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FULL LENGTH ARTICLE

Microvesicles (MIVs) secreted from adipose-derived stem cells (ADSCs) contain multiple microRNAs and promote the migration and invasion of endothelial cells

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Abstract Extracellular vesicles (EVs) such as microvesicles (MIVs) play an important role in intercellular communications. MIVs are small membrane vesicles sized 100—1000 nm in diameter that are released by many types of cells, such as mesenchymal stem cells (MSCs), tumor cells and adipose-derived stem cells (ADSC). As EVs can carry out autocrine and paracrine functions by controlling multiple cell processes, it is conceivable that EVs can be used as delivery vehicles for treating several clinical conditions, such as to improve cardiac angiogenesis after myocardial infarction (MI). Here, we seek to investigate whether ADSC-derived MIVs contain microRNAs that regulate angiogenesis and affect cell migration of endothelial cells. We first characterized the ADSC-derived MIVs and found that the MIVs had a size range of 100—300 nm, and expressed the MIV marker protein Alix. We then analyzed the microRNAs in ADSCs and ADSC-derived MIVs and demonstrated that ADSC-derived MIVs selectively released a panel of microRNAs, several of which were related to angiogenesis, including two members of the let-7 family. Furthermore, we demonstrated that ADSC-derived MIVs promoted the cell migration and invasion of the HUVEC endothelial cells. The PKH26-labeled ADSC-derived MIVs were effectively uptaken into the cytoplasm of HUVEC cells. Collectively, our results demonstrate that the ADSC-derived MIVs can promote migration and invasion abilities of endothelial cells, suggesting pro-angiogenetic potential. Future studies should focus on investigating the roles and mechanisms through which ADSC-derived MIVs regulate angiogenesis.

Introduction

Increasing evidence indicates that intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell-cell contact or transfer of secreted molecules, namely extracellular vesicles (EVs) such as exosomes and microvesicles (MIVs).[1—4] EVs are composed of a lipid bilayer containing transmembrane proteins, RNA, DNA, lipids, and metabolites that can be transferred to recipient cells and exert functional effects on target molecules either immediately or after EV fusion and/or endosomal uptake. EVs, also called microvesicles, ectosomes, or microparticles, can be formed and released by budding from the cells' plasma membrane and display a large range of sizes (100—1,000 nm in diameter). Conversely, exosomes are vesicles <150 nm in diameter and are enriched in endosome-derived components.[1—4]

EVs can serve autocrine and paracrine functions by controlling multiple cell processes in development, proliferation, migration, and pathology.[1—4] EVs are formed by multiple biogenesis mechanisms, which is likely to affect their cargo content. Furthermore, the release of extracellular vesicles (EVs) is a highly conserved process exploited by diverse organisms as a mode of intercellular communication.[1—4] It is conceivable that EVs can be used as delivery vehicles for treating several clinical conditions, such as to improve cardiac angiogenesis after myocardial infarction (MI). MI is the major cause of mortality in the world.[5—7] For example, China has 23 million patients with cardiovascular diseases and approximately 3 million patients die from this condition each year.[8,9] Thus, in addition to conventional therapies,[10,11] novel and efficacious treatments for cardiovascular diseases are needed, such as stem cell therapy.[12—14] The recovery of myocardial damage after MI is closely related to the state of blood vessels,[15] and stem cell therapy may promote angiogenesis and improve the blood supply.[16,17]

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells that can be easily isolated from adipose tissue, and have become the main source of stem cell-based therapies.[18—20] Several studies examined the potential effects of ADSCs on angiogenesis,[21—23] and potential application in cardiovascular diseases.[24] ADSCs can promote angiogenesis by differentiating into endothelial cells. ADSCs can also secrete factors, such as vascular endothelial growth factor-A (VEGF-A) and angiogenin (ANG),[19] to influence the tissue microenvironment and promote angiogenesis, suggesting that the use of ADSCs is safe and feasible.[25]

In this study, we seek to investigate whether ADSC-derived MIVs contain microRNAs that regulate angiogenesis and affect cell migration of endothelial cells. ADSC cells can secrete different sizes of MIVs with diameters ranging from 30 nm to 1 μm packed in lipid vesicles.[26—28] MIVs from several cells, such as mesenchymal stem cells and cancer cells, can promote angiogenesis by stimulating endothelial cells or changing the microenvironment.[29] As discussed above, MIVs play an important role of transferring information between cells,[30] and contain proteins, microRNAs, and mRNAs, which are secreted by cells in a selective manner.[26] MIVs were shown to play a role in the transfer of information by releasing their content into the target cells, and then induce epigenetic changes in the target cells.[31]

