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M1 macrophage-derived exosomes moderate the differentiation of bone marrow mesenchymal stem cells

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Abstract: Differentiated macrophages have been proven to participate in the development of mesenchymal stem cells in different tissues. However, the regulatory processes remain obscure. Exosomes, which are key secretions of macrophages, have attracted increasing attention. Therefore, macrophage-derived exosomes may modulate the development of Bone marrow mesenchymal stem cells (BMMSCs). Different culture conditions were used to induce M1 polarization in THP1 cells. Subsequently, exosomes derived from unpolarized (M0) and polarized (M1) macrophages were isolated, BMMSCs were cultured with normal complete medium or inductive medium supplemented with M0 or M1 derived exosomes, and the osteogenic capacity of the BMMSCs was measured and analyzed. Finally, molecular mechanism associated with Akt and RUNX2 was investigated. Alizarin red staining and WB experiments showed that M1 macrophages could promote the osteogenic differentiation of BMMSCs better than M0 macrophages. Then, exosomes derived from M0 and M1 macrophages were successfully isolated and analyzed by electron microscopy and WB experiments. We concluded that media containing M1-derived exosomes promoted the osteogenic differentiation of BMMSCs better than media containing M0-derived exosomes. In addition, M1-derived exosomes could activate Akt and increase RUNX2 levels to promote osteogenesis. Our data demonstrated that exosomes derived from M1 macrophages induced osteogenesis by activating Akt and increasing RUNX2 level.

Introduction

During the previous time, stem cell therapy has become the focus of regenerative medicine and tissue engineering (Wei *et al.*, 2013). BMMSCs, due to their advantages, stand out from a variety of stem cells and are the most promising option for allogeneic and autologous transplantation (Hu *et al.*, 2018; Ma *et al.*, 2018). However, the clinical application of BMMSCs is associated with is associated with many challenges, such as low dose, inefficiency and poor survival rates (Regmi *et al.*, 2019). Considerable efforts have improved the regeneration of mesenchymal stem cells (Fahy *et al.*, 2014). At present, the importance in regulation of macrophages in the BMMSCs has been widely recognized (Pajarinen *et al.*, 2019; Zhang *et al.*, 2017b).

Macrophages, as important components of the innate immune system, were recently discovered to participate in tissue regeneration (Yu *et al.*, 2016). Under various stimuli, macrophages can change from an unpolarized (M0) to a differentiated state (M1 or M2) and play significant roles in many stages of tissue healing. M1 macrophages generally participate in wound inflammation and play a proinflammatory role. M2 macrophages, on the other hand, have anti-inflammatory functions and induce tissue healing (Mcdonald *et al.*, 2014; Zhang *et al.*, 2017b). In the past, numerous reports have demonstrated the regulatory effects of macrophages on mesenchymal stem cells (Maxson *et al.*, 2012; Pajarinen *et al.*, 2019; Sesia *et al.*, 2015; Zhang *et al.*, 2017a).

Exosomes are single-membrane vesicles that have the same topology as the cell, and diameters of ~30 to ~200 nm, and are important regulators of critical physiological and pathological processes (Kalluri and Lebleu, 2020; Lee *et al.*, 2019). Exosomes take up nucleic acids, lipids, proteins and other substances, which can be released into the extracellular environment and internalized by target cells, subsequently regulating cellular physiological and

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pathological processes (Zhang *et al.*, 2019). In fact, there is increasing evidence suggesting that exosomes released from macrophages are important regulators of many biological activities (Li *et al.*, 2019; Poltavtseva *et al.*, 2019; Wei *et al.*, 2019). However, the underlying mechanism of macrophage-derived exosomes with different phenotypes in regulating BMMSCs is still unclear.

RUNX2 is an important transcriptional regulator of osteogenesis (Chava *et al.*, 2018). RUNX2 deficient mice fail to develop bone cells (Thacker *et al.*, 2016). The differentiation experiment demonstrates that RUNX2 is an important transcription factor in bone development. RUNX2 has important regulatory effects on the differentiation of osteoblasts, the maturation of chondrocytes, the differentiation of osteoclasts, and the secretion of extracellular matrix (Chava *et al.*, 2018; Kim *et al.*, 2018).

RUNX2 is primarily used for mesenchymal cell commitment to osteoblastic cell lines, which are necessary for the development of immature osteoblasts but not for the further maturation of osteoclasts (Omar *et al.*, 2011). This finding indicates that RUNX2 is essential for osteogenesis. However, this difference is mainly due to differences in the cellular environment, as glucose appears to induce bone maturation by regulating the synthesis of Type I collagen (Ikebuchi *et al.*, 2018; Omar *et al.*, 2011). This finding suggests that specific regulatory signals are requires for the different commitment processes.

Based on the published works, we hypothesized that exosomes were the key mediators of macrophage on BMMSCs. We also further elucidate the role of exosomes from type macrophages in the differentiation of BMMSCs. The results are prospected to improve understanding of interaction between macrophages and BMMSCs and help to better regulate the effectiveness of mesenchymal stem cells in tissue regeneration.

