The Role of Apoptotic Bodies From Different Stages of Apoptosis in Maintaining the Vitality of BMSCs

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Abstract

Background: Apoptosis is a complex process of autonomously and orderly programmed cell death. During apoptosis, bone marrow mesenchymal stem cells (BMSCs) generate many vesicles termed apoptotic bodies (ABs). The formation of ABs is closely related to the process of apoptosis. ABs have been found to act as containers to transmit the nucleic acid, protein, and lipid signals to target cells to promote . However, whether the composition and function of ABs are related to the process of apoptosis remains elusive.

Methods: After STS induced BMSCs apoptosis, ABs derived from apoptotic BMSCs were extracted, characterized. Then the effects of proliferation, migration, and osteogenic differentiation of ABs from different stages of apoptosis on normal BMSCs were evaluated in vitro.

Results: We confirmed that apoptotic BMSCs generated abundant ABs. These ABs were engulfed by normal BMSCs and promoted the proliferation, migration, and osteogenic differentiation of recipient cells. Besides, ABs from the middle stage of apoptosis were the most efficient at enhancing the capacity of proliferation, migration, and osteogenic differentiation of BMSCs.

Conclusions: This study aims to understand the detailed biological role of ABs from different stages of apoptosis in promoting the activity of BMSCs. This study reveals a previously unknown relationship between the function of ABs and the process of dying BMSCs on promoting the viability of BMSCs.

1. Introduction

Apoptosis, involving distinct cell shrinkage, chromatin condensation, and plasma blebbing, is a common phenomenon in embryonic development, cell differentiation, tissue regeneration, and other physiological processes, as well as in tumor, immune deficiency, organ atrophy, and other pathological processes[1–3]. Apoptotic bone marrow mesenchymal stem cells (BMSCs) have been found to initiate a regenerative response after injury and promote the proliferation of uninjured cells to replace damaged cells and maintain tissue homeostasis[4, 5]. This phenomenon is named apoptosis-induced compensatory proliferation (AiP)[4]. In several model organisms, AiP exerts this effect by secreting mitogenic signals to recipient cells[5]. Recent evidence suggests that most cells constantly release extracellular vesicles (EVs), which are believed to augment intercellular communications upon exposure to noxious stimuli[6]. During the process of apoptosis, a series of membrane-wrapped structures named apoptotic bodies (ABs) are formed through cell membrane budding and blebbing[7] and then are engulfed and digested by target cells. ABs have been found to play an important role in intercellular communication in proliferation, regeneration, immune modulation, tumors, and related disease settings[8–10].

Apoptosis can be traditionally divided into biochemically and morphologically distinct phases in which the obvious changes have taken place in genes and proteins. In the first, pro-apoptotic stimuli trigger activation of the central molecular machinery of apoptosis (initiation phase). In the second, committed, or effector phase, the molecular executioner (caspase family) machinery becomes fully activated as shown

in changes in nuclei. In the third, or degradation phase, do the hallmarks of apoptosis become evident. These include morphologic changes and DNA fragmentation[11]. It has been found apoptotic membrane protrusions formed microtubule spikes, apoptopodia, and beaded apoptopodia at the early of apoptosis. After that formed the large sizes of ABs [12]. The formation of ABs is related to the process of apoptosis. During the process of apoptosis, ABs encapsulate the remaining different components of dead cells including proteins (e.g., from the nucleus, mitochondria, and plasma membrane), lipids, and nucleic acids (e.g., mRNA, long non-coding RNA, rRNA, miRNA, or fragments of these RNA molecules)[13, 14]. Less is known about the relation between the process of apoptosis and the function of ABs. Whether these ABs formed at different stages of apoptosis have different effects on BMSCs remains elusive.

In this study, we focused on exploring the effects of ABs from different stages of apoptosis on the proliferation, migration, and differentiation of BMSCs. These findings suggest the relationship between the process of apoptosis and ABs in maintaining BMSCs stem cell properties.

2. Materials And Methods

2.1 Reagents and chemicals

Staurosporine (STS) (#9953) and phalloidin (#8953) were purchased from Cell Signaling Technology (Danvers, Ma, USA). Annexin V (556420) and propidium iodide (PI) (556463) were purchased from BD Pharmingen (San Jose, CA, USA). BCA Protein Assay Kit (KGP902) and CCK-8 kit (KGA317s-1000) were purchased from KeyGEN BioTECH (Jiangsu, China). DAPI was purchased from Solarbio (Beijing, China). Histone 3 (PA5-16183) and Complement C3b (PA5-21349) were purchased from Thermo (Carlsbad, CA, USA). CD63 (510953) and Gapdh (200306) were purchased from Zen Bioscience.