Here, we characterized the ADSC-derived MIVs and found that the MIVs had a size range of 100—300 nm, and expressed the protein Alix. We further analyzed the microRNAs in ADSCs and ADSC-derived MIVs and demonstrated that ADSC-derived MIVs selectively released microRNAs, and were rich in microRNAs related to angiogenesis, including two members of the let-7 family. To

verify the role of ADSC-derived MIVs in angiogenesis, we demonstrated that ADSC-derived MIVs promoted the cell migration and invasion of the HUVEC cells. Furthermore, using PKH26-labeled ADSC-derived MIVs we revealed that the labeled ADSC-derived MIV were uptaken into the cellular cytoplasm of HUVEC cells. Collectively, these results indicate that ADSC-derived MIVs can promote the migration and invasion abilities of endothelial cells, suggesting pro-angiogenetic potential. Future studies should focus on investigating the roles and mechanisms through which ADSC-derived MIVs regulate angiogenesis.

Materials and methods

Cell culture and chemicals

Human ADSCs were cultured in human ADSC growth medium (Cyagen, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, and 100 μg/ml glutamine. Human umbilical vascular endothelial cells (HUVEC) were cultured in DMEM (Hyclone, USA) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in the humidified atmosphere containing 5% CO_2 at 37 °C. Unless indicated otherwise, all chemicals were purchased from Sigma—Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA).

MIV isolation

MIV isolation was carried out as previously described.[26—28] Human ADSCs were cultured as described above and allowed to reach 80% confluence in T75 flasks. The cells were then washed three times with PBS, and incubated with fresh human ADSC growth medium containing 10% FBS. After 48 h incubation the conditioned media were collected, and the MIVs were collected by centrifugation. Briefly, the collected media were centrifuged at 300×*g* for 10 min at 4 °C, 1200×*g* for 20 min at 4 °C, and 10,000×*g* for 30 min at 4 °C to eliminate cell debris; and the supernatants were then collected and ultracentrifuged at 100,000×*g* for 1 h at 4 °C, followed by being washed in PBS, and ultracentrifuged at 100,000×*g* for 1 h at 4 °C. The precipitation was designated as MIVs, which were resuspended with sterile PBS and stored at −80 °C prior to the use.

Morphological and size analyses of MIVs

Scanning Electron Microscopy was used to determine the morphology of MIVs. Briefly, the isolated MIVs were resuspended in 2.5% glutaraldehyde, stored at 4 °C overnight, and then ultracentrifuged at 100,000×*g* for 1 h at 4 °C. The supernatant was discarded. The MIVs were resuspended and ultracentrifuged sequentially in the presence of 15%, 30%, 60% and 80% ethanol to be dehydrated. After the last centrifugation step, the MIVs were resuspended in 0.5 ml of 100% ethanol and stored at room temperature overnight, and then subjected to Scanning Electron Microscopy (SEM; Nova NanoSEM 450 instrument; FEI) on aluminum substrate after gold-palladium sputtering.

For the size distribution analysis, the MIVs were resuspended in 1 ml PBS and subjected to the Nano Particle Size Analyzer (PSA NANO2590; Malvern, United Kingdom) according to the manufacturer's instructions.

Western blotting analysis of MIV marker protein Alix

Protein lysate was prepared from MIVs and ADSCs using the protein extraction reagent (Applygen, Beijing, China) according to the manufacturer's instructions. The protein lysates were subjected to SDS-PAGE, followed by being transferred onto a polyvinylidene difluoride (PVD) membranes (Millipore, Bedford, MA, USA). The membranes were blocked and then incubated with anti-Alix or β-actin antibody (1:500 dilution; Abcam) at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h followed by enhanced chemiluminescence (Thermo Fisher).

MicroRNA microarray analysis of MIVs

Total RNA samples were prepared from the ADSCs (control group, n = 3) and the MIVs (experimental group, n = 3) using TRIzol (Invitrogen) according to the manufacturer's instructions. The gene expression differences between groups were analyzed using DEG-seq. The standard default was set at fold change >2 or fold change <0.05, false discovery rate (FDR) < 0.05. Differentially expressed microRNAs (dif-microRNAs) were selected and then combined with the microRNA target gene prediction database miRbase, and TargetScan to predict the target gene(s) of the dif-microRNAs.

Gene ontology (GO) analysis and pathway analysis

GO analysis was used to analyze the main function of the differentially expressed genes. Generally, Fisher's exact tests were used to classify the GO category, and the FDR was calculated to correct the p-value. Enrichment provided a measure of the significance of the function, and the threshold of significance was defined as a p-value <0.01.

The pathway analysis identifies the pathways, in which the differentially expressed genes play roles. The pathways were annotated using KEGG pathway database. The Fisher's exact tests were used to identify significantly enriched pathways. The resulting p-values were adjusted using the BH FDR algorithm. Statistical significance was defined as a p-value <0.05.