Materials and Methods

Isolation and culture of BMMSCs

Approximately female C57BL/6 mice aged 5 weeks were purchased from the Experimental Animal Center of the Shenzhen University and were sacrificed and the femurs and tibias dissected. After two washes with PBS, bone marrow cells were flushed from the bones into 10-cm-culture dishes using MEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin and streptomycin (Sigma Aldrich). Then, the dishes were incubated at 37°C, 5% CO_2 incubator. Cells from the 2nd or 3rd passage were used in the analysis.

Characterization of BMMSCs and macrophages by flow cytometry

To analyze the expression of surface markers characteristic of BMMSCs or and macrophages, FACS was performed using specific fluorochrome-conjugated monoclonal antibodies corresponding to each cell type. Briefly, 1 × 106 cells were washed with 10% FBS/PBS and centrifuged at 300 × g for 5 min at room temperature to pellet the cells. Subsequently, primary antibodies purchased from Abcam, anti-CD90 (ab225), anti-CD29 (ab183666), anti-CD34 (ab81289), anti-CD45 (ab10558), anti-CD63 (ab213090), and anti-iNOS (ab115819) at a concentration of 2 mg/mL for the identification of BMMSCs or macrophages. Subsequently, unbound antibody was removed by washing with 2 mL of 10% FBS/PBS, and pellets were resuspended in 500 μL PBS and examined by flow cytometry, with 10,000 events recorded for each condition. Flow cytometry data was analyzed using BD CellQuest™ Pro software Version 5.1 (BD Bioscience).

THP-1 cell culture, macrophage differentiation and polarization

Human leukemia monocytic THP-1 cells were purchased from Procell (Cat. CL-0233, Wuhan, China). THP-1 cells were maintained in RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamine. THP-1 monocytes (Mo) were differentiated into resting macrophages (M0) using 100 nM phorbol 12-myristate 13-acetate (PMA, Solarbio, P6741) for 72 h followed by 24 h in PMA-free medium (PMA-resting, PMAr). For M1 polarization M0 macrophages were further cultured in M1-polarization medium containing 100 ng/mL LPS (Sigma-Aldrich) and 20 ng/mL interferon gamma (IFNγ, Solarbio, P00041) for 24 h.

Oil Red O staining

The BMMSCs induction for 18 days, cells were washed once with PBS, fixed by treatment with 4% paraformaldehyde for 30 min at room temperature, and then stained with Oil Red O solution (Jiancheng Biotechnology, Nanjing, China) for 60 min at room temperature. A microscope was used to observe the stained cells.

ALP staining

BMMSCs were seeded into 24-well plates at a density 1 × 104 cells/cm^2 until 70% confluence was reached. Then, the old complete medium was removed, and fresh medium containing 0.1 μmol/L dexamethasone (Cat# RASMX-90021, Oricell, Cyagen Biosciences) was added to the plates. All groups were cultured for 3 days prior to ALP staining. The cells were washed thrice with PBS and fixed by treatment with 4% Paraformaldehyde Fix Solution (Cat# E672002, Sangon Biotech, Shanghai, China) for 1 min. Then, the cells were incubated with ALP for 20 min at 25°C in the dark and observed under a light microscope.

Isolation and identification of exosomes

After the cells reached 80% confluence, serum-free culture medium was added, and the supernatants were collected after culturing for 24 h. Then, the exosomes were isolated from the supernatants through traditional ultracentrifugation: 2,000 × g for 30 min to remove the cells and debris, centrifugation at 10,000 × g for 30 min to remove the subcellular components, and centrifugation at 100,000 × g for 70 min to obtain the exosomes. Finally, the exosomes were resuspended in 0.01M PBS, centrifuged at 100,000 × g for 70 min for purification, and preserved in a freezer at –80°C. A Zeta View system was used to measure the exosome concentration and size distribution, and transmission electron microscopy was used to detect exosome morphology. The exosome surface markers were analyzed by Western blotting.

Exosome treatment

Exosomes were isolated from 5 × 106 M0 and M1, BMMSCs were planted into 6-well plates one day before treatment. When the cells grew at about 70% of confluent, 200 μg of exosomes were directly added into cells. Two days after treatment, cells were collected for the following experiments.

RNA extraction and quantitative real-time PCR

Total cellular RNA was isolated using a single-step method with TRIzol (Invitrogen) according to the manufacturer's instructions. First-strand complementary cDNA was synthesized from 1 μg of total RNA according to the manufacturer's instructions. The reaction was terminated by heating at 70°C for 5 min. The single-strand cDNA was quantified by spectrophotometer so as to use 10 ng of cDNA in each Real-Time PCR well.

Quantitative real-time PCR was performed using the Applied Biosystems StepOne™ Real-Time PCR System and the Fast SYBR® Green Master Mix reagent. The quantification of gene expression for each target gene and reference gene was performed in separate tubes. The relative expression level of the target gene was normalized to that of the endogenous reference GAPDH gene and the 2−ΔΔCt cycle threshold method was used to calculate the relative expression levels of the target genes defined by the primers.

