ORIGINAL ARTICLES

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Exosomes from gingival mesenchymal stem cells enhance migration and osteogenic differentiation of pre-osteoblasts

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Gingival mesenchymal stem cells (GMSCs) have great potential in bone tissue regeneration. However, it is not well known how on exosomes derived from GMSCs affect the functions of bone-related cells. In this study, we explored the impact of GMSCs-derived exosomes (GMSCs-Exos) on pre-osteoblast MC3T3-E1 proliferation, migration and osteogenic differentiation. Results of CCK-8 assay showed that GMSCs-Exos had no effect on proliferation of pre-osteoblasts. Further, we found that GMSCs-Exos promoted the migration of pre-osteoblasts and osteogenic differentiation of MC3T3-E1 as revealed by enhanced Alizarin red staining, elevated alkaline phosphatase (ALP) activity and upregulated expression of osteogenic genes. This study provides new insights into the potential exosome-mediated paracrine mechanism of GMSCs in bone regeneration.

1. Introduction

Periodontitis, one of the most common chronic inflammatory diseases, is caused by periodontopathic bacteria. It is characterized by progressive destruction of connective tissue and tooth-supporting structures including the gingiva, periodontal ligament, and alveolar bone (Tonetti et al. 2018). The main goal of periodontal treatment is to reconstruct the lost bone tissue (Reynolds et al. 2010).

In recent years, mesenchymal stem cells (MSCs) have shown promising results in bone tissue regeneration in both animal and human studies (Bartold et al. 2019; Castillo-Cardiel et al. 2017; Gjerde et al. 2018; Jin and Lee 2018). Among them, GMSCs have been the focus of most studies because of their unique advantages (Xudong and Zhengguo 2020). Compared to other MSCs, GMSCs are abundant and easy to isolate through minimally invasive procedures (Grawish 2018; Venkatesh et al. 2017). Moreover, GMSCs have a stable morphology and maintain their MSC characteristics for long (Jin et al. 2015; Sun et al. 2019). These suggest that GMSCs-based therapy is a promising alternative for bone regeneration. Several animal studies have been performed to test the performance of GMSCs transplantation in bone tissue regeneration (Al-Qadhi et al. 2020; Wang et al. 2011; Xu et al. 2014). Interestingly, the conditioned medium derived from GMSCs was found to increase bone regeneration *in vivo* (Diomede et al. 2018). However, the mechanism underlying these processes is not fully understood.

Accumulating evidence suggests that MSCs enhance tissue regeneration through paracrine pathways (Gnecchi et al. 2016). Paracrine is an essential mode of intercellular communication, achieved through secreted cytokines, growth factors and packaged mediators (Grellier et al. 2009). Exosomes are nanosized lipid bilayer vesicles secreted by almost all human cell types. They are involved in cell-to–cell communication and intracellular signaling (Zhao et al. 2019). Exosomes convey various bioactive cargos from their donor cells including proteins, nucleic acids and lipids. In addition, they transfer these molecular contents to adjacent or distant recipient cells, thereby regulating the biological behavior of receptor cells (Hessvik and Llorente 2018; Ludwig and Giebel 2012).

Based on the above findings, we hypothesize that GMSCs might promote bone regeneration through their exosome-mediated regulatory effect on cells residing in the bone microenvironment. In this study, we isolated exosomes from GMSCs and investigated the effect of GMSCs-Exos on proliferation, migration and differentiation of pre-osteoblast MC3T3-E1. We find for the first time that GMSCs-derived exosomes regulates bone regeneration via a paracrine mechanism.

2. Investigations and results

2.1. Identification of GMSCs

Light microscopy inspection showed the typical fibroblast-like, spindle-shaped morphology of isolated GMSCs at the third culture passage (Fig. 1A). Meanwhile, Alizarin Red S and Oil Red O staining confirmed the multi-lineage differentiation capacity of GMSCs, similar to other MSCs (Fig. 1B). Furthermore, flow cytometry analysis revealed that GMSCs expressed the MSC markers such as CD90 and CD105 but did not express hematopoietic stem cell markers CD34 and CD45 (Fig. 1C). These results indicated that the isolated cells were stem cells of mesenchymal origin and were determined to be GMSCs.

