Osteoclast-Derived Extracellular miR-106a-5p Promotes Osteogenic Differentiation and Facilitates Bone Defect Healing

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Research

Keywords: Small extracellular vesicles, miRNA, osteogenic differentiation, bone remodeling, intercellular communication

Posted Date: October 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-940184/v1>

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Abstract

Small extracellular vesicles (sEVs) are considered to play critical roles in intercellular communications during normal and pathological processes since they are enriched with miRNAs and other signal molecules. In bone remodeling, osteoclasts generate large amounts of sEVs. However, there is very little research about whether and how osteoclast-derived sEVs (OC-sEVs) affect surrounding cells. In our study, microarray analysis identified miR-106a-5p highly enriched in OC-sEV. Further experiments confirmed that OC-sEVs inhibited *Fam134a* through miR-106a-5p and significantly promoted bone mesenchymal stem cell (BMSC) osteogenic mineralization in vitro. Next, we prepared sEV-modified demineralized bone matrix (DBM) as a repair scaffold, and used a calvarial defect mouse model to evaluate the pro-osteogenic activities of the scaffold. In vivo result indicated DBM modified with miR-106a-5p-sEVs showed an enhanced capacity of bone regeneration. This important finding further emphasizes that sEV-mediated miR-106a-5p transfer play critical roles in osteogenesis and indicate a novel communication mode between osteoclasts and BMSCs.

Introduction

Bone is a dynamic organ that undergoes constant remodeling throughout the lifespan. Bone remodeling is precisely orchestrated by osteoclast bone resorption and osteoblast bone formation, so as to generate new bone to replace old bone at discrete temporary anatomic structures called basic multicellular units (BMUs) [1, 2]. In the process of bone remodeling, intercellular cytokines associate the bone resorption of osteoclasts with the bone formation of osteoblasts, which also contribute to the migration and differentiation of their respective precursors [3–5]. With the stimulation of two essential factors receptor activator for macrophage-colony stimulating factor (M-CSF) and nuclear factor- κ B ligand (RANKL), monocytes/macrophages differentiate and fuse into multinucleated mature osteoclasts with strong bone resorption activity [6–8]. Subsequent bone resorption results in the bone matrix releasing insulin-like growth factor type 1 (IGF-1) and transforming growth factor- β (TGF- β), further recruiting Bone Mesenchymal Stem Cells (BMSC) to form new bone. [9, 10]. Previous studies reported that the bone resorption function of osteoclasts from osteopetrotic patients and mice is impaired, suggesting that mature osteoclasts may regulate bone formation independent of bone resorption activity. [11, 12]. However, the specific cell signaling mechanisms of which osteoclasts regulate osteogenesis of BMSCs in a non-resorption-dependent manner remains unclear.

MicroRNAs (miRNAs) are endogenous small non-coding RNA capable of binding to the 3'UTR of target mRNAs and forming RNA-induced silencing complex (RISC) to further facilitate mRNAs degradation. [13]. miRNAs play critical regulatory roles in many physiological and pathological conditions such as embryo development [14], tumor progression [15], immune regulation [16] and other biological processes [17]. In recent year, the miRNA is detected and quantified in human serum, urine and plasma, promoting many studies about using miRNA as a potential biomarker to diagnose various diseases [18, 19]. During bone remodeling, miRNAs is also involved in multiple processes, such as regulates differentiation of bone cells and mediates intercellular communications between bone cells [4, 20]. miRNAs are first found to be

present in the cytoplasm. Recent studies reported that they are protected from RNase degradation and exist in the extracellular space under the protection of extracellular vehicles (EVs).[21, 22]. According to Minimal Information 2018 for Studies of Extracellular Vesicles (MISEV2018), EVs are widely comprised of two subtypes according to their size, large EVs (IEVs; also known as microvesicles) and small EVs (sEVs; also known as exosomes), the diameter of sEVs is less than 200 nm, while IEVs are generally greater than 200 nm [23]. Recent studies have showed that EVs serving as intercellular communication tools carried various RNA species such as messenger RNA (mRNAs), miRNAs and long non-coding RNAs (lncRNAs), and these cargos can be transferred from parental cells into recipient cells for performing biological function [24, 25]. In bone microenvironment, EVs are also important mediators of signal transduction among bone cells [26]. A large number of EVs are released by active osteoclasts, subsequently affecting the surrounding environment. It has been demonstrated that osteoclast-derived EVs couple bone resorption and bone formation via RANKL reverse signaling [4, 27]. However, few studies reported the role of osteoclast-derived EV on osteogenic differentiation of BMSCs.

In this study, we revealed a novel mode of osteoclast-BMSC communication mediated by OC-sEVs. We suggested that OC-sEVs deliver miR-106a-5p to recipient BMSCs and promote osteogenic differentiation and mineralization via suppressing *Fam134a*. This new mode of osteoclast-BMSC communication was further confirmed *in vivo*.

Materials And Methods

Reagents

Mouse macrophage RAW264.7 cell line and human embryonic kidney 293 (HEK293) cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured with Alpha minimal essential Medium (α -MEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Hyclone, Thermo Scientific, MA, USA) in a 37°C incubator with 5% CO₂. Recombinant mouse RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Alizarin stain kit were obtained from Solarbio Life Sciences (Beijing, China). Antibodies against Histone 3 (bs-17422R), CD81 (bs-2489R), TSG101 (bs-1365R), LaminA/C (bs-1839R), Osteocalcin (bs-0470R), LC3 (bs-8878R), SQSTM1/P62 (bs-2951R) and FAM134A (bs-14725R) and β -actin (bs-0061R) were purchased from Bioss Antibodies (Beijing, China).