ALP: Forward Primer 5' -ACCACCACGAGAGTGA-ACCA-3', Reverse Primer 5'-CGTTGTCTGAGTACCAG-TCCC-3'; Osterix: Forward Primer 5'-CCTCTGCGG GACTCAACAAC-3'Reverse Primer 5'-AGCCCATTAGTG-CTTGTAAAGG-3'; Runx2: Forward Primer 5'-TGCTTA-CTGTCATGGCGGGTA-3', Reverse Primer 5'-TCTCAG-ATCGTTGAACCTTGCTA-3'; AKT: Forward Primer 5'-AGCGACGT GGCTATTGTGAAG-3', Reverse Primer 5'-GCCATCATTCTTGAGGAGGAAGT-3', BMP2: Forward Primer 5'-ACCCGCTGTCTTCTAGCGT-3', Reverse Primer 5'- TTTCAGGCCGAACATGCTGAG-3'.

Immunoblotting analysis

The protein samples were isolated from BMMSCs by using lysis buffer (P0013G, Beyotime, Shanghai, China). Protein lysates were quantified using Bradford protein assay kit (ab102535, Abcam, Cambridge, UK), and then 20 μg protein was performed electrophoresis on SDS-PAGE (10%; Beyotime, Shanghai, China) and then transferred on PVDF membranes (Millipore, Billerica, MA, USA). Subsequently, the PVDF membranes were incubation with ALP (1:1000, ab229126, Abcam, Cambridge, UK), Osterix (1:1000, ab229258, Abcam, Cambridge, UK) and Runx2 (1:1000, ab76956, Abcam, Cambridge, UK), CD9 (1:500, ab263024, Abcam, Cambridge, UK), CD63 (1:500, ab134045, Abcam, Cambridge, UK), AKT (1:1000, ab8805, Abcam, Cambridge, UK), p-AKT (1:1000, ab8933, Abcam, Cambridge, UK), BMP2 (1:1000, ab14933, Abcam, Cambridge, UK) and GAPDH (1:5000, ab8245, Abcam, Cambridge, UK) at 4°C overnight. Then it was incubated with secondary antibody by using HRP goat anti-rabbit/mouse IgG antibodies (1:20000, ab8245, Abcam, Cambridge, UK). Enhanced chemiluminescence detection system (Millipore, Massachusetts, USA) was used for photographing of the protein peptides on the PVDF membranes.

Alizarin Red S staining

Cells were seeded into 24-well plates at a density of 1 × 104 cells/cm^2 overnight. The following day (Day 0), media were changed to DMEM, 10% FBS, 1% penicillin-streptomycin, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate (Control media) or DMEM, 10% FBS, 1% penicillin-streptomycin, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, 100 ng/mL BMP-2 (Osteogenic media). Media were changed every 72 h. After 27 days, cells were washed with PBS, fixed with 10% neutral buffered formalin for 30 min, stained with 2% Alizarin Red stain for 45 min, rinsed three times with distilled water, air-dried and imaged using an inverted microscope equipped with a color camera.

BMMSCs transmission electron microscopy

The exosomes were immediately fixed with 4% glutaraldehyde for 2 h at 4°C after isolation, washed 3 times with 0.1 mol/L PBS, fixed with 1% osmium tetroxide for 2 h, and dehydrated with conventional ethanol and acetone. Exosomes was impregnated with epoxy resin, embedded, and polymerized. After that, 0.5-μm-thick semi-thin sections were prepared and the ultra-thin sections of 60 nm were prepared after localization of the light microscope. The sections were stained with uranium dioxide acetate and lead citrate and visualized under an electron microscope.

Statistical analysis

All statistical analyses were performed with SPSS 19.0 (Chicago, IL) software or GraphPad Prism 8 software (San Diego, CA, USA). Results were repeated at least three times and are shown as mean ± SEM. We combined the quantitative data from three biological replicates and analyzed the results with Statistical significance between groups was determined by Student's *t*-test or one-way analysis of variance (ANOVA). $P < 0.05$ were considered statistically significant.

Results

Effects of macrophages on the differentiation of BMMSCs

To examine the effect of macrophages on osteogenic differentiation, we first induced THP1 cells to differentiate into functional macrophages. After treatment with different cytokines, cellular morphology was examined. THP1 cells treated with LPS and IFN-γ displayed higher cell numbers than the control and PMA treated groups, suggesting that the proliferation of macrophages was enhanced (Fig. 1A). In addition, flow cytometry was performed to analyze the cell surface markers on macrophages. Compared to control and PMA treated THP1 cells, LPS and IFN-γ treated macrophages showed significant increase in CD68 and iNOS (specific markers of M1 macrophages) (Fig. 1B). These results suggested that THP1 cells were successfully induced to undergo M1 polarization by LPS and IFN-γ.

