



Original Article

Detection of MicroRNAs in Extracellular Vesicles Secreted by Umbilical Cord-derived Mesenchymal Stem Cells

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Abstract: Extracellular vesicles (EVs) are emerging as a potential candidate for disease treatment due to their function of bioactive cargoes. Recently, mesenchymal stem cells (MSC)-derived EVs have shown their capacity to replace parental cells as their similar functions to MSCs. The therapeutic effects of EVs depend on their cargoes, such as DNA, miRNA, proteins, and lipids. In this study, we expanded umbilical cord-derived MSCs (UCMSCs) for EV release. Additionally, we evaluated the expression level of several microRNAs in three EV populations, including apoptotic bodies (AB), microvesicles (MV), and exosomes (EX). The results show that UCMSCs released three EV types: AB, MV, and EX into culture media. The three EV populations were different in morphology and size. Three EVs were detected to carry microRNAs, such as hsa-miR-320, hsa-miR-181b, and hsa-miR-140. Among these microRNAs, hsa-miR-140 expressed with the greatest level, followed by hsa-miR-181b and hsa-miR-320. The results of this study provide more knowledge about UCMSC-derived EV miRNAs and reveal the potential role of UCMSC-EVs associated with detected miRNAs.

Keywords: Extracellular vesicles, umbilical cord-derived mesenchymal stem cells, microRNAs.

1. Introduction

Mesenchymal stem cells (MSCs) are pluripotent adult stem cells isolated from mature tissues and able to renew and

differentiate into different cell lines such as chondrocytes, osteocytes, and adipocytes [1]. In addition, MSCs released a large number of growth factors, chemokines, and cytokines that had the ability to influence neighboring cells. In fact, these secretory factors are capable of increasing angiogenesis, reducing apoptosis and fibrosis, enhancing neuronal survival and

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differentiation, stimulating extracellular matrix regeneration, limiting inflammation, and modulating immune responses [2]. In this way, MSCs directly or through paracrine secretion stimulated the regeneration of damaged cells, reduced tissue damage, and ultimately accelerated organ repair. Besides, molecules secreted by MSCs worked as mediators that activated target cells or stimulated neighboring cells to secrete activity factors [3]. Recently, evidence showed that MSCs, including UCMSCs, secreted many extracellular vesicles (EVs) involved in tissue regeneration through the transfer of information to damaged cells or tissues, and biological activity is similar to MSCs [4, 5].

Extracellular vesicles (EVs) surrounded by a lipid bilayer are released from various cell types. They have also been detected in cell culture media [2, 6, 7] as well as body fluids, such as saliva [8], plasma [9], breast milk [10], and amniotic fluid and urine [11]. EV can be classified into three categories of apoptotic body, microvesicle or ectosome, and exosomes. Exosomes (EX) are the product of a complex process starting from an endocytic vesicle formation that will then be undergone development to form a multivesicular body and then releasing exosomes to the extracellular environment by the fusion of multivesicular membrane with the cellular membrane. Exosomes are the smallest population with a size range from 30 nm to 250 nm. The microvesicles or ectosomes are shed directly from the extracellular membrane at the sites happening the transition of phosphatidylserine. Microvesicles (MV) have irregular morphology and size ranges from 100 nm to 1000 nm. The largest population is apoptotic bodies (AB) that have size from 1000 to 5000 nm and are product of apoptosis [4]. Among three EV populations, exosomes is the most attracted by scientists due to their important roles in many biological processes, for example, enhancing osteoclastogenesis [12], alleviating liver fibrosis [13], promoting angiogenesis [14], promoting cutaneous wound healing [15], and skin rejuvenation [16]. However, evidence

suggested that not only exosomes, microvesicles, apoptotic bodies are also able to regulate various pathways. The mechanism under this modulation is that EVs carry many bioactive molecules and transfer these to the target cells.

