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Original Article

BMSC-derived microvesicles assist the migration and homing of multiple myeloma XG-7

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Abstract: Microvesicles (MVs) have been demonstrated to be implicated in the communication between bone marrow stromal cells (BMSCs) and cancer cells resulting in the promotion of the developments of diverse cancer. This study aimed to investigate the effects of BMSC-derived MVs on migration and homing of multiple myeloma (MM) cells. We used differential ultracentrifugation to obtain MVs confirmed by nanoparticle tracking analysis and observed that BMSC-derived MVs could fuse with MM cells. Further, it could also be observed that BMSC-derived MVs could (i) promote the proliferation of MM cells within at least 48 h, (ii) facilitate the migration of MM cells through transwell chamber confirmed by scratch test, (iii) increase the levels of CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt, β-catenin proteins by 1.92-, 1.50-, 2.80-, 2.00-, 2.27-, 2.67-, 1.72-, 2.53-, 6.10-, 2.84- and 3.72-fold which were MM-related. In a rat model, we found that MM cells could accumulate in several internal organs, i.e. heart, lung and spleen after co-incubated with BMSC-derived MVs. These results suggested that BMSC-derived MV could be a promising potential drug target for MM treatment as it could be helpful in the migration and homing of MM cells.

Keywords: Microvesicles, bone marrow stroma cell, multiple myeloma, migration, homing

Introduction

Multiple myeloma (MM) is the second most frequent hematological malignancy characterized by the accumulation of malignant plasma cells within bone marrow (BM) compartment and devastating bone lesions. Men are more inclined to be suffered with MM than women, and the age-adjusted incidences vary globally from 6/100000 per year in USA and most developed countries to 1/100000 per year in China [1]. Although in the past decade, the median survival of MM patients has been greatly improved from 3 to 6 years as a result of the advents of several potent anti-MM drugs, especially lenalidomide and bortezomib, and drug combinations [2], the supportive roles of BM microenvironment in heterogeneous features of MM including differentiation, migration, proliferation, prognosis, and drug resistance make it mostly incurable in current stage [3].

The BM microenvironment can be divided into a cellular compartment which consists of hematopoietic cells (e.g. myeloid/T/B/NK cells and osteoclasts) and non-hematopoietic cells (e.g. bone marrow stromal cells (BMSCs), fibroblasts, and blood vessels), and a non-cellular compartment which is composed of the extracellular matrix (ECM), oxygen, and the liquid milieu (cytokines, growth factors, and chemokines) [3, 4]. These microenvironment compartments can work together and make diverse impacts on MM development. The trafficking processes that MM cells home to BM and interact directly with ECM and BMSC promote the metastasis/migration of MM to new BM sites [4], the communications of which may be mediated by a type of membrane vesicle termed microvesicle (MV) [5].

MVs are small membrane-covered heterogeneous cell fragments with a diameter of 0.1-1 μm and can be secreted by all types of eukary-

otic cells including red blood cells, lymphocytes, endothelial cells as well as tumor cells constitutively or upon stimulation [6-9]. Mounting evidences demonstrated that MV could be potential biomarkers for prognostic and therapeutic purposes in many sorts of cancer [10-12]. Although MV originated from MM cells was believed to participate in the promotion of angiogenesis [5], however, the roles of MV secreted from BMSCs in the regulation of MM progression remained largely unknown.

This study aimed to isolate BMSCs-secreted MV and investigate their promotion on migration and homing of MM cells via CCK8 test, scratch assay, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunoblotting.

Materials and methods

Cell, BMSC, mice, reagents and antibodies

MM cell XG-7 was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). The BMSC was obtained from a MM patient who signed informed consent. The whole experiment was preapproved by medical ethics committee of Taishan Medical University. The 6-week-old C57/BL6 mice were purchased from the Animal Center of Taishan Medical University. The CCK8 kit, protein marker, pancreakin and DEME medium were acquired from KeyGEN (Nanjing, Jiangsu, China). SDS-PAGE loading buffer and fetal calf serum (FCS) were purchased from CWBIO (Beijing, China) and MRC (Shanghai, China) respectively. The primary anti-human CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt, β-catenin and GAPDH antibodies and the second anti-human antibody were purchased from Sigma (Shanghai, China).