Subsequently, to investigate the effect of differentiated macrophages, including the M0 and M1 types, on the osteogenic differentiation abilities of BMMSCs, BMMSCs were cocultured with different types of differentiated macrophages. After 14 days of culture, ALP activity analysis was performed to analyze the extent of osteogenesis as shown in Suppl. Fig. 1. Quantitative analysis revealed that M1 macrophages but not M0 macrophages increased the ALP activity of BMMSCs in

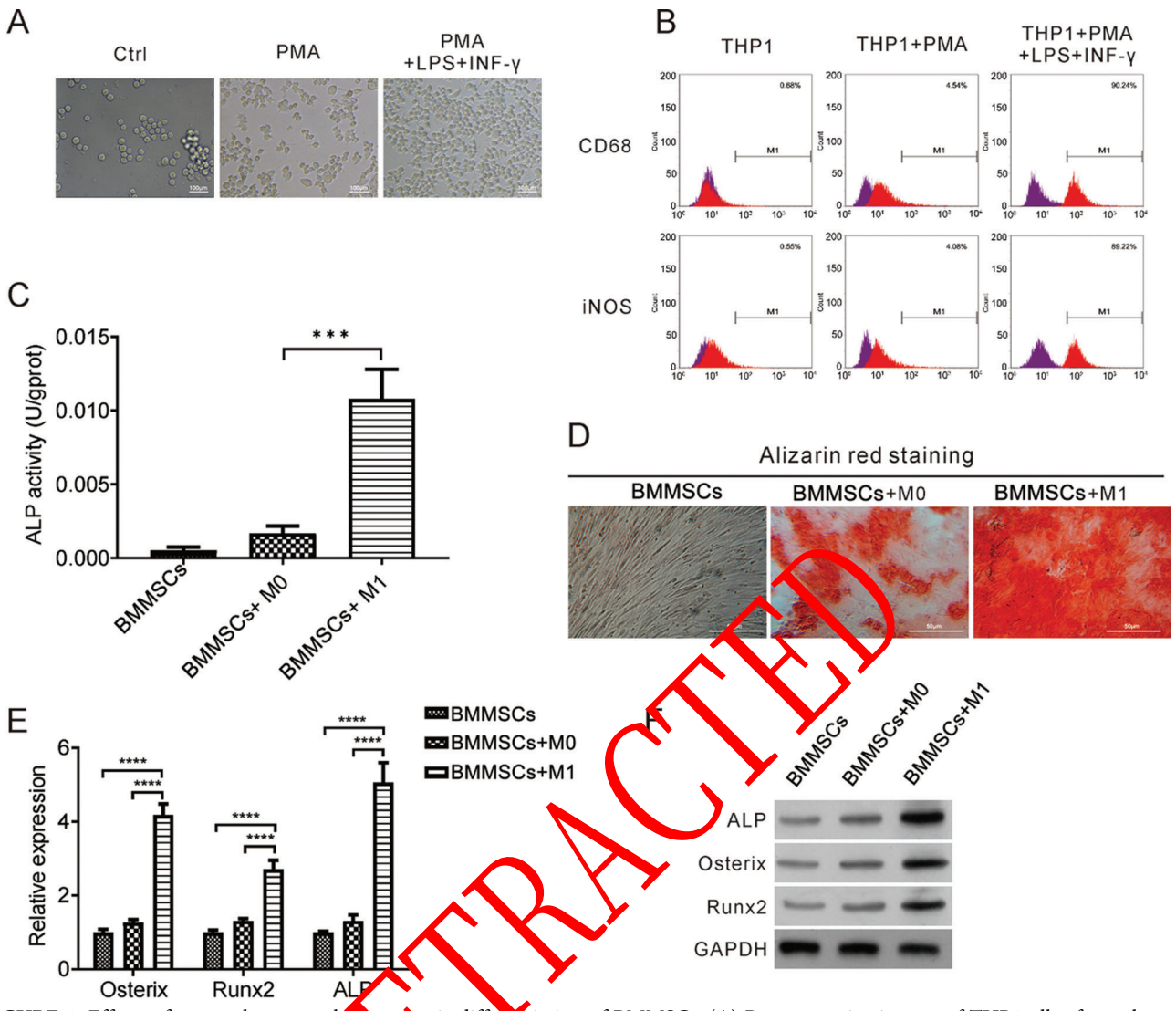

FIGURE 1. Effects of macrophages on the osteogenic differentiation of BMMSCs. (A) Representative images of THP1cells after culture in various conditions. (B–C) Flow cytometric and statistical analysis of CD68 and iNOS (M1 marker) in the indicated treated or unstimulated cells. (D) Alizarin red S staining of cells under different culture conditions. (E–F) Expression levels of osteogenesis-related genes (ALP, Osterix and Runx2) in BMMSCs. $^{**}P < 0.005$; $^{***}P < 0.001$ *vs.* BMMSCs; Ctrl, control; M0 unpolarized macrophages; M1, polarized macrophages; PMA, phorbol 12-myristate 13-acetate.

the coculture system. This result was further confirmed by Alizarin red S staining. The results showed that BMMSCs cultured with M1 macrophages formed the highest number of positively stained cells. In contrast, M0 macrophages showed little influence on the development of BMMSCs (Fig. 1D). In addition, the mRNA and protein levels of osteogenesis related genes were analyzed after cells were subjected to osteogenic induction for 7 days. The data showed that BMMSCs co-cultured with M1 macrophages had significantly increased mRNA and protein levels of ALP, Osterix and Runx2 (Figs. 1E and 1F). These results suggested that M1 macrophages could promote the osteogenesis of BMMSCs.

