVs are a positive regulator of angiogenesis. To the best of our knowledge, this is the first study demonstrating the modulation of endothelial cell angiogenesis by miRNA transfected from H-EVs.

OM-MSC-EVs do not only carry diverse sets of proteins [16], but also contain non-coding RNA and DNA, among which miRNAs are of particular interest. The miRNAs are a class of small non-coding RNA molecules 19–25 nucleotides in length that regulate gene expression by binding to the 3’-UTRs of target mRNA [45]. It is generally accepted that miRNAs exert critical effects on cellular processes, such as proliferation, stemness, apoptosis, invasion, and metastasis [46, 47]. However, the effects of miRNAs secreted by human hypoxic OM-MSC-EVs on endothelial cell angiogenesis are poorly understood. Using deep miRNA-seq analysis, 19 miRNAs differentially expressed between H-EVs and N-EVs were identified. The miR-612 is one of the upregulated miRNAs after hypoxia. Indeed, GO and KEGG analyses of miRNA patterns indicated that processes predominant in hypoxia were related to vesicular trafficking and positive regulation of cell communication, as well as pathways related to angiogenesis. Interestingly, miR-612 was involved in the VEGF signaling pathway. Previous research suggested a role for miR-612 in tumorigenesis. miR-612 has been shown to exhibit tumor-suppressing activity in multiple cancers by regulating major tumor-related biological behaviors. However, the role of miR-612 in angiogenesis remains unknown. The current study demonstrated that miR-612 expression was upregulated in human hypoxic OM-MSCs and H-EVs. In addition, miR-612 expression was remarkably enhanced in endothelial cells, indicating that miR-612 can be transferred from OM-MSC-EVs to recipient cells. To confirm the role of miR-612 in this process, EVs were obtained from OM-MSCs that were pretreated with an anti-miR-612 oligonucleotide (OM-MSC-EVsanti-miR-612) or with a scrambled construct as the control (OM-MSC-EVNC). Functional assays showed that the angiogenic ability, proliferative ability, and motility of HBMECs were decreased in response to the treatment with the OM-MSC-EVsanti-miR-612. Thereafter, the miR-612 mimics and inhibitor were used by directly transfecting miRNA into HBMECs. Indeed, miR-612 knockdown in HBMECs partially diminished their pro-angiogenic activity. The results were consistent with those from knocking down miR-612 in OM-MSC-EVs. Moreover, HBMECs were transfected with miR-612 agomir and antagonim for the gain- and loss-of-function investigation. The miR-612 agomir greatly promoted the number of HBMEC-formed tubes and angiogenesis in Matrigel plugs, whereas antagonim-612
Fig. 9 (See legend on previous page.)
significantly attenuated the pro-angiogenic effect. These findings illustrated that miR-612 plays crucial roles in the pro-angiogenic activity of OM-MSC-EVs. However, the effects induced by the specific inhibitor targeting miR-612 were notably reversed by H-EVs and N-EVs in vitro and in vivo. Interestingly, the H-EV-induced angiogenesis in HBMECs was markedly augmented. These findings suggest that miR-612 is one of the critical mediators in H-EV-induced regulation of HBMEC characteristics. Other signaling molecules may also be involved in this process, which warrants a further investigation.

TP53 is a central component of most cellular stress responses [48]. After activation, TP53 can positively or negatively regulate the expression of numerous target genes involved in various essential cellular processes, including cell proliferation, survival, and angiogenesis [49, 50]. To further determine the role and molecular mechanism of EV-transfected miR-612 during angiogenesis, computational bioinformatics were used to predict whether TP53 is a potential target of miR-612. PCR, western blot, and luciferase assays confirmed that miR-612 bound directly to the 3’UTR of TP53 mRNA and inhibited its translation. Previous studies have found that TP53 can suppress angiogenesis by transcriptional repression of VEGF expression through regulation of HIF-1α [51, 52]. In accord with these published findings, results of the present study showed that the TP53 expression levels were significantly decreased, whereas the activities of HIF-1α-VEGF signaling were markedly augmented in HBMECs stimulated by the miR-612-containing H-EVs and N-EVs. Furthermore, HBMECs were transfected with pcDNA3.1-TP53 and TP53 siRNA to up-regulate and down-regulate TP53, respectively, to investigate its effect on angiogenesis. As a result, TP53 overexpression attenuated the pro-angiogenic activity of miR-612, indicating that controlling TP53 expression is at least partly responsible for how miR-612 promotes angiogenesis. Collectively, these findings suggest that TP53 is a strong mediator in this signaling axis. To the best of our knowledge, the results from both in vitro and in vivo data suggested for the first time that miR-612 likely contributes to the process of angiogenesis. The detailed mechanisms underlying how TP53 regulates VEGF remain largely unclear. The study of angiogenesis will lead to a better understanding of various physiological and pathological processes, such as vascular disease, wound healing, and tumorigenesis [53]. Although the present study demonstrated a potential role of hypoxic OM-MSC-derived miR-612, further research is required in order to determine the overall importance of miR-612 compared to the wider secretome, as well as the mechanisms behind EV-induced mRNA expression. Further investigation of H-EV-transferred miRNAs might focus on the therapeutic modulation of diseases involving angiogenesis.

There are several limitations of our current study. Firstly, it is possible that our in vivo experiments were not sufficient since we were not able to promote angiogenesis in specific models of ischemic disease. A future study will examine specific ischemic disease models in depth, such as cerebral ischemia and ischemia following intracerebral hemorrhage. In addition, while numerous studies have investigated the effects of MSC-EVs on vascular endothelial cell proliferation by using CCK-8, additional experiments are required to confirm the effects [20, 37]. This will be further illustrated in future models of OM-MSC-EVs for specific ischemic diseases. Secondly, since no studies have reported that miR-612 has pro-angiogenic effects, we used agonim and antagonim to overexpress and knock down miR-612 in target cells. To determine whether miR-612 has an angiogenesis-promoting effect. Future work will use EVs collected from knockdown or over-expressed OM-MSCs to directly interact with in vivo models.

Altogether, the present findings demonstrate that H-EVs markedly enhance angiogenesis. In addition, the miR-612 may play a crucial role in the process of H-EV-dependent regulation of angiogenesis. The study results also suggest that EVs are important mediators of OM-MSC function and can be utilized as a novel therapeutic nano-delivery system for ischemic disease. Therefore, hypoxic preconditioning of MSC-derived EVs represents a novel strategy for the clinical treatment of ischemic diseases with stem cell-derived products.

**Abbreviations**

OM-MSCs: Olfactory mucosa mesenchymal stem cells; EVs: Extracellular vesicles; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; miRNAs: MicroRNAs; CCK-8: Cell counting kit-8; qRT-PCR: Quantitative real-time PCR; siRNAs: Small interfering RNA; HIF-1α: Hypoxia-inducible factor 1-alpha; VEGF: Vascular endothelial growth factor.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12951-021-01126-6.

**Additional file 1:** Figure S1. Characterization of N-OM-MSCs and H-OM-MSCs and EVs uptake assay. Figure S2. Hypoxia Leads to Changes in H-EVs and N-EVs miRNA Profiles. Figure S3. Evaluation of TP53 transfection efficiency and CCK-8 analysis.

**Additional file 2:** Table S1. Differentially expressed miRNA(|log2FoldChange|> 2, pvalue < 0.05).

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