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Distinct Mirna Expression Patterns of Extracellular Vesicles Derived From 4 Types of Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are known to reside in the stromal fraction of many tissues and have multiple differentiations. MSCs isolated from different sources have functional heterogeneity that is controlled by dynamic interactions between extracellular signaling, epigenetic, transcriptional and post-translational regulation. Extracellular vesicles (EVs) derived from MSCs are one of the main factors responsible for the therapeutic effect of MSCs. We propose here that EVs from different types of MSCs maintain the imparity. Recent studies have revealed that microRNAs (miRNAs), one of the integral cargoes of EVs, play a crucial role in translational regulation. We thus examined the miRNAs expression patterns in EVs derived from mesenchymal stem cells isolated from the bone marrow (BM), adipose tissue (AT), Wharton's jelly (WJ) and human exfoliated deciduous teeth (SHED) and summarized the biological functions of common and specific miRNAs as well as their target genes in physiological and pathological processes. The data presented here help compare the biological properties and potential of EVs from these distinct MSC populations.

Keywords: Mesenchymal stem cells; Extracellular vesicles; Bone marrow-mesenchymal stem cells; Adipose tissue-mesenchymal stem cells; Stem cells from human exfoliated deciduous teeth; Wharton's Jelly-derived mesenchymal stem cells; MicroRNAs

Introduction

Mesenchymal stem cells (MSCs) are a population of fibroblast-like cells derived from nearly all tissues in adult (adipose tissue, bone marrow, exfoliated deciduous teeth, peripheral blood) and fetal tissues (placenta, Wharton's jelly and umbilical cord) [1,2]. These cells have the capability of differentiating into fat, bone, cartilage, muscle and neurons depending on the stimulus and culture conditions [3]. MSCs isolated from various tissue sources exhibit significantly different morphologies, differentiation capabilities, and gene expression. MSCs from different tissues are widely known to not be biologically equivalent and to have variable self-renewal and multipotent capabilities. Bone marrow (BM)-MSCs were first described by Friedenstein et al. and are usually considered to be the gold standard [4,5]. Adipose tissue (AT)-MSCs, also known as adipose-derived mesenchymal stem cells (ADSCs), have strong proliferation, repair and regeneration abilities [6,7]. Previous researches has revealed that Wharton's Jelly (WJ)-derived MSCs have more powerful immunosuppressive and therapeutic activities [8-10]. Stem cells from human exfoliated deciduous teeth (SHED) were reported to differentiate into neural cells and express neuronal markers under neural induction [11,12].

MSCs are emerging as a novel powerful tool for the treatment of various diseases. MSC EVs function as an extension of MSC's biological roles. They exert specific effects on their microenvironment and play important roles in intercellular communication in both healthy and diseased tissues.

MicroRNAs (miRNAs) were firstly identified in 1993 [13] and are small non-coding small RNAs of approximately 20-22 nucleotides. MiRNAs regulate approximately 30-70% gene expression through binding to the 3' untranslated region (UTR) of target mRNAs. As one of the cargo content of EVs, miRNAs are key contributors to the overall biological function of EVs and the source cells. They are known to post-transcriptionally regulate the expression of genes involved in the differentiation pathways of MSCs [14-17].

Methods

Culture of BM-MSCs, AT-MSCs, WJ-MSCs and SHED

Ethical approval (IRB No.18000015) was obtained from Tsukiji clinic cosmos. Four types of mesenchymal stem cells were donated by four individuals. Bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived mesenchymal stem cells (AD-MSCs) were obtained from the patients who received medical treatment of injecting cells for ameliorating structural changes associated with skin aging before the enforcement of the Act on the Safety of Regenerative Medicine on November 25, 2014 and consenting full-term provided. BM-MSCs were isolated according to the method described by Pittenger *et al*. [18]. AD-MSCs were isolated according to the method described by Zuk PA *et al*. [19]. Wharton's jelly mesenchymal stem cells (WJ-MSCs) were extracted from the human umbilical cord (UC). The UC was obtained from the patients who provided full consent, and the UC was taken immediately after natural childbirth. WJ-MSCs were isolated according to the method described by Sarugaser *et al*. [20]. Stem cells from human exfoliated