Isolation of BMSC-derived MVs

The isolation was in accordance to the procedures described previously with less modifications [5]. Briefly, the BMSCs (1×10^7) were firstly cultured in 20 mL serum-free DMEM medium for 24 h at 37°C and centrifugated at 1000× g for 5 min to collect the supernatant. Subsequently, the supernatant was centrifuged respectively at 4000× g for 1 h at 4°C to remove the cellular debris and at 16000× g for 1 h at 4°C to remove the exosomes existent in the resultant supernatant. The precipitates contained MVs which was washed for several times and resuspended in PBS. Another round of centrifugation was employed to remove the remaining exosomes at 16000× g for 1 h at 4°C. The isolated MVs were stored in PBS at 4°C for use.

Nanoparticle tracking analysis (NTA) of BMSC-derived MVs

BMSC-derived MVs were isolated and collected as described above, and the experiment followed the procedures in a previous report [13]. Briefly, the particle size distribution and quantity of BMSC-derived MVs were processed at a range of concentration of 4×10^8-8×10^8 cells/mL by NanoSight LM10 with NTA 2.2 analytical software according to the manufacturer's instructions (NanoSight, UK).

Membrane interaction between BMSC-derived MVs and MM cells

As previously reported [14], the BMSC-derived MVs were stained with 1 μmol/L anti-CD138-FITC (Lab-bio, Beijing, China), and then incubated with XG-7 cells for 24 h at 37°C. After washed twice with sterile PBS, the XG-7 cells were counterstained with DAPI (Beyotime, Shanghai, China) and visualized using confocal laser scanning microscope (CLSM, NikonA1Si, Nikon, Tokyo, Japan).

Proliferation of MM cells incubated with BMSC-derived MVs

One hundred microliter XG-7 cells (5×10^5 cells/mL) were seeded into each well of a 96-well microplate (Corning, NY, USA) for 24 h of incubation at 37°C in a 5% CO_2 atmosphere (v/v). Then, 10 μL of MM-MVs were added at a final concentration of 5 μg/mL and cultured for an additional 12, 24, 36, or 48 h. After incubations, each well was added 10 μL CCK8 solution and cultured for another 2 h according to the product's instructions. Finally, optical density was detected by a microplate reader (Sanco Instruments, Shanghai, China) at 450 nm.

Transwell migration assay

The transwell (Corning, NY, USA) migration assay was performed as previously described

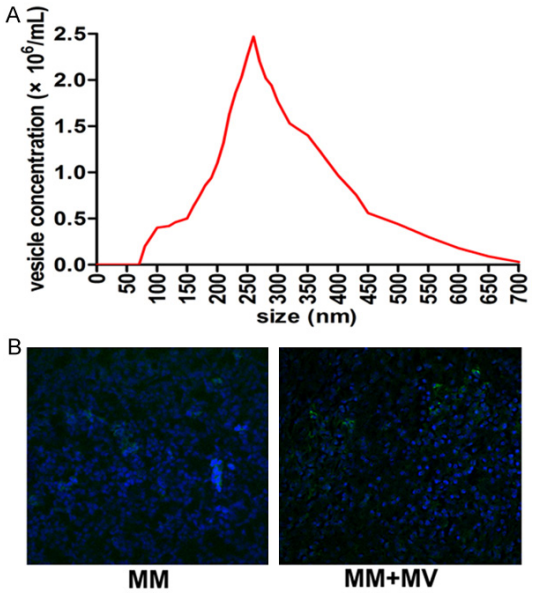

Figure 1. Isolation of BMSC-derived MVs by differential ultracentrifugation. A. Size distributions of BMSC-derived MVs were identified using the NTA. B. BMSC-derived MVs were taken up by XG-7 cells.

