M2 macrophage-derived exosomes carry miR-142-3p to restore the differentiation balance of irradiated BMMSCs by targeting TGF-β1

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Research Article

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Abstract

**Purpose** Radiotherapy is essential to cancer treatment, while it inevitably injures the surrounding normal tissues, and bone tissue is one of the most common sites prone to irradiation. Bone marrow mesenchymal stem cells (BMMSCs) are sensitive to irradiation and the irradiated dysfunction of BMMSCs may be closely related to irradiation-induced bone damage. Macrophages play an important role in stem cell function regulation, bone metabolic balance and irradiation response, but the effects of macrophages on irradiated BMMSCs are still unclear. This study aimed to investigate the role of macrophages and macrophage-derived exosomes in restoring irradiated BMMSCs function.

**Methods** The effects of macrophage conditioned medium (CM) and macrophage-derived exosomes on osteogenic and fibrogenic differentiation capacities of irradiated BMMSCs were detected. The key microribonucleic acids (miRNAs) and targeted proteins in macrophage-derived exosomes were also determined.

**Results** The results showed that X-ray irradiation significantly inhibited the proliferation of BMMSCs. Additionally, it caused a differentiation imbalance of BMMSCs, with decreased osteogenic differentiation and increased fibrogenic differentiation. M2 macrophage-derived exosomes (M2D-exos) inhibited the fibrogenic differentiation and promoted the osteogenic differentiation of irradiated BMMSCs. We identified that miR-142-3p was significantly overexpressed in M2D-exos and irradiated BMMSCs treated with M2D-exos. After inhibition of miR-142-3p in M2 macrophage, the effects of M2D-exos on irradiated BMMSCs differentiation were eliminated. Furthermore, transforming growth factor beta 1 (TGF-β1), as a direct target of miR-142-3p, was significantly decreased in irradiated BMMSCs treated by M2D-exos.

**Conclusion** This study indicated that M2D-exos could carry miR-142-3p to restore the differentiation balance of irradiated BMMSCs by targeting TGF-β1. These findings pave the way for a new, promising, and cell-free therapeutic method to treat radiation-induced bone damage.

Introduction

Radiotherapy, either alone or in combination with surgery and chemotherapy, has become one of the most important methods for cancer treatment. It is estimated that approximately 10 million patients with cancer worldwide received radiotherapy annually[1, 2]. Radiotherapy increases the cure rate of malignant tumors, but also destroys normal tissues. Compared with other tissues, bone tissues absorb 30–40% more radiation energy, thereby making them the most commonly irradiated tissues[3]. However, the prevention and treatment of radiation-induced bone damage still remains a clinical challenge. Radiation-induced bone damage includes bone loss, bone fragility, bone fracture, and osteonecrosis[4, 5]. As one of the most severe types of radiation-induced bone damage, the pathology of osteoradionecrosis of the jaw (ORNJ) after radiotherapy in the head and neck region is also characterized by excessive fibrotic accumulation, in addition to bone loss and bone necrosis[6, 7]. However, the mechanisms underlying this irradiation-induced bone damage and fibrosis are not fully understood.
BMMSCs are multidirectional differentiation-capable stem cells that are widely distributed in bone marrow, and were recently thought to be the main target of irradiation in bone tissues[8, 9]. BMMSCs can differentiate into osteoblasts, adipocytes, chondrocytes, and so on, and the differentiation ability of BMMSCs is greatly affected by the surrounding microenvironment. Studies showed that the osteogenic differentiation of BMMSCs significantly decreased after irradiation, which was an important cause of irradiation-induced bone damage[10]. In the irradiation microenvironment, BMMSCs also showed a potential to differentiate into myofibroblasts, the main effector cells of fibrosis, by a process called fibrogenic differentiation. The abnormal proliferation of myofibroblasts after irradiation is the culprit of radiation-induced fibrosis [11]. Mesenchymal stem cells from different origins are usually used for radiation protection. However, the potential risk of fibrogenic differentiation hinders stem cell therapy. In the acute inflammatory response induced by irradiation at the beginning of the initial stage, cells begin to secrete cytokines, such as fibroblast growth factor-β, transforming growth factor-β-1 (TGF-β1), tumor necrosis factor-α, and interleukins, thereby leading to the aggregation and transdifferentiation of fibroblasts and BMMSCs into myofibroblasts[12], which are responsible for the characterization of fibrosis in many irradiation-induced bone injury diseases. In summary, restoring the normal function of BMMSCs is important to treat irradiation-induced bone injury diseases.

After irradiation damage, various immune cells were recruited to the irradiated field sites, which started the repair process and regulated bone homeostasis[13]. As a key player in the immune system, macrophages have been widely involved in regulating the inflammatory responses and promoting tissue injury and repair. Previous studies have found that macrophages play an important role in regulating the irradiation protection of specific cells[14–16]. In addition, macrophages displayed the strongest interaction with BMMSCs among all the immune cells and had regulatory effects on BMMSCs differentiation function[17]. However, the role of differently polarized macrophages in regulating the function of irradiated BMMSCs, especially in osteogenic and fibrogenic differentiation ability, has not been explored.

Exosomes, double membrane-structured vesicles with 30–150 nm in diameter, are a component of paracrine secretion that contain functional messengers, such as ribonucleic acids (mRNAs), miRNAs and proteins. Furthermore, they are cytoprotective and promote tissue repair[18]. Among the contents encapsulated by exosomes, miRNAs are the most widely studied, and many studies have confirmed that miRNAs in exosomes played a regulatory role in the balance of bone metabolism[19] and fibrogenic differentiation[20]. Therefore, further identification of the molecular mechanisms that regulate the osteogenic and fibrogenic differentiation of BMMSCs after irradiation is critical for treating or preventing ORNJ and other types of radiation-induced bone damage. This study investigated the effects of different polarized macrophage-derived exosomes on irradiated BMMSCs, and the underlying mechanisms.

