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Cells Isolated from Human Bone Marrow, Plays a Key Role in Osteosarcoma Growth

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KEYWORDS Exosome. Mesenchymal Stem Cells. miRNA. Osteosarcoma

ABSTRACT In the current study, the researchers cultured the hBMSCs in α-DMEM supplemented with foetal bovine serum. After an incubation period of 48 hours the exosome-containing cell culture supernatants were collected and subjected to PEG-based vesicle enrichment for purification. The enriched exosomes derived from hBMSCs, with or without miR-6087, were used to treat osteosarcoma (U2OS) cells. The cell viability was tested using Transwell invasion assay, CCK-8 test and scratch migration assay. The Western blotting and RT-PCR were employed to determine the levels of HSP70/ALIX and miR-6087 in the exosomes. The HBMSC derived exosomes exhibited significant impact on the growth of U2OS cells. The growth of tumour cells was notably inhibited when miR-6087 in exosomes was suppressed. This study marks the initial instance where the researchers successfully showcased the ability of exosome derivatives from hBMSCs to prevent the U2OS cells proliferation, through the modulation of miRNA-6087. The findings present a novel perspective on the involvement of hBMSCs in tumour advancement.

INTRODUCTION

In this study, the researchers investigated the secretion of miRNA by hBMSCs through exosomes and their effects on human U2OS cells and their growth. Osteosarcoma (OS) is considered as a primary tumour with high malignant potential, frequently occurring among children and young individuals (Ottaviani and Jaffe 2009). Its resistance to chemotherapy is the major reason that OS treatment fails (Ahmed et al. 2014; Constantinidou et al. 2013). Hence, there is an immediate need to uncover the underlying molecular mechanisms of OS and identify a suitable therapeutic approach for its treatment, such as genetic intervention (Miao et al. 2013). Osteosarcoma, a cancerous bone tumour with a significant unstable gene, primarily affects individuals in their twenties and thirties (Kubota et al. 2013). Since the 1970s, patients diagnosed with localised disease have achieved a 70 percent rate of event-free survival (EFS) over a period of five years after the application of pre- and postoperative chemotherapy. Compared to individuals diagnosed with a localised disease, patients suffering from metastatic OS exhibit 5-year EFS rates that do not exceed 20 percent (Mialou et al. 2005).

Microvesicles (MVs) are released by cells in various conditions (Pittenger et al. 1997; Méndez-Ferrer et al. 2010; Salem and Thiemermann 2010). Recent studies have increasingly shown that MVs have the ability to transport DNA, RNAs, and proteins to the neighbouring cells (Johnson and Dorshkind 1986; Pittenger MF et al. 1997; Campagnoli et al. 2001; Lee et al. 2004). MVs consist of different types of vesicles, including exosomes (30-200 nm) (Campagnoli et al. 2001). Non-coding RNAs called microRNAs (miRNAs) play a negative regulatory role in mRNA transcription levels, thereby influencing gene expression. Several studies have demonstrated the involvement of miRNAs in cellular processes such as cell proliferation and apoptosis (Calin and Croce 2000; Esquela-Kerscher and Slack 2006; Sun and Lai 2013; Rider et al. 2016; Shinagawa et al. 2010; Lin et al. 2019). Han et al. (2014) previously suggested distinct profiles of serum-derived miRNAs in different cancer types (Yu and Zhang 2014; Nawaz et al. 2016). Notably, miR-6087 has

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been found to be significantly enriched in hBMSCs-EXO according to previous research (Baglio et al. 2015), and it is considered an important miRNA species involved in tumorigenesis (Yoo et al. 2012).

The MVs derived from the cells of malignant tumours have been involved in the early detection and signal transduction of cells, which is now a controversial topic. In recent times, numerous research notices have indicated the probable utilisation of MVs as harmless and capable carriers for drug delivery (Campagnoli et al. 2001; Gradilla et al. 2014; Yang et al. 2010). Johnson and Dorshkind (1986) discovered that miRNA is present in MVs secreted by hBMSCs, which could potentially interact with the internal environment and target cells in a specific manner (Méndez-Ferrer et al. 2010; Fu et al. 2013) The components of RNA-induced silencing complex (RISC) to target cells can be transferred via MVs as stated by Pittenger MF (1997) and Lee et al. (2004) hereby implying their involvement in gene silencing induced by miRNA (Zhao et al. 2014; Teng et al. 2015; Rider et al. 2016).