2.2. Characterization of GMSCs-exos

TEM images revealed that the isolated exosomes displayed a cup-shaped morphology (Fig. 2A). In addition, western blot analysis of GMSCs-Exos revealed the presence of the typical exosomal markers CD9, CD63 and TSG101 (Fig. 2B). The peak size of GMSCs-Exos was 128 nm in diameter as determined by NTA (Fig. 2C).

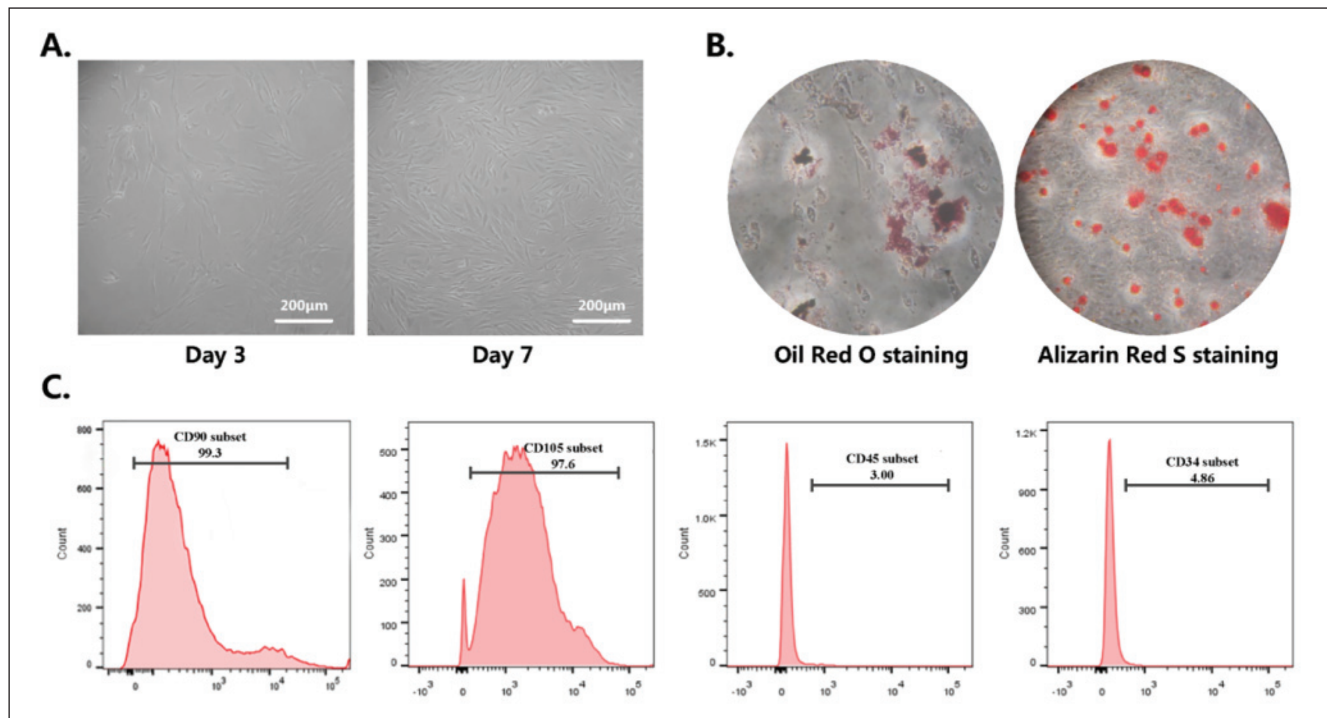

Fig. 1: Identification of human GMSCs. (A) An optical microscopic view of cultured primary GMSCs at day 3 and day 7. (B) The differentiation ability of GMSCs towards osteogenesis-like or adipogenesis-like cells as determined by Oil Red O staining and Alizarin Red S staining. (C) Flow cytometry analysis of mesenchymal stem cell surface markers (CD90 and CD105) and negative markers (CD34 and CD45) in cultured GMSCs.