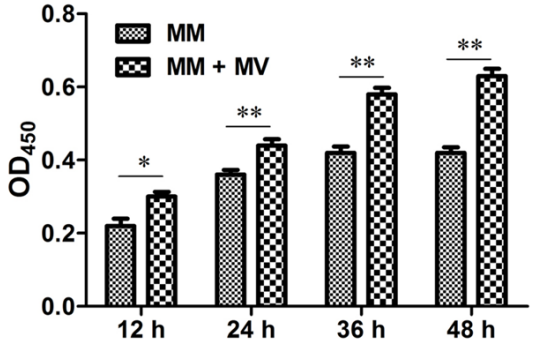

Figure 2. Proliferation of XG-7 cells (5×10^5 cells/mL) treated with/without 5 μg/mL BMSC-derived MVs were assessed using a CCK8 kit after 12, 24, 36, and 48 h of incubations. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

with minor modifications [15]. The cells were collected by digestion, rinsed three times by DEME medium and resuspended to the final concentration of 5×10^5 cells/mL. The top chambers were seeded with 200 μL of XG-7 cell suspension, and the bottom chambers were added in 600 μL of DMEM medium supplemented with 10% FCS and 5 μg/mL fibronectin. Then, 5 μg/mL BMSC-derived MVs was added to the top chamber. After 12, 24, 36, and 48 h of incubations at 37°C, the MM cells migrated to the bottom chamber were fixed by pre-cold methanol for 30 min, stained by DAPI for 10 min, photographed and counted.

Scratch test

The scratch test was performed to evaluate the in vitro migration potential of XG-7 cells after incubated with/without BMSC-derived MVs as previously reported with a few modifications [16]. XG-7 cells were pre-incubated with BMSC-derived MVs for 24 h, suspended in DEME medium and centrifuged to remove BMSC-derived MVs at 4000× g for 1 h. The obtained XG-7 cells were washed three times by DEME medium and resuspended to the final concentration of 5×10^5 cells/mL for use. The XG-7 cells were seeded in 6-well plates at a density of 500000 cells/well and cultured to reach 80-90% confluence. Then, the cell monolayers were scratched by sterile pipette tips. The wounded monolayers were washed three times with PBS to remove debris and scratched cells, and incubated in serum-free DEME medium at 37°C 5% CO_2 for 24 h to allow cells to migrate into the wounded area. After 24 h of incubation, the images were recorded.

Western blotting

The procedures were in accordance to a previous study with a few modifications [8]. The target proteins were extracted by EpiQuik Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA) and quantified by a BCA Protein Quantification Kit (vazyma, Nanjing, Jiangsu, China) with bovine serum albumin (BSA) as standard. The sample (= 50 mg) containing target protein were processed by electrophoretic separation before being transferred to PVDF membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked by 5% milk solution for 2 h at room temperature and washed three times by TBST (Sigma-Aldrich, USA). The membrane was respectively incubated with the primary monoclonal mouse anti-human CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt, β-catenin and GAPDH (Ebiosciences) at 4°C overnight at a dilution of 1:500. The membrane was immersed in PBST containing 0.5% tween-20 with three times, and then incubated with the second anti-human antibody conjugated with horseradish peroxidase (ZSGB-BIO, Beijing, China) at 1:500 for 90 min at room temperature. The proteins were