In this research, the researchers have observed that hBMSC-derived exosomes play a crucial role in promoting the growth of U2OS cells. By inhibiting miR-6087 within these exosomes derived from hBMSCs, the researchers have witnessed a significant reduction in tumour growth. As a result, the researchers propose an audacious hypothesis that targeted modulation of miR-6087 could potentially contribute to the inhibition of osteosarcoma growth in clinical settings.

METHODS AND MATERIAL

Cell Cultivation

Mesenchymal stem cells derived from human marrow of bone were cultured in a 37°C incubator with 5 percent CO_2 using DMEM (BI, Israel) supplemented with 10 percent foetal bovine serum (FBS, Hyclone, USA).

QRT-PCR

The assays were performed in order to measure the levels of mature miRNAs, with minor modifications as previously mentioned (Fatima and Nawaz 2017; Nawaz et al. 2016). The RNA was totally extracted following instructions using the TRIzol Reagent (Servicebio). The miRNA was quantified using Taqman microRNA probes (ABI7500). The trials and experiments executed triplicate. During the course of the reactions, threshold was predetermined and applied to verify the (CT) data, and the average CT values were computed using three replicates of RT-PCR assays. The CT analysis was performed for comparison between the experimental reactions and the control group. U6 snRNA served as the internal reference for miRNAs.The normalised relative levels of gene expression were determined by applying the equation 2ΔÄCT, where ΔCT represents the difference between the CT values of the target gene and control. The primers for miRNA-6087 and U6 were designed using the following sequences: 52 -CTCGCTTCGGCAGCACA-32 (sense) and 52 -AACGCTTCACGAATTTG CGT-32 (antisense) for U6; 52 ACACTCCAG CTGGGTGAGGCGGGGGG-32 (sense) and 52 -TGGTGTCGTGGAGTCG -32 (antisense) for miRNA-6087.

Transfection

The hBMSCs planted in the six well plate were kept for 24 hours following the completion of the transfection experiment. The hsa-miR-6087-inhibitors, were obtained from GeneChem (Shanghai, China). These lentiviral particles were then introduced into hBMSCs following the instructions provided by the manufacturer. The PCR analysis was conducted on the harvested cells 72 hours post-transfection.

Extraction of Microvesicles from Media

They were kept on the culture medium of MSCs using a previously described method (Campagnoli et al. 2001). The supernatant of cell cultures containing vesicles were subjected to centrifugation at 2,000 g for a period of 30 minutes, and an additional centrifugation step at five hundred grams for removal of debris. Following the completion of centrifugation, a 16 percent PEG solution was added to media with volumetric 1:1 ratio. The obtained mixture was then incubated at a temperature of 4 °C for a duration of 12 hours. Subsequently, on the following day, the samples underwent centrifugation at a speed of 3,214 g (Eppendorf) for an hour while maintaining a temperature of 4 °C. The liquid was eliminated,

and the centrifuge tube was turned upside down for a duration of 5 minutes, occasionally tapped to eliminate any surplus PEG. The bottom of the centrifuge tube contained the sediment, which was resuspended in one mL of free particle PBS with (pH 7.4), subjected to purification using, and incubated overnight at a temperature of 4 °C. The procedures were repeated as performed on the previous day for all samples. They were subsequently utilised promptly or preserved in liquid nitrogen after being agitated at room temperature for a maximum duration of 30 minutes.

Electron Microscopy for Transmission Purposes

After immersing the samples in a droplet of PBS buffer with 2.5 percent glutaraldehyde at a temperature of 4 °C, the samples were placed on copper wire for a duration of 20 minutes to allow excess absorption, followed by negative staining using phosphotungstic acid for one minute at a temperature of 25°C. Finally, observation was conducted through a FEI Tecnai T20 TEM operating at an acceleration voltage of 120 kV.