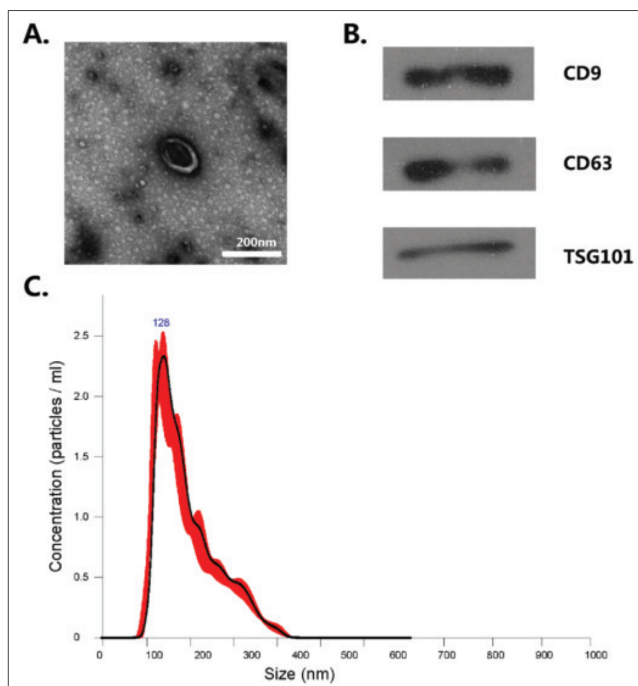

Fig. 2: Characterization of GMSCs-Exos. (A) Representative TEM images showing the morphology of GMSCs-Exos. (B) Expression of exosomal marker proteins (CD9, CD63 and TSG101) by western blotting. (C) The average size of exosomes was measured by NTA.

2.3. *Effect of GMSCs-Exos on proliferation of pre-osteoblasts*

Proliferation of MC3T3-E1 cells at 1, 3, 5 and 7 days was evaluated by CCK-8. However, there was no significant difference between the proliferation level of GMSCs-Exos treatment group and control group as illustrated in Fig. 3. This result suggests that GMSCs-Exos had no effect on proliferation of pre-osteoblasts.

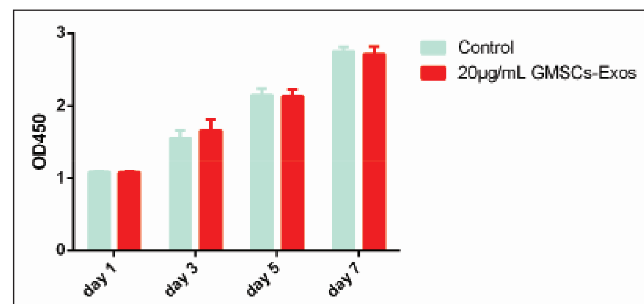

Fig. 3: Effect of GMSCs-Exos on proliferation of pre-osteoblast, analyzed by the CCK-8 assay.

2.4. *GMSCs-Exos promote migration of pre-osteoblasts*

Migration of precursor cells towards wound sites is prerequisite for bone healing. To assess pro-migration ability of GMSC-Exos on pre-osteoblasts, MC3T3-E1 cells received 20 µg/mL GMSCs-Exos or an equal volume of PBS as control for a series of *in vitro* functional assays. The transwell assay showed that more cells treated with GMSCs-Exos migrated to the lower chamber compared to the control group as shown in Fig. 4A. Quantitative results of migratory cells also showed similar trends as in Fig. 4B. The effect of GMSCs-Exos on migration of pre-osteoblasts was further confirmed using the scratch assay, which is another method for evaluating cell migration. Exosomes treatment markedly enhanced the motility of MC3T3-E1 cells, as determined by the representative photomicrograph and quantitative data (Fig. 4C-D).