Isolations of BMMs and BMSCs

In present study, BMMs were used studying osteoclast differentiation and BMSCs were used investigating osteogenic differentiation. The detailed processes of BMMs and BMSCs separation have been described in our previous studies [28, 29]. Briefly, 8 week old male C57BL/6 mice were euthanatized and mouse femur and tibia were dissected under aseptic conditions. The medullary cavity of tibia and femur were washed with α -MEM to collect the bone marrow cells (BMCs). After BMCs were cultured in α -MEM for 24

hours, the non-adherent cells were removed through renewing complete medium (α -MEM, 10% FBS, 1% penicillin-streptomycin) to obtain BMSCs. As for BMMs generation, collected BMCs were stimulated with 50 ng/ml M-CSF for 96 hours to obtain BMMs.

Osteoclast differentiation and TRAP staining

To generate osteoclasts 5×10^3 BMMs were seeded in 96-well plates and cultured with α -MEM which contains 50 ng/ml M-CSF, 100 ng/ml RANKL 10% FBS and 1% penicillin-streptomycin for 96 hours. For TRAP staining, culture medium was removed and cells were washed three times with phosphate-buffered saline (PBS, Hyclone, Thermo Scientific, MA, USA) and fixed in 4% paraformaldehyde (PFA, Biosharp, Anhui, China) for 20 mins. PFA were removed and cells were washed with PBS one more time. Subsequently, cells were stained with TRAP staining solution (Sigma-aldrich, Shanghai, China) for 1 hour. TRAP assay kit (Beyotime Biotechnology, Jiangsu, China) was used to detect relative TRAP activity according instruction manual.

Separation of OC-sEVs

Osteoclast-derived sEVs were collected through centrifugation with a series of speed gradients as previously described with some modifications [30]. Briefly, culture medium was removed and osteoclasts were washed three times with PBS. For sEVs isolation, osteoclasts were cultured in α -MEM for 24 hours to get supernatant. To remove the dead cells and apoptotic bodies, the supernatant was centrifuged at $1,500 \times g$ for 15 mins and $3,000 \times g$ for 15 mins. To remove IEVs, the supernatant was centrifuged at $18,000 \times g$ for 30 mins. Finally, sEVs were pelleted via centrifugation at $110\,000 \times g$ for 70 mins. The pellets were washed in 50 ml PBS and the centrifuged again at $110\,000 \times g$ for 70 mins. The separated sEV were resuspended in PBS for experiments. All centrifugation operations were performed at 4°C using Optima XE-90 (Beckman Coulter).

Characterization of OC-sEVs

For transmission electron microscopy (TEM) analysis, the 20 μl separated sEVs were dropped on each formvar copper grid and fixed with 20 μl 4% PFA. After fixation, the liquid on the surface of copper grids were removed by absorbent paper and uranyl acetate (Electron Microscopy Sciences) were dropped on formvar copper grids for staining. After 10 mins at room temperature, uranyl acetate was removed and formvar copper grids were naturally dried for observation. Digital images were obtained under FEI Tecnai microscope at an accelerating voltage of 80 kV. For nanoparticle tracking analysis (NTA) analysis, the size distribution and concentration of separated sEVs was examined using a ZataView (Particle 140 Metrix, Germany).

miRNA microarray analysis

The sEVs secreted by osteoclasts at each differentiation stages were isolated by ultracentrifugation. miRNeasy Mini Kit (Qiagen) was used to extract total RNA from the isolated sEVs and purity of RNAs were examined by Agilent 2100 Bioanalyzer before microarray analysis. Mouse miRNA Microarray Kit (Agilent, V3) was used and we added 100 ng total RNA in each sample. According to manufacturer's instructions, the RNA in samples were first phosphorylated and treated with cyanine 3-cytidine biphosphate. Subsequently, the samples were vacuum dried for 2 hours at 50°C and hybridized to microarray surface for 20 hours at 55°C on a shaker with 20 RPM. The glass slides then were washed and scanned with Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software v11.0.1.1 (Agilent Technologies) was used to extract data and GeneSpring GX software (Agilent Technologies) was used to normalize and analyze. We used R v3.6.0 to convert data into heatmap.

Osteogenic differentiation

For osteogenic differentiation, 1×10^6 BMSCs were seeded in 24-well plates and 5×10^6 BMSCs were seeded in 6-well plates. When BMSCs covered all the bottoms of each well and stopped proliferating, osteogenic induction was performed. The osteogenic medium contains 2mM β -glycerophosphate (Aladdin, Shanghai, China), 100 μ M ascorbic acid (Aladdin, Shanghai, China) and 10 nM dexamethasone (Aladdin, Shanghai, China). In order to generate sEVs-contained induction medium, 1×10^9 OC-sEVs were added to per ml osteogenic medium. Subsequently, BMSCs in 24-well plates were cultured with sEVs-contained osteogenic medium for 7 days to extract total RNA. BMSCs in 6-well plates were cultured with sEVs-contained osteogenic medium for 14 days to extract total protein. Total protein and RNA extracted from BMSCs were used to assess the protein and mRNA expression of osteogenic markers by western blots and qPCR analysis. For detecting ALP activity and assessing mineralization, BMSCs cultured with sEVs-contained osteogenic medium for 14 days in 24-well plates were washed by PBS three times and fixed in 4% PFA for 30 mins. Alkaline phosphatase assay kit (Beyotime Biotechnology) and 1% Alizarin Red-S (Solarbio Life Sciences) were used to detect ALP activity and assess mineralization according to manufacturer's protocols.