**Materials And Methods**

**Reagents and Antibodies**
Recombinant rat macrophage colony-stimulating factor (M-CSF) was purchased from Novoprotein Technology Co., Ltd. (Suzhou, China). Recombinant rat interleukin-4 (IL-4) and interferon-gamma (IFN-γ) were purchased from PeproTech Corporation (New Jersey, USA). Cell Counting Kit-8 (CCK-8) and lipopolysaccharides (LPS) were purchased from PlantChemmed (Shanghai, China). PKH26 was purchased from Umibio Co., Ltd. (Shanghai, China). Rabbit anti-Runx-2, anti-cluster of differentiation 81 (CD81), and anti-tumor susceptibility gene 101 (TSG101) were purchased from Abcam (Massachusetts, USA). Alkaline phosphatase (ALP) antibody and antibodies against F4/80, β-actin, collagen type (COL), CD29, CD34, CD44, CD90, and collagen type (COL) were purchased from GeneTex Corporation (Texas, USA). Alpha-smooth muscle actin (α-SMA) was purchased from Cell Signaling Technology (Danvers, USA). The phycoerythrin CD86 rat monoclonal antibody, HRP-conjugated GAPDH Monoclonal antibody and CD206 monoclonal antibody were purchased from Proteintech Group, Inc. (Chicago, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from the Invitrogen Gibco Corporation (Gaithersburg, USA). β-glycerophosphate, dexamethasone, and ascorbic acid streptomycin were purchased from the Sigma-Aldrich corporation (Shanghai, China). The 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) ALP chromogenic kit was purchased from the Biyuntian Institute of Biotechnology (Shanghai, China). An alkaline phosphatase detection kit was purchased from Jiancheng Bioengineering (Nanjing, China). Alizarin red dye was purchased from BioTek Instruments (San Jose, USA). Universal miRNA extraction kit, GoldenstarTM RT6 cDNA Synthesis Kit and miRNA Universal SYBR qPCR Mix kit were purchased from Tsingke Biotechnology Co., Ltd. TRIzol Reagent, PrimeScript TM RT Master Mix kit and TB Green® Premix Ex Taq™ II kit were obtained from Takara Biomedical Technology (Beijing) Co., Ltd. (Tokyo, Japan). Radioimmunoprecipitation assay strong lysis solution (RIPA) was purchased from Zhonghui Hecai corporation (Shaanxi, China). Tris-buffered saline with 0.1% Tween-20 (TBST) was purchased from Proandy corporation (Shaanxi, China). Lipofectamine 3000 was purchased from Thermo Fisher Scientific (Waltham, USA).

**Isolation And Culture Of Bmmscs And Bmdms**

The male Sprague-Dawley (SD) rats (2–3 weeks of age) used in the experiment were all obtained from the Laboratory Animal Center of the Fourth Military Medical University and approved by the Welfare and Ethics Committee of the Laboratory Animal Center of the Fourth Military Medical University. Femurs and tibias of SD rats were used to obtain bone marrow, and the whole bone marrow adhesion method was used to culture BMSCs as described in previous study[21]. The cells were cultured in DMEM complete medium (include 10% FBS and 1% penicillin-streptomycin) at 37°C in a 5% CO₂ incubator, and the medium was refreshed every 3 days. Cells at passages 3–5 (P3–P5) were used in this study. The multi-differentiation potential and surface markers were detected by osteogenesis-induction, adipogenesis-induction, chondrogenesis-induction and flow cytometric analysis to identify BMSCs.

To obtain primary bone marrow derived macrophages (BMDMs), the bone marrow cells were isolated from femurs and tibias of rats, then cultured with DMEM supplemented by 10% FBS, 1% penicillin-
streptomycin, and stimulated by 30 ng/mL M-CSF for 7 days. M1 and M2 macrophages polarization was initiated by 100 ng/mL LPS and 30 ng/mL IFN-γ, or 20 ng/mL IL-4 for 24 h, respectively. The macrophages were identified by flow cytometric analysis and Real-time Quantitative polymerase chain reaction (RT-qPCR).

**Osteogenic Differentiation Induction**

The P3-P5 BMMSCs were cultured with osteogenesis-induction medium when they reached 60%-70% confluence, and the medium was refreshed every 3 days. The osteogenesis-induction medium was composed of DMEM, 10% FBS, 1% penicillin-streptomycin, 10 mM β-glycerophosphate, 10 nM dexamethasone, and 50 µg/mL ascorbic acid. After 7 days of induction, ALP staining and ALP activity analysis was performed. On day 21 of induction, alizarin red staining was performed.

**Adipogenic And Chondrogenic Differentiation Induction**

The P3-P5 BMMSCs at 80–90% confluence were cultured with adipogenesis-induction medium, which was composed of DMEM, 10% FBS, 1% penicillin-streptomycin, 10 µg/mL insulin, 0.1 mmol/L 3-isobutyl-1-methylxanthine, 50 µg/mL indomethacin, and 10 nM dexamethasone. The medium was refreshed every 3 days. The adiopogenesis-induced cells were fixed and stained with Oil red O after 21 days of induction. The P3-P5 BMMSCs were transferred into a centrifuge tube with cell numbers of 4×10^5, and centrifuged at 250 × g for 4 min. And then a chondrogenesis-induction kit was used to induce the BMMSCs following the manufacturer's instructions. When the induced cells formed into a cartilage pellet, it was fixed and stained with alcian blue solution.