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deciduous teeth (SHED) were obtained from the patient consenting full-term provided from the deciduous teeth immediately. SHED were isolated according to the method described by Gronthos *et al*. [21] and Miura *et al*. [22]. Each cell type was cultured with Dulbecco's modified Eagle's medium with 4500 mg/L of glucose, 584 mg/L of L-glutamine, 110 mg/L of sodium pyruvate, and 3700 mg/L of sodium bicarbonate (DMEM D6429; Sigma-Aldrich, St. Louis, MO, USA) containing 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of the antibiotic-antimycotic amphotericin B (100 x; Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (S1820 FBS; Biowest Nuaillé, France). When the cells proliferated to 80-100% confluency, they were passaged using TrypLE™ Select (Gibco™) and passaged until P4. All P4 passaged cells were used for collected conditioned medium (CM).

EV purification

Prior to CM collection, MSCs were washed twice with PBS, and the medium was switched to fresh serum-free medium (Dulbecco's modified Eagle's medium with 584 mg/L of L-glutamine (DMEM D6429; Sigma-Aldrich, St. Louis, MO, USA). After incubation for 48 h, the CM was collected and centrifuged at 2,000 × g for 10 min at 4°C. To thoroughly remove cellular debris, the supernatant was filtered through a 0.22-µm filter (Millipore). The CM was then used for EV isolation. To prepare EVs, CM was ultracentrifuged at 35,000 rpm using a SW41Ti rotor for 70 min at 4°C for 3 times. The pellets were washed with 11ml PBS, ultracentrifuged at 35,000 rpm using the SW41Ti rotor for 70 min at 4°C. After ultracentrifugation, they were resuspended in PBS. The EVs yield per 10^6 MSCs per day was 1-4 × 10^8 particles as determined by NTA, or 1-4 ug protein, as determined by the Bradford method.

RNA extraction and miRNA analysis

Total RNA was extracted from EVs using QIAzol reagent and the miRNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of extracted RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc. USA) and the Agilent Bioanalyzer system (Agilent Technologies, USA), as recommended. Total RNA was labeled with cyanine 3 (Cy3) using the miRNA Complete Labeling and Hyb Kit (Agilent Technologies) as instructed by the manufacturer. Briefly, total RNA was dephosphorylated by incubating with Calf Intestinal Alkaline Phosphatase (CIP) Master Mix at 37°C for 30 min. Dephosphorylated RNA was denatured by incubating with DMSO at 100°C for 5 min and then immediately transferred to ice for 2 min. After addition of a ligation master mix for T4 RNA Ligase and Cyanine 3-Cytidine bisphosphate (Cy3-pCp), the RNA was incubated at 16°C for 2 h. Labeled RNA was dried using a vacuum concentrator at 55°C for 1.5 h and then hybridized onto Agilent SurePrint G3 Human miRNA 8x60K arrays at 55°C for 20 h. After washing, the microarrays were scanned using an Agilent DNA microarray scanner. The intensity values for each scanned feature were quantified using Agilent Feature Extraction software version 10.7.3.1, which performs background subtractions.

Microarray data analysis

The intensity values of the miRNA microarray were log2-transformed and imported into Partek Genomics Suite 6.6 (Partek Inc, Chesterfield, MO, USA). For gene expression analysis, fold changes were calculated for each analysis. Unsupervised clustering and heat map generation were performed with sorted or whole datasets based on the Euclidean distances of the average linkage clustering with selected probe sets using Partek Genomics Suite 6.6.