Viability of Cells

The cell proliferation capacity was assessed using the CCK-8 assay. The U2OS cells and normal exosomes were added together and cultured with 400 μg/mL as well as the downregulated exosomes with 400 μg/ml miRNA-6087 respectively, in a 96-well plate at a density of 3×103 cells per well. The experimental group was treated with normal exosomes and miRNA-6087 downregulated exosomes, while the control group received an equivalent amount of exosome-depleted medium. The viability of cells was assessed at 6, 12, and 24 hours post-treatment. Among both the groups, the experimental group was treated with miRNA-6087 downregulated exosomes and normal exosomes, whereas the control group received an equivalent amount of exosome-depleted medium. The viability of cells was assessed at 6, 12, and 24 hours post-treatment.

Migration of Scratch

In the current study, briefly, the U2OS cells were seeded at a density of 1×105 cells per well in a 6-well plate and incubated under standard conditions at 37 °C with 5 percent CO_2. When the floor area was occupied by 90 percent of cells, a sterile 2 μL scratch was created. The wells were washed twice using PBS, and the PBS was then removed using100 μL pipette. The 400 μg/mL exosomes, with either presence or absence of down regulated miRNA-6087, along with equivalent volume of conditioned medium were introduced as experimental controls onto the cells. The scratch gap area was observed and documented at 6, 12, or 24 hours using a microscope.

Transwell Infiltration

A diluted ECM glue solution of 40 μl was introduced into each chamber 5 hours prior to cell seeding in the invasion assay test. Following that the U2OS cells were plated with a density of 8×104 cells, which included a 24-well with a size of 8 μm in the upper chamber. They were re-cultured with exosomes at a concentration of 400 μg/mL, either in their original form or with reduced levels of miRNA-6087. The control group was provided with an equivalent medium that lacked exosomes. The relevant growth media (600 μL), which was suspended with Transwell as a chemoattractant, supplemented with 30 percent FBS was introduced. They were treated with methanol for a duration of 20 minutes and then subjected to staining using a solution containing 0.1 percent crystal violet for a period of 15 minutes. Following this, the cells were incubated in a CO2 incubator at a temperature of 37 °C for 24 hours. The work conducted a microscopic analysis (20X magnification) to quantify the extent of cell invasion into the membrane. Five random fields were observed per well.

Analysis Using the Western Blot Technique

Alix and Hsp70 protein levels were assessed using Western blot analysis, with GAPDH serving as the internal control. Immunoblots underwent blocking using a buffer solution (PBS with 5 percent fat-free dried milk) at a temperature of 25 °C for one hour. Subsequently, they were incubated overnight at 4 °C with antibodies against Alix and Hsp70 (Abcam, diluted to a ratio of 1:1000), as well as GAPDH (Abcam, diluted to a ratio of 1:2000).

Quantitative Analyses

Five distinct experiments were used to obtain all the data. The mean values ± S.E. were used to represent the data.

RESULTS

Relevant to the Enrichment of hBMSCs-exo, PEG-based Vesicle Concentration Demonstrates Remarkable Effectiveness

The work initially cultured human bone marrow-derived mesenchymal stem cells in a laboratory setting (Fig.1). As shown in Figure 1, the cell morphology of the third generation human bone marrow-derived MSCs was observed after culturing for 2, 7, and 20 days, and under an optical microscope, the cells grew in a fibroblast-like, spindle-shaped morphology. Figure B shows characterisation analysis of MSCs from human bone marrow examined at passage 3 showed the presence of CD29 and CD105 markers in human bone marrow-derived MSCs, indicating positive expression. In contrast, CD45 and CD34 markers were not observed. Figure C shows the changing trend of the number of human bone marrow-derived MSCs in the first and third generations with the culture time. Subsequently, the researchers opted for a PEG-based technique to isolate exosomes. In the present study, the researchers observed the cup shaped vesicles measuring 100 nm in diameter (Fig. 2). As shown in Figures 1 and 3, transmission electron microscopy images displayed