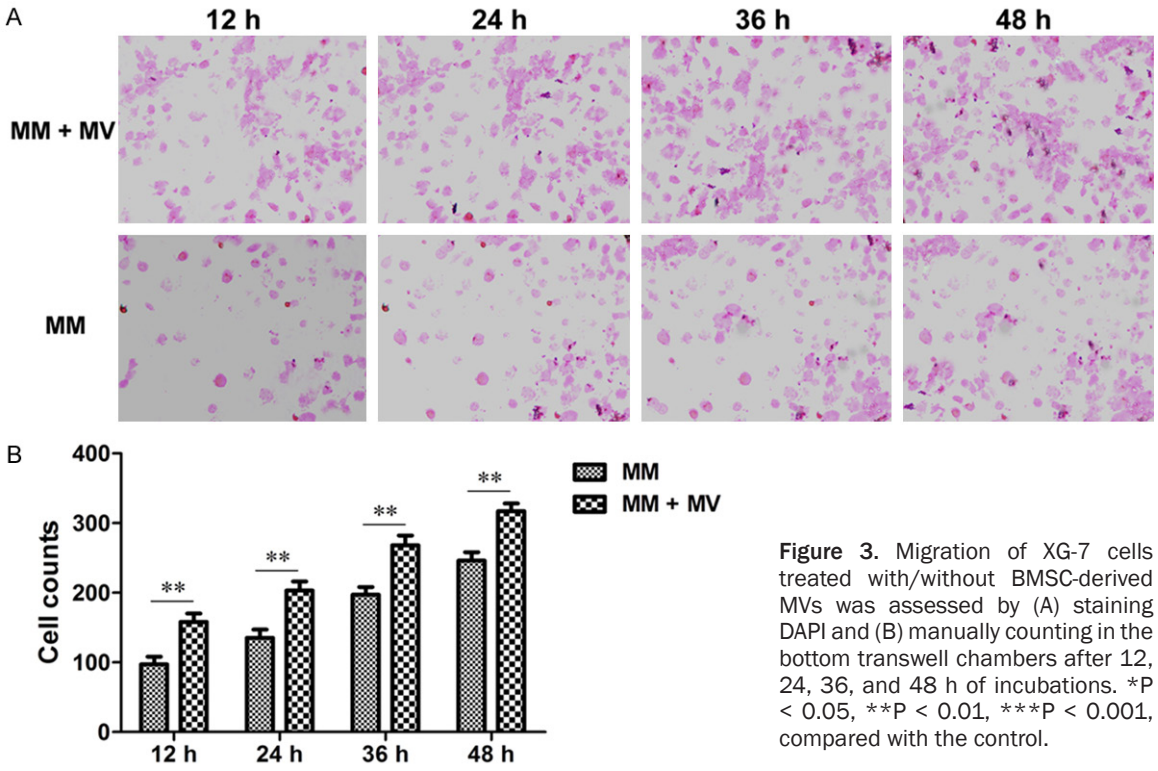

Figure 3. Migration of XG-7 cells treated with/without BMSC-derived MVs was assessed by (A) staining DAPI and (B) manually counting in the bottom transwell chambers after 12, 24, 36, and 48 h of incubations. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

exposed in ECL developing solution followed by the product's instructions (BestBio, Shanghai, China). The strip gray levels were quantified using a Quantity one v4.62 software by normalizing to GAPDH as an internal control.

Rat model

The animal experiment was performed as previously described with minor modifications [17]. Briefly, the GFP-tagged MM cells coincubated BMSC-derived MVs were intravenously injected into 6-week-old C57/BL6 mice. After three days, the mice were sacrificed, and the heart, lung and spleen were taken out, waxed, fixed by 4% paraformaldehyde immediately, sliced, dewaxed and hydrated. Then the sections were stained by DAPI and imaged by CLSM.

Statistical analysis

All experiments were performed triplicate in three independent occasions. The data were presented as the mean ± standard deviation, processed by SPSS 17 (SPSS, Chicago, IL, USA), and evaluated using a two-tailed Student's t-test. The following significance levels were used: *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Isolated membrane vesicles from BMSC were MV

The membrane vesicles isolated from BMSC by differential ultracentrifugation were firstly validated by NAT and CLSM to examine the size and fusion features. As shown, the isolated vesicles had a wide diameter range of 70-650 nm and their average size was 250 nm (**Figure 1A**), confirming that these BMSC-derived vesicles were MV. After co-incubation with BMSC-derived MV tagged by anti-CD138-FITC, it could be observed that they could be taken up by MM cells (**Figure 1B**).