Results & Discussion

Common miRNAs in BM-MSCs, AT-MSCs, WJ-MSCs and SHED

Using miRNA microarray, the expression levels of 2551 miRNAs were examined from EVs derived from BM-MSCs, AT-MSCs, WJ-MSCs and SHED. We employed a criterion of signal intensity > 3 at a log2 value for each miRNA expression as being highly expressed. Ninety-one miRNAs were commonly detected in 4 types of MSC EVs (Figure 1). Table 1 lists 8 miRNAs that are biologically well-documented. Several studies have suggested that miR-199-3p is involved in the regulation of the cell cycle, cancer progression,

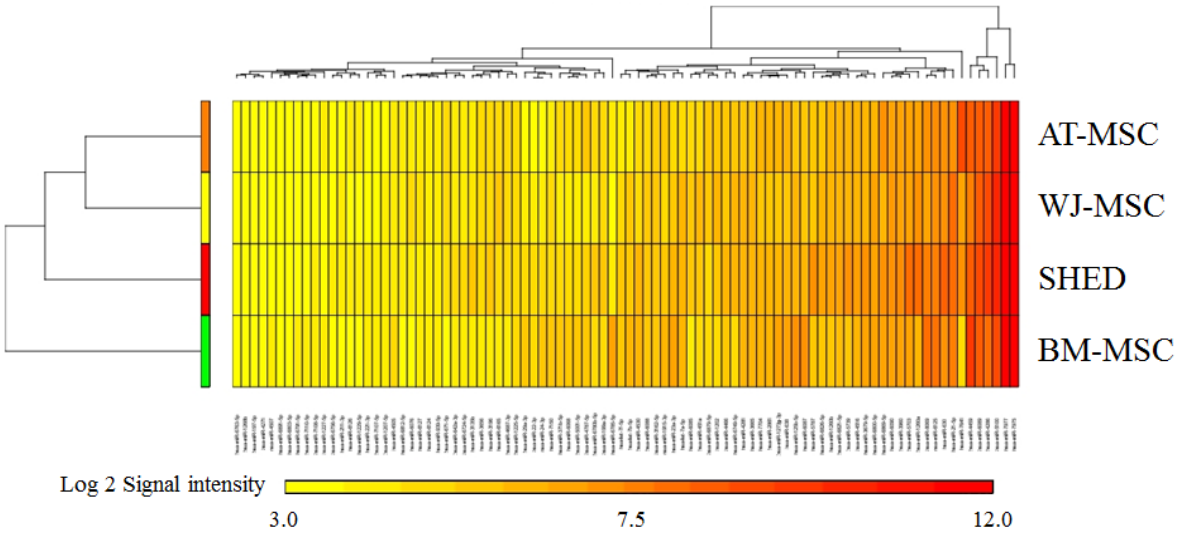

Figure 1: Commonly detected miRNAs in BM-MSCs, AT-MSCs, WJ-MSCs and SHED. With the criterion of signal intensity more than 3 at log2 ratio, 91 miRNAs are selected as positively-detected miRNAs in 4 types of MSCs. The selected 91 miRNAs are used to visualize as a heatmap

Common miRNAs			
miRNA	**Target genes**	**Function**	**References**
miR-199a-3p	CD44, c-MYC, cyclin D1, EGFR, HIF1α	↓cell-cycle progression ↓cancer cell proliferation ↑adipocyte differentiation	[23] [24]
miR-24-3p	H2AX MYC, E2F2 CCNB1, CDC2, p27Kip1, VHL	↑DNA repair ↓cell-cycle progression ↑cell proliferation ↑terminal differentiation	[25] [26]
miR-29a-3p	ZFP36, VDAC1, VDAC2, CDC42	↓inflammation ↑cancer cell proliferation	[27] [28]
miR-23a-3p	HAS2, SMAD3 E-cad PDCD4	↑senescence ↑cell motility ↓cancer cell apoptosis ↑cancer cell proliferation	[29] [30] [31]
miR-638	BRCA1 CDK2 DACT3	↓cancer cell proliferation ↓DNA repair ↑myeloid differentiation ↑autophagy	[32] [33] [34]
miR-125b-5p	Smad4 HMGA2 MCL1	↑pre-adipocyte differentiation ↓proliferation ↑senescence ↑proliferation	[35] [36] [37]
miR-630	IGF1R	↓cell motility	[38]
miR-21-5p	PTEN, Bcl2, hMSH2, PDCD4 JAG1	↑cancer cell proliferation ↑dendritic cell differentiation ↑inflammation	[39] [40] [41]