**Alp Activity, Alp Staining And Alizarin Red Staining**

After 7 days of osteogenesis induction, the supernatant of BMMSCs was collected and the ALP activity was detected by using an ALP Detection Kit according to the manufacturer's instructions. The optical density (OD) value of the supernatant was measured at 520 nm using a spectrophotometer (Epoch spectrophotometer, USA). ALP staining was performed on day 7 of osteogenesis induction by using the BCIP/NBT alkaline chromogenic phosphatase kit. After 3 weeks of the osteogenesis induction of BMMSCs, alizarin red staining was performed. BMMSCs were fixed with 4% paraformaldehyde for 20 min and incubated with 1% alizarin red staining solution for 30 min. The images of stained mineralized nodules were obtained using an inverted light microscope (Olympus, Tokyo, Japan). Then 2% cetylpyridinium chloride was used to dissolve the stained mineralized nodules, and OD value of the supernatant solution was determined at 560 nm using a spectrophotometer.

**Flow cytometric analysis**
The BMMSCs were identified by the surface makers including CD45, CD44, CD34, CD31, CD90, and CD29. For macrophages, surface makers CD11b, F4/80, CD86 and CD206 were used for M0, M1 and M2 macrophage identification. The cells were incubated with polyclonal antibodies of these surface makers in the dark at 4°C for 30 min. Subsequently, flow cytometry (Beckman Coulter, USA) was used to detect the positively stained cells.

**Macrophage Conditioned Medium (Cm) Preparation**

M1 and M2 macrophages were cultured with DMEM complete medium at 37°C in a 5% CO₂ incubator for 24 h, and the medium supernatant was collected. To remove debris and cells, the medium supernatant was centrifuged at 2,000×g for 30 min at 4°C and then filtered through a sterilized 0.22-µm filter. After these protocols, the obtained fluid was defined as macrophage conditioned medium (CM). The CM derived from M1 and M2 macrophages was respectively termed as CM-M1 and CM-M2. The CM-M1 and CM-M2 was stored at -80°C before use.

**Purification, Characterization And Uptake Of Exosomes**

Before exosomes collection, the culture medium of M1 and M2 macrophages was replaced with an exosome-depleted complete medium. After 24 h, the obtained cell supernatant was centrifuged at 1,000 × g for 10 min and then filtered using a 0.22-um filter. This process was followed by an ultra-centrifugation at 100,000 × g at 4°C for 70 min. Subsequently, the supernatant was discarded and the precipitates were resuspended in PBS. The medium was ultra-centrifuged at 100,000 × g for another 70 min to purify the exosomes. The isolated exosomes were resuspended in PBS and stored – 80°C for further use. For identification of collected exosomes, the morphology was observed by transmission electron microscopy (TEM, JEOL, Japan), the distribution size was assessed by Nanoparticle Tracking Analysis (NTA, NanoFCM, China), and the specific surface markers (TSG 101, CD81) were detected by western blot. To confirm exosomes were uptaken by irradiated BMMSCs, PKH 26 was used to label the exosomes. Briefly, the exosomes were incubated in 1 mL of diluted C solution containing 5 µM PKH26 dye for 5 min. The labeling reaction was stopped by adding 10 mL PBS. It was followed by ultra-centrifugation at 100,000 × g for 70 min at 4°C and suspension in 100 µL PBS. The irradiated BMMSCs were treated with these labeled exosomes for 12 h and then analyzed by confocal microscope (NiKon, Japan).

**Immunofluorescence Staining Analysis**

Cells were washed thrice with PBS, fixed with 4% paraformaldehyde for 30 min, permeabilized using 0.05% Triton X-100 for 2 min, and blocked with 5% goat serum for 1 h. Subsequently, the cells were incubated overnight at 4 °C using specific antibodies against CD86 (1:200), and CD206 (1:200). Furthermore, the secondary antibodies included donkey anti-mouse Alexa Flou 488 (1:400) and Goat
anti-rabbit Alexa Fluor 594 (1:400). The cytoskeleton was stained with the ghost pen cycloppeptide, and cell nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI).

**Bmmscs Irradiation And Treatment**

BMMSCs at 70–90% confluence were irradiated by X-rays using an RS2000 X-ray Biological Irradiator (RAD SOURCE, USA) at a voltage of 160 kV and a current of 25 mA. The radiation dose rate was 1.20 Gy/min, and the radiation dose was 2, 6, and 10 Gy, respectively. After 1 d, 3 d, 5 d, and 7 d of radiation, CCK-8 assay was used to detect cell proliferation of irradiated BMMSCs. Briefly, BMMSCs with a density of 5x10³ cells/well were added to 96-well plates, and incubated with 10 µL of CCK-8 solution at 37 °C for 1 h. The OD value was measured at 450 nm using a spectrophotometer. The Nanog and octamer-binding transcription factor 4 (OCT-4) mRNA expression of irradiated BMMSCs was detected by RT-qPCR at 48 h after radiation. The most proper radiation dose was chosen according to the CCK-8 assay and the RT-qPCR results.

To evaluate the effect of macrophage CM on irradiated BMMSCs, the cultured BMMSCs were divided into blank group (BMMSCs without any treatment), irradiation group (Irradiated BMMSCs), CM-M1 group (Irradiated BMMSCs + CM-M1 treatment) and CM-M2 group (Irradiated BMMSCs + CM-M2 treatment). After treatment for 48 h, the expression of α-SMA and Col of BMMSCs in all groups were detected by western blot and RT-qPCR. After treatment and osteogenesis induction for 7 d, ALP staining and ALP activity detection were performed in all groups, and the expression of Runx-2, ALP and Col were detected by western blot and RT-qPCR. After treatment and osteogenesis induction for 21 d, alizarin red staining was performed in all groups. Notebly, the CM was mixed 1:1 with osteogenesis-induction medium.