Characterization of different macrophage-derived exosomes

It has been reported that exosomes participate in the osteogenesis of BMMSCs (Ekstrom *et al.*, 2013; Wei *et al.*, 2019). Toinvestigate the underlying mechanisms of macrophages affect the osteogenesis of BMMSCs, we focused our attention on exosomes derived from macrophages. First, exosomes were isolated from M0 or M1 macrophages. Transmission electron microscopy (TEM) analysis showed that the isolated exosomes were small round nanometer sized particles with bilayer membranes (Fig. 2A). Moreover, markers of exosomes, such as CD9 and CD63, were all expressed on the M0 and M1-derived exosomes (Fig. 2B). These results ensured the successful isolation of exosomes from M0 and M1 macrophages.

Effects of macrophage-derived exosomes on the differentiation of the BMMSCs

To determine whether macrophage-derived exosomes affect the osteogenic differentiation of BMMSCs, an ALP activity assay was first conducted. The results revealed that ALP activity was dramatically increased by supplementation with M1-derived exosomes. However, M0 derived exosomes induced consistently low ALP activity (Fig. 3A). The effects of exosomes on BMMSC differentiation were also investigated by Alizarin red S staining. The results

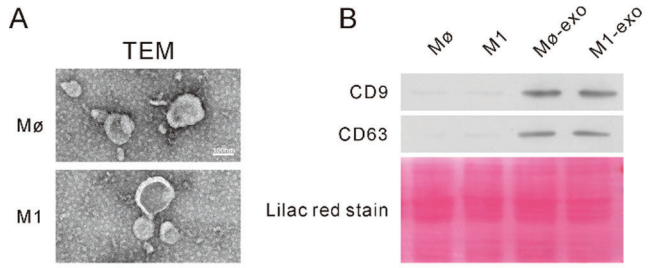

FIGURE 2. Characterization of different macrophage-derived exosomes (A) TEM analysis of M0 and M1-derived exosomes. TEM, Transmission Electron Microscope; M1, polarized macrophages; PMA, phorbol 12-myristate 13-acetate (B) The exosome marker (CD9 and CD63) in the M0 and M1-derived exosome.

showed that cells cultured with M1-derived exosomes had the highest number of positively stained cells, suggesting that these cells have the most robust differentiation potential. In contrast, cells cultured with M0-derived exosomes showed fewer positively stained cells, revealing low differentiation potential (Fig. 3B). In addition, the gene expression analysis of osteogenic differentiation-related genes, including Osterix, RUNX2 and ALP, further confirmed the difference in osteogenesis promotion in the M0 and M1 groups (Figs. 3C and 3D).

Osteogenesis is associated with M1-derived exosome-mediated activation of Akt and BMP2 in differentiation

Recent studies have revealed that the Akt signaling pathway plays a role in BMMSC differentiation. Moreover, since BMP2 has been confirmed to be an important substrate of Akt, we examined whether M1-derived exosomes participate in the Akt signaling pathway and regulate BMP2 during BMMSCs differentiation. To experimentally verify this hypothesis, we examined the phosphorylation of Akt and BMP2 expression levels during the differentiation of BMMSCs to examine downstream signaling pathways in the osteogenesis process. Our data showed that M1-derived exosomes activated Akt (phosphorylation level) as shown in Suppl. Fig. 2 and increased the expression of BMP2 (Figs. 4A and 4B). In conclusion, these findings suggest that M1 macrophage derived exosomes may promote the differentiation of BMMSCs through the Akt-BMP2 pathway.

Discussion

Macrophages have been proved to play an important role in host defending and regulate a range of physiological and pathological events (Krzyszczyk *et al.*, 2018; Li *et al.*, 2019). Macrophages can participate to regulate the microenvironment through differentiating into functional cells (Murray and Wynn, 2011).

Macrophages are a cell type that can be differentiated into a series of effector subtypes based on local cells and secreted signals. As we all know, macrophages play an active regulatory role in the process of fracture healing. However, the basic mechanism of this beneficial effect is still unclear. In order to fully explore the role of M1 macrophages in bone formation and explore the underlying underlying mechanisms, we focus here on the effect of M1-Exos on osteoblast differentiation.