RNA isolation, reverse-transcription PCR (RT-PCR), and quantitative real-time PCR (qPCR)

Total RNA was extracted from ADSC-derived MIVs and ADSCs using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified using a Nano drop spectrophotometer (ND-1000, Nanodrop Technologies), and subjected to reverse transcription using a reverse transcription kit (TaKaRa, Otsu, Japan) to synthesize complementary DNA (RT-PCR) according to the manufacturer's

instructions. The RT-PCR products were used for qPCR analysis: 3.6 μl RNase-free H_2O, 0.2 μl forward primer (5 μM), 0.2 μl reverse primer (5 μM), 5.0 μl of 2× SYBR Green Supermix,; and RT-PCR cDNA, 1.0 μl. The qPCR cycling conditions were as follows: 95 °C for 20 s, 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and 70 °C for 1 s. All reactions were done in triplicate.

Cell wounding/migration assay

HUVEC cells were seeded in 6-well cell culture plates and allowed to reach 80% confluence, and then the monolayer culture was scratched using sterile 200 μl pipette tips. The culture medium was changed to remove any floating cells. The fresh culture medium was mixed with or without the MIVs. Microphotographs of wound closure were taken at 0 h and 24 h after cell wounding. The locations for microphotographs were marked on the plates. The % of gap remaining was also quantitatively calculated.

Boyden chamber transwell invasion assay

HUVECs were seeded onto the ECM-coated transwells of 24-well plates (Sigma Millipore), and cultured in EBM-2 + 0.25% BSA medium with or without ADSC-derived MIVs. After 24 h, the non-invading cells inside the transwells were gently removed with Q-tips, and the invading cells were fixed with pre-chilled 100% ethanol, and stained with propidium iodide. The numbers of invading HUVECs were then counted under a fluorescent microscope.

Uptake of the PKH26-labeled MIVs by HUVEC cells

MIVs were labeled with the PKH26 red fluorescent linker kit (Sigma, USA) according to the manufacturer's instructions. The labeled MIVs were resuspended and incubated with HUVEC cells at 37 °C for 12 h. After 12 h the cells were washed twice with PBS twice, and then fixed with methanol for 20 min. The fixed cells were further stained with DAPI for 20sec. The stained cells were then subjected to confocal microscopy to determine the uptake of PKH26-labeled MIVs into HUVEC cells.

Results

Isolation and characterization of the MIVs derived from ADSC cells

The MIVs secreted from ADSC cells were obtained by centrifugation as described previously.[26–28] Nanoparticle tracking analysis, SEM, and Western blotting analysis were performed to confirm the size and identity of the isolated MIVs. Briefly, a nanoparticle tracking analysis revealed that the MIV size distribution ranged from 100—300 nm particles (Fig. 1A), which were further confirmed by SEM imaging to show that nearly all particles were small vesicles (Fig. 1B). Furthermore, the expression of the MIV proteins β-actin and Alix was confirmed by using Western blotting (Fig. 1C). Collectively, these data confirm that the isolated MIVs products exhibit the characteristics of MIVs.

Microarray analysis of the ADSC-derived MIVs

The MIVs are closed vesicles and consist of molecules secreted by their originating cells, including proteins, mRNAs, and microRNAs, which together play an important role in the transfer of information between cells.[29] To explore potential contents of the MIVs, we conducted microRNA microarray analysis by comparing the samples from ADSCs and ADSC-derived MIVs. Our results revealed that 32 microRNAs were expressed differentially between ADSCs and ADSC-derived MIVs (Table 1). Among them, nine were downregulated in ADSC-derived MIVs compared with ADSCs, whereas 23 were upregulated (fold change>2 or