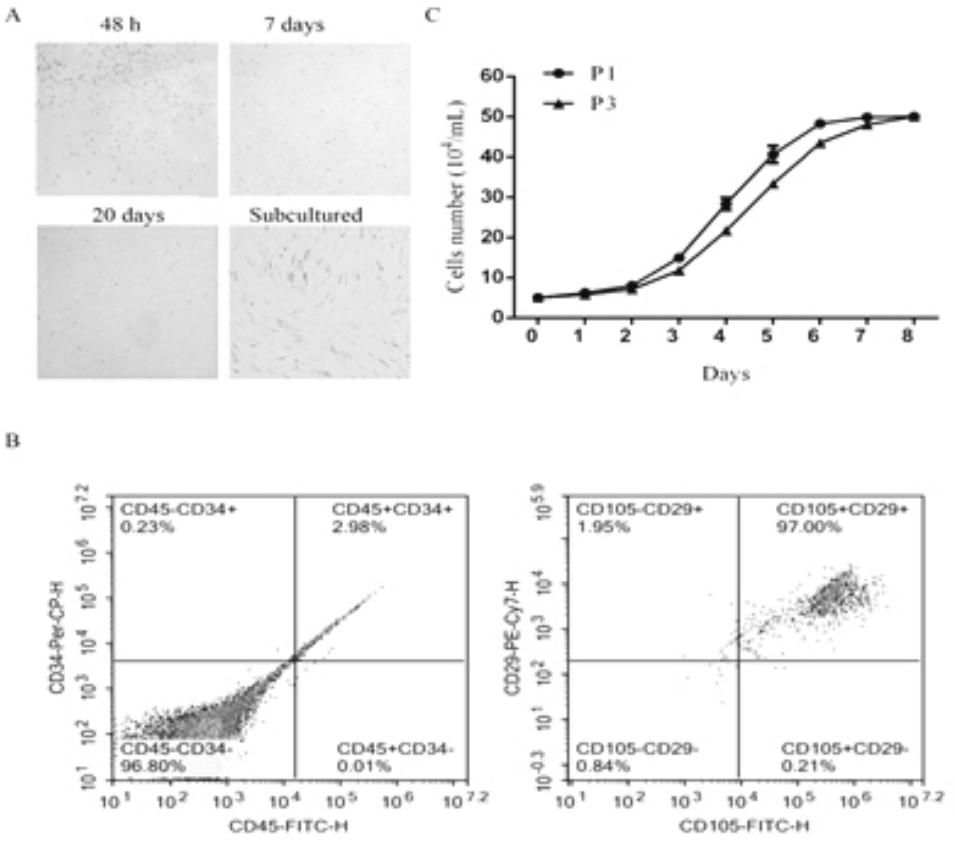

Fig. 1. Analysis of mesenchymal stem cells derived from the bone marrow of human individuals

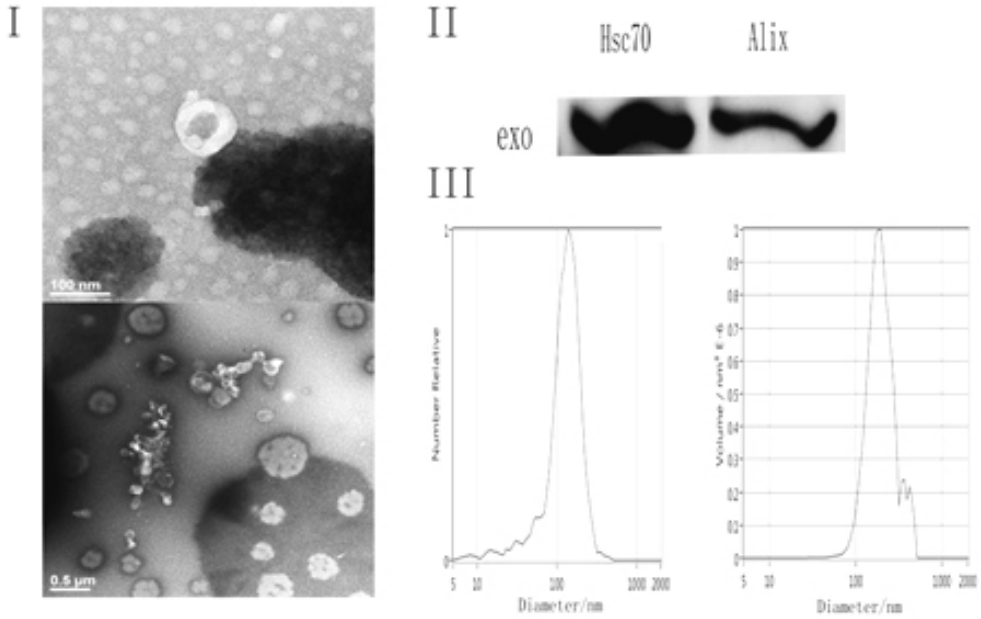

Fig. 2. Identification and characterization of exosomes released by MSCs derived from human bone marrow

the presence of characteristic small circular nanoparticles in exosomes obtained from MSCs, with sizes ranging between 40 to 150 nm. The provided scale bars represent measurements of 100 nanometers and 0.5 micrometers. In Figure 2, Western blotting was employed to detect the expression of Hsp70 and ALIX in exosomes derived from hBMSCs.

