**Original Article**

**Let-7a inhibits migration of melanoma cells via down-regulation of HMGA2 expression**

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**Abstract:** This study aimed to investigate the effects of exosomes derived from BM-MSCs transduced with let-7a on B16f10 cells and BM-MSCs. BM-MSCs were transduced with let-7a and the exosomes of them were isolated for further culture of B16f10 cells and BM-MSCs. The migration of B16f10 cells were detected by transwell, proliferation of B16f10 cells and BM-MSCs was examined by MTT method, HMGA2 expression was measured by western blot. In addition, the let-7a secreted level in exosomes and IGF level were measured by RT-PCR and ELISA respectively. Our results showed that the level of let-7a in exosomes derived from Let-7a-transduced BM-MSCs was increased after treated by exosomes. HMGA2 in B16f10 cells was down-regulated and cell survival rate of BM-MSCs was decreased. However, neither cell survival rate of B16f10 cells nor IGF-1 secreted by B16f10 cells in different groups had significant differences. In conclusion, Let-7a contained in exosomes can inhibit the migration of Melanoma cells and inhibit the proliferation of BM-MSCs.

**Keywords:** Exosome, let-7a, MSC, melanoma, HMGA2

**Introduction**

Stem cell-based therapy has shown promise in animal models and some clinical patients. This novel organ regeneration method can enhance tissue repair through multipotent-differentiating potential and paracrine effects and reduce the risk of immune rejection due to tissue compatibility. These stem cells such as Mesenchymal stem cells (MSCs) are relatively safe. However, the risk of stem cell transplantation such as increasing tumor migration couldn’t be ignored either. Therefore, therapeutic strategies avoiding direct use of living stem cells are more likely to provide a safer way to prevent disease progression [1].

Exosomes are vesicles of the multi-vesicular body, a complex intracellular organelle involved in endocytosis. The diameter of exosomes is typically 40-100 nm. After multi-vesicular bodies fused with plasma membrane, exosomes are released from the cells into the extracellular environment [2, 3]. Initially, exosomes became of interest since they were reported to play a role in antigen presentation [4]. It has also been demonstrated that exosomes can be used as a cell-free vaccine with therapeutic effects in cancer [5]. More recently, the finding that exosomes shuttle genetic materials, such as mRNAs and micro-RNAs (miRNAs), shed new light on the role of exosomes in cell-to-cell communication [6]. The properties of exosomes reflect the specialized properties of their original cells and the environment. Such novel mechanisms of intercellular communication raise the possibility that the transfer of genetic information, biological factors or therapeutic substances via exosomes may modulate cellular activities in recipient cells.

Previous studies showed that MSCs secrete exosomes with biochemical and biophysical function [7-9]. In this study, we hypothesized that exosomes derived from BM-MSCs can exert a paracrine effect on the migration of B16f10 cells.
Materials and methods

Animals

Healthy SD rats (110-120 g) were purchased from the Animal Institute of the First Affiliated Hospital of Zhengzhou University and maintained under specific pathogen-free conditions at 20 ± 2°C and 45 ± 5% humidity. All animal handling and experimental procedures were performed in accordance with the Guidelines of the Care and Use of Laboratory Animals published by the China National Institute of Health.

Cell preparation

For BM-MSC isolation, whole bone marrow (BM) aspirates from the limbs of normal SD rats were obtained as previously described (Reference). Different passages of BM-MSCs were cultured in DMEM containing 10% certified fetal calf serum qualified for mesenchymal cells. Cells were sustained in culture flasks and cultured in DMEM culture medium (Hyclone, USA) supplemented with 10% fetal calf serum (FBS, Gibico, Australia) and digested with 0.25% trypsin plus 0.02% EDTA.

When the cells reached 30%-40% confluence, the cells were transfected with LV-rno-Let-7a or LV-rno-Let-7a-inhibitor at the suitable MOI.

B16-f10 (melanoma) cells, a rat melanoma cell strain, were provided by the Department of Cell Lab of Xiangya University. Cells were sustained in culture flasks and cultured in DMEM culture medium (GNM12800, Gibico, Australia) supplemented with 10% fetal calf serum (FBS, Gibico, Australia) and digested with 0.25% trypsin plus 0.02% EDTA.