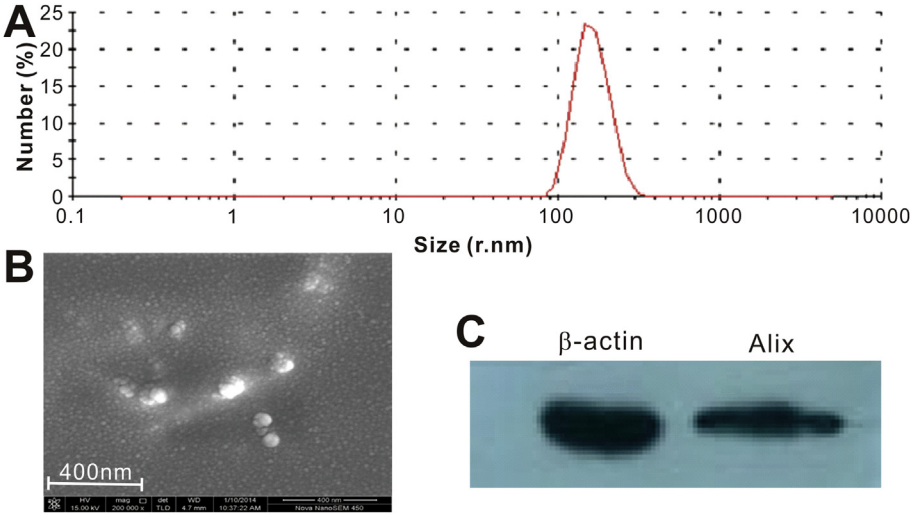

Figure 1 Identification and characterization of the ADSC-derived MIVs. (**A**) The size distribution of the MIVs isolated from ADSCs. (**B**) The morphology of the MIVs isolated from ADSCs, as determined by using an electron microscope. Representative images are shown. (**C**) Western blotting analysis of the presence of the MIV marker protein Alix (β-actin as a control) in MIVs isolated from ADSCs.

Table 1 List of the microRNAs that are differentially expressed in ADSCs *vs.* ADSC-derived MIVs (n = 3 per group).

microRNA	Expression in ADSC	Expression in MIV	Log^2 FC	P-Value
Hsa-miR-199a-3p	22.6	1.98	3.51	0.00005
Hsa-miR-4284	19.34	1.1	4.13	0.00006
Hsa-let-7f-5p	20.12	1.35	3.89	0.00006
Hsa-let7i-5p	26.41	3.9	2.76	0.00015
Hsa-miR-125b-5p	22.43	2.69	3.06	0.00097
Hsa-miR-23a-3p	18.33	1.76	3.38	0.00037
Hsa-miR-19b-3p	19.14	3.62	2.4	0.0035
Hsa-miR-15b-5p	18.32	3.74	2.3	0.00579
Hsa-miR-20a-5p	19.67	4.43	2.15	0.00657
Hsa-miR-4466	0	20.02	—	0.00001
Hsa-miR-5787	0	19.53	—	0.00001
Hsa-miR-3656	1.5	20.07	−3.74	0.00001
Hsa-miR-6068	0	17.99	—	0.00001
Hsa-miR-1246	1.96	19.74	−3.33	0.00001
Hsa-miR-2861	5.5	24.5	−2.16	0.00002
Hsa-miR-4687-3p	4.24	21.48	−2.33	0.00004
Hsa-miR-6088	4.86	21.21	−2.13	0.00009
Hsa-miR-4787-5p	2.88	16.9	−2.56	0.00016
Hsa-miR-1268a	2.43	16	−2.72	0.00016
Hsa-miR-574-5p	4.15	18.94	−2.19	0.0002
Hsa-miR-762	1.05	12.26	−3.54	0.00028
Hsa-miR-1225-5p	3.6	15.71	−2.12	0.00082
Hsa-miR-1207-5p	5.08	17.01	−1.74	0.001656
Hsa-miR-4763-3p	2.73	12.79	−2.22	0.002046
Hsa-miR-638	10.92	24.58	−1.17	0.002379
Hsa-miR-1915-3p	7.69	19.94	−1.38	0.002757
Hsa-miR-574-3p	3.4	13.22	−1.96	0.00325
Hsa-miR-1234-5p	10.75	23.66	−1.14	0.00324
Hsa-miR-6124	2.24	10.66	−2.25	0.004623
Hsa-miR-4443	9.97	21.2	−1.09	0.006794
Hsa-miR-4505	5.58	14.05	−1.33	0.013636
Hsa-miR-3934-5p	6.24	14.67	−1.23	0.015819

FC, fold change.

fold change <0.5; P-value <0.05; FDR <0.05) (Table 1). Thus, these results suggest that the release of MIVs from ADSCs may occur in a specific and organized manner, and that the bioactive molecules may be packaged into the MIVs selectively from ADSCs.

Gene ontology (GO) and pathway analyses of the target genes for the MIV-associated microRNAs

We selected the MIV-associated microRNAs (dif-microRNAs), and then used the microRNA target gene database miRbase to predict the potential target genes of the dif-microRNAs. The potential target genes were then selected to obtain the significant GO using GO analysis. The results were divided into three groups, biological process, cellular components and molecular function (data not shown). The biological processes included the positive regulation of transcription from the RNA polymerase II promoter, nervous system development, the fibroblast growth factor receptor signaling pathway, hemophilic cell adhesion, cell migration, cell differentiation, and angiogenesis ($P < 0.01$). The cellular components contained neuronal cell bodies, dendrites, plasma membrane, tight junctions, and growth cones ($P < 0.01$). Kinase activity, protein binding, protein kinase activity, vascular endothelial growth factor receptor-2 binding, transferase activity, and β-catenin binding ($P < 0.01$) were included in the molecular function group. These results suggest that ADSC-derived MIVs may regulate cell differentiation, cell migration, cell adhesion, the cell cycle, and angiogenesis through the bioactive molecules they contain.