MicroRNAs (miRNAs) are small non-coding RNAs that play roles in targeting mRNAs for cleavage or translational repression [17]. miRNAs are generally 19-24 nucleotides long and estimatedly regulate more than 60% of the translation of protein-coding genes [18, 19]. After Validi et al., [20] found 121 miRNAs in exosomes released from mast cells, many other studies have found miRNAs in EV isolated from different sources; for example, blood cells, immune cells, and body fluids [20, 21]. Recently, Than et al., [7] reported the miRNAs detected from all three AB, MV, and EX from keratinocytes. Because miRNAs are often functional and can be transferred to target cells through the EV mechanism, EV miRNAs could be a potential source of efficient diagnostic and disease-specific therapy.

Importantly, the cargoes in EVs are not identical between the three EV types and EVs released by different cell types. The components carried by EV depend on the difference in conditions such as the secreting cell origin, culture conditions, physiological or pathological condition of the cells, etc. Additionally, these components determine the roles of EVs. Therefore, this study investigated the microRNAs, including hsa-miR-320, hsa-miR-181b, and hsa-miR-140, present in three EV populations, including apoptotic bodies, microvesicles, and exosomes, secreted by UCMSCs. Different miRNAs have been reported in UCMSC-derived EVs [22, 23], but these three miRNAs of hsa-miR-320, hsa-miR-181b, and hsa-miR-140 have not been detected in EV released by human UCMSCs previously; they have only been reported in EVs secreted by human adipose- and bone marrow-derived MSCs [24, 25]. These miRNAs have also been illustrated their involvement in

osteoarthritis. Thus, data from this study is important to reveal the potential roles of UCMSC-derived EVs in osteoarthritis treatment.

2. Materials and Methods

2.1. Umbilical Cord-derived Mesenchymal Stem Cell Expansion

UCMSCs were received from the EV groups at passage two (P2). The cells were thawed in the water bath (37 °C) and seeded into T75, or T225 cell culture flasks (Nunc, Thermo Scientific, Massachusetts, United States) containing DMEM/F12 (Dulbecco's Modified Eagle's Medium, Gibco, USA) supplemented with 10% FBS (Foetal Bovine Serum, Gibco, USA) with a density of 375×10^3 cells/cm² as passage 3. The culture flask was surface-coated with CTS™ CELLstart™ substrate (Gibco, Massachusetts, USA) diluted in PBS at the rate of 1: 300 and washed twice with PBS before cell seeding. UCMSCs were then incubated at the condition of 5% CO₂/37 °C for the expansion. When cells reach 80% confluency, they were split using CTS™ TrypLE™ Select Enzyme (Thermo Fisher Scientific, USA) for the next passage. At the culture of P5, UCMSCs were incubated to reach 80% confluency and released EVs into conditioned media (supernatant) that were harvested for further EV isolation. The UCMSCs were split for marker analysis.

2.2. Human Mesenchymal Stem Cells Marker Analysis

The UCMSCs at P5 were harvested following supernatant collection using CTS™ TrypLe™ Select Enzyme (Thermo Fisher Scientific, USA). Then they were subjected to marker analysis using Human MSC Analysis Kit (BD Biosciences, California, USA). The kit includes the MSC positive cocktail (FITC CD90, PerCP-CyTM5.5 CD105, and APC CD73) and the MSC negative cocktail (PE: CD45, CD34, CD11b, CD19, and HLA-DR). Staining was

performed according to the manufacturer's instructions. Flow cytometry was performed using a Beckman Coulter flow cytometer equipped with Navios software.