Table 1: Common miRNAs.

differentiation and inflammation. MiR-199a-3p exerts the suppressive functions in prostate cancer by targeting CD44 and several mitogenic molecules including c-MYC, cyclin D1 and EGFR [23]. These results presented here offer a possibility for developing a miR-199a-3p delivery system by EVs into anti-PCa replacement therapeutics. Additionally, miR-199a-3p functions as an obesity-associated miRNA that is induced during adipogenesis [24]. MiR-24 upregulation in post-replicative cells reduced the H2AX levels, make cells vulnerable to DNA damage, and inhibited cell cycle progression by down-regulating of multiple E2F- and MYC-regulated genes [25,26]. MiR-29a has been repeatedly implicated in apoptosis, but its pro and anti-apoptotic functions have not been elucidated [27,28]. MiR-23a-3p has been shown to cause cellular senescence by targeting HAS2 [29]. Overexpression of miR-23a-3p decreases E-cadherin expression and significantly reverses osthole-mediated inhibition of cell invasion, while silencing of miR-23a-3p partially inhibits cell motility [30]. MiR-23a/b significantly suppresses PDCD4 expression and enhances tumor growth in gastric cancer [31]. MiR-638 which can reduce the proliferation, invasion, and DNA repair capabilities and is associated with downregulation of BRCA1 expression [32]. In addition, miR-638 regulates proliferation and myeloid differentiation by targeting *CDK2 [33,34]*. Restoring the miR-638 expression levels by delivering EVs may contribute to the treatment of acute myeloid leukemia treatment. MiR-125b-5p has been suggested to serve as an important positive regulator in adipocyte differentiation partially by down-regulating Smad4 [35]. These results indicated that miR-125b-5p encapsulated in the EVs of MSCs may be a potential target in obesity and metabolic diseases. Several reports have shown that miR-21-5p is able to regulate the expression of other target genes such as PTEN, MSH2, Cdc25A, SPRY2 and PDCD4, and therefore is associated with a worse response to therapy in other tumor types [36-41].

Specific miRNAs in AT-MSCs, BM-MSCs, SHED and WJ-MSCs

Specific miRNAs in AT-MSCs: To identify cell-type specific miRNAs, we excluded miRNAs that were not detected in any of the 4 types of MSC EVs. There were 397 miRNAs left that were expressed in at least one sample. Among these miRNAs, specifically expressed miRNAs in each type of MSCs were selected based on expression levels. 4, 25, 29 and 35 miRNAs were selected as cell-type specific miRNAs in EVs derived from AT-MSC, WJ-MSC, SHED and BM-MSC, respectively (Figure 2). We also summarized these findings in Table 2 by showing biologically well-documented miRNAs. In Table 2, miR-424-5p, which was specifically expressed in AT-MSC EVs, possessed diverse functions in different biological processes. In the metastatic process of hepatocellular carcinoma, miR-424-5p reversed EMT by directly targeting ICAT, and further reconstituted the E-cadherin/β-catenin complex on the cell membrane [42]. MiR-424-5p was frequently upregulated in pancreatic cancer and modulated the ERK1/2 signaling pathway by negatively regulating SOCS6 [43]. MiR-424, which is induced by hypoxia in human endothelial cells, targeted cullin 2, a scaffolding protein critical to the assembly of the ubiquitin ligase system. Thus, miR-424 stabilizes HIF-α isoforms and promotes angiogenesis [44]. MiR-424 also participates in osteogenic differentiation [45].