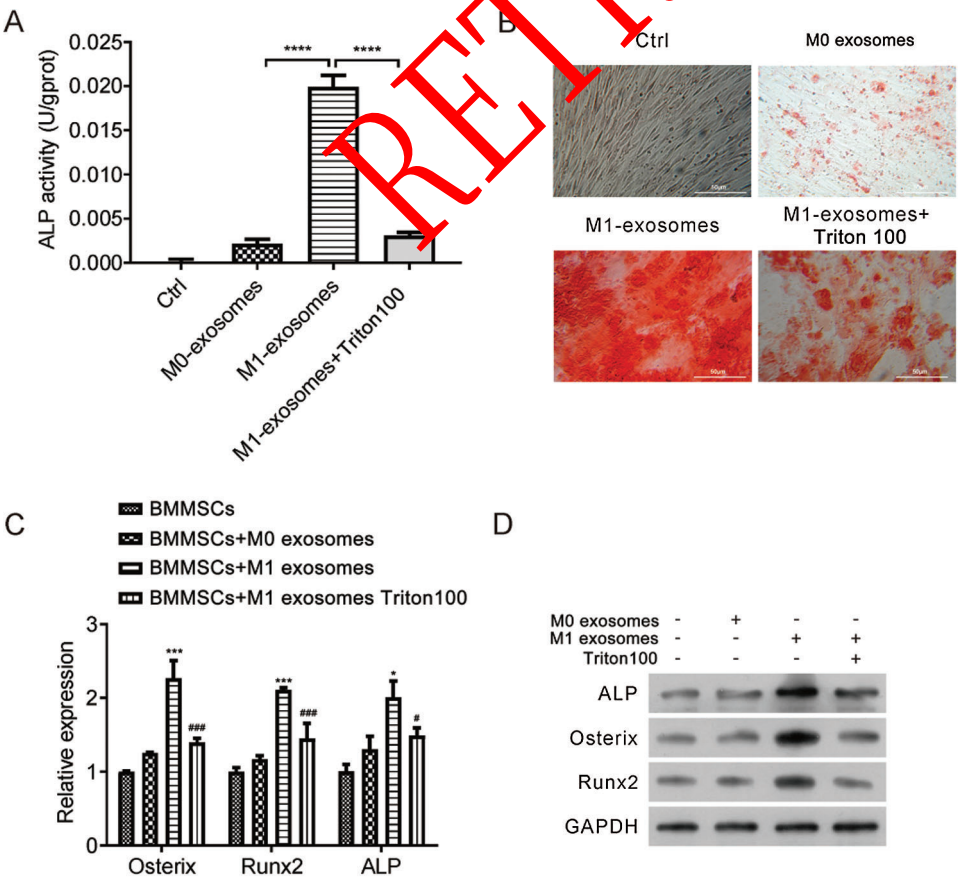

FIGURE 3. Effects of macrophage derived exosomes on the osteogenic differentiation of BMMSCs. (A) Statistical analysis of the ALP activity of M0 or M1-derived exosome-treated BMMSCs. (B) Alizarin Red S staining of M0 or M1-derived exosome-treated BMMSCs. (C–D) Expression levels of osteogenesis-related genes (ALP, Osterix and Runx2) in M0 or M1-derived exosomes treated BMMSCs. $^{*}P < 0.05$, $^{****}P < 0.001$ *vs.* M0 exosomes; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$ *vs.* M1 exosomes; Ctrl, control; M0 unpolarized macrophages; M1, polarized macrophages.

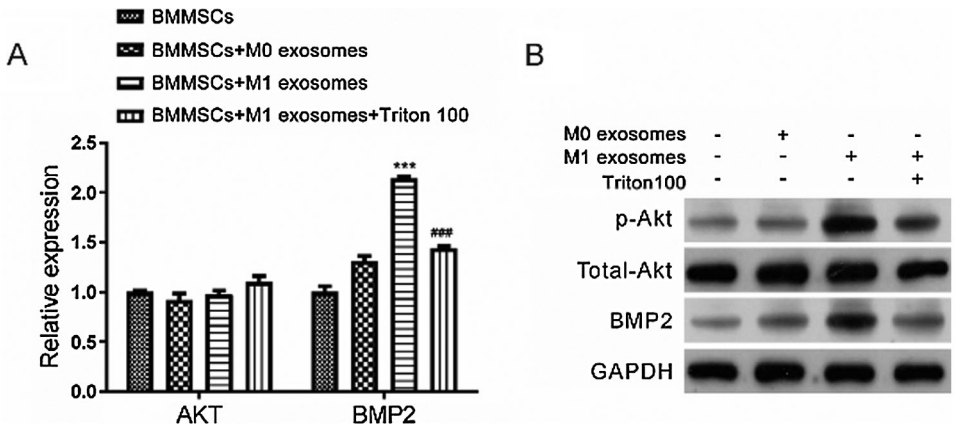

FIGURE 4. Osteogenesis is associated with M1-derived exosome-mediated activation of Akt and BMP2 during differentiation. (A) Expression levels of Akt and BMP2 in M0 or M1-derived exosome-treated BMMSCs. (B) Protein levels of Akt and BMP2 in M0 or M1-derived exosome-treated BMMSCs. $^{***}P < 0.005$ *vs.* M0 exosomes; $^{\#\#\#}P < 0.005$ *vs.* M1 exosomes.

Exosomes are small vesicles that are secreted into circulation by a range of cell types *in vivo*, whereupon they can be internalized by proximal or distal cells. The small molecules within these exosomes can then regulate the functionality of recipient cells upon internalization, and thereby conducting a communication among various cells and organs. In this study, we found that exosomes derived from M1 can promote the osteogenic differentiation of BMMSCs and may be involved in the regulation of AKT/RUNX2.