2.3. Cellular Senescence Analysis of Human Mesenchymal Stem Cells

UCMSCs at the P5 cultured in DMEM/F12 supplemented with 10% FBS were analyzed for cellular senescence using Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Missouri, USA). Cells were seeded in a six-well plate at 0.2×10^6 cells/well and incubated overnight at 37 °C and 5% CO₂. After incubation, the culture medium was removed, and cells were washed twice with 1X PBS (Gibco™, USA). Next, cells were fixed with 1X Fixation Buffer for 6 min at room temperature prior to being rinsed three times with 1X PBS and then incubated in Staining Mixture overnight at 37 °C without CO₂. The cells were washed twice with 1X PBS and stained with DAPI Staining Solution (Abcam, Cambridge, UK) for 5 min at room temperature. After staining, cells were washed twice with 1X PBS, examined under an inverted microscope IX73 (Olympus, Tokyo, Japan), and images were captured with a digital color camera. The images were semi-qualitatively analyzed with Image J. Software (version 1.46r).

2.4. EV Isolation

The supernatant from UCMSC cultures containing EV was centrifuged at $300 \times g$ for 10 min at 4 °C to remove cell debris. The supernatant was collected and subjected to a centrifuge at $2,000 \times g$ for 10 min to collect apoptotic bodies, followed by a centrifuge at $16,500 \times g$ for 30 min at 4 °C to collect microvesicles. The supernatant after microvesicle collection was centrifuged at $100,000 \times g$ for 90 min at 4 °C (Optima XPN-100 Ultracentrifuge, Beckman Coulter, California, USA) to collect exosomes. The EV pellets were resuspended in 100 µL PBS and stored at -80 °C for further uses.

2.5. Extracellular Vesicles Morphology Analysis

Transmission electron microscopy (TEM) was used to analyze the individual morphology and morphological homogeneity of each EV population. EVs were fixed using 4% paraformaldehyde (Sigma-Aldrich, Germany) and then deposited on Formvar-carbon coated grids (TED PELLA Inc., CA, USA) and allowed to stand for 20 min in a dry environment. The grids were washed with PBS and then were stained with uranyl-oxalate, pH 7 (4% uranyl acetate (w/v), pH 4 in 0.15 M oxalic acid with ratio 1: 1). Finally, the grids were allowed to dry at room temperature. Imaging was performed using a JEOL 1100 TEM (JEOL Ltd.) for different magnification levels at 80 kV.

2.6. MicroRNA Analysis

2.6.1. Total RNA Extraction

Total RNA was extracted using the Trizol™ method following the manufacturer's protocol. Suspensions of whole cells, ABs, MVs, and EXs, were dissolved in Trizol™ reagent (Thermo Fisher Scientific, USA) in the volume ratio of 1: 9 (100 µL vesicles: 900 µL Trizol). Chloroform was then added to the sample and vortexed vigorously before being centrifuged at $12,000 \times g$ for 5 min for the aqueous phase collection. Then, isopropanol was added and incubated at $-20\text{ }^{\circ}\text{C}$ for 1 hour (or overnight). After incubation, the samples were centrifuged at $12,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, and RNA pellets were washed with RNase-free 75% ethanol twice. The RNA pellets were allowed to air dry, and the dried pellets were resuspended in the range of 10 - 20 µL RNase-free water (Invitrogen, USA). Total RNA concentration was determined using Nanodrop® ND-1000 (Thermo Scientific) spectrometer.

2.6.2. cDNA Synthesis

Extracted total RNAs (described above) were used as template RNAs to prepare complementary deoxyribonucleic acid (cDNA) using miScript II RT kit (QIAGEN, Germany) following the manufacturer's instructions. All reagents and template RNA were mixed to create a 10 µL for a half-reaction (2 µL 5X miScript Hispec Buffer, 1 µL 10X miScript Nucleics Mix,

1 µL miScript Reverse Transcriptase Mix, and 6 µL template RNA). The mixture was incubated in a Bio-Rad T100™ Thermal Cycler (Bio-Rad) at $37\text{ }^{\circ}\text{C}$ for 1 hour, $95\text{ }^{\circ}\text{C}$ for 5 min, and then $6\text{ }^{\circ}\text{C}$ to protect cDNA from degradation.