To investigate the effect of M1 macrophage-derived exosomes (M1D-exos) and M2 macrophage-derived exosomes (M2D-exos) on irradiated BMMSCs, the cultured BMMSCs were divided into blank group (BMMSCs without any treatment), irradiation group (Irradiated BMMSCs), M1-exos group (Irradiated BMMSCs + M1D-exos) and M2D-exos group (Irradiated BMMSCs + M2D-exos). For exosomes treatment, 2 µg exosomes were added to 1 × 10⁵ irradiated BMMSCs[22]. The assessment methods were similar to the CM treatment mentioned above.

**Cell Transfection**

M2 macrophages were tansfected with 100 nM inhibitor-142-3p (M2 miR-142-3p inhibitor) and inhibitor-NC (M2 NC inhibitor) by Lipofectamine 3000 Kit according to the manufactures’ instructions. The exosomes extracted from the transfected M2 macrophages were termed as M2D-exos NC inhibitor and M2D-exos miR-142-3p inhibitor. To verify the effects of miR-142-3p in M2D-exos on irradiated BMMSCs, the cultured BMMSCs were divided into IR group (Irradiated BMMSCs), IR + M2-exos group (Irradiated...
BMMSCs + M2D-exos), IR + M2D-exos NC inhibitor group (Irradiated BMMSCs + M2D-exos NC inhibitor) and IR + M2D-exos miR-142-3p inhibitor group (Irradiated BMMSCs + M2D-exos miR-142-3p inhibitor).

**Rt-qpcr**

For mRNA detection, TRIzol Reagent was used for total RNA isolation of cells, and PrimeScript TM RT Master Mix kit was used for cDNA synthesis. For miRNA detection, Universal miRNA extraction Kit was used to extract miRNAs of cells and exosomes, followed by cDNA synthesis using GoldenstarTM RT6 cDNA Synthesis Kit, based on the manufacture's instructions. RT-qPCR was conducted using the TB Green Premix Ex Taq Kit. PCR reactions were performed by Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). All results were calculated by 2 (−ΔΔCT), and normalised to GAPDH or U6. All primer sequences were listed in the Supplementary Table S1.

**Western Blot**

The total protein of cells was extracted using the RIPA buffer containing 1% protease inhibitor. Furthermore, 20 µg protein was separated via 4–20% SDS-PAGE at 160 V for 40 min, and transferred to a polyvinylidenedifluoride (PVDF) membranes at a current of 400 mA for 30 min. The membranes were blocked with 5% milk, and then incubated with primary antibodies for GAPDH (1:5000), β-actin (1:1000), COL (1:1000), α-SMA (1:1000), TGF-β1 (1:1000), ALP (1:1000), Runx-2 (1:500), COL (1:1000), TSG101 (1:500) and CD81 (1:500), at 4 ℃ for 18 h. Thereafter, the membranes were washed three times in TBST, followed by incubation with HRP-conjugated species-matched secondary antibodies at room temperature for 1 h. Finally, blots were detected using a chemiluminescence imaging system (Bio-Rad, USA). The expression level of target proteins was normalized to GAPDH or β-actin.

**Statistical Analyses**

All experiments were performed at least thrice, the number of experimental replicates is denoted by n. Results were analyzed using the GraphPad Prism 8.0.2 software (GraphPad Software Inc.) and were presented as the mean ± standard deviation. The differences between two groups and multiple groups were analyzed by the t-test and one-way analysis of variance (ANOVA). A value of p < 0.05 was considered statistically significant.

**Results**

**Identification of BMMSCs and BMDMs**

The flow cytometry results showed that BMMSCs were positive for surface markers including CD29 (99.5%), CD44 (98.9%) and CD90 (98.4%), and negative for CD34 (1%), CD31 (0.8%) and CD45 (0.9%) (Supplementary information, Fig. S1A). In addition, alizarin red staining and oil red O staining after
Osteogenesis and lipogenesis induction of BMMSCs showed significant red mineralized nodules (Fig. S1B) and red lipid droplets (Fig. S1C), and the cartilage pellet was stained clear blue by alcian blue solution (Fig. S1D). These results confirmed that the cultured cells were BMMSCs.

The flow cytometry results showed that the positive rate of cell surface markers in BMDM, was 82.2% (F4/80) and 90.1% (CD11b) (Fig. S1E). The positive rate of CD86 was significantly increased after 24 h of treatment with LPS and IFN-γ (Fig. S1F). The positive rate of CD206 was also significantly increased after 24 h treatment of IL-4(Fig. S1G). RT-qPCR results showed that TNF-α and IL-1β were significantly increased after LPS and IFN-γ treatment, while Arg-1 and CD206 were significantly increased after IL-4 treatment (Fig. S1H). These results showed that M1 and M2 macrophages were successfully cultured and polarized.

Irradiation Induced Differentiation Imbalance Of Bmmscs

In order to select the appropriate radiation dose, BMMSCs were irradiated with different radiation doses (0, 2, 6, 10Gy). The CCK-8 results showed that the proliferation rate of irradiated BMMSCs with 6 and 10 Gy radiation doses was significantly lower than that of 0 Gy (Fig. 1A). However, BMMSCs showed negative growth after 5 days of irradiation at 10 Gy (Fig. 1A). The RT-qPCR results showed that the expression levels of stemness maintenance markers, OCT-4 and Nanog, were decreased with increasing doses of radiation (Fig. 1B-C). Based on these results, 6 Gy radiation dose was chosen in follow-up experiment, because this dose achieved a certain degree of radiation damage to BMMSCs without completely destroying cell proliferation and stemness.