Specific miRNAs in BM-MSCs: EVs secreted by BM-MSCs, the most widely studied mesenchymal stem cells, expressed several distinct miRNAs (Figure 2D). Almost all of the miRNAs listed were reported to be correlated with differentiation. MiR-140-5p has been revealed to directly repress BMP2 and inhibited osteogenic lineage commitment in undifferentiated human MSCs [49]. MiR-140-5p has been shown to inhibit TGFBRI in hepatocellular carcinoma (HCC), and its overexpression may suppress HCC growth and metastasis [50]. In ovarian cancer, miR-140-5p increases PDGFRα expression, and enhances ovarian cancer cell proliferation [51]. Overexpression of

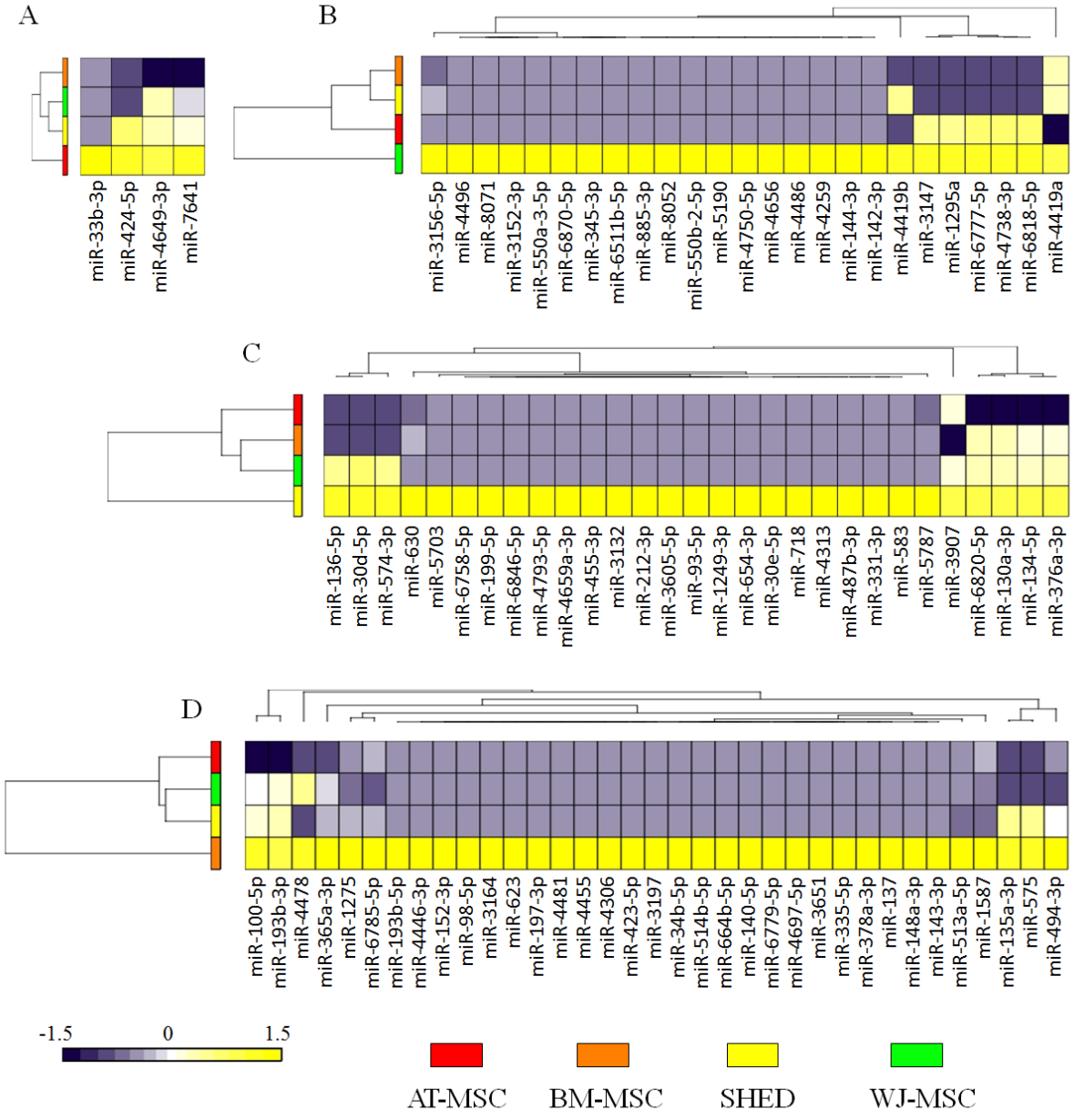

Figure 2: Specific expression of miRNAs in BM-MSCs, AT-MSCs, WJ-MSCs and SHED.
Based on selection criterion of fold change > 2 in the comparison with others, highly-expressed miRNAs in each type of MSCs are calculated. A-D, as specific miRNA sets, the heatmaps are produced in 4 types of MSCs, respectively

miR-140-5p disrupts autophagy in colorectal cancer stem cells through both Smad2 and ATG12, and impacts the downstream regulators of autophagy, such as cathepsin B, cathepsin S, and immunity-related GTPase family M [52]. MiR-335-5p has been previously reported to regulate the osteogenic, chondrogenic and adipogenic differentiations of MSCs [52-55]. Meanwhile, miR-378a-3p has also been associated with differentiation of myoblasts, osteoblasts, and adipocytes [56,57]. MiR-378a-3p induces adipogenesis by targeting MAPK1 [58]. By targeting Gli3, miR-378a-3p suppresses the activation of hepatic stellate cells during liver fibrogenesis [59]. MiR-137 is an essential regulator of neurogenesis during multiple developmental stages. It negatively regulates cell proliferation and accelerates neural differentiation of embryonic neural stem cells through a regulatory loop with nuclear receptor TLX and LSD1 [60]. Together with miR-124, miR-137 induces the differentiation of adult mouse neural stem cells, mouse oligodendroglioma-derived stem cells and human glioblastoma multiforme-derived stem cells as well as glioblastoma multiforme cell cycle arrest [61,62]. By reducing both the mRNA and protein expression levels of Cdc42, miR-137 inhibits proliferation, induces G1 cell cycle arrest, and blocks invasion of the colorectal cancer cells [63].