In Comparison to hBMSCs, Exosomes Derived from hBMSCs Exhibited a Notably Elevated Expression of miRNA-6087

Q-PCR technology was utilised to analyse the expression of miRNA-6087 in both hBMSCs and hBMSC-exo, revealing a noteworthy increase in miRNA-6087 expression within exosomes compared to hBMSCs. Hence, it is the researchers' contention that there was a noticeable occurrence of miRNA-6087 enrichment within the exosomes, which could potentially contribute to the development of a malignant phenotype in U2OS cells (Fig. 3). The invasion, migration as well the proliferation, of OS cells can be significantly enhanced by exosomes released from hBMSCs. However, exosomes obtained from hBMSCs with reduced levels of miRNA-6087 do not exhibit the same promoting effects.

To further investigate this, the researchers performed a migration assay from scratch. The scratch was almost completely filled by U2OS cells treated with exosomes within 24 hours. The migrated U2OS cells were observed to be limited to only 10 percent and 20 percent of the scratch after a 24-hour incubation period (Fig. 4). As shown in Figure A and B, migration assay was conducted to evaluate the impact of hBMSC-exosomes interference, down-regulation of miR-6087, and control group at 6, 12, and 24 hours. The wound healing assay revealed enhanced migratory capacity of cells in the hBMSC-exosomes group. Compared to the control group and the group with down-regulated miR-6087, there was a significant disparity in the percentage of wound closure (n=3 per group). The blank control and miR-6087 down-regulated groups exhibited reduced cell migration ability. As shown in Figure C, the U2OS cell line is subjected to co-culture with different concentrations of hBMSC-exosomes (400μg/ml) for varying time intervals (0, 6, 12, and 24 hours). Subsequently, CCK-8 analyses

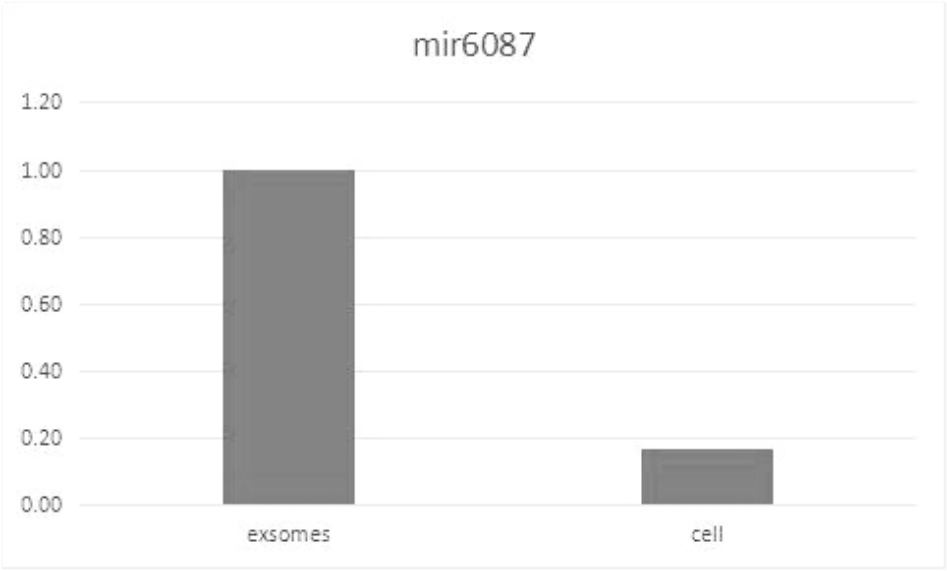

Fig. 4. Rejuvenation and growth of U2OS cells are stimulated by hBMSC-derived exosomes