2.5. *GMSCs-Exos promote the osteogenic differentiation of pre-osteoblasts*

To evaluate whether GMSCs-Exos can promote pre-osteoblast osteogenic differentiation, MC3T3-E1 cells were cultured in the presence or absence of 20 µg/mL GMSC-Exos under osteogenic induction. After 14 days of stimulation, ALP staining was performed, and its activity measured. GMSCs-Exos enhanced pre-osteoblast ALP

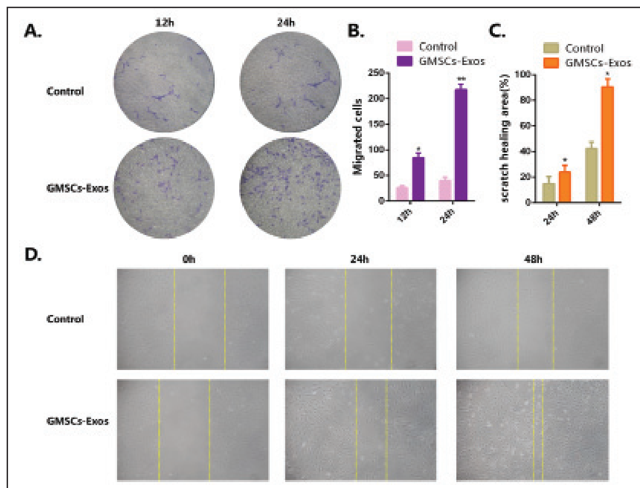

Fig. 4: GMSCs-Exos promote pre-osteoblast migration. (A) Representative images of migrated MC3T3-E1 cells migrating through a transwell chamber. (B) Quantitative analysis of the migrated cells. *$P < 0.05$, **$P < 0.01$ compared with the control group. (C) The typical pictures of scratch wound healing assay. (D) Quantitative analysis of percent migration in scratch assay. *$P < 0.05$ compared with the control group

activity significantly as shown in Fig. 5A-B. The staining result with Alizarin Red S also showed a higher induction of mineralization in the GMSCs-Exos treated group than in the control (Fig. 5C). Furthermore, quantitative measurement of alizarin red S staining confirmed the above results (Fig. 5D). Figure 5E shows the mRNA expression of osteogenic differentiation-related genes determined by real-time PCR. Enhanced expression of *BMP2, OCN, RUNX2 and OPN* genes was also observed after GMSCs-Exos treatment. Consequently, our findings suggest that GMSCs-Exos can enhance the osteogenic differentiation of pre-osteoblast.

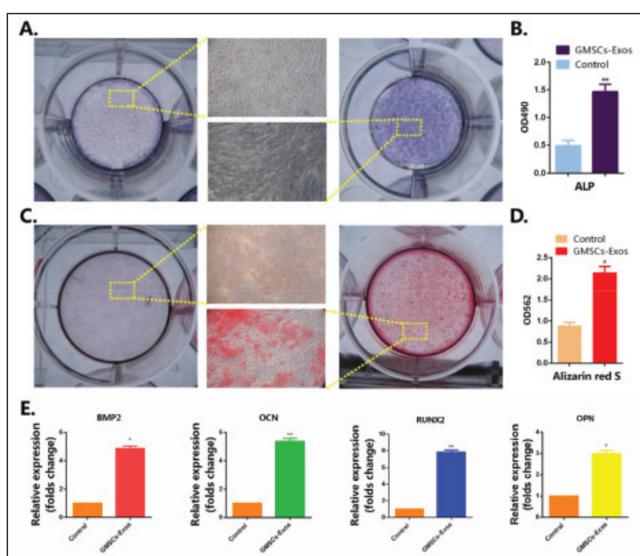

Fig. 5: GMSCs-Exos promotes the osteogenic differentiation of pre-osteoblasts. (A) Alkaline phosphatase staining. (B) Quantitative alkaline phosphatase activity assay. (C) alizarin red S staining images. (D) Quantitative analysis of alizarin red S staining at absorbance of 562 nm. (E) mRNA expression of osteogenic differentiation-associated genes detected with quantitative real-time PCR. *$P < 0.05$, **$P < 0.01$ compared to the control group.