Generation of sEV-modified DBM scaffolds

Decalcified bone matrix (DBM) was prepared in our study based on demineralized bovine limbs as previously reported [31]. we cut DBM into 2.5 mm cubes to fill the calvarial defect. Before sEVs coated, the DBM were immersed in 75% ethanol for 2 hours, washed three times with PBS and then incubated with 10 μ g/mL fibronectin (Sigma, Shanghai, China) overnight at 37 °C. The DBM was immersed in sEV-containing medium (50 μ g sEVs suspended in 50 μ L α -MEM) and incubated at 37°C for 6 hours. Subsequently, The DBM was taken out and stored at -80°C.

Animal experiments

We evaluated the bone regeneration potency of sEV-modified DBM using a calvarial defect mouse model. After 8 week old male C57BL/6 mice were anesthetized by intraperitoneal injection of pentobarbital (Sigma Aldrich, 4mg/100g), a sagittal median incision of 1.5 cm was made and a 2.5 mm-sized defect was created on the right side of the calvarial bone using a dental micro-drill. Afterwards, the sEV-modified scaffolds were implanted into the bone defects. After 4 weeks, mice were euthanized according to the AVMA guidelines for the euthanasia of animals. the calvarial bones of mice were removed for subsequent experiments. All mice were obtained and fed in the animal facility of Third Military Medical University. The Institutional Animal Care and Use Committee of the Third Military Medical University reviewed and approved all experimental protocols.

Histological TRAP and IHC evaluation

Calvarial bones were collected at the euthanized time and fixed in 4% PFA, washed three times and decalcified in 10% EDTA solution for 2 weeks. Decalcification solution was renewed every two days. After dehydrated by ethanol gradient, decalcified calvarial bones were embedded in paraffin and sectioned into 5 μ m by microtome for subsequent TRAP and IHC staining. For histological TRAP stain, sections were incubated with TRAP stain solution (Sigma-aldrich, Shanghai, China) according to the manufacturers' instructions. The expressions of osteocalcin and FAM134A were detected by IHC assessment. Firstly, sections were incubated with primary antibodies of osteocalcin (1:300) and FAM134A (1:300) at 4°C overnight. Afterwards, EnVision+ system HRP kit (Dako, Sweden) containing biotinylated secondary antibody was used to develop color and nuclei were counterstained with hematoxylin. Finally, the washed sections were taken using an optical microscope. The German semi-quantitative scoring system was used to analysis slice data, which was described particularly in our previous studies [28].

Luciferase reporter assay

1×10^5 cells were seeded and cultured into 24-well plates until reaching 70% confluence. Lipofectamine 2000 Reagent (Invitrogen) was used to transfected 1.5 μ g constructed pGL3-basic luciferase reporter plasmid per well (Promega) or 1.5 μ g control luciferase plasmid (Promega) into the cells with 0.15 μ g pRL-SV40 plasmid (Promega) according manufacturer's instructions. Dual-luciferase reporter assay system (Promega, USA) was used to detect luciferase and *Renilla* activities of transduced cells after 48 hours.

Western blot analysis

Cells or sEVs were lysed in RIPA lysis buffer mixing with protease phosphatase inhibitor (Beyotime Biotechnology, Jiangsu, China). The lysis system was incubated on ice for 30 mins, and ultrasonic lysed

every 10 mins. Subsequently, the supernatant was carefully removed and transferred into a new microfuge tube after centrifugation at 13000 g for 15mins. The BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China) was used to detect the concentration of proteins according manufacturer's instructions. Each sample (50 µg) was diluted in loading buffer (Beyotime Biotechnology, Jiangsu, China) and subjected to a standard SDS-PAGE followed by transferred onto polyvinylidene fluoride membranes (Immobilon™-PSQ Membranes, Sigma-Aldrich, China). After blocking in 5% skim milk and washing in Tris Buffered Saline Tween buffer, proteins were detected using the following antibodies: Histone 3 (bs-17422R) at a 1:1000 dilution, CD81 (bs-2489R) at a 1:1000 dilution, TSG101 (bs-1365R) at a 1:1000 dilution, LaminA/C (bs-1839R) at a 1:1000 dilution, and β-actin (bs-0061R) at a 1:2000 dilution. Corresponding secondary antibodies against primary antibodies were used by an hour of incubation (1:2000). Blots against β-actin served as loading control. Super ECL Plus (Bioground, Chongqin, China) and Immun-Star HRP (BioRad) were used to detect chemiluminescent signals.

qPCR analysis

Total RNAs from cells or sEVs were extracted using Trizol reagent (Life Technologies, NY, USA). The ultra-low volume spectrometer (BioDrop µLite, Cambridge, England) was used to measure concentrations and purity of the RNA samples by detecting OD260 and OD280. PrimeScript™RT reagent kit (Takara, Nojihigashi, Japan) was used to synthesize cDNA. SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and specific primers (listed in supplement table 1) were used to further amplification process. Real-time PCR process was carried out on CFX96™ Real-Time PCR System instrument (Bio-Rad).