Pathway analysis was used to identify the pathways modulated by the differentially expressed genes. The pathway annotations of the microarray genes were downloaded from KEGG; and Fisher's exact test was used to identify significantly enriched pathways. The microRNAs in ADSC-derived MIVs play roles in several different pathways, such as proteoglycans, cancer pathways, the MAPK signaling pathway, endocytosis, the Wnt signaling pathway, the VEGF signaling pathway, the Ras signaling pathway, and chronic myeloid leukemia (Fig. 2). It is conceivable that these pathways may play important roles in tumor development, angiogenesis, apoptosis, cell adhesion, and the transfer of information between cells.

Regulatory networks of MIV-associated microRNAs and their target genes

The relationships between the microRNAs and the genes they regulated were investigated by calculating their differential expression values according to the interactions between the microRNAs and genes in the Sanger microRNA database. The MicroRNA-target gene networks were then built, which connect 17 upregulated microRNAs and nine downregulated microRNAs and their potential target genes (Supplemental Figure 1). As an example, we highlighted a simple network formed by the up-regulated hsa-miR-1915-3p and its target genes (Fig. 3A), and an extended network

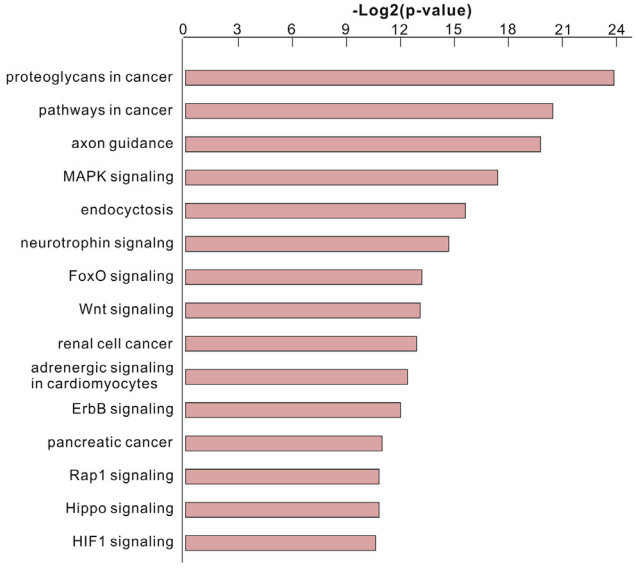

Figure 2 The KEGG pathway analysis of potential target genes of the differentially expressed microRNAs in MIVs.