BMSC-derived MVs promote the proliferation of MM cells in vitro

As proliferation is one the important steps for angiogenesis, we evaluated the proliferation potential of MM cells after co-incubation with BMSC-derived MVs. It could be notified that the quantity of MM cells alone increased till 36 h when they stopped growing. In comparison, the MM cells incubated with BMSC-derived MVs proliferated evidently in a time-dependent manner within 48 h (P < 0.05 at 12 h and P <

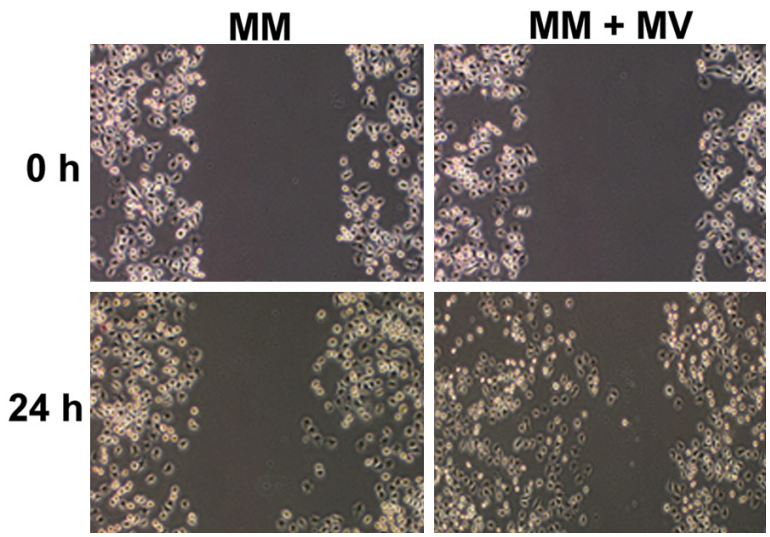

Figure 4. Representative images of the migrations of XG-7 cell treated with/without BMSC-derived MVs for 24 h.

0.01 at 24, 36, 48 h, **Figure 2**), indicating that BMSC-derived MVs had the potential to promote the proliferation of MM cells.

BMSC-derived MVs accelerate migration of MM cells in vitro

Migration is a critical capability for the metastasis and invasion of MM cells, and the transwell assay and scratch test were employed to survey the migration potential of MM cells after co-incubation with BMSC-derived MVs. Along with the extension of time, the MM cells penetrating the well increased. Compared with the control, the MM cells with incubation with BMSC-derived MVs increased remarkably ($P < 0.01$ at 48 h, **Figure 3A** and **3B**). In scratch test, BMSC-derived MVs treated MM cells presented evident potential of migration after 24 h of incubation as migrated cells filled up the blank between the two sides of cells (**Figure 4**). These results suggested that BMSC-derived MVs could accelerate the migration of MM cells.

BMSC-derived MVs increase the levels of MM-related proteins and migration and homing of MM cells in vivo

We further investigate the expressions of MM-related proteins and used a rat model to examine the effects of BMSC-derived MVs on the migration and homing of MM cells. The expressions of several MM-related proteins implicated in the migration and homing of MM cells were analyzed and displayed significant promotions after co-incubated with BMSC-derived MVs (**Figure 5A**). In details, the levels of eleven proteins, i.e. CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt and β-catenin, were increased respectively by 1.92-, 1.50-, 2.80-, 2.00-, 2.27-, 2.67-, 1.72-, 2.53-, 6.10-, 2.84- and 3.72-fold (**Figure 5B**). As exhibited, it could be seen that the GFP-tagged MM cells treated by BMSC-derived MVs have been migrated into several main internal organs, i.e. heart, lung and spleen (**Figure 6**). The results indicated that BMSC-derived MVs could alter the expressions of many types of proteins and facilitate the migration and homing of MM cells via at least Wnt-β-catenin and Notch-hes pathways.