Lentivirus construction and transfection

In our study, mimics and inhibitors of miR-106a-5p were purchased from GenePharma (Shanghai, China). The constructed lentivirus vectors which express miR-106a-5p or inhibit miR-106a-5p were obtained from Genechem (Shanghai, China). We logged in the National Center for Biotechnology Information and used BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure inhibitors and products of inhibitory vector only targeted miR-106a-5p. Before transfection, 1×10^6 cells were seeded and cultured in 6-well plates until reaching 70% confluence. Complete medium was replaced to transfection medium containing a-MEM, 10% FBS and lentivirus vectors with 20 MOI (Multiplicity of Infection). After incubation for 48 hours at 37°C, the transfection medium was replaced to selection medium containing a-MEM, 10% FBS and 5 mg/ml puromycin for obtaining stable transduced cells. For rescue experiments, Lipofectamine 2000 Reagent (Thermo Scientific, MA, USA) was used to transfect *Fam134a* expressing plasmids (GeneCopoeia) to cells which were cultured with miR-106a-5p-sEVs or stably expressed miR-106a-5p. Exo-Fect Exosome Transfection Kit (System Biosciences) was used to load mimics or inhibitors into OC-sEVs. miR-106a-5p mimic (5' to 3'): AAAAGUGCUUACAGUGCAGGUAGACCUGCACUGUAAGCACUUUUUU; miR-106a-5p inhibitor (5' to 3'): CUACCUGCACUGUAAGCACUUUU.

Statistical analysis

All representative data comes from experiments with similar results which were repeated at least three times and we used means $\pm$ standard deviation to present the collected data. Independent unpaired two-tailed Student's t-tests were used to analyze the difference within the two groups and one-way ANOVA followed by Student-Newman-Keuls post hoc tests were used to detect differences in multiple groups. When p value < 0.05 , the difference was considered to be statistically significant. * ($p < 0.05$) or ** ($p < 0.01$) were used to indicate the significances of difference within the two groups.

Results

OC-sEVs promote osteogenic differentiation of BMSCs

We first extracted BMMs from the femur and tibia marrow of 8-week old mice, and induced differentiation to obtain mature osteoclasts for 96 hours. Osteoclast differentiation was confirmed by TRAP staining, showing that TRAP⁺ multinucleated cells increased significantly after 96 hours of induction (**Figure 1a, b**). With the increase of induction time, osteoclast-specific genes continue to be up-regulated, analyzed by gene array. (**Figure 1c**). We separated OC-sEVs from serum-free media and characterized the structure and content of OC-sEVs according to Minimal Information 2018 for Studies of Extracellular Vesicles (MISEV2018) guidelines proposed by the International Society for Extracellular Vesicles (ISEV) [23]. sEVs were collected through a series of microfiltration and ultracentrifugation steps and further characterized by transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blot analysis of some relevant biomarkers. Under transmission electron microscopy, extracellular vesicles presented a classic saucer-shaped double-layer membrane structure (**Figure 1d**). And the diameter data of most sEVs were around 110 nm (**Figure 1e**). Moreover, western blot analysis of TSG101, CD81, LaminA/C protein and Histone 3 expressions revealed the efficient separation of OC-sEVs: TSG101 and CD81 were abundant in OC-sEV as EV markers, while the nucleoproteins LaminA/C and Histone 3, which are considered to be EV contaminating proteins, were absent (**Figure 1f**). (**Figure 1f**).

We next sought to explore the effects of OC-sEVs on osteogenic differentiation. BMSCs were stimulated with osteogenic medium contained OC-sEVs (1×10^9 per ml) for 21 days, subsequently followed by Alizarin red staining for detection of osteogenic mineralization. Quantification analysis revealed significantly increased calcium nodules in OC-sEVs-cultured BMSCs (**Figure 1g**). Besides, qPCR analysis results showed significant upregulation of osteogenic differentiation related markers—such as *Alpl*, *Sp7*, *Col1a1* and *Runx2* in BMSCs cultured with OC-sEVs. (**Figure 1h**). GW4869 is an inhibitor which has been reported to prevent the production and release of sEV in a variety of cell types. [32]. GW4869 was used to pretreat osteoclasts and then extracted the osteoclast conditioned medium (OC-CM). The number of sEVs secreted by osteoclasts pretreated with GW4869 decreased significantly, which confirmed by significantly reduced expression of CD81 and TSG101 (**Supplementary Figure 1**). We next assessed the osteogenic potency of OC-CM and results showed that OC-CM markedly induced osteogenesis characterized by increased calcium nodules as well as expression level of *Alpl*, *Sp7*, *Col1a1* and *Runx2* in BMSCs.

However, BMSCs cultured with OC-CM from GW4869 pretreated osteoclasts appeared no enhancement bone formation. The results suggested that OC-sEV is a key factor in promoting osteogenesis (**Figure 1g, h**). In brief, OC-sEVs as an essential factor promoted BMSCs osteogenesis chartered by increased mineralization and upregulated osteogenic markers.

miR-106a-5p derived from OC-sEVs can be transferred to BMSCs and induce osteogenic differentiation