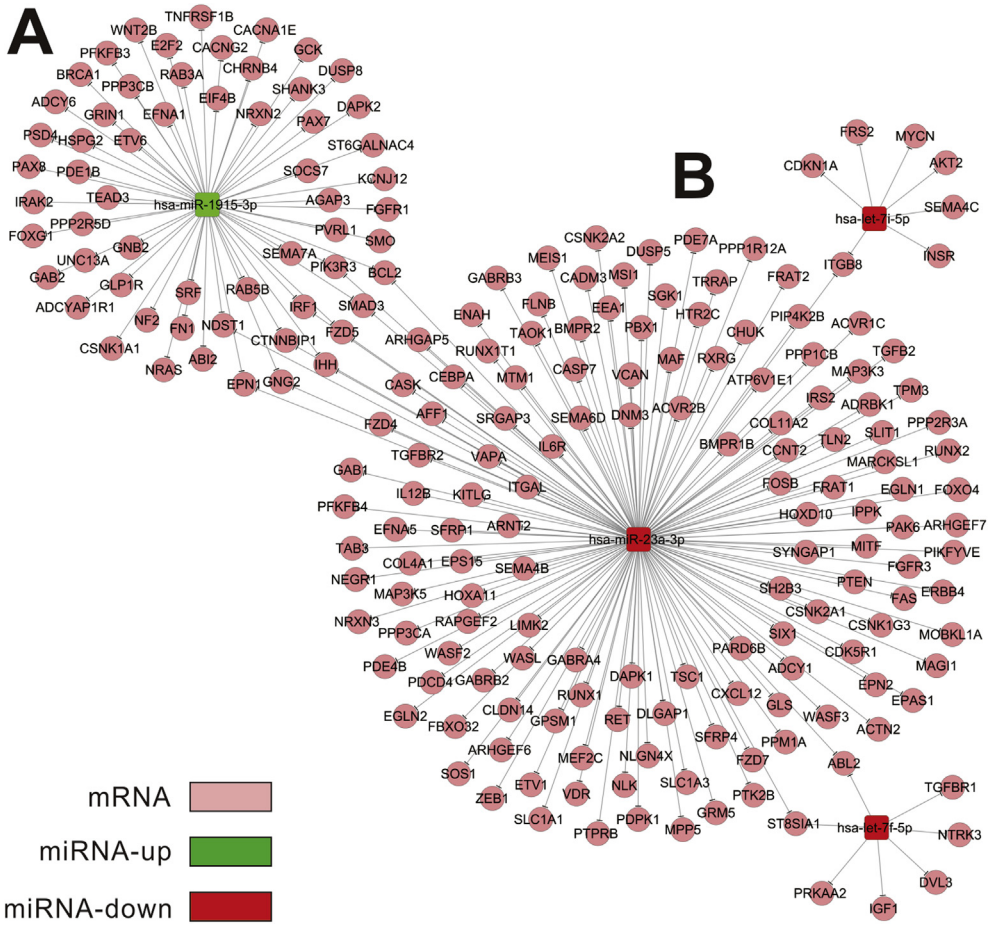

Figure 3 Representative networks of the microRNA up-regulated (**A**) and down-regulated (**B**) target gene pathways.

formed by three down-regulated miRNAs, hsa-miR-23a-3p, hsa-let-7i-5p, and hsa-let-7f-5p and their target genes (Fig. 3B). Collectively, these microRNAs are linked to the dysregulation of transcription in cancer, non-small cell lung cancer, the VEGF signaling pathway, acute myeloid leukemia, the Wnt signaling pathway, adipocytokine signaling, the MAPK signaling pathway, and the Ras signaling pathway. They also play roles in a number of biological processes, including dilated cardiomyopathy, tumor development, angiogenesis, and cell adhesion.

ADSC-derived MIVs promote cell migration of human endothelial cells

Two members (let-7i-5p and let-7f-5p) of the let-7 family, which have been shown to play an important role in angiogenesis, were identified in the microRNA-target gene network. To verify the results of the microarrays, we conducted qPCR analysis and found that the let-7i-5p was differentially expressed in ADSCs and ADSC-derived MIVs (Fig. 4A), which are consistent with the microarray results. Thus, these results confirm that the microRNAs present in the MIVs were derived from a cellular origin of ADSCs. To investigate the effects of ADSC-derived MIVs on HUVECs, we labeled ADSC-derived MIVs with PKH26 and then used them to treat HUVECs. Our results revealed that ADSC-derived MIV were located in the cytoplasm of HUVEC cells (Fig. 4B), suggesting that the ADSC-derived MIVs may be uptaken by HUVEC cells.

The microarray analysis, GO analysis, and pathway analysis revealed that ADSC-derived MIVs may play a role in angiogenesis. We hypothesized that ADSC-derived MIVs may directly affect HUVECs. To test this hypothesis, we investigated whether ADSC-derived MIVs affected the migration of HUVECs. HUVECs treated with ADSC-derived MIVs exhibited enhanced migration compared with the control group (Fig. 5A panel *a*). Quantitative analysis indicates that approximately 31% of the wounded gap remained open in the no MIVs group, compared with <5% gap open in the +MIVs group ($p < 0.01$) (Fig. 5A, panel *b*). Similarly, the Boyden chamber transwell invasion assay revealed that ADSC-derived MIVs significantly promoted the invasion capability of the HUVEC cells (Fig. 5B). Collectively, these results suggest that ADSC-derived MIVs may have potential to promote angiogenesis by enhancing both migration and invasion of HUVEC cells.

Discussion

The proangiogenic potential of ADSC-derived MIVs may be explored as therapeutic strategies for clinical conditions such as myocardial infarction (MI). MI is a serious form of

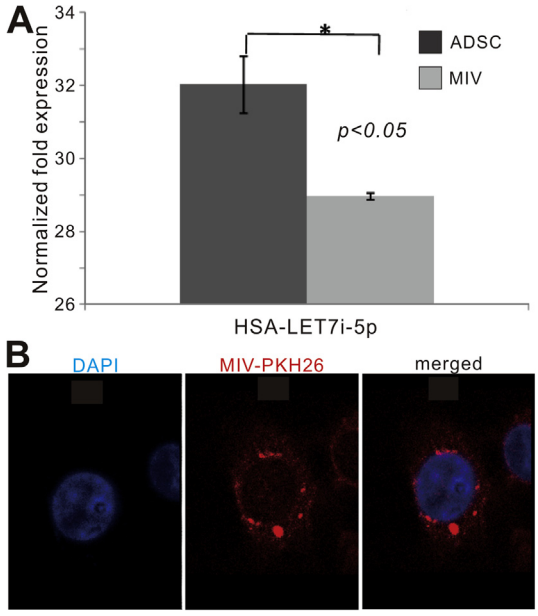

Figure 4 MIVs can be uptaken by HUVECs. (**A**) the qPCR analysis of the expression of let-7i in ADSCs and ADSC-derived MIVs. *p $<$ 0.05. (**B**) the MIVs were labeled with PKH26 (red), and then incubated with HUVECs. HUVEC nuclei were labeled with DAPI (blue), and examined under a fluorescence microscope. Representative images are shown.