Discussion

Deciphering mechanisms of migration and homing of MM cells is critical in the treatment and prognosis of MM. BMSCs have long been believed to participate in regulating the processes of migration and homing of diverse cancer cells including MM cells via releasing cytokines and growth factors [17-20], and the metastasis and development of MM will be delayed and ceased without the assistance of BMSCs [4]. Although several reports demonstrated that exosomes derived from BM mesenchymal stromal cell and BMSC could promote the advance and drug-resistance of MM cells [17, 21], the effects of BMSC-derived MV on MM development have yet not been discussed.

The signal transduction and material exchange between BMSCs and MM cells have been thought to depend on membrane vesicles which comprised MV and exosomes [22]. Although these two vesicles contains analogous membrane proteins and receptors and execute similar intercellular missions, MV are distinct from exosomes in at least two aspects: one is that they include different sizes with 30-100 nm for exosomes and 100-1000 nm for MV,

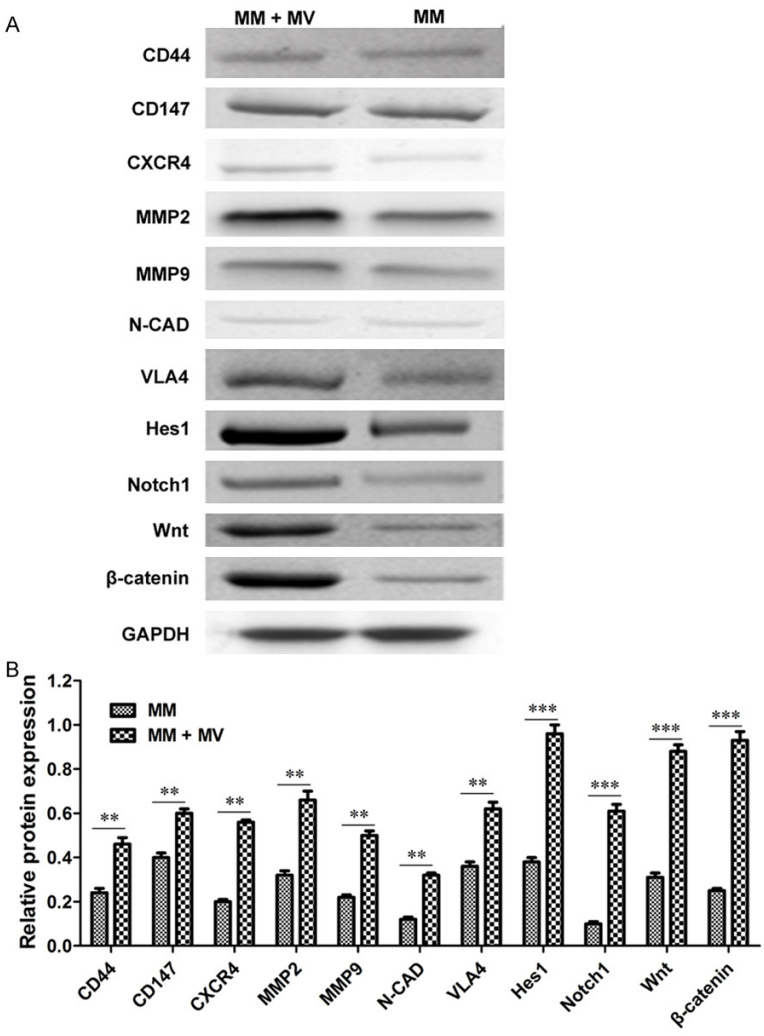

Figure 5. (A) Representative images and (B) relative expressions of protein (CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt and β-catenin) by western blotting after 24 h of incubation with/without BMSC-derived MVs in XG-7 cells. GAPDH was set as the internal control. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