2.6.3. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has been used to identify the presence of specific miRNAs, including hsa-miR-140, hsa-miR-320, and hsa-miR-181b (Qiagen, Germany) in three populations of UCMSC-derived EVs. Details of primers are described in Table 1.

All samples were prepared in triplicate in a reaction mix with a total volume of 10 µL. Each well in a MicroAmp® Fast optical 96-well plate (Applied Biosystems™, China) contained 5 µL SYBR Green (Qiagen, Germany), 1 µL 10X miScript Universal Primer (Qiagen, Germany), 1 µL 10X miScript Primer Assay (Qiagen, Germany), 1.5 µL RNase-free water (Invitrogen, USA), and 1.5 µL cDNA template (1 ng of cDNA). The qRT-PCR of samples was performed using an Applied Biosystems 7500 Block (Applied Biosystems™) under the following conditions: holding stage ($95\text{ }^{\circ}\text{C}$ for 15 min); 3 step-cycling stage ($94\text{ }^{\circ}\text{C}$ denaturation for 15 seconds; $55\text{ }^{\circ}\text{C}$ annealing for 30 seconds; $70\text{ }^{\circ}\text{C}$ extension for 34 seconds); and melt curve stage ($95\text{ }^{\circ}\text{C}$ for 15 seconds; $60\text{ }^{\circ}\text{C}$ for 1 min; $95\text{ }^{\circ}\text{C}$ for 30 seconds and $60\text{ }^{\circ}\text{C}$ for 15 seconds) and 40 cycles. The qRT-PCR data were analyzed for cycle threshold (Ct) values inversely proportional to target DNA copy number in the sample, and RNU6 was used as an internal control gene.

3. Results and Discussion

3.1. Morphology and Marker Expression of UCMSCs

To analyze morphology and surface phenotype of UCMSCs, the cell was cultured in conventional cell culture media DMEM/F12 supplemented with 10% FBS. Images under the inverted microscope showed a typical heterologous morphology of spindle-shaped

(Figure 1A). Additionally, the surface phenotype analysis using flow cytometry showed that positive markers, including CD90 ($94.1 \pm 1.84\%$), CD105 ($93.87 \pm 2.55\%$), and CD73 ($95.18 \pm 3.09\%$), were greater than 90%, and negative markers, including CD45, CD34, CD11b, CD19, and HLA-DR, were lower than 2% ($0.76 \pm 0.35\%$) (Figure 1B). These data indicate that UCMSCs cultured at passage 5 to secrete EV are consistent and express the typical characteristics of MSCs regarding morphology and surface markers.

3.2. Cellular Senescence

To evaluate the physiological characteristics of UCMSC at the time of EV release, the cellular senescence of UCMSCs at P5 was examined.

The obtained data show few cells positive with senescence-associated β -galactosidase activity (blue areas - yellow arrows) (Figure 2). The majority of cells are negative with cellular senescence signals. These data indicate that almost all UCMSCs at the culture for EV secretion remain in the normal physiology of MSCs.

Table 1. MiRNA primer sequence for qRT-PCR

MiRNA name	MiRNA primer sequence	miScript Assay Cat#
hsa-miR-320	5' AAAAGCUGGGUUGAGAGGGGCGA	MS00014707
hsa-miR-181b	5' CUCACUGAACAAUGAAUGCAA	MS00042252
hsa-miR-140	5' CAGUGGUUUUACCCUAUGGUAG	MS00003500