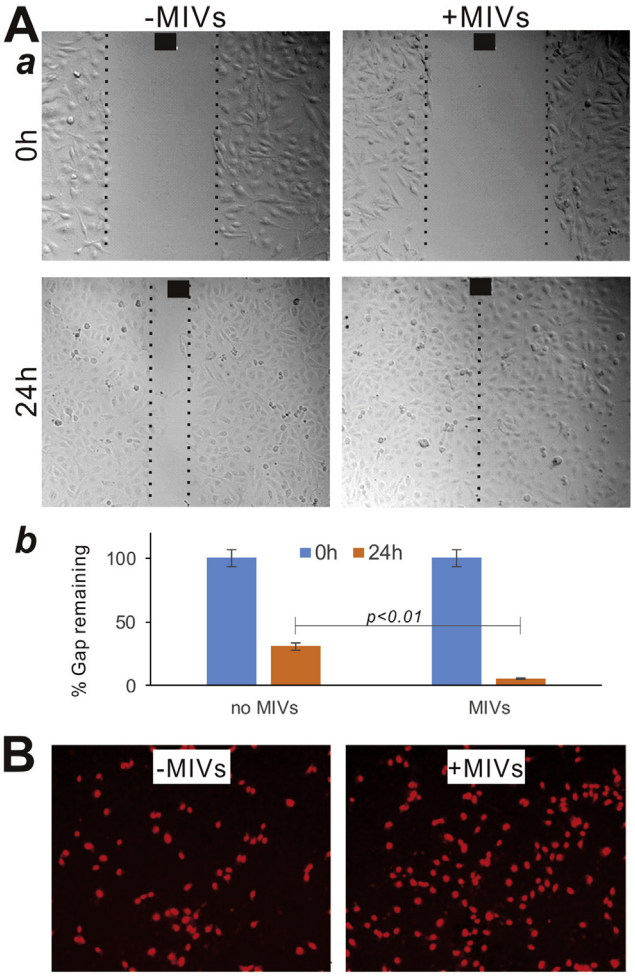

Figure 5 ADSC-derived MIVs promote the cell migration of HUVECs. (**A**) The effect of MIVs on cell wound heading. Subconfluent HUVECs were scratched (0 h) and maintained with (+MIVs) or without MIVs (-MIVs). Wounding areas were photographed again in 24 h (***a***). Black squares represent the reference points. Representative images are shown. The % of gap remaining was quantitatively calculated and graphed (***b***). (**B**) Boyden Transwells invasion assay was carried out using HUVECs by incubating the cells with (+MIV) and without (-MIV) MIVs. Representative images are shown.

acute coronary syndrome (ACS) with myocardial ischemic necrosis, and is caused by a sharp reduction or interruption in coronary arterial blood due to thrombus formation in the intravascular tissues or persistent spasm in the coronary artery. Because the underlying pathology of MI is coronary artery lesion, the main treatment is coronary artery reperfusion.[32] Only a limited number of patients can undergo CABG surgery, and up to 30% of patients do not have transplantable veins.[6,33] Novel treatments have been introduced in recent years, including cooling and stem cell transplantation.[34,35] The treatment of MI using stem cell transplantation involves the transplantation of stem cells into damaged cardiac muscle where they differentiate into cardiac muscle or endothelial cells or affect the microenvironment.[36–39] The repair of the myocardium is closely related to the blood supply,[15] and successful treatment depends on either coronary recanalization or building a novel vascular access. Successful cases of treatment using stem cells are not rare. For example, patients with lymphatic leukemia and myeloid leukemia have been treated successfully using hematopoietic stem cells, those with Parkinson's disease and stroke have been treated with mesenchymal stem cells.[40,41] Additional studies have shown positive effects of stem cell treatment in MI, and follow-up data suggest that there are also considerable long-term effects.[8–10] The use of stem cell therapy for MI might involve the promotion of angiogenesis.[42]

Stem cells can regenerate blood vessels after differentiating into endothelial cells and vascular smooth muscle cells or by promoting angiogenesis in the areas surrounding the damaged myocardium via paracrine mechanisms.[43,44] Mallela et al investigated the effects of stem cells in angiogenesis and the role of natriuretic peptide receptor A (NPRA) in tumor development.[45] Furthermore, Matsumura et al indicated a role for bone marrow mesenchymal stem cells in angiogenesis by differentiating into endothelial cells and vascular smooth muscle cells.[46] Stem cells can promote angiogenesis via paracrine pathways by secreting VEGF, rich cysteine protein 61 (Cyr61), and other factors.[47] However, there are several concerns over the use of stem cell-based therapies. The first problem is the selection and collection of stem cells. While bone marrow mesenchymal stem cells are the most widely used adult stem cells, there are disadvantages associated with their use, including low recovery rate, high morbidity and adverse effects associated with harvest, and potential host immune response if allotransplantation is used. Furthermore, there are some issues associated with stem cell therapy, including the induction of differentiation and potential tumorigenesis.[48]