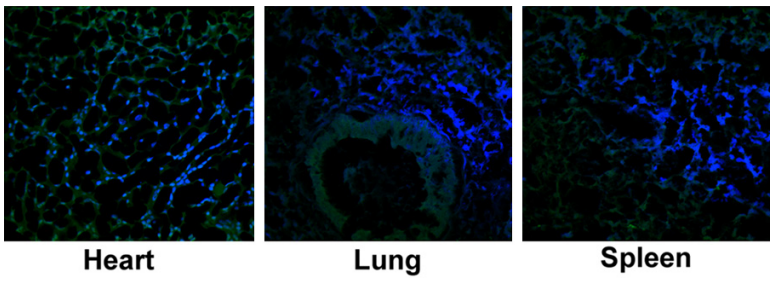

Figure 6. CLSM images of heart, lung and spleen sections after GFP-tagged XG-7 cells were co-incubated with/without BMSC-derived MVs for three days in a mice model.

the other is that their origins are different in which MV bud directly from the plasma membrane, whereas exosomes are released when the multivesicular endosome is fused with the plasma membrane [22]. Actually, there has been no convincing report to clarify that all MV were above 100 nm in size [23]. On the contrary, some vesicles less than 100 nm (and even smaller) have been observed to bud from the plasma membrane [24]. Thus, differential centrifugation alone is unable to fully isolate MV from exosomes as the sizes of both vesicles are overlapped. Instead, differential ultracentrifugation can obtain high purity of MV [23]. In this study, we employed NAT to observe quantities of relatively larger vesicles (an average size of 250 nm) with a wider size distribution (= 70-650 nm) isolated by differential ultracentrifugation, indicating that the acquired vesicles were MV instead of exosomes (**Figure 1A**).

The smaller membrane vesicles, exosomes, derived from BMSC have been demonstrated to be different in the compositions compared with those derived from MM, leading to the communication between BMSC and MM via direct membrane fusion [17]. As for BMSC-derived MV, we observed that they could be taken up by MM cells (**Figure 1B**), resulting in the release of several critical factors, such as possibly free miRNA [25], from a higher malignancy of MM cells to a lower one. After 24 h of incubation with BMSC-derived MV, MM cells acquired significant proliferation (P < 0.01), while the evaluations of optical density (OD) showed that the growth of MM cells came to a standstill after 36 h of incubation alone (**Figure 2**).

Further, we also observed that BMSC-derived MV could notably promote the migration and metastasis of MM cells in vitro and in vivo (**Figures 3**-**5**).

Although the specific molecules that could be possibly transported into MM cells from BMSC-derived MV were not determined, the expressions of a group of MM-related proteins, i.e. CD44, CD147, CXCR4, MMP2, MMP9, N-cadherin, VLA4, Hes1, Notch1, Wnt and β-catenin, responsible for migration and homing of MM cells were analyzed in this study. It could be observed that BMSC-derived MVs were able to significantly facilitate the expressions of the eleven proteins in MM cells compared with those without MVs treatment ($P < 0.01$ or $P < 0.001$). In terms of the previous reports, it was convincing that these proteins were also associated with the roles of MV in the advancement of diverse cancers. As described, the uptake of MV was mediated by CD44 receptors [26], MM-derived MVs are enriched in CD147 expression [27], MMP2 and MMP9 were internal proteins of MM in charge of degrading collagens and facilitate tumor growth [28], while the expressions of CXCR4, VLA4 and N-cadherin were also necessary for migrations of diverse cancers [29-31]. What is more, Wnt-β-catenin and Notch-hes signaling cascades were also implicated in the regulations of migration and homing of MV-treated cancer cells [32]. In addition, the MV-treated MM cells had improved potential of homing compared with the control as the GFP-transfected MM cells were accumulated in the main internal organs (heart, lung and spleen) in the rat experiment (**Figure 2**). We hypothesized that BMSC-derived MVs had a comprehensive and diverse influences on the development of MM, a process which involved a large quantity of proteins and many signaling pathways.

In conclusion, this study demonstrated that BMSC-derived MV could be a promising potential drug target for MM treatment as it could be helpful in the migration and homing of MM cells. However, the specific proteins as well as miRNAs contained in BMSC-derived MV should be clarified in the subsequent experiments, which may be beneficial for providing a deep insight into the mechanism of how BMSC-derived MV mediates the communication between BMSC and MM cells.

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Disclosure of conflict of interest

None.

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