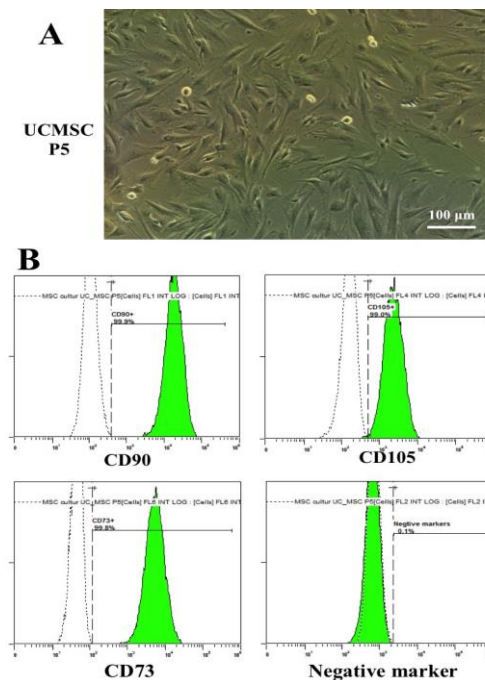


Figure 1. Human primary mesenchymal stem cells isolated from the umbilical cord at passage 5 cultured in DMEM/F12 supplemented with 10% FBS. (A) Typical morphology of UCMSCs captured under Nikon Inverted Microscope Eclipse Ti-S, objective 10X; and (B) immune-phenotypes of UCMSCs at P5, including positive markers (CD90, CD105, and CD73) and negative markers (CD45, CD34, CD11b, CD19, and HLA-DR) were analyzed.

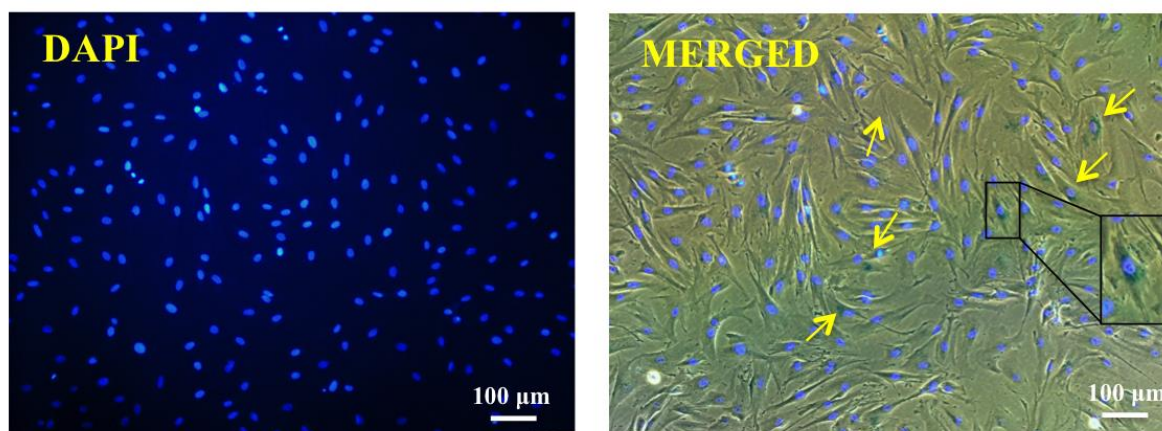


Figure 2. Cellular senescence of UCMSCs at passage 5 cultured in DMEM/F12 supplemented with 10% FBS was analyzed by Senescence Cells Histochemical Staining Kit (Sigma-Aldrich). The results show that several cells expressed cellular senescence signals. A) DAPI staining for nuclear detection; B) The cells expressed nucleus in blue and senescence signals in green (yellow arrows). The images are representative of 3 biological samples.

3.3. EV Morphology

In order to separate the three EV populations from UCMSC cultures, differential centrifugation has been applied with different g-forces. EV pellets were examined under the transmission electron microscope and photographed. Data showed that three populations of AB, MV, and EX had been characterized with different morphology and size (Figure 3). AB population has an irregular shape with a size range from 700 nm to 2.5 μm; MV has an irregular morphology, unsmooth surface, and size from 300 nm to 700 nm; and EX has typical cup-shaped morphology with the size from 50 nm to 250 nm. These data indicate that three EV populations have been isolated successfully from UCMSC culture media.