ADSCs have been shown to differentiate into endothelial cells and vascular smooth muscle cells to promote angiogenesis.[37,38,49,50] Unlikely many other sources of mesenchymal cells, ADSCs are derived from adipose tissue with easy access and rich resources, and may replace bone marrow stem cell as the mainstay of stem cell transplantation.

ADSCs can also release MIVs, which vary in sizes with diameters ranging from 30—1000 nm. Many types of cells can secret MIVs, including ADSCs, tumor cells, bone marrow mesenchymal stem cells, and HUVECs. MIVs contain numerous bioactive molecules, including proteins, mRNAs, and microRNAs, which are released from cells to play an important role in the transfer of information.[51] Body fluids such as blood, urine, and cerebrospinal fluid can transport MIVs to target cells.[52] Therefore, MIV may be used as disease diagnostic markers in body fluids.

Since MIVs are secreted by cells with the release of bioactive molecules selectively packaged by cells into MIVs, it is conceivable that MIVs may replace its origin cells to play biological functions of cells. It was shown that mesenchymal stem cell-derived MIVs could protect the heart by replacing mesenchymal stem cells in this cardiac protective function.[53] Similar observations were reported regarding the role of tumor cell-derived MIVs in angiogenesis.[54] MIV-based therapy has more advantages than stem cell therapy, as MIVs do not need to be induced and do not solicit host immune response. Furthermore, their smaller sizes mean that it is easier for MIVs to reach the target cells than stem cells.[55]

Although MIVs contains numerous types of bioactive molecules, one of the most important biomolecules that are released by MIVs is microRNAs, which are short non-coding RNAs (sized 20—25 nucleotides) that regulate gene expression at the post-transcriptional level and hence modulate biological processes including the cell cycle, differentiation, apoptosis, and angiogenesis.[56,57] Since Lin-4 and let-7 were the first and second microRNAs discovered in *Caenorhabditis elegans*, respectively,[58,59] more than 2000 microRNAs have been identified in humans to date. It was reported that microRNAs may play an important role in angiogenesis by regulating signal pathways, HUVEC migration, and HUVEC proliferation and differentiation.[60,61]

The let-7 microRNA family have 13 members in humans including let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, let-7i, mir-98, and mir-202. Angiogenesis plays a critical role in the formation of blood vessels and the maintenance of homeostasis. HUVEC is the key regulator of vascular biology, including angiogenesis. Let-7 plays an important role in angiogenesis, and is expressed at high levels in HUVEC. Let-7 has been shown to play an important role in angiogenesis.[62] However, the exact mechanism by which let-7 promotes angiogenesis is not fully understood. Several angiogenesis-related factors including platelet reaction protein, matrix metalloproteinnase-1, and VEGF-2 may be involved in let-7-mediated angiogenesis.[63—65] The hypoxia-inducing factor 1α/let-7/Argonaut 1/VEGF signaling pathway may play a role in hypoxia-induced angiogenesis.[66] It was reported that let-7i decreased in co-cultured glioma and brain capillary endothelial cells, as well as in co-cultured neural stem cells and endothelial cells.[67—69] Nonetheless, the potential roles of let-7 in both tumor angiogenesis and brain angiogenesis via neural stem cells should be further investigated.[68]

In summary, we investigate the potential use of ADSC-derived MIVs as a replacement of stem cell therapy to promote angiogenesis. We characterized the MIV products using electron microscopy, nanoparticle tracking analysis, and Western blotting. We found that the MIV products had a size range of 100—300 nm, and expressed the protein Alix. We further analyzed the microRNAs in ADSCs and ADSC-derived MIVs using microRNA, GO, signaling pathway analysis, as well as microRNA-target-gene network analysis. Our results demonstrated that ADSC-derived MIVs selectively released microRNAs, and were rich in microRNAs related to angiogenesis, including two members of the let-7 family. To verify the role of ADSC-derived MIVs in angiogenesis, we analyzed the effect of ADSC-derived MIVs on HUVEC migration and invasion. Our results indicate that ADSC-derived MIVs can promote the migration and invasion abilities of endothelial cells. Furthermore, PKH26-labeled ADSC-derived MIVs were effectively uptaken into the cellular cytoplasm of HUVEC cells. Future studies should be directed to investigate the role and mechanism through which of ADSC-derived MIVs regulates angiogenesis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2019.04.005.

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