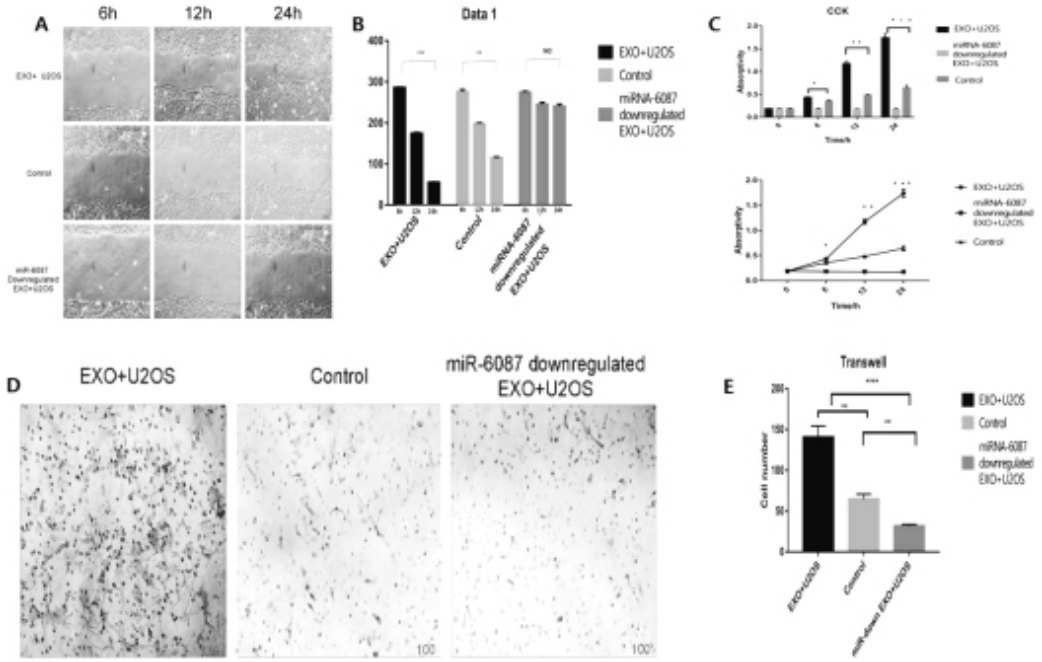

Fig. 4. Rejuvenation and growth of U2OS cells are stimulated by hBMSC-derived exosomes

were performed on each group consisting of three replicates. As shown in Figures D and E, U2OS cells were added to the upper chamber of a transwell system, which had a membrane coated with matrigel. The cancer cells were exposed to exosomes at a concentration of 400μg/ml, or treated with exosomes that had been modified to downregulate miR-6087 expression (also at a concentration of 400μg/ml). A control group was included using an equal volume of medium depleted of exosomes. After a duration of 24 hours, the quantity of cells that moves to the lower chamber of the membrane with pores sized at 8 μm is assessed through visual inspection and cell counting. Each group consisted of three replicates.

****p-value less than 0.001, ***p-value less than 0.005, **p-value less than 0.01, *p-value less than 0.05, NO: no statistically significant findings.

DISCUSSION

The hBMSCs cells target the tumour microenvironment, which could potentially enhance the effectiveness of chemotherapy in individuals diagnosed with OS as stated by Tu et al. (2016), suggesting that hBMSCs are integral targets to repress the intercellular communication in the process of carcinogenesis (Melzer et al. 2016). The presence of hBMSCs in the tumour microenvironment could potentially facilitate the migration and invasion of OS cells. The growth of OS may be influenced by hBMSCs, which have been found to play a supportive role. The link between miRNA composition of the EVs released from hBMSCs are said to be closely linked with each other (Vallabhaneni et al. 2016). According to the report, miRNAs have been identified as promising and innovative therapeutic targets for addressing OS (Jiang et al. 2014). The latest literature indicates that extracellular vesicle mediated miRNA promotes ferroptosis and inhibits proliferation, migration, and invasion of osteosarcoma by regulating ZEB1 (Jiang et al. 2023). It has been reported that the proliferation of active osteosarcoma cells is specifically promoted by mesenchymal stem cells, while relatively stationary tumour cells are unaffected. This discovery brings new hope for post-tumour resection treatment (Avril et al. 2016).