3.4. Detection of MicroRNAs in Three EV Populations

For the purpose of investigating microRNAs carried by EVs, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was employed to quantify target miRNAs of interest, including hsa-miR-140, hsa-miR-181b, and hsa-miR-320, present in

each EV population. Resulted data are represented by $2^{-\Delta\Delta C_t}$ values, which indicate the changes in the level of miRNA expression.

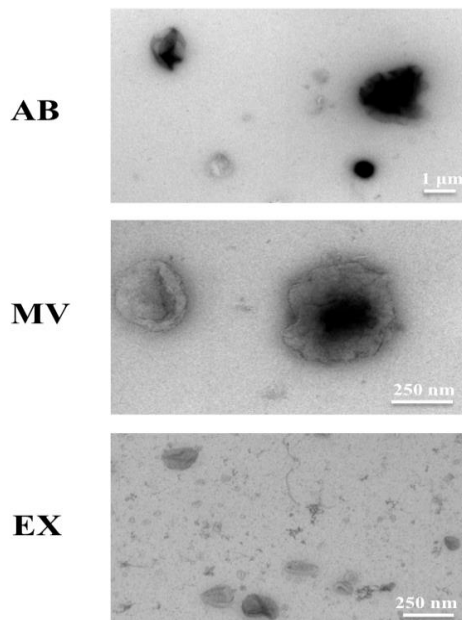


Figure 3. EV morphology imaged under the transmission electron microscope. Data show the heterogeneous morphology of AB and MV and cup-shape morphology of EX.

RNU6 was used as endogenous control, and the miRNA expression levels in EVs were normalized to parental cells. The results show that all three miRNAs were detected in three EV populations released from UCMSCs and UCMSC parental cells (Figure 4). The fold changes of hsa-miR-140 and hsa-miR-181b were greater than the parental cells meaning that these miRNAs expressed higher in EVs compared to the secreting cells. On the other hand, the expression level of hsa-miR-320 was lower in the three EV populations.

There was no difference in a target miRNA among the three EV populations. However,

there was a different association with the expression levels of different miRNAs in each EV population. In detail, hsa-miR-140 showed a greatest increase in the miRNA expression in MV population (p-value < 0.001), followed by hsa-miR-181b (p-value < 0.05) and then hsa-miR-320 (p-value < 0.05). Additionally, the hsa-miR-181b was expressed higher than hsa-miR-320 in EX population (p-value < 0.05). The difference of miRNAs in AB was of no statistical significance.

Data from this indicate the different expressions of target miRNAs in each EV population.

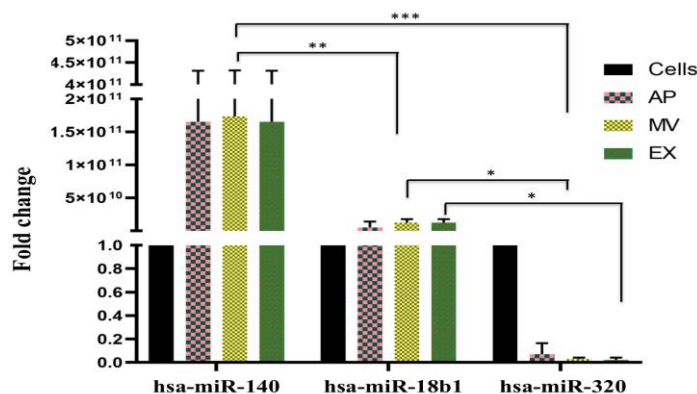


Figure 4. microRNA expression in three EV populations (AB, MV, and EX). The miRNA expression levels are represented by $2^{-\Delta\Delta C_t}$ values with 1 ng of input cDNA. miRNA expression was normalized to parental cells, while much more hsa-miR-140 and hsa-miR-181b detected in all three EV populations and greater than parental cells, the expression level of hsa-miR-320 is lower than the parental cell. Additionally, hsa-miR-140 expressed the greatest in the MV population. The results were averages of 3 biological samples. Statistical significance was determined by ANOVA and T-Test and is determined by: * where p-value < 0.05; ** where p-value < 0.01; and *** where p-value < 0.001.