Transwell migration assay

The transwell migration assay was performed on B16f10 cells using a 24-well culture plate with Boyden chamber containing a polycarbonate filter with an 8 mm pore size (Corning Costar, Corning, NY). B16f10 cells were serum-starved for 24 h in DMEM prior to initiation of the experiment. B16f10 cells and BM-MSCs transfected with let-7a and let-7a-inhibitor were cultured to a confluence of 90-100% and washed with serum-free conditioned medium and cultured in serum-free conditioned medium for 12-24 h. Then the supernatants were centrifuged at 2500 rpm for 10 min to remove cells and cell debris and ordinate cultured medium combined with IGF1 (novoprotein) were collected in the same volume. The lower chambers of corresponding group were filled with 600 ml of the conditioned medium from above-mentioned. After that, 100 ml of suspension liquid from B16f10 cells (10^6/chamber) mixed with 300 ml serum-free conditioned medium were added to the upper chamber of each well. After that, the plate was cultured at 37°C, 5% CO_2 for 24 h to allow cell migration through the membrane. Migratory cells were fixed in 4% paraformaldehyde and stained with crystal violet. The images were captured using Microscope (Leica, China).

MTT

The B16-F10 cells were seeded into 96-well culture plates with 5000/well and cultured in different medium including Ordinary culture medium, cell culture fluid of BM-MSCs, BM-MSCs transfected with let-7a/let-7a-inhibitor. After cultured in 37°C, 5% CO_2 for 24 h, 48 h, 96 h, 20 μl of thiazolyl blue tetrazolium bromide (5 mg/ml, MTT, Amresco, USA) was added for a further 3 h-culture in a humidified atmosphere containing 5% CO_2 at 37°C. Medium was discarded and the cells were harvested in 150 μl DMSO (Amresco, USA) and shaken for 10 min. Cell lysate was analyzed for MTT activity to indicate cell proliferation by measuring the absorbance at 492 nm. The same steps were applied to the BM-MSCs transfected with let-7a, let-7a-inhibitor to detect the proliferation.

Enzyme-linked immunosorbent assay (ELISA)

To verify the secretion of IGF-1 by BM-MSCs, cell supernatants from BM-MSCs and BM-MSCs transfected with let-7a/let-7a-inhibitor were collected for the detection of IGF-1 levels by ELISA. Quantitative analysis of IGF-1 levels was performed by IGF-1-specific ELISA kits (Boster Biological Technology Co, LTD). Measurements of IGF-1 were made on the culture medium from each well (The cell supernatants were prepared after 72-h incubation).

Immunofluorescence

After cultured in supernatants collected from groups mentioned above for 48 h, B16f10
cells were seeded into 24-well plate and fixed in 4% paraformaldehyde for 30 min. After washing by PBS, they were incubated with bovine serum albumin (BSA, 5%, Genview) for 1 h, washed by PBS for three times (5 min per each) and with antibodies against IGFR (1:200 dilution, rabbit, Boster) for 24 h overnight at 4°C. Goat anti rabbit (1:200 dilution, Invitrogen, USA) were used to label the primary antibodies for 2 h at room temperature, and then washed in PBS for three times and dripped by DAPI Fluoromount-GTM. Photos were taken under a fluorescence microscope (Thermo, USA).

Isolation of exosomes

BM-MSCs were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-Glutamine centrifuged at 10,000 g overnight to eliminate preexisting bovine-derived exosomes. Exosomes derived from Ordinate culture medium, BM-MSCs or BM-MSCs transfected with let-7a and let-7a-inhibitor cultured for 48 h were isolated using ExoQuick-TCTM (System Bioscience, Mountain View, CA) according to the manufacturer’s protocol. In brief, cell culture supernatants were harvested and centrifuged at 3,000 g for 15 min to remove cells and cell debris. 1 ml of ExoQuick-TC Exosome Precipitation Solution was added to 5 ml of the supernatants and the mixture was refrigerated overnight. Then, the mixture was centrifuged at 1,500 g for 30 min and the supernatants were aspirated. The residual solution was centrifuged at 1,500 g for 5 min and removed. The exosome pellet was resuspended in the appropriate buffer for further labeling or RNA analysis.