In recently published works, macrophage-derived exosomes have been revealed to regulate many physiological processes (Pajarinen *et al.*, 2019; Saha *et al.*, 2016; Sesti *et al.*, 2015). For example, M1-derived exosomes are important for intestinal stem cell proliferation and self-renewal, subsequently maintaining intestinal homeostasis (Saha *et al.*, 2016). Moreover, it has been reported that exosomes derived from M1 macrophages can induce endothelial cell proliferation and migration to accelerate wound repair (Hu *et al.*, 2018; Li *et al.*, 2019). However, the regulation of BMMSCs properties by M1-derived exosomes remains unclear. Thus, exosomes were isolated from M0 or M1 macrophages. Different methods, including TEM and NTA staining, were used to identify these exosomes. Subsequently, we found that exosomes secreted by different types of macrophages had different effects on BMMSCs differentiation. The Alizarin red S staining and osteogenic gene expression results revealed that only M1-derived exosomes markedly induced the differentiation of BMMSCs. In contrast, M0 derived exosomes exhibited little effect on osteogenic differentiation. This result revealed that only exosomes derived from polarized M1 macrophages influenced the differentiation of BMMSCs. This observation indicates that exosomes not only mediate the microenvironment during pathophysiological processes, but also play a key role during tissue repair, such as during BMMSCs differentiation. In general, our current data indicate that M1-derived exosomes may be used as therapeutic agents to improve the properties of BMMSCs in the regenerative microenvironment.

However, the precise mechanism by which polarized macrophages affect osteogenesis is unclear. In the current study, we mechanistically demonstrated that M1-derived exosomes regulate the Akt signaling pathway, which is critical in osteogenic commitment (Xu *et al.*, 2019; Yu *et al.*, 2016). Moreover, our results suggest that BMP2 is a metabolically regulated substrate of M1-derived exosomes and that this regulation occurs mainly through Akt phosphorylation. This is the first report to show a direct relationship between macrophages and Akt mediated BMP2 function in osteogenesis. Furthermore, to examine the functional relationship between M1-derived exosomes and BMP2 in the differentiation of BMMSCs, distinctive models were used in this study. These findings correlate with the well thought hypothesis that M1-derived exosomes activate Akt and increase BMP2 levels in osteogenesis, which is beneficial for maintaining bone health. In addition, it has also been confirmed that BMP2 is a target of M1-derived exosomes and may act as a regulatory connection between macrophages and osteoblast development. Our macrophage differentiation studies revealed that the M1 microenvironment plays a key role in osteogenesis. Although the mRNA levels of BMP2 were increased during osteogenesis, it should also be carefully considered that M1-derived exosomes mediate Akt activation and may maintain the homeostasis of osteocytes by stabilizing the BMP2 protein. This finding was evident from the fact that there was persistent Akt activity at all times, as seen in our study. However, there may be other regulatory pathways at the translational or posttranslational level. We will further elucidate the regulatory mechanism between M1-derived exosomes and osteogenesis.

Availability of Data and Materials: The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions: (I) Conception and design: CGD; (II) Administrative support: CGD; (III) Provision of study materials or patients: TLW, XZ, CHY, WCL; (IV) Collection and assembly of data: TLW, XZ, HTL, YZW; (V) Data analysis and interpretation: ZKK, ZJH, JZL, HRT; (VI) Manuscript writing: CGD, TLW, XZ; (VII) Final approval of manuscript: All authors.

Ethics Approval: All operation on mice were pre-approved by the Animal Care and Use Committee of the Shenzhen University General Hospital (S2019-101607).