In fact, MV-mediated small RNAs have long been regarded as more effective than conventional delivery methods like liposomes and viruses (Dudakovic et al. 2014). One explanation is that these MVs originate from human cells, which inherently possess the capacity to decrease toxicity and improve immune cell tolerance (Théry et al. 2006; Dudakovic et al. 2014). It was also reported that the transfection of HUVECs with mimics of both the control and hsa-miR-6087 did not result in a significant alteration in endoglin mRNA expression. In contrast to the upregulation of mRNA expression, the presence of hsa-miR-6087 reduced endoglin protein levels than controls (Yoo et al. 2012).

The present study found that hBMSC-exo can enhance U2OS cells' invasion, proliferation, and migration. Numerous reports have suggested that exosomes include a multitude of small molecules, including basic molecules such as RNA, proteins, and lipids. In previous studies, considerable attention has been given to investigating the correlation between miRNA and the oncogenic characteristics of OS, leading to significant insights into the impact of miRNAs on the proliferation of OS cells. Therefore, exosomes are the essential carriers for the intercellular communication and they could potentially serve as a means for miRNA to modulate target cells.

Additionally, miRNA-6087 was considerably enriched in the exosomes, and the exosomes exhibited a remarkable inhibitory effect on U2OS cells' invasion, proliferation, and migration, in which miRNA-6087 was downregulated. When U2OS cells were exposed to exosomes obtained from hBMSCs that had undergone downregulation of miRNA-6087, there was a notable decrease in U2OS cells' invasion, proliferation, and migration. Even the cellular function of U2OS was inhibited. Therefore, the researchers have determined that the miRNA-6087 present in exosomes derived from human bone marrow mesenchymal stem cells is found to be crucial for regulating the migration, invasion, and proliferation of U2OS cells.

Restrictions

Similar to numerous other research studies, the present investigation possesses several limitations. These include the following:

1. The researchers solely conducted in vitro experiments and aspire to conduct future in vivo investigations.

2. The researchers aim to investigate the impact of miR-6087 upregulation on hBMSC-exo, as it has been primarily associated with downregulation. This approach allows the researchers to explore the potential effects resulting from increased levels of miR-6087.
3. The researchers plan to replicate the findings in additional OS cell lines as part of the ongoing research, considering that the current study was limited to a single cell line.
4. The downstream molecular mechanism was not investigated in the current study, however, the researchers plan to allocate more focus towards this aspect in future research.
5. It is crucial to validate the findings by examining the clinical specimens. Additionally, conducting gene sequencing on these clinical specimens would greatly contribute to further research on osteosarcoma.

CONCLUSION

In the current study the researchers investigated the ability of hBMSCs in tumour advancement and the researchers conclude that downregulation of miRNA-6087 expression in extracellular vesicles derived from hBMSCs can inhibit tumour cell proliferation. The findings implicate a novel perspective in investigating the enhancement of tumour growth with involvement of hBMSCs.

RECOMMENDATIONS

In the follow-up research, the researchers will focus on in vivo experimental research, using multiple cell lines for experiments, studying the downstream molecular mechanism and gene sequencing of clinical specimens.

LIST OF ABBREVIATIONS

TNFR: Tumour necrosis factor receptor
RISC: RNA-induced silencing complex
MVs: Microvesicles
miRNAs: MicroRNAs
RT-PCR: Reverse Transcription polymerase chain reaction
OS: Osteosarcoma
ALIX: ALG-2-interacting protein X
HSP70: Heat Shock Protein 70
hBMSCs: mesenchymal stem/stromal cells

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

COMPETING INTERESTS

No conflicts of interest exist in this manuscript.

CONSENT FOR PUBLICATION

Manuscript is approved by all authors for publication.

AVAILABILITY OF DATA AND MATERIALS

The data and materials of this experiment are available.

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Not applicable.

AUTHOR CONTRIBUTIONS

Yuan Niu was responsible for conception and design. Yibiao Zhou was responsible for manuscript writing. Xiaowei Yang and Sheng Huang were responsible for collection and assembly of data. Xuqiang Liu and Bin Zhang were responsible for data analysis and interpretation. All authors were responsible for manuscript writing. All authors were responsible for the final approval of the manuscript.

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