Cellular uptake of exosomes

Isolated exosomes were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO). In brief, 1 ml PKH26 was added to exosomes collected above in a total volume of 1 ml of diluent and incubated for 20 min at room temperature. 1 ml fetal bovine serum (FBS) was added to stop labeling and the mixture was added into 12 ml phosphate buffered saline (PBS) and centrifuged at 4000 g for 70 min at 4°C. The pellet containing PKH26-labeled exosomes was resuspended in 2 ml of conditioned medium (exosome-removed serum).

B16F10 cells were previously cultured to 60% confluence, pre-labeled with Hoechst 33342 (Leagene, China) and the medium was replaced by medium containing PKH26-labeled exosomes and cells were incubated at 37°C with 5% CO₂. Cellular uptake of BM-MSC-derived exosomes was observed after 24 h incubation under microscope (Thermo, USA). The same steps were applied to the BM-MSCs transfected with let-7a, let-7a-inhibitor to observe its uptake of BM-MSC-derived exosomes.

Isolation of let-7a in exosomes by Reverse Transcribed-PCR (RT-PCR)

Exosomes were isolated from cultured medium of BM-MSCs and BM-MSCs transfected with let-7a, let-7a-inhibitor and the let-7a was isolated using the Trizol Reagent (Ambion, Life technologies). MiRNA was stem-loop reverse transcribed (RT) with the Ultra SYBR Mixture (With ROX, ComWin Biotech, China) and real-time PCR amplifying was performed with the Reverse Transcriptase M-MLV (RNase H-, TaKaRa, China). Relative expression was calculated based on 2-∆∆Ct method.

Western blot

B16F10 melanoma cells in the exponential phase of growth were plated into a 6-well plate and treated with medium with exosomes isolated above. After treatment, cells were collected and washed with PBS. The total protein was extracted using a RIPA lysis buffer kit (Dingguochangsheng Technology, BeiJing, China), on ice for 30 min. The lysates were collected and centrifuged at 12,000 g for 30 min at 4°C. Protein concentrations were detected using a bicinchoninic acid protein assay kit (Genview, Shang-Hai, China). Aliquots of the lysates were boiled for 5 min, electrophoresed on 10% SDS-PAGE gels and transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membrane was blocked with 5% Skim milk powder (Amresco, USA) at room temperature for 2 h and then probed with primary antibodies against HGMA2 at a 1:50 dilution (Bioss, China) and GADPH at a 1:1000 dilution (Good Here Technology, China) overnight at 4°C, washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for three times followed by incubation of the membrane with the goat-anti-rabbit secondary antibodies at a 1:5,000 dilution (Jackson, USA) for 1 h at room temperature. Washing with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for another three times,
protein bands were detected using enhanced chemiluminescence (Genview, China). The bands were quantified with ImageJ software.

Statistical analysis

Data were presented as mean ± SEM. Differences between groups were analyzed by ANOVA. A P value of less than 0.05 was considered statistically significant.

Results

Let-7a transduction significantly inhibits B16f10 cells migration

We used transwell plate to co-culture B16f10 cells with BM-MSCs and showed that B16f10 cells can migrate more than cultured alone when co-cultured together with BM-MSCs (Figure 1). We then transfected the BM-MSCs
Let-7a suppresses migration in melanoma cells

**Table 1.** MTT data of different groups (% ± s, n = 4)

<table>
<thead>
<tr>
<th>Group</th>
<th>24h</th>
<th>48h</th>
<th>96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.40 ± 2.96</td>
<td>51.40 ± 5.12</td>
<td>78.42 ± 5.51</td>
</tr>
<tr>
<td>MSC</td>
<td>26.82 ± 2.68</td>
<td>52.87 ± 2.12</td>
<td>78.55 ± 1.83</td>
</tr>
<tr>
<td>Let-7a</td>
<td>23.08 ± 1.98</td>
<td>44.00 ± 4.94</td>
<td>76.52 ± 2.60</td>
</tr>
<tr>
<td>Let-7a-inhibition</td>
<td>22.68 ± 2.25</td>
<td>50.48 ± 2.91</td>
<td>78.55 ± 6.27</td>
</tr>
</tbody>
</table>