To explore the functional molecules responsible for the potency of OC-sEVs to induce osteogenic differentiation, we extracted total RNAs of sEVs from osteoclast and BMMs, and analyzed the expression profile of miRNAs using miRNA microarray. The results showed that the expression levels of miR-18a-5p, miR-185-5p, miR-142-3p, miR-106a-5p and miR-132-3p were significant in OC-sEV. (**Figure 2a**). We transfected each miRNA mimic into BMSCs and cultured them with osteogenic medium to investigate whether these sEV-derived miRNAs can promote osteogenesis. The following alkaline phosphatase (ALP) assay at 14 days revealed an increase in ALP activity in BMSCs overexpressing miR-142-3p and miR-106a-5p. (**Figure 2b**). Alizarin red staining at 21 days showed that miR-106a-5p expression induced extensive mineralized nodules, suggesting the involvement of miR-106a-5p in osteogenic differentiation of BMSCs (**Figure 2c**). Since studies demonstrated that miRNA can be transferred between cells and play important roles in intercellular communications, we hypothesized whether miR-106a-5p can be transferred from osteoclasts to BMSCs via OC-sEVs. To address this issue, miR-106a-5p was knockdown or overexpressed in BMMs using lentivirus transfection system, and qPCR was performed to validate the transfection efficiency in cells and secreted sEVs. Intriguingly, after transfection with overexpression or knockdown lentivirus, the expression of miR-106a-5p in OCs and OCs-sEVs was synchronously up-regulated or suppressed. (**Supplementary Figure 2a, b**). Moreover, we found miR-106a-5p expression was upregulated in OC-sEVs during osteoclast differentiation, and further confirmed that OC-sEVs can protect miRNA from degradation by RNase. (**Supplementary Figure 2c**). Next, we added miR-106a-5p overexpression or knockdown EVs (miR-106a-5p-sEVs or si-miR-106a-5p-sEVs) obtained by transfected OCs into osteogenic medium to culture BMSCs. The qPCR results revealed that miR-106a-5p derived from sEVs (miR-106a-5p-sEVs) only upregulated the expression of miR-106a-5p in BMSCs but not pri-miR-106a-5p. (**Figure 2d and e**). Moreover, miR-106a-5p-sEVs induced the formation of extensive mineralized nodules, while si-miR-106a-5p-sEVs dampened osteogenic mineralization(**Figure 2f**). (**Figure 2f**). The similar regulatory effect was also confirmed at the expression level of osteogenic-related mRNA. (**Figure 2g**).

Fam134a is a functional target of miR-106a-5p involving in osteogenic differentiation

We utilized mRNA target-predicting algorithm (TargetsCan7.2, miRDB and miRWalk) to perform *in silico* prediction and identify *Fam134a* as a potential downstream target of miR-106a-5p. It has been

demonstrated that overexpression of *Fam134a* suppressed differentiation of immortalized human mesenchymal stem cell line UCB408E6E7TERT-33 [33]. Here we also investigated the effects of *Fam134a* expression on osteogenic differentiation of BMSCs through knockdown or overexpressing *Fam134a* (**Figure 3a**). After knockdown of *Fam134a* and osteogenic induction for 14 days, the ALP activity of BMSCs increased, while the result of the *Fam134a* overexpression group was opposite (**Figure 3c**). These results suggested that *Fam134a* level negatively regulate the osteogenic differentiation of BMSCs, which is consistent with previous studies. Furthermore, miR-106a-5p was also suppressed or overexpressed in BMSCs (**Supplementary Figure 3**). qPCR results revealed that overexpression of miR-106a-5p reduced the expression of osteogenic markers while knockdown of miR-106a-5p significantly upregulated the expression of osteogenic markers (**Figure 3d**). To confirm whether miR-106a-5p binds to *Fam134a*, we constructed luciferase reporter plasmids inserted *Fam134a* 3' UTR and then co-transfected with miR-106a-5p mimics. Subsequently, real-time fluorescence was detected to evaluate the binding, and result was normalized by *Renilla* luciferase plasmid (**Figure 3e**). Consistent with *in silico* prediction, the fluorescence level of BMSCs co-transfected with miR-106a-5p mimics and *Fam134a* 3'UTR was suppressed. (**Figure 3f**). Moreover, the *Fam134a* level in BMSCs was suppressed by miR-106a-5p overexpression, whereas knockdown of miR-106a-5p resulted in a significant upregulation of *Fam134a* (**Figure 3g**). Together, these findings indicate that miR-106a-5p regulates osteogenic differentiation through silencing *Fam134a* in BMSCs.

OC-sEVs-delivered miR-106a-5p silences *Fam134a* during osteogenic differentiation of BMSCs

We next pre-transfected BMSCs with *Fam134a* 3' UTR plasmids and then cultured with miR-106a-5p-sEVs for further detecting luciferase activity. The results revealed miR-106a-5p-sEVs but not mimic-NC-sEVs dampened luciferase activity. Moreover, pre-transfecting miR-106a-5p inhibitor can reverse this phenomenon suggesting that *Fam134a* in BMSCs can be suppressed by miR-106a-5p derived from OC-sEVs (**Figure 4a**). Not only that, culturing with miR-106a-5p-sEVs significantly down-regulated the expression of *Fam134a* but up-regulated the expression *Alpl*, *Sp7*, *Col1a1* and *Runx2* in BMSCs.. However, restoration the *Fam134a* expression in sEV-cultured BMSCs rescued *Fam134a* level and diminished ability of miR-106a-5p-sEVs to promote osteogenesis (**Figure 4b and c**). Annexin V is reported to be an inhibitor of EV internalization through blocking phosphatidylserine at the EV surface [34]. Notably, Annexin V or miR-106a-5p inhibitor abolished ability of miR-106a-5p-sEVs to promote osteogenesis and rescued the expression of *Fam134a* in BMSCs. We also reintroduced *Fam134a* plasmid lacking the 3' UTR region to BMSCs, which also restored *Fam134a* expression and prevented miR-106a-5p-sEVs from promoting osteogenesis (**Figure 4b and c**). Furthermore, Alizarin red staining results revealed that similar with *Fam134a* restoration, preincubation of miR-106a-5p-sEVs with miR-106a-5p inhibitor or Annexin V markedly reduced the area mineralized nodules (**Figure 4d and e**). These evidences demonstrate that OC-sEVs-delivered miR-106a-5p targeted the *Fam134a* in BMSCs and further strongly promote osteogenesis. According to the functional characteristics of FAM134 proteins (FAM134A,