Specific miRNAs in SHED: SHED have been identified as a novel population of postnatal stem cells that are capable of differentiating into neural cells, odontogenic cells, and adipocytes. MiR-199b-5p is a positive erythroid regulator during erythroid differentiation and is dependent on the binding of GATA-1 and NF-E2 to its gene locus

Distinct miRNAs				
miRNA	**Source**	**Target genes**	**Function**	**References**
miR-424-5p	AT-MSCs	ICAT SOCS6 CUL2 FGFR1	↓EMT ↑cancer proliferation ↑angiogenesis ↑differentiation	[42] [43] [44] [45]
miR-423-3p	BM-MSCs	AdipoR2 p21Cip1/Waf1	↓cancer proliferation ↑cancer proliferation ↑cell growth and cycle	[46] [47] [48]
miR-140-5p	BM-MSCs	BMP2 TGFBR1, PDGFRA Smad2	↓differentiation ↓cancer proliferation ↑cancer proliferation ↓cell-cycle progression	[49] [50] [51] [52]
miR-335-5p	BM-MSCs	DKK1, Daam1, ROCK1	↑differentiation (osteoblast, chondrocyte, terminal)	 [53] [54] [55]
miR-378a-3p	BM-MSCs	HDAC4, MoyD, MHC, GalNT-7 MAPK1 Gli3	↑differentiation (myoblast, osteoblast, adipocyte) ↑angiogenesis ↓liver fibrosis	 [56] [57] [58] [59]
miR-137	BM-MSCs	LSD1, CDK6, Ezh2, Jarid1b CDC42, CDK6	↓cell proliferation ↑neuronal and glial differentiation ↓cell-cycle progression	[60] [61] [62] [63]
miR-199b-5p	SHED	HES1 c-Kit	↓cancer proliferation ↑erythroid differentiation	[64] [65]
miR-455-3p	SHED	Runx2	↑chondrogenic differentiation	[66]
miR-212-3p	SHED	RFXAP SGK3 SOX11	↑immune tolerance ↓cancer proliferation ↓neuronal differentiation	[67] [68] [69]
miR-144-3p	WJ-MSCs	ABCA1 c-Met PTEN, Smad4	↑inflammation ↑apoptosis ↑cell growth ↓osteoblast differentiation	[70] [71] [72] [73]
miR-142-3p	WJ-MSCs	APC TBP2 CSF1 IL6st, cAMP/PKA CD133, ABCG2, Lgr5	↑differentiation (osteoblast, myeloid, erythroid, macrophage) ↓macrophage differentiation ↑cancer proliferation ↓cancer proliferation	 [74] [75] [76] [77] [78] [79] [80]