**Table 2.** Cell proliferation rate of B16 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>24h</th>
<th>48h</th>
<th>96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSC</td>
<td>27.17 ± 0.41</td>
<td>38.03 ± 0.68</td>
<td>72.28 ± 1.66</td>
</tr>
<tr>
<td>Let-7a</td>
<td>23.52 ± 0.71</td>
<td>34.48 ± 0.68</td>
<td>65.33 ± 0.99</td>
</tr>
<tr>
<td>Let-7a-inhibition</td>
<td>30.92 ± 0.83</td>
<td>42.37 ± 0.98</td>
<td>81.78 ± 2.31</td>
</tr>
</tbody>
</table>

**Figure 3.** Cell proliferation of MSCs and B16 cells by MTT. The B16-F10 cells were seeded into 96-well culture plates and cultured in different medium including Ordinary culture medium, cell culture fluid of BM-MSCs, BM-MSCs transfected with let-7a/let-7a-inhibitor. After cultured in 37 °C, 5% CO₂ for 24 h, 48 h, 96 h, thiazolyl blue tetrazolium bromide was added for MTT analysis.

**Figure 4.** IGF-1 level secreted by cells in different groups. Cell supernatants from BM-MSCs and BM-MSCs transfected with let-7a/let-7a-inhibitor were collected for the detection of IGF-1 levels by ELISA (Boster Biological Technology Co, LTD).

Let-7a transduction does not influence proliferation of B16f10 cells but inhibits BM-MSCs proliferation

Cell survival rate of B16f10 cells and BM-MSCs cultured in different supernatants were detected by MTT method and we found that BM-MSCs proliferation after transduced by let-7a was decreased while let-7a-inhibitor-BM-MSCs’ proliferation was up-regulated (Figure 3). However, no effect of let-7a transduction on the proliferation of B16f10 cells was observed.
Let-7a suppresses migration in melanoma cells

IGF1 slightly influences migration of B16f10 cells

We previously demonstrated that IGF-1 was a probable participative factor in BM-MSC-melanoma microenvironment as confirmed by several studies concerning BM-MSC-secreted factors’ function on different tumor cells and also had predictable binding sites with the majority of let-7 family. So we detected the levels of secreted IGF-1 through supernatants collected from ordinate culture medium and BM-MSCs transduced with let-7a/let-7a-inhibitor by ELISA and found slightly elevated IGF-1 secretion from BM-MSCs without significant difference (Figure 4). We further added IGF1 factor (200 ng/ml) into ordinate culture medium in the transwell plate as chemotactic factor to detect the variation of B16f10 cells’ migration and found they slightly enhanced the migration comparing with ordinate medium but less obvious than supernatants from BM-MSCs transfected with let-7a-inhibitor.

At the same time, we use immunofluorescence to observe the expression of IGFR on B16f10 cells after treated with supernatants from cells identical with above mentioned and no obvious difference was found (Figure 5).

Let-7a transduction can up-regulate let-7a secretion of exosomes by BM-MSCs

We then isolated exosomes from different groups and added them back to BM-MSCs and B16f10 cells, for analysis of the vesicle-shaped exosomes’ swallowing by BM-MSCs and B16f10
Let-7a suppresses migration in melanoma cells

Using PCR, we detected that let-7a levels varied according to the transduction of let-7a and let-7a-inhibitor, in other words, let-7a expression notably up-regulated through let-7a transduction (Figure 7). In addition, we replaced the supernatants applied in above procedures with corresponding exosomes (exosomes mixed in the same culture medium with the same volume) and also obtained consistent experiment results, further confirming that exosomes were the main functional elements.

**HMGA2 expression decreases after treated by let-7a-exosomes**

After all the steps above, we used immunoblotting method to detect the protein expression changes, and found that after treated by BM-MSC-derived exosomes, HGMA2 expression in B16f10 cells was up-regulated compared to ordinate-medium group (Figure 8), while HGMA2 of B16f10 cells in let-7a-exosomes was down-regulated and up-regulated in let-7a-inhibition compared to be cultured using BM-MSCs-derived exosomes, consistent with previous study revealing HMGA2 is one of direct targets of let-7a through Luciferase reporter assays [10].