FAM134B and FAM134C), we hypothesized that FAM134A may be involved in the regulation of autophagy in BMSCs and further detected the expression of related markers (LC3-I, LC3-II and p62). We detected the autophagy markers of BMSCs after osteogenesis induction 7 days and the results suggested that inhibiting the expression of FAM134A up-regulated the expression level of autophagy-related markers in BMSCs, whereas, overexpression of FAM134A did not significantly change the autophagy level (**Figure 4f**). In addition, the treatment of miR-106a-5p-sEVs down-regulated the expression of FAM134A in BMSCs, and the expression of autophagy-related markers was up-regulated correspondingly (**Figure 4g**).

OC-sEVs-mediated miR-106a-5p transfer promotes bone defect repair *in vivo*

We established a calvarial defect mice model to further evaluate the pro-osteogenic effect of miR-106a-5p-sEV *in vitro*. Subsequently, we used the demineralized bone matrix (DBM) as a scaffold which was coated with miR-106a-5p-sEVs to fill the bone defect. In previous reports, using bone defect models to evaluate angiogenesis and osteogenesis abilities of DBM scaffold “loading cells or sEVs” was considered reliable [35, 36]. After sEV-modification, no significant differences were observed in macroscopic appearance and wet weight between the groups (**Figure 5a, b**). Cranial drilling was conducted and scaffold was filled into the defect area. The mice were euthanized at 4 weeks after the operation, and their craniums were removed for further micro-computed tomography (micro-CT) scanning analysis to evaluate bone regeneration of defect repair area. Results showed that bone regeneration significantly increased in miR-106a-5p-sEVs coated DBM scaffold group characterized by the highest bone mineral density (BMD) and bone volume density (BV/TV) (**Figure 5c, d**). These results suggested the bone defect healing potency of DBM modified with miR-106a-5p-sEVs.

To investigate the effects of sEV-modified scaffold on osteoblasts and osteoclasts on the surface of repair area, we performed immunohistochemistry of osteocalcin and histological TRAP staining on decalcified calvarial sections. Importantly, higher TRAP⁺ cell ratio and osteocalcin expression presented in miR-106a-5p-sEVs scaffold group, indicating an active bone remodeling process (**Figure 6a and b**). However, pre-transfection of miR-106a-5p inhibitor abrogated the reparation of miR-106a-5p-sEVs scaffold characterized by lower TRAP⁺ cell ratio and osteocalcin expression (**Figure 6a-c**). Moreover, Compared with the control group “FAM134A expression in miR-106a-5p-sEVs scaffold group significantly reduced” while pre-transfecting miR-106a-5p inhibitor can rescue the expression level of FAM134A. (**Figure 6a, c**). Altogether, these evidences indicated that OC-sEVs-mediated miR-106a-5p transfer promotes bone regeneration in calvarial defect mice.

Discussion

In this study, we presented evidences that the pro-osteogenic miRNAs can be transferred from osteoclasts to BMSCs through the release and internalization of sEVs. This intercellular transfer of sEVs contributed to osteogenic differentiation of BMSCs and resulted in accelerated bone defect healing. Our identification

of this sEV-mediated miRNA transfer between osteoclasts and BMSCs may provide a novel osteoclast-osteoblast coupling mode mediated by sEV.

Increasing evidences suggest the involvement of EVs in a diverse of intracellular or intercellular signal transduction. In bone remodeling microenvironments, EVs derived from various bone cells (osteoclasts, osteoblasts, osteocytes, BMSCs and immune cells) carried specific proteins, miRNAs, lncRNAs, circRNAs and other growth factors [26]. For instance, osteoclast-derived EVs carry RANK, while osteoblast-derived EVs carry RANKL, Osteocrin and so on. [37–39]. After EVs are internalized by adjacent bone cells, these EV-carried bioactive molecules further affect the growth, differentiation and death of neighboring cells. Studies reported that EVs from BMSCs upregulate the expression of transforming growth factor beta 1 (TGFβ1) and bone morphogenetic protein 9 (BMP9) in bone marrow stromal cells, resulting in increased osteogenic differentiation activities [40–42]. *In vivo*, MSCs-derived EVs also showed a significant positive regulation on angiogenesis and osteogenesis during bone remodeling [43, 44]. Our group also found that osteoclast-derived apoptotic bodies promote osteogenic differentiation of preosteoblasts through vesicular RANK-mediated RANKL reverse signaling [27, 28]. In addition to delivering cytokines, bone cells-derived EVs are also reported to be involved in the regulation of osteogenic differentiation through miR-375, miR-218 and miR-214-3p transfer [45–47]. BMSCs-sEV can transfer miR-151-5p to surrounding BMSCs ameliorating osteopenia and rescuing damaged osteoblast differentiation. [48]. Our work suggested a new path OC-sEVs facilitate osteogenesis by transferring miR-106a-5p from osteoclasts to BMSCs explaining the conversion from bone resorption to bone formation.