Alpha-smooth muscle actin (α-SMA) is a marker protein for myofibroblasts, and COL III is a common marker of fibrosis. The mRNA and protein expression levels of α-SMA and COL III in BMMSCs were significantly increased after irradiation (Fig. 1D-F). The mRNA and protein expression levels of Runx-2, ALP and COL I in BMMSCs were significantly decreased after irradiation. The results confirmed that an irradiation dose of 6 Gy inhibited the osteogenic differentiation of BMMSCs (Fig. 1G-I), which was also confirmed by ALP staining, ALP activity and alizarin red staining (Fig. 1J-M). These results were in accordance with the pathological phenomenon that radiation-induced necrotic bones were surrounded with myofibroblasts and fibrotic matrix. In a word, irradiation caused a differentiation imbalance of BMMSCs, with increased fibrogenic differentiation and decreased osteogenic differentiation.

Cm-m1 And Cm-m2 Couldn’t Reverse The Differentiation Imbalance Of Irradiated Bmmscs

The effects of CM-M1 and CM-M2 on irradiation-induced differentiation imbalance of BMMSCs were investigated. CM-M1 treatment significantly reduced the expression of α-SMA and COL in irradiated BMMSCs, whereas CM-M2 treatment significantly promoted the expression of α-SMA and COL in irradiated BMMSCs (Fig. 2A-C). Western blot and RT-qPCR results showed that the expression levels of
osteogenesis-related genes in irradiated BMMSCs, including COL I, RUNX2, and ALP, were significantly increased after CM-M2 treatment (Fig. 2D-G). The CM-M1 treatment had no significant effect on the expression levels of these osteogenesis-related genes. Furthermore, compared with irradiation group, the ALP activity of the CM-M2 group was significantly increased (Fig. 2H-I), and the formation and staining degree of calcium nodules were also increased in the CM-M2 treatment group (Fig. 2J-K). These results indicated that CM-M2 treatment promoted the osteogenic differentiation and fibrogenic differentiation capacity of irradiated BMMSCs, whereas CM-M1 treatment inhibited the fibrogenic differentiation capacity without affecting the osteogenic differentiation capacity. In one word, both CM-M1 and CM-M2 couldn’t completely reverse the differentiation imbalance of irradiated BMMSCs.

Macrophage-derived Exosomes Were Internalized By Irradiated Bmmscs

TEM results showed that the extracted M1D-exos and M2D-exos had a cup or ball shape (Fig. 3A). The results of the NTA showed that the diameters of these extracted particles were ranged from 40 to 150 nm (Fig. 3B). Western blot further proved that the isolated exosomes were positive for specific surface protein markers, such as TSG101 and CD81 (Fig. 3C). To test whether the irradiated BMMSCs could internalize these exosomes, the PKH26-labeled exosomes (Red) were co-cultured with irradiated BMMSCs. The nucleus and cytoskeleton of irradiated BMMSCs were stained with DAPI (Blue) and ghostly cyclopeptide (Green). After 12 h, exosomes labeled with red fluorescence were mainly distributed in the cytoplasm surrounding the nucleus of irradiated BMMSCs under confocal microscopy, which indicated that irradiated BMMSCs exhibited efficient uptake of M1D-exos and M2D-exos (Fig. 3D).

M2d-exos Improved The Differentiation Imbalance Of Irradiated Bmmscs

The irradiated BMMSCs were co-cultured with M1D-exos and M2D-exos for 48 h to detect the effects of these exosomes on irradiated BMMSCs. Western blot and RT-qPCR results showed that both M1D-exos and M2D-exos significantly inhibited the expression of α-SMA and COL in irradiated BMMSCs (Fig. 4A-C). After 7 days of osteogenesis induction, the expression of COL I, Runx-2 and ALP were significantly increased in the M2D-exos group compared to the irradiation group (Fig. 4D-F). However, M1D-exos treatment had no obvious effect on the expression of these osteogenesis-related genes. ALP staining, ALP activity and alizarin red staining also showed the same trends as the expression of osteogenesis-related genes (Fig. 4G-J). These results confirmed that the M2D-exos inhibited the fibrogenic differentiation capacity and promoted the osteogenic differentiation capacity of irradiated BMMSCs, which successfully restored the differentiation function of the BMMSCs suffered from radiation damage.

M2d-exos Delivered Mir-142-3p Into Irradiated Bmmscs
The above experiments confirmed the role of macrophages and macrophage-derived exosomes in the differentiation imbalance of irradiated BMMSCs. Additionally, it was found that M2D-exos inhibited fibrogenic differentiation and alleviated radiation-induced osteogenic inhibition. A review of previous miRNAs revealed that four types of miRNAs were related to osteogenic differentiation and fibrosis, including miR-21-5p, miR-142-3p, miR-29a-5p and miR-22-3p. The expression levels of these four miRNAs in M1D-exos and M2D-exos were detected by RT-qPCR. The RT-qPCR results showed that miR-21-5p and miR-142-3p were significantly upregulated in M2D-exos compared to M1D-exos (Fig. 5A). After irradiated BMMSCs were cocultured with M2D-exos, RT-qPCR showed that the expression level of miR-142-3p was significantly increased (Fig. 5B). So miR-142-3p was further studied. RT-qPCR assays showed that the expression levels of miR-142-3p in M2 macrophages and M2D-exos were significantly decreased after M2 macrophages transfection with inhibitor-142-3p (Fig. 5C-D). Furthermore, the high expression of miR-142-3p in irradiated BMMSCs treated with M2D-exos could be effectively inhibited when M2 macrophages were transfected with inhibitor-142-3p (Fig. 5E). These results suggested that M2D-exo could deliver miR-142-3P into irradiated BMMSCs.