3. Discussion

The ultimate goal of periodontal treatment is to regenerate the damaged tooth-supporting tissues, especially the lost bone tissue (Reynolds et al. 2010). GMSCs have shown the potential to repair bone defects in animal models. In addition to the aforementioned advantages, GMSCs are considered ideal for bone tissue regeneration (Shi et al. 2019). Apart from GMSCs, the conditioned media from GMSCs also improve bone or periodontal regeneration (Diomede et al. 2018; Qiu et al. 2020). This implies that GMSCs may support osteogenesis by secreting some important substances. Exosomes are important mediators of intercellular communication which have been extensively studied (Mathieu et al. 2019). Therefore, we speculate that exosomes might mediate the regulatory roles of GMSCs in bone-related cells.

Although many studies have reported that stem cell-derived exosomes have diverse effects on cell biological behavior, fewer studies have investigated the role of GMSCs-Exos (Li et al. 2019; Zhao et al. 2018, 2019). GMSCs-Exos regulate macrophage polarization under inflammatory conditions as well as promote Schwann cell proliferation (Rao et al. 2019; Wang et al. 2020). To our knowledge, no study has explored the relationship between GMSC-Exos and bone-related cells.

In this study, we successfully isolated and characterized exosomes from GMSCs using ultracentrifugation method. Among the diverse extraction methods, ultracentrifugation is well recognized as the gold standard of exosome isolation (Yang et al. 2020). Additionally, the method yields greater purity and less protein contamination. Generally, at least two different techniques should be used to confirm the existence of exosomes. In our study, TEM, western blot and NTA were adopted to identify GMSCs-Exos. The isolated exosomes showed typical cup-shaped morphology with sizes less than 100 nm and high expression of CD9, CD63 and TSG 101. These results were consistent with those from previous reports (Q. Shi et al. 2017).

To determine the effect of GMSCs-Exos on bone-related cells, 20 μg/ml GMSC-Exos, based on previous studies (Wang et al. 2020), were used to treat MC3T3-E1. The MC3T3-E1 is a pre-osteoblastic cell line commonly used in studying osteogenic development. Proliferation, migration and differentiation of osteoprogenitor cells are known to serve important roles in osteogenesis (Oryan et al. 2015). In addition, transwell and scratch-wound assays revealed that GMSCs-Exo promoted the migration of MC3T3-E1. Furthermore, GMSCs-Exos significantly increased the differentiation of pre-osteoblasts as determined by real-time PCR, ALP activity and Alizarin Red S staining. However, CCK-8 analysis showed that GMSCs-Exos had no significant effect on MC3T3-E1 proliferation. Zhang et al. demonstrated that exosomes derived from bone bone marrow mesenchymal stem cells had a pro-proliferative effect on MC3T3-E1 as well as positive effects on migration and differentiation (Zhang et al. 2020). A different study by Xu et al. (2018) showed that exosomes from myoblasts can promote pre-osteoblasts MC3T3-E1 differentiation. These different effects may be explained by differences in exosomal origin which lead to different contents that activate different signals of target cells. These reports therefore indicate that exosomes can influence the biological activity of osteoprogenitor cells although their precise impacts are highly dependent on the exosome contents.

Results from this pilot study should however be considered as preliminary. Further research based on tools, such as microRNA array or proteomics analysis should be conducted to determine which specific contents in GMSCs-Exos exerts the modulatory effects observed here. Could it be microRNA, specific proteins or other molecules? It is worth noting that *in vivo* animal models had demonstrated that GMSCs-Exos are able to promote the regeneration of tissues including nerve, skin, gingival and taste buds (Kou et al. 2018; Rao et al. 2019; Shi et al. 2017; Zhang et al. 2019). However, there is no direct evidence that GMSCs-Exo affect bone regeneration. Therefore, further *in vivo* experiments are required to elucidate the exact mechanisms by which GMSCs-Exos regulate bone regeneration.