**Discussion**

It has long been believed that BM-MSCs could influence proliferation, migration or invasion of tumor cells [10-15]. In this study, we found that B16f10 cells co-cultured with BM-MSCs penetrated more than in ordinate medium, reflecting the promoting migration function of BM-MSCs to melanoma (B16f10 cells). Many investigations have explored the role of the BM-MSC
Let-7a suppresses migration in melanoma cells

microenvironment in releasing relevant cytokines and growth factors such as IGF-1, VEGF, IL-6. Insulin-like growth factor (IGF-1), plays an important role in the prevention of apoptosis and stimulating of migration of tumor cells [16-20]. On the other hand, through let-7 family transduction, we found that after transduced with let-7a, B16f10 cells co-cultured in transwell plate with corresponding MSCs penetrated less than control group (MSCs without any treatment), while other members in let-7 family didn’t show significant changes. As a member of let-7 family, IGF-1 may play the part of promoting melanoma (B16f10) migration, so we detected the secreting level of IGF-1 by BM-MSCs and IGFR expression of IGFR on B16f10 cells in different groups. Surprisingly, no significant differences were found.

Exosomes, derived from the exocytosis of intraluminal vesicles (within multivesicular bodies, MVBs), and released into the extracellular space when fused with the plasma membrane [21, 22] came into our notice then. Most cell types release exosomes through this mechanism including hematopoietic cells, reticulocytes, B- and T-lymphocytes, dendritic cells, mast cells, platelets, intestinal epithelial cells astrocytes, neurons and tumor cells [23]. More recently, quite a few research have found that exosomes shuttle genetic materials, such as mRNAs and micro-RNAs (miRNAs) [6]. Combined with what we have found above, we proposed a hypothesis that let-7a may be packaged in exosomes and produce further influence on downstream B16f10 cells.

With a series of procedures carried out in our study, significant up-regulation of let-7a in exosomes from let-7a-transducted-MSCs were detected; exosome-uptake by B16f10 cells and BM-MSCs were captured by microscope; furthermore, HMGA2 expression on B16f10 cells after treatment of let-7a-exosomes were down-regulated notably, suggesting the decreased migration of B16f10 cells; besides, proliferation of BM-MSCs were inhibited after let-7a-exosomes treatment.

In this study, we found let-7a could decrease the migration through secreted exosomes. Similar to a report from Wu et al. [24], HMGA2, predicted as the target of let-7a, could be down-regulated in melanoma cells (B16f10) after treated by let-7a-exosomes. The downside of the design, however, was let-7a-exosomes may have some inhibiting effects on BM-MSCs themselves. In contrast, neither cell survival rate of B16f10 cells nor IGF-1 secreted by B16f10 cells in different groups had significant differences. To sum up, through our study we conclude that BM-MSCs could enhance migration of B16f10 cells, which reflects the potential risk of direct MSC transplantation from the side.

In response, interest is growing in using exosomes as biological delivery vehicles. Exosomes are taken up by acceptor cells, whereby cellular processes can be altered [25]. There is also evidence that exosomes do not elicit acute immune rejection, and as they are non-viable, they do not have a risk of tumor formation [25, 26]. Combined the latest research progress with our own study, we believe exosomes will undoubtedly have attractive research prospect with their characteristics. For example, the properties of them reflect the specialized prop-

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**Figure 8.** HMGA2 expression in B16f10 cells after treated by different groups of exosomes. RNA and protein were isolated from B16f10 cells for analysis of HMGA2 mRNA expression by RT-PCR and protein expression by western blot.
properties of their original cells and the environment, they could also serve as carrier which could package themselves with target drugs [27-32], or else we could regulate components contained in exosomes especially miRNAs so as to reach different aims [33-40]. Moreover, exosomes from MSCs with feature of more generous secretion, more types and wilder application, and may probably have wider research significance.

In conclusion, Let-7a contained in exosomes can inhibit the migration of Melanoma cells and inhibit the proliferation of BM-MSCs.

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Disclosure of conflict of interest

None.

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