**M2d-exos Carrying Mir-142-3p Improved The Differentiation Imbalance Of Irradiated Bmmscs By Targeting Tgfβ-1**

To predict the potential target genes of miR-142-3p, we used Target Scan and MicroRNA (http://www.microrna.org) for analysis. The results showed that miR-142-3p directly targeted transforming growth factor-β1 (TGF-β1) (Fig. 6A). Western blot and RT-qPCR results showed that expression levels of TGF-β1, α-SMA, and COL III were significantly decreased in IR + M2D-exos group and IR + M2D-exos NC inhibitor group, compared with IR group (Fig. 6B-C). Furthermore, the expression levels of TGF-β1, α-SMA, and COL III were significantly increased in IR + M2D-exos miR-142-3p inhibitor group, compared with IR + M2D-exos group (Fig. 6B-C). The expression of osteogenesis-related genes and proteins, including Runx-2, ALP, and COL I, were significantly increased in IR + M2D-exos group and IR + M2D-exos NC inhibitor group, compared with IR group. Moreover, the expression of these osteogenesis-related genes and proteins were significantly decreased in IR + M2D-exos miR-142-3p inhibitor group, compared with IR + M2D-exos group (Fig. 6D-E). The ALP staining, ALP activity and alizarin red staining showed the same trends with the expression of osteogenesis-related genes and proteins (Fig. 6F-I). These results indicated that M2D-exos could transfer miR-142-3p to improve the differentiation imbalance of irradiated BMMSCs by downregulating TGF-β1 expression.

**Discussion**

Irradiation-induced bone damage is a common complication after radiotherapy for cancer treatment, and it has become an increasingly important clinical challenge with the prolonged survival of cancer patients. However, there is still a lack of effective strategies for the prevention and treatment of irradiation-induced bone damage, leading to a high risk of fragility bone fractures and osteonecrosis[23, 24]. BMMSCs were previously thought to be the main targets of irradiation, which caused the inhibition of bone formation.
Macrophages have been proven to reduce the radiosensitivity of cells[26]. However, the interaction between macrophages and irradiated BMMSCs remains unclear. In the current study, we investigated the effects of macrophages and macrophages-derived exosomes on irradiated BMMSCs, and the findings confirmed that M2D-exos could carry miR-142-3p to restore the differentiation balance of irradiated BMMSCs through promoting osteogenic differentiation and inhibiting fibrogenic differentiation via targeting TGF-β1. Our findings provided a novel therapeutic strategy for irradiation-induced bone damage and offered a reference for further studies.

BMMSCs are self-renewal and multi-directional differentiation pluripotent stem cells, which are widely distributed in bone marrow and have the ability to regulate bone metabolism and promote bone regeneration[27, 28]. As main stem cells with active proliferative capacity in bone tissues, BMMSCs were sensitive to irradiation and the inhibition of osteoblast differentiation of irradiated BMMSCs was thought to be the key elements of irradiation-induced bone damage, including irradiation-induced bone loss, bone fracture and osteonecrosis [29, 30]. Except for this, BMMSCs were usually used as stem cell therapy in many diseases. Based on the regenerative and anti-inflammatory functions, BMMSCs and mesenchymal stem cell (MSCs) from other origins were previously proved to have positive effects on the prevention and treatment of irradiation-induced diseases, such as irradiated pneumonia, irradiated pulmonary fibrosis, irradiated liver injury and so on[31–33]. Similarly, BMMSCs were also used to treat fibrotic diseases, such as idiopathic pulmonary fibrosis, non-alcoholic fatty liver fibrosis, skin fibrosis and so on, due to the immunomodulatory function of BMMSCs to the recipient cells[34–36]. However, in recent years, researchers found that BMMSCs could differentiate into myofibroblasts which is the key effector cell in fibrosis, in particular microenvironment including irradiation injury[37, 38]. This fibrogenic differentiation phenomenon makes MSCs a potential danger to aggravate fibrosis stimulated by irradiation or other factors[39]. In this study, we found that X-ray irradiation suppressed the proliferation of BMMSCs in a dose-dependent manner. Furthermore, at a dose of 6 Gy, irradiation significantly inhibited the osteogenic differentiation and mineralization, while significantly promoted the fibrogenic differentiation with high expression levels of COL  and α-SMA mRNA and protein after irradiation. These findings of differentiation imbalance in irradiated BMMSCs in our study confirmed the concerns of fibrotic stimulation potential of BMMSCs in irradiated microenvironment, which were proposed by other researchers in previous studies. Therefore, it is essential to restore the differentiation balance in BMMSCs after irradiation to treat irradiation-induced bone damage, other irradiation-induced diseases or fibrosis.

Macrophages play a key role in the immune system and have been widely used to regulate inflammatory responses and promote the repair of tissue damage. Generally, M1 macrophages help to clean wounds and play an inflammatory role. In contrast, M2 macrophages are anti-inflammatory and promote tissue repair. In the past few years, several studies have confirmed the regulatory role of macrophages in MSCs[40]. In the other way, studies also found that BMMSCs had a significant impact on macrophage polarization in the process of in vitro culture. To avoid the influence of this interaction between BMMSCs and macrophages, M1 and M2 macrophages conditioned medium (CM-M1, CM-M2) were used in most previous studies to investigate effects of macrophages polarization on the in vitro cell behavior of BMMSCs. In CM-M1 and CM-M2, cytokines secreted by macrophages are major contributor to regulate
function of BMMSCs. The effects of macrophages on irradiated BMMSCs haven't been studied further, while the effects of macrophages on normal BMMSCs have been studied a lot. Previous studies have shown that M2 macrophages can promote osteogenic differentiation of BMMSCs. It has also been studied that M2 macrophages promoted the differentiation of myofibroblasts through secreting TGF-β1. Similarly, the current study found that CM-M2 significantly promoted the osteogenic differentiation and fibrogenic differentiation of irradiated BMMSCs. And for M1 macrophages, many studies showed that it could inhibit osteogenesis and osteogenic differentiation of BMMSCs, and it could reduce fibrotic accumulation[41, 42]. In our study, we found that CM-M1 had no significant effects on the osteogenic differentiation of irradiated BMMSCs, but it could inhibit fibrogenic differentiation of irradiated BMMSCs. So, neither CM-M1 nor CM-M2 could reverse the differentiation imbalance of irradiated BMMSCs, other effective methods should be searched further.