4. Experimental

4.1. Isolation, culture and characterization of GMSCs

Human gingival tissues were obtained from healthy adult volunteers (18–25 years old) with no gingival inflammation. Approval was obtained from the ethical committee of the Renmin Hospital of Wuhan University. Human GMSCs were isolated and

cultured as described in a previous study with slight modifications (Zhang et al. 2019). Briefly, gingival tissues from the maxillary molar region were separated, minced and digested in phosphate-buffered saline (PBS) containing 2 mg/mL dispase II (Roche Diagnostics, USA). They were incubated overnight at 4 °C and 4 mg/mL collagenase type I (Beijing Solarbio Science & Technology, China) at 37 °C for 2 h. After this, they were filtered through a 70-μm cell strainer and the cell suspension transferred to 25 mm^2 culture flask containing α-Minimum Essential Medium (MEM) (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA), 1% penicillin–streptomycin solution (Corning, NY, USA) at 37°C in 5% CO^2 and 95% O_2. The GMSCs at passage 3 were used for further experiments.
Flow cytometry analysis, adipogenic and osteogenic differentiation were performed to identify the GMSCs. Approximately 1×10^6 GMSCs were trypsinized and washed to obtain single-cell suspensions. The cells were then incubated with monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-human antibodies for CD34 (cat. no. 560942), CD45 (cat. no. 555482), CD90 (cat. no. 555595) and CD105 (cat. no. 561443) (BD Biosciences, USA) at room temperature for 30 min. Cells incubated with isotypematched normal IgG were used as controls, and the suspension was analyzed via flow cytometer (FACSort, BD Biosciences). The GMSCs were cultured in osteogenic inductive medium containing 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich Co., USA) to conduct osteogenic differentiation. After 28 days of incubation, mineral deposition was detected by Alizarin Red staining (Cyagen Biosciences Inc. China). Additionally, GMSCs were cultured in adipogenic induction medium containing 10 μL/mL insulin, 1 μM dexamethasone, 0.5 mM indomethacin, and 60 μM 3-isobutyl-1-methylxanine (Sigma-Aldrich Co., USA) for adipogenic differentiation. After 2 weeks, Oil Red O staining (Cyagen Biosciences Inc. China) was performed to detect the formation of oil globules.

4.2. Isolation of exosomes by ultracentrifugation

As previously reported (Wang et al. 2020), the GMSCs were gently rinsed three times with PBS when they reached a sub confluent state (80-90%).The culture medium was then replaced with serum deprivation medium for 48 h. Subsequently, the culture supernatant was collected and subjected to gradient centrifugation (300 × g for 10 min, 2,000 × g for 10 min, and 10,000 × g for 30 min) to remove residual cells and debris. Exosomes were pelleted by ultracentrifugation (Beckman SW32 Ti swinging bucket rotor, CA, USA)) twice at 100,000 × g for 70 min. The final pellets were re-suspended in PBS for further experiments.

4.3. Identification of GMSC-Exos

The morphology of exosomes was observed with using transmission electron microscopy (TEM). The samples were loaded onto a carbon-coated electron microscopy grid (200 mesh), stained with 2% aqueous solution of uranylacetate for 10 s, air-dried then observed using transmission electron microscopy (HT7700, Hitachi, Japan). The size distribution and concentration of GMSCs-Exo were measured by nanoparticle tracking analysis (NTA) using a NanoSight NS300 instrument (Malvern Instruments, Malvern, UK) according to the manufacturer's instructions. The characteristic surface marker proteins of exosomes were analyzed by western blot. Briefly, the total protein concentration of exosomes was initially quantified using a BCA protein Assay kit (Thermo Fisher Scientific, MA, USA). The GMSCs-Exos lysates were then separated by 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore) for 1 h. The membrane was blocked with 1% bovine serum albumin (Gibco, Grand Island, NY, USA) to prevent non-specific binding. Subsequently, the blots were incubated with primary antibodies at 4 °C overnight-followed by incubation with the horse radish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The antibodies including anti-CD9 (ab92726), anti-CD63 (ab134045), anti-TSG101 (ab30871) and goat anti-rabbit IgG H&L (ab205718) were obtained from Abcam. The immunoreactive bands were revealed via an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, UK).