4. Discussion

In an attempt to investigate the miRNAs present in EVs released from UCMSCs, the cells were maintained in conventional media. Data from cell cultures showed the normal physiological state of UCMSCs at the time point of EV secretion (Figures 1, 2). Additionally, the success in EV isolation using differential centrifugation showed three populations of AB, MV, and EX. These three EV populations expressed different morphology and size range (Figure 3). The EV results herein

are also consistent with the previous investigation [7].

Numerous reports on the positive effects of EVs from MSCs in the treatment have been published [15, 16]. The effect of EVs is determined by their cargoes, which can be lipids, proteins, or microRNAs [29]. It is noted that the EV secretion and EV cargoes depend on many factors, such as secreting parental cell sources, culture conditions, stimulative factors, EV isolation techniques [30]. For example,

dendritic cells exposed to lipopolysaccharide produce EVs expressed more immunogenic activity [31]. Or stimulation of toll-like receptors can induce HEY cells to release exosomes with pro-inflammatory activity [32]. Additionally, isolation techniques affect the EV purity and isolation performance [30, 33]. However, this field is encountering challenges in developing stable secreting cell sources for particular therapeutic agents as well as isolation techniques that meet the requirement of large scale.

Interestingly, we have detected several microRNAs, including hsa-miR-140, hsa-miR-181b, and hsa-miR-320, present in three UCMSC-derived EV populations. The detection of miRNAs has been reported previously in EVs released by skin keratinocytes [7] or exosomes isolated from plasma of healthy people or cancer patients [34, 35]. The detection of miRNAs in EVs is important to direct further investigations of EVs according to their miRNA contents. According to Qiu et al., (2018), MSC-derived EVs can regulate outcomes of renal, liver, heart, and brain diseases by transferring their miRNA contents [36]. This current study has identified hsa-miR-140, which expressed the greatest compared to the two others of hsa-miR-181b and hsa-miR-320 in all three EV populations. All these three miRNAs have been reported in exosomes released by human adipose-derived MSCs [24], but not UCMSCs. Although this hsa-miR-140 has not been reported yet in UCMSC-derived EVs somewhere else, the miRNA was found with high expression in UCMSC-derived EVs herein. On the other hand, hsa-miR-181b, which is expressed highly in three EV populations from this current study, has also been identified in EVs released by keratinocytes [7]. Moreover, the hsa-miR-320, which was detected in exosomes isolated from plasma of healthy subjects and patients with ovarian cancer [34, 35], was detected with very low abundance in EVs released by UCMSCs in this study. Those miRNAs have been reported to be involved in osteoarthritis biology; for instance, miR-140 could attenuate the progression of osteoarthritis by down-regulating chondrocyte

senescence and apoptosis, promoting cartilage formation, and inhibiting cartilage degeneration [27, 37]. Additionally, the miR-320 could also reduce cartilage degeneration, or miR-181 reduce oxidative cartilage stress, consequently protecting osteoarthritis from the progression. Due to the detection of these miRNAs in UCMSC-derived EVs in this study, these EV miRNAs should be further investigated for their roles in osteoarthritis condition, from *in vitro* to *in vivo* models.

5. Conclusion

In this study, we successfully detected miRNAs in three EV populations that originated from human UCMSCs. UCMSCs secreted AB, MV, and EX that carried hsa-miR-140, hsa-miR-181b, and hsa-miR-320. hsa-miR-181b was expressed with the greatest levels in all three EVs, followed by hsa-miR-181b, and the lowest expression level belongs to hsa-miR-320. The results herein are preliminary data that requires more investigations on other miRNAs and proteins in EVs. For future perspectives, more studies should be performed to investigate the roles of these EV miRNAs in regulating different biological processes.

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