Exosomes are small vesicles secreted by a range of cells and acted as important intercellular and inter-organ communication tools through transferring small molecules including proteins and nucleic acids. Therefore, it is popular to use exosomes as ideal nanomaterials for delivering the regulatory substances to the targets to treat diseases in recent years[43]. In addition, exosomes derived from macrophages are key regulatory factors to adjacent cells, including BMMSCs[44]. Therefore, macrophage-derived exosomes should be considered to better explain the mechanism of crosstalk between macrophages and BMMSCs. According to previous studies, M1D-exos could inhibit osteogenesis [45], while we found that M1D-exos had no effects on the osteogenic differentiation of irradiated BMMSCs. And M1D-exos previously have been proved to be effective anti-fibrotic therapy [46]. Consistently, the current study showed that M1D-exos significantly reduced the differentiation of BMMSCs into myofibroblasts after irradiation. These findings indicated that M1D-exos could not completely restored the differentiation balance of irradiated BMMSCs, as M1D-exos had positive effects on inhibiting fibrogenic differentiation, while had negative effects on promoting osteogenic differentiation. M2 macrophages play a key role in tissue damage repair. M2D-exos were used to be effective bone regeneration and bone repair tools through promoting osteogenic differentiation of BMMSCs, proliferation of osteoblasts and osteocytes and bone mineralization [45]. Our study found that M2D-exos could significantly promote osteogenesis of irradiated BMMSCs, which indicated that M2D-exos could also act as effective bone regenerative tools in irradiation microenvironment. However, the role of M2D-exos in fibrosis was ambiguous and even contradictory in previous studies. Some studies showed that M2D-exos stimulated fibrogenic differentiation of fibroblasts, stem cells and endothelial cells into myofibroblasts, promoted myofibroblasts proliferation and secretion, and accelerated extracellular matrix accumulation, finally leading to tissue fibrosis[47–49]. On the contrary, some studies found that M2D-exos acting as immunomodulatory factors, could migrate inflammation, reduce the pro-inflammatory factors, and restore the normal function of recipient cells, finally promoting tissue repair in a regular way but not a fibrotic way[50]. These different roles of M2D-exos in fibrosis may be related to different pro-fibrotic irritants, different organs and tissues, different microenvironment and different action timing. Our study found that M2D-exos could significantly inhibit the differentiation of BMMSCs into myofibroblasts after irradiation, which indicated that M2D-exos may play an antifibrotic role in irradiation microenvironment.
The findings in our study showed that M2D-exos successfully restored the differentiation function of irradiated BMMSCs with promoting osteogenic differentiation and inhibiting fibrogenic differentiation, which provided a cell-free and less harmful way to treat irradiation-induced bone damage. Nevertheless, we only performed in-vitro study, the role of M2D-exos in irradiation-induced fibrosis and irradiation-induced bone damage needed to be studied further in vivo.

Among the components of macrophage-derived exosomes, miRNA is the most important factor. miRNA regulates target cell-related genes by combining with mRNA in target cells. The results of this study found that miR-142-3p was abundant in M2D-exos and could be transferred into irradiated BMMSCs. And we also found that inhibition of miR-142-3p could inhibit the effects of M2D-exos on irradiated BMMSCs, which indicated that miR-142-3p played a key role in M2D-exos restoring irradiated BMMSCs function. In previous studies, miR-142-3p was proved to have effective anti-fibrotic role in hypoxia/reoxygenation-induced cardiac fibrosis, liver fibrosis, idiopathic pulmonary fibrosis, nonalcoholic fatty liver disease and skin scars, through inhibiting myofibroblasts differentiation and proliferation, suppressing TGF-β1 expression and so on [51–54]. Previous studies in bone diseases found that miR-142-3p could inhibit osteoclastogenesis, promote osteoblast activity and matrix mineralization in bone healing process, and induce osteogenic differentiation of BMMSCs, which indicated that miR-142-3p had a positive role in bone regeneration and bone repair[55–57]. Although the results of miR-142-3p inhibiting fibrogenic differentiation and promoting osteogenic differentiation of irradiated BMMSCs in our study were in consistent with previous cognition of miR-142-3p effects, we believe that mi-RNA in M2D-exos were more abundant than miR-142-3p. Therefore, further in-vivo and in-vitro studies are needed to determine the specific mechanism of M2D-exos in preventing and treating irradiation-induced bone damage.

Conclusion

In conclusion, this study indicated that M2 macrophage-derived exosomes (M2D-exos) restored the differentiation balance of irradiated BMMSCs by promoting osteogenic differentiation and inhibiting fibroblastic differentiation. This positive effects of M2D-exos were mediated partially by delivering miR-142-3p into irradiated BMMSCs and suppressing the expression of TGF-β1 (Fig. 7). Our findings provide new insight into the prevention and treatment of irradiation-induced bone damage.