4.4. Proliferation assay

The effect of GMSCs-Exos on proliferation of pre-osteoblasts was determined by a cell counting kit-8 assay (CCK-8; Dojindo, Japan). In brief, MC3T3-E1 were seeded into 96-well plates at 5×10^3 cells/well and cultured in a medium supplemented with or without GMSCs-Exos. After incubation for the indicated time, the medium was removed then washed with PBS. Thereafter, a CCK-8 solution (10 μL) and 100 μL of fresh culture medium was added to each well and incubated at 37 °C for 1 h. Absorbance was measured using a microplate reader at a wavelength of 450 nm.

4.5. Migration assay

To conduct a scratch wound assay, MC3T3-E1cells were plated into a 6-well plate at 1×10^5 cells/well and incubated for 12 h. Linear scratches were created by a 1000 μL sterile pipette. The cell debris were then washed gently with PBS and changed to the complete medium supplemented with or without GMSCs-Exos. Cells were photographed at 0 h, 24 h and 48 h post-wounding. The area of the wound was quantified by Image J and normalized against wound area at 0 h. To perform the transwell assay, 1×10^4 cells were plated into the upper compartment of a transwell chamber with 8 μm pore filters (Corning, NY, USA). 700 μL of complete medium containing GMSCs-Exos or not was then added to the lower chamber. After incubation for 12 h and 24 h, the non-migrated cells from the upper surface of the filter membranes were gently wiped off by a cotton swab. Subsequently, the membranes were fixed in 4% paraformaldehyde for 10 min and stained with crystal violet for 5 min. The level of migration was evaluated by counting the number of stained cells in five randomly selected microscopic fields per well.

4.6. qRT-PCR

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was then synthesized from 1 μg of total RNA using Oligo dT primer and PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). Thereafter, Quantitative RT-PCR was performed using SYBR PCR master mix in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA). The relative level of miRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method, using β-actin as the reference. The primer sequences used in this study are shown in the Table.

Table: Primer sequences used in the qRT-PCR

Primer	Forward 5'-3'	Reverse 5'-3'
BMP2	AAGCGTCAAGCCAAACACAAAC	GCCACGATCCAGTCATTCCAC
RUNX2	AACTTCCTGTGCTCCGTGCTG	TCGTTGAACCTGGCTACTTGG
OCN	GGACCATCTTTCTGCTCACTCTG	GTTCACTACCTTATTGCCCTCCTG
OPN	CTCAGAAGCAGAATCTC	ATGGTCTCCATCGTCATCAT
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

4.7. ALP assays

MC3T3-E1 cells were plated into a 24-well plate at a density of 5×10^4/well. After 24 h, the culture media were replaced with an osteogenic inductive medium containing different concentrations of GMSCs-EXos. After culturing for 14 days, ALP staining was performed using the cALP Stain Kit (Nanjing, Jiancheng, China) according to the manufacturer's protocols. For ALP quantitative analysis, MC3T3-E1 cells were lysed with 1% TritonX-100 (Beyotime, Beijing, China). Next, 50 μL of p-nitrophenyl phosphate substrate solution, 80 μL of glycine buffer and 50 μL of cell lysate were added to a 96-well plate. The mixture was incubated for 30 min and the absorbance measured at 405 nm after adding 20 μ L of 5 M NaOH.

4.8. Alizarin red S staining

The MC3T3-E1 cells were cultured in an osteogenic induction medium and treated with GMSCs-Exos as mentioned above. 21 days after incubation, the formation of mineralized nodules associated with osteogenic activity was assessed through alizarin red S staining. The cells were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min and stained with alizarin red solution (Cyagen Biosciences Inc. China) for 15 min. The mineralized nodules were then viewed and photographed after rinsing with distilled water. Subsequently, 10% cetylpyridinium chloride (Sigma-Aldrich Co., USA) was used to quantitatively determine mineralized nodules then the absorbance value at 562 nm was determined using a microplate reader.

4.9. Statistical analysis

All data are presented as mean ± SD (standard deviation). Statistical analyses were performed using GraphPad Prism 6.0. Comparisons between two groups were performed by t-test. $P<0.05$ was considered significant.

Conflicts of interest: None declared.

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