Table 2: Distinct miRNAs.

[64,65]. MiR-455-3p activates early chondrogenesis by inhibiting the expression of Runx2 [66]. MiR-212-3p increases SOX11 expression and halts the process of neurogenesis at the stage of immature neurons as well as impairs the final maturation of neurons into mature granule cells in the chronic phase of epilepsy [66-80].

Specific miRNAs in WJ-MSCs: Previous studies have revealed that WJ-MSCs exhibited more powerful proliferative, immunosuppressive and therapeutic activities compared to MSCs derived from adult BM or AT. MiR-144-3p negatively regulates osteogenic differentiation and proliferation of murine MSCs by targeting Smad4 [73]. MiR-142-3p has been shown to promote myeloid differentiation in hematopoietic stem/progenitor cells, osteoblast differentiation in the human fetal mesenchymal precursor cells, and erythroid differentiation in human embryonic stem cells [74-76]. In addition, miR-142-3p improves the macrophage differentiation potential of human peripheral blood monocytes [77]. However, it prevents macrophage differentiation of both canonical and non-canonical modulation of the gp130 and C/EBPβ signaling during tumor-induced myelopoiesis [78]. The findings from recent studies on miR-142-3p expression and its functions in cancer are somewhat diverse. For instance, miR-142-3p is upregulated in T-cell acute lymphoblastic leukemia and acts as an oncogene by targeting the cAMP/PKA pathway [79]. On the other hand, this miRNA has been reported to inhibit the growth of colon cancer cells accompanied by the downregulation of CD133, Lgr5 and ABCG2 [80].

Conclusion

MicroRNAs have been recently recognized as molecular regulators at the posttranscriptional level in a variety of biological processes. By repressing and activating mRNA translation and stability, miRNAs are involved in inflammation, apoptosis, angiogenesis, cell growth and mobility. MSCs are defined as an archetype of multipotent somatic stem cells. Furthermore, the subpopulations of MSCs isolated from different tissues have various characteristics. Based on their accessibility, expandability and multipotentiality, MSCs hold a promise for future stem cell-based therapy strategies. MSCs EVs have been considered to be an extension of the biological roles of MSCs. In this study, we have analyzed the miRNA expression profiles of EVs derived from human BM-MSCs, AT-MSCs, WJ-MSCs and SHED and summarized the molecular mechanisms and target genes of these functional miRNAs. The common and distinct miRNAs help compare the characteristics and therapeutic potential of EVs secreted by these four types of MSCs. Our results provide a foundation for a deeper and more precise

understanding of MSC EVs and highlight their biochemical potential to restore tissue homeostasis.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from Tsukiji clinic cosmos (IRB No.18000015) by No. 1 in 2015.

Consent For Publication

Not applicable

Availability of Data and Material

All the data was shown in the supplementary material.

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Authors' Contributions

Toshimitsu Itaya provided the culture medium of 4 types of MSCs. Maki Abe extracted the EVs from MSCs. Yueyuan Zhou analyzed the miRNAs and wrote the paper. Yusuke Yamamoto, Takahiro Ochiya and hongdang Xiao reviewed and edited the manuscript. All authors read and approved the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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