Declarations

Statements & Declarations

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Conflict of interest The authors have no relevant financial or non-financial interests to disclose in connection with this manuscript.
**Author contributions** All authors contributed to the general study conception and design. The material preparation and data collection were performed by Chong Huang, Lu Zhao, Yun Xiao, Zihao Tang, Li Jing and Kai Guo. The data analysis and visualization were performed by Chong Huang, Lu Zhao, and Chunlin Zong. The first draft of the manuscript was written by Chong Huang and Lu Zhao. The manuscript was revised by Lei Tian and Chunlin Zong. All authors commented on previous versions of the manuscript, gave valuable suggestions for corrections, and approved the final manuscript. Lei Tian and Chunlin Zong supervised the research project.

**Data availability** The data that support the findings of this study are available from the corresponding author, LT, as well as CZ, upon reasonable request.

**Ethical approval** All animal research protocols in this study were approved by the Welfare and Ethics Committee of Laboratory Animal Center of the Fourth Military Medical University (approval number: IACUC-20210971)

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Figures
Figure 1

Irradiation induced differentiation imbalance of BMMSCs. A, Proliferation of BMMSCs irradiated at different doses was detected by CCK-8 assay; B-C, mRNA levels of OCT-4 and Nanog in BMMSCs irradiated at different doses were detected by RT-PCR; D-F, The expression levels of COL I and α-SMA were detected by Western blot and RT-qPCR; G-I, The expression levels of COL I, Runx-2 and ALP were analyzed by Western blot and RT-qPCR; J-K, ALP staining and ALP activity of irradiated BMMSCs after 7 days of
osteogenesis induction (Scale bar = 200 μm); L-M, Alizarin red staining and quantitative results of irradiated BMMSCs after 21 days of osteogenesis induction (Scale bar = 200 μm). Data was represented as mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, n=3.
Effects of the macrophage-CM on irradiated BMMSCs. A-C, The expression levels of COLIII and α-SMA in irradiated BMMSCs cultured with CM-M1 or CM-M2 for 48 h; D-F, The expression levels of COL I, Runx-2 and ALP in irradiated BMMSCs cultured with CM-M1 or CM-M2 after osteogenesis induction for 7 days; G-H, ALP staining and ALP activity detection in irradiated BMMSCs cultured with CM-M1 or CM-M2 after osteogenesis induction for 7 days (Scale bar = 200 μm); I-J, Alizarin red staining and quantification in irradiated BMMSCs cultured with CM-M1 or CM-M2 after osteogenesis induction for 21 days (Scale bar = 200 μm). Data was represented as the mean ± SD; * p < 0.05, ** p < 0.01, *** p<0.001, n=3.
Figure 3

The identification and internalization of M1D-exos and M2D-exos. A, Images of M1D-exos and M2D-exos under TEM (Scale bar = 100 nm); B, Size distribution of M1D-exos and M2D-exos by NTA analysis; C, Western blot detection of TSG 101 and CD81 in M1D-exos and M2D-exos; D, Irradiated BMMSCs internalization of PKH-26-labeled exosomes under confocal microscopy (Scale bar = 40 um).
Figure 4

M2D-exos improved the differentiation imbalance of irradiated BMMSCs. A-B, The expression levels of COL I and α-SMA proteins were detected by western blot; C, The expression levels of COL I and α-SMA mRNA were detected by RT-qPCR; D-E, The expression levels of COL I, ALP and Runx-2 proteins were detected by western blot; F, The expression levels of COL I, ALP and Runx-2 mRNA were detected by RT-qPCR; G-H, ALP staining and ALP activity; I-J, Alizarin red staining and quantification. Data was represented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, n=3.
Figure 5

M2D-exos delivered miR-142-3P into irradiated BMMSCs. A, Expression levels of miR-21-5p, miR-142-3p, miR-29a-5p and miR-22-3p in M1D-exos and M2D-exos were detected by RT-PCR; B, Expression levels of miR-142-3p and miR-21-5P in irradiated BMMSCs co-cultured with M2D-exos; C, The expression levels of miR-142-3p in M2 macrophages, M2 NC inhibitor and M2 miR-142-3p inhibitor; D, The expression levels of miR-142-3p in M2D-exos, M2D-exos NC inhibitor and M2D-exos miR-142-3p inhibitor; E, The expression
levels of miR-142-3p in BMMSCs (Blank group), irradiated BMMSCs (IR group), IR+M2D-exos NC inhibitor group and IR+M2D-exos miR-142-3p inhibitor group. Data was represented as the mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001, n=3.

Figure 6
M2D-exos carrying miR-142-3P improved the differentiation imbalance of irradiated BMMSCs by targeting TGFβ-1. A, Complementation relationship between miR-142-3p and TGF-β1 base sequence; B-C, The expression levels of TGF-β1, α-SMA, and COL III were detected by Western blot and RT-qPCR in IR group, IR+M2D-exos group, IR+M2D-exos NC inhibitor group and IR+M2D-exos miR-142-3p inhibitor group; D-E, The expression levels of TGF-β1, Col I, ALP and Runx-2 were detected by Western blot and RT-qPCR in IR group, IR+M2D-exos group, IR+M2D-exos NC inhibitor group and IR+M2D-exos miR-142-3p inhibitor group; F-I, ALP staining (Scale bar = 500 μm), ALP activity and alizarin red staining(Scale bar = 200 μm) in IR group, IR+M2D-exos group, IR+M2D-exos NC inhibitor group and IR+M2D-exos miR-142-3p inhibitor group. Data was represented as the mean ± SD. * p < 0.05, ** p < 0.01; *** p<0.001, n=3.
Figure 7

Schematic diagram showing M2 macrophage-derived exosomes carrying miR-142-3p improved the differentiation imbalance of irradiated BMMSCs, through promoting osteogenic differentiation and inhibiting fibrogenic differentiation by targeting TGF-β1.

Supplementary Files
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- Table1.pdf
- FigS1.pptx