miRNA is an important intercellular communication tools, since EV-mediated miRNAs horizontal transfer plays critical roles in regulating biological functions of recipient cells. In recent years, a number of miRNAs have been identified to be involved in the osteogenic differentiation of MSCs, raising the possibility that osteogenic differentiation of MSCs may also be affected by bone cells-derived miRNAs during bone remodeling [49, 50]. In this study, we found miR-106a-5p is highly enriched in OC-derived sEVs and induce the osteogenic differentiation of BMSCs. So far, a few studies have revealed the role miR-106a-5p in a variety of biological processes including tumor development, immune regulation and epithelial mesenchymal transition [51, 52]. A recent study also reported that miR-106a-5p is involved in modulating the osteogenic differentiation of periodontal ligament stem cells through an endogenous competitive RNA mechanism [53]. However, it is little reported that miR-106a-5p can induce osteogenesis of BMSCs. In our research, it was confirmed that OC-sEV riched in miR-106a-5p can mediate the osteogenic differentiation of BMSCs and *Fam134a* is identified as a target of miR-106a-5p through *in silico* prediction. *Fam134a* (also known as metastasis associated genes 2, MAG-2) is widely expressed in various types of tissues including tumors and is considered to be closely correlated with tumor proliferation, invasion and metastasis [54]. As the member of reticulophagy regulator, FAM134A can also binds to the LC3/ GABARAP protein on the endoplasmic reticulum membrane stabilizing and regulating the structure of the endoplasmic reticulum [55]. A recent study also found that *Fam134a* negatively regulates osteogenic differentiation of UCB408E6E7TERT-33 cells and is involved in tumor-induced osteoblastic destruction [33, 56]. One step further, we speculate that FAM134A may act as a negative factor inhibiting osteogenic differentiation of BMSCs by regulating the stability of the endoplasmic

reticulum structure and the level of autophagy [55, 57]. The detecting result of autophagy markers preliminarily confirmed our conjecture and provided research Interests for further exploration of Fam134a in bone remodeling and metabolism.

Gene therapy relies on nano-sized vectors to efficiently deliver the specific cargos to designated destinations, which has been widely used in many genetic diseases [58]. Since miRNAs play crucial roles in regulating gene expression in cells, miRNAs are considered to be promising therapeutic targets in gene therapy. Recent years, several miRNA-based gene therapeutics have been applied to some diseases which may be caused by abnormal expression of a specific miRNA [59]. In these previous studies, miRNA can be efficiently and accurately delivered by designed adenoviral vectors and other non-viral vectors. However, miRNA degradation, poor biocompatibility and non-selective cytotoxicity are still the major defects of these vectors. Therefore, cell-derived sEV has become a preferred choice for nano-sized miRNA delivery, because of its advantage as naturally produced biological vector [60]. Moreover, genetic modification and electroporation as two main strategies for effectively loading cargos into EVs are currently adopted and applied [61, 62]. It has been reported that siRNA aggregates on one side of cell or EV during electroporation, resulting in decreased loading efficiency [63]. Whether other RNAs such as mRNAs and miRNAs can be enriched in EVs through electroporation is unclear. In this study, we overexpressed miR-106a-5p in osteoclasts using a lentivirus, which produces mature miR-106a-5p in recipient cells. This loading strategy based on genetic modification has been used to incorporate mRNAs, miRNA and small hairpin RNAs into EVs [48, 64]. It has been reported that sEVs derived from miR-940-overexpressed prostate cancer cells are also enriched with miR-940 and exacerbate tumor-induced osteoblastic destruction [33]. Another study demonstrated that by overexpressing miR-25-3p in donor cells, sEVs are also successfully enriched with high level of miR-25-3p and contribute to pre-metastatic niche formation [65]. Here, we also showed that miR-106a-5p can be enriched in osteoclast-derived sEVs by overexpressing the miR-106a-5p in the parental cells and are protected from RNase degradation.

In conclusion, our findings suggest that miR-106a-5p carried by OC-sEVs propagate osteogenic signals to BMSCs and promote osteogenesis *in vitro* and *vivo*. This research provides a novel osteoclast-osteoblast communication mode mediated by sEV during bone remodeling.

Declarations

Authors' contributions

Y.W. conceived and designed the study, Y.W, H.A, and Y.Z. performed experiments; Y.W. analyzed the data; J.X. provided advice and technical assistance; and Y.W. wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by the Postgraduate tutor team building project of Chongqing (4142Z242).

Competing interests

The authors report no conflict of interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

All authors agree to be published.

Ethics approval and consent to participate

Not applicable.

Acknowledgements

Not applicable.

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Figures

Figure 1

OC-sEVs promote osteogenic differentiation of BMSCs (a) TRAP staining of BMMs stimulated with 50 ng/ml M-CSF and 100 ng/ml RANKL for 96 hours. Scale bar = 200 μ m. (b) Quantification of osteoclasts (TRAP+) in per well (96-well plate), n = 5. (c) Heatmap showed the gene profile with the different expression level between BMMs and OCs under the stimulation of RANKL. (d) TEM showed that OC-sEVs were characterized by the saucer-shaped double-layer membrane structure. Scale bar = 100 nm. (e) NTA data showed the diameter of most OC-sEVs was around 110 nm. (f) Western blot results showed the CD81, TSG101 and β -actin exist both in OCs lysate and purified OC-sEVs, the LaminA/C and Histone 3 only exist in OCs lysate. (g) Alizarin-red staining of BMSCs cultured with OC-sEVs or OC-CM, and quantification analysis of calcium deposit in each group. Scale bar = 100 μ m, n = 5. (h) qRT-PCR showed the relative expression of Alpl, Sp7, Col1a1 and Runx2 in BMSCs incubated with OC-sEVs, OC-CM and OC-CM pretreated by with GW4869, n = 3. The data in the figures represent the averages $\pm$ SD. Stsatically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 2