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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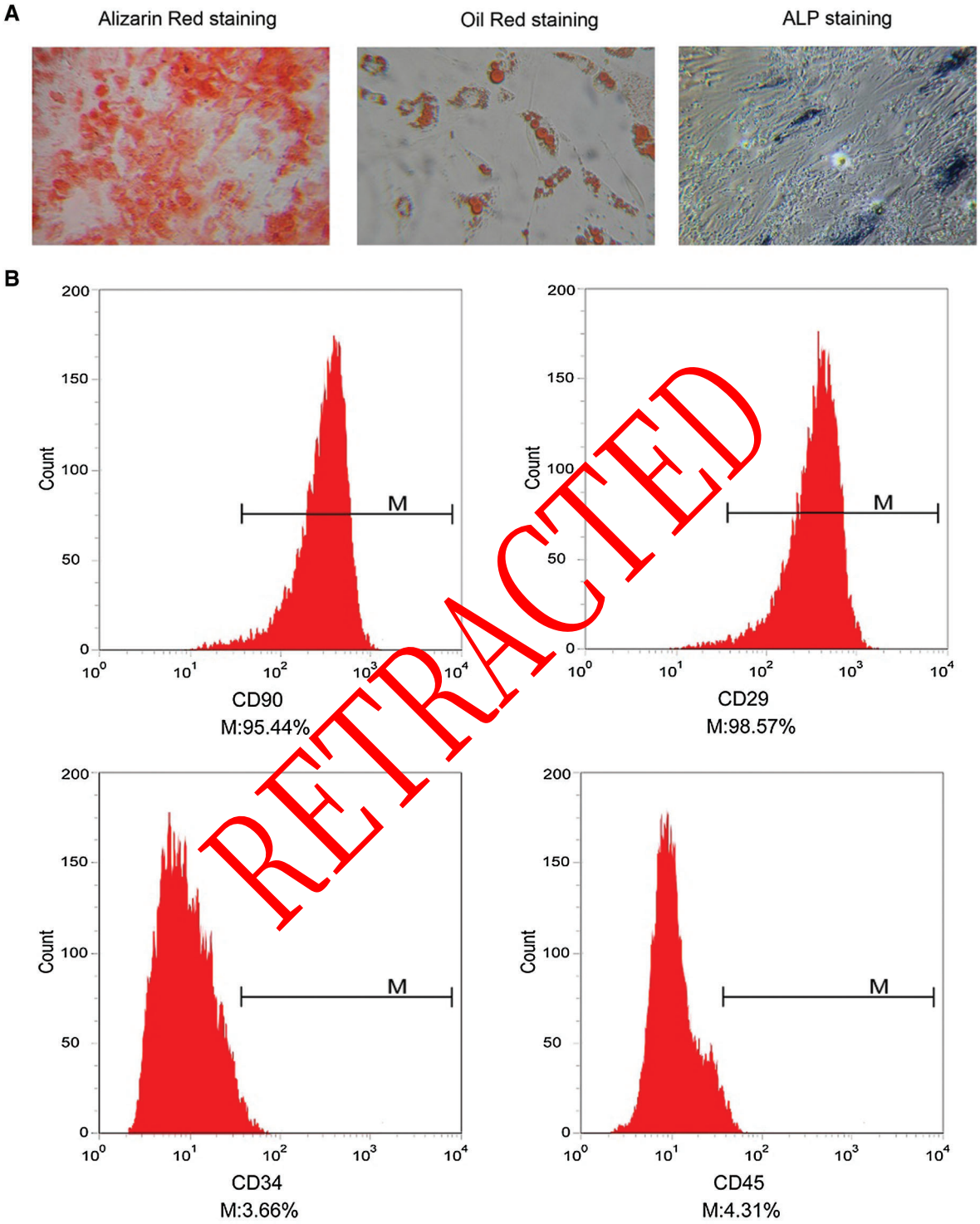
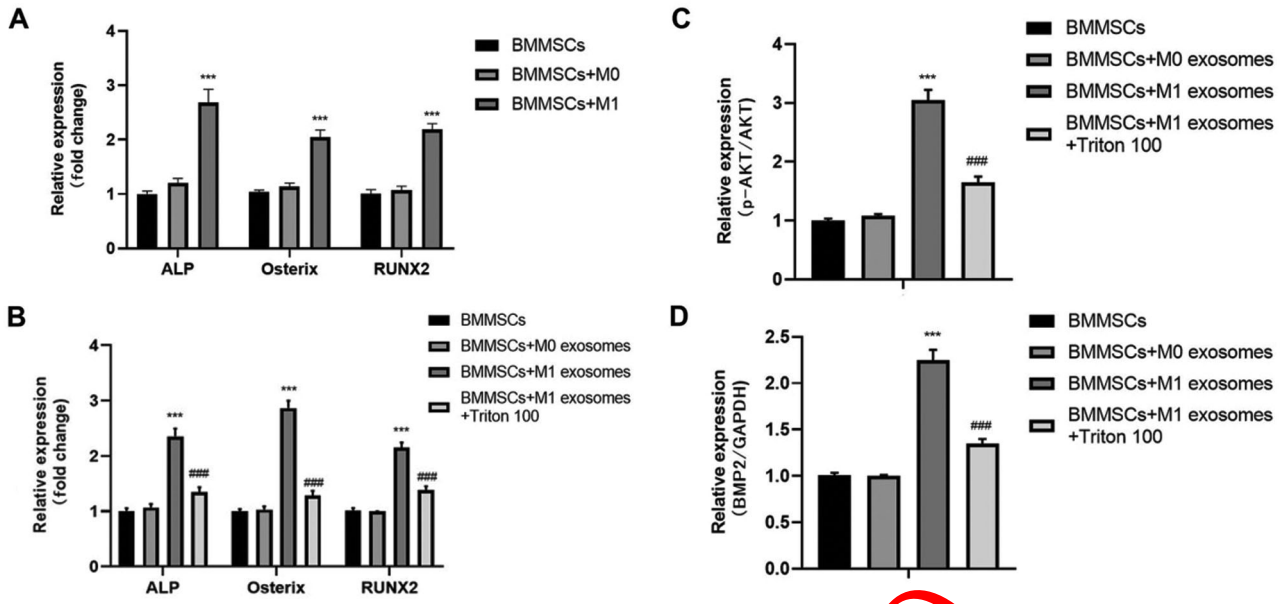
Appendix



SUPPLEMENTARY FIGURE 1. BMMSCs cell identification. (A) The results of Alizarin Red Oil Red O and ALP staining. (B) Identification of BMMSCs by flow cytometry.



SUPPLEMENTARY FIGURE 2. The quantitative analysis histogram of western blot. $^{***}P < 0.005$; $^{###}P < 0.005$.