miR-106a-5p derived from OC-sEVs can be transferred to BMSCs and induce osteogenic differentiation (a) Heatmap showed the miRNAs expression profile in BMM-sEVs secreted during osteoclastogenesis. Using BMM-sEVs (0 hours) as a normalization control, red color means higher expression, while blue color means lower expression (b) Overexpression of either miR-142-3p or miR-106a-5p increased the ALP activity, $n = 3$. (c) Alizarin red staining showed that overexpression of miR-106a-5p induced the mineralization of BMSCs. Scale bar = 100 μm , $n = 5$. Relative expression levels of (d) miR-106a-5p and (e) pri-miR-106a-5p in BMSCs cultured with miR-106a-5p-sEVs or si-miR-106a-5p-sEVs over time, $n = 3$. (f) Alizarin red staining of BMSCs cultured with miR-106a-5p-sEVs, si-miR-106a-5p-sEVs or control sEVs, and quantification analysis of calcium deposit in each group. Scale bar = 100 μm , $n = 5$. (g) qRT-PCR showed the relative expression of *Alpl*, *Sp7*, *Col1a1* and *Runx2* in BMSCs incubated with miR-106a-5p-sEVs or si-miR-106a-5p-sEVs, $n = 3$. The data represent the means $\pm$ standard deviation. * ($p < 0.05$) or ** ($p < 0.01$) were used to indicate the significance of the difference within the two groups.

Figure 3

Fam134a is a functional target of miR-106a-5p involving in osteogenic differentiation (a) qRT-PCR showed the relative expression levels of Fam134a in BMSCs which were overexpressed or knockdown Fam134a, n = 3. (b) ALP activity of BMSCs which were overexpressed or knockdown Fam134a, n = 3. (c) qRT-PCR showed the relative expression levels of Alpl, Sp7, Col1a1 and Runx2 in BMSCs which were overexpressed or knockdown Fam134a, n = 3. (d) qRT-PCR showed the relative expression levels of Alpl, Sp7, Col1a1 and Runx2 in BMSCs which were overexpressed or knockdown miR-106a-5p, n = 3. (e) Construction of Fam134a 3' UTR wild type (wt) and mutant type (mut) luciferase reporter vectors. (f) Relative luciferase activity of reporter containing the 3' UTR of Fam134a upon co-transfection with miR-106a-5p mimics, relative to transfection with negative control miRNA, n = 5. (g) qRT-PCR showed the relative expression levels of Fam134a in BMSCs which were overexpressed or knockdown miR-106a-5p, n = 3. The data represent the means $\pm$ standard deviation. * ($p < 0.05$) or ** ($p < 0.01$) were used to indicate the significance of the difference within the two groups.

Figure 4

OC-sEVs-delivered miR-106a-5p silences Fam134a during osteogenic differentiation of BMSCs (a) Relative luciferase activity of Fam134a in BMSCs which were incubated with OC-sEVs, mimic-NC-sEVs and miR-106a-5p-sEVs pre-transfected with miR-106a-5p inhibitor or not, n = 5. (b) qRT-PCR showed the relative expression levels of *Alpl*, *Sp7*, *Col1a1* and *Runx2* in BMSCs after variously treated (OC-sEVs, mimic-NC-sEVs, miR-106a-5p-sEVs, miR-106a-5p-sEVs + Annexin V, miR-106a-5p-sEVs pre-transfected

with miR-106a-5p inhibitor and miR-106a-5p-sEVs + Fam134a), n = 3. (c) qRT-PCR showed the relative expression levels of Fam134a in each group, n = 3. (d) Alizarin red staining of BMSCs in each group. Scale bar = 100 μ m, n = 5. (e) Quantification analysis of calcium deposit of BMSCs in each group, n = 5. (f) Western blot results showed the FAM134A, LC3-I/LC3-II, P62 and β -tubulin expression level in BMSCs with treatment. (g) Western blot results showed the FAM134A, LC3-I/LC3-II, P62 and β -tubulin expression level in BMSCs cultured different sEVs. The data represent the means $\pm$ standard deviation. * ($p < 0.05$) or ** ($p < 0.01$) were used to indicate the significance of the difference within the two groups.

Figure 5

OC-sEVs-mediated miR-106a-5p transfer promotes bone defect repair in vivo (a) The sEV-modified DBM scaffolds before implantation. Vehicle represents DBM scaffold without sEVs modification. (b) Wet weight of the sEV-modified DBM scaffolds in each group, n = 5; (c) Micro-CT images of calvarial bone defect stuffed with sEV-modified DBM scaffolds after 4 weeks. (d) Bone formation ratio, bone mineral density (BMD) and bone volume density (BV/TV) of total defect repair area in each group, n = 5. The data represent the means $\pm$ standard deviation. * (p < 0.05) or ** (p < 0.01) were used to indicate the significance of the difference within the two groups.

Figure 6

OC-sEVs-derived miR-106a-5p promotes bone defect repair through inhibiting FAM134A (a) Histological TRAP staining and immunohistochemistry staining (IHC) of OCN and FAM134A in bone defect sections. Scale bar = 50 μ m. (b) Number of osteoblasts (N.Ob) and number of osteoclasts (N.Oc) on bone surface (BS) counted basing on TRAP and IHC staining images, n = 5. (c) Semi-quantitative analysis of OCN and FAM134A in each group, n = 5. The data represent the means $\pm$ standard deviation. * (p < 0.05) or ** (p < 0.01) were used to indicate the significance of the difference within the two groups.

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