2.2 Cell culture

BMSCs were isolated from femurs and tibias of 8-weeks male mice and 4-weeks male rats as previously described [15]. The hindlimbs were removed, and then BMSCs were separated by flushing marrow cavities and cultured at a density of 1.5×10^6 cells per 10 cm culture dish (Corning, NY, USA) in α -modified Eagle's medium (α -MEM; HyClone), 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 U/mL streptomycins. BMSCs were cultured at 37°C in 5% CO₂ for 10 days. The medium was replaced every 3 days. Cells were digested and passaged once for further experimental use by using 0.25% trypsin (Invitrogen, USA) at 80–90% confluence.

2.3 Apoptosis rate analysis

STS (500 nM for working solution) induced BMSCs apoptosis for 2 h, 4 h, 6 h, and 12 h. 5 μ l Annexin V and 10 μ l PI were stained with apoptotic BMSCs for 30 min, and the apoptotic ratio was detected by Flow cytometry (BD Accuri C6 flow cytometer). Cells positive for FITC Annexin V and negative for PI (Q3) are undergoing apoptosis. Necrotic cells stain negative for FITC Annexin V and positive for PI (Q1). Cells

positive both for FITC Annexin V and PI (Q2) are either in the end stage of apoptosis or dead. Alive cells are negative for both FITC Annexin V and PI (Q4).

2.4 Isolation of ABs

ABs were isolated using sequential centrifugation followed by a standard literature protocol[16–18]. Briefly, after 500 nM STS treated for 2 h, 4 h, 6 h, and 12 h respectively, the supernatants were centrifuged at 800× g for 10 min to remove cell debris. The ABs-rich supernatant was centrifuged at 16000× g for 30 min to pellet the ABs. The pellet was suspended in ice-cold PBS and was centrifuged at 16000× g for 30 min again to remove the contaminated protein. All centrifuge processes were performed at 4°C to prevent the inactivation of ABs. The purified ABs were resuspended in 100 µL ice-cold PBS and stored at – 80°C immediately for future analysis.

2.5 Characterization of ABs

The morphology of ABs was observed by Transmission Electron Microscopy (TEM). The expression of gapdh, H3, C3b, and CD63 in ABs was detected by Western blot. Flow Cytometry (Attune® NxT, ThermoFisher, CA, USA) were used to analyze the number of ABs and special marker of apoptosis (Annexin V). 1 µm- and 4 µm-size calibration beads were used to gate ABs. FCM calculated the positive ratio of Annexin V in ABs. The concentration of ABs was measured in terms of their protein content determined by the bicinchoninic protein assay method using the manufacturer's protocol (BCA Protein Assay Kit, KeyGEN BioTECH). 10 µg/µl were used as a work concentration in the following experiments.

2.6 ABs labeling and cellular engulfment assay

ABs were labeled with 10 µl membrane-labeling dye PKH67 (MKCK0731, Sigma, Massachusetts, USA) at 37°C for 20 min according to the manufacturer's protocol. BMSCs were cultured on confocal dishes and treated with 4×10^6 PKH67-labeled ABs per 1×10^6 BMSCs for 24 h. Cells were fixed in 4% paraformaldehyde, stained with phalloidin, and DAPI and imaged by confocal microscopy (Olympus FV1000, Japan).

2.7 Cell proliferation assay

BMSCs at a density of 2×10^3 / well were inoculated in 96-well plates (Corning, NY, USA) and co-cultured with PBS or ABs from different stages of apoptosis. At the designated times (1, 2, 3, 4, and 5 days), cell proliferation ability was assessed using the cell counting kit-8 (CCK8) assay according to the manufacturer's instructions. The absorbance value at 450 nm was detected to analyze cell proliferation viability.

2.8 Wound healing assay

For the wound healing assay, BMSCs were seeded on 24-well plates (Corning, NY, USA). At 90% confluence, scratch wounds were created by pipette tips scratching the cell monolayer. BMSCs were continuously incubated with PBS or 10 µg/ml ABs from different stages of apoptosis for 24 h. 4% paraformaldehyde fixed for 30 min and 0.05% crystal violet stained. Pictures of scratch were taken under

a microscope and the motility of cells was compared by the degree of confluence from one side of the scratch to the other.

2.9 Boyden chamber assay

For the cell migration assay, 1×10^6 BMSCs were plated into the upper chamber of a 24-well 8 μm pore size transwell device (Corning Incorporated, Corning, NY, USA). 10 $\mu\text{g/ml}$ ABs from different stages of apoptosis were added to the lower chamber as a chemoattractant, while PBS were added as a control group. 24 h after incubation at 37°C , cells on the top surface of the chamber were removed and cells on the underside of the chamber were fixed in 4% paraformaldehyde for 30 min, stained with 0.05% crystal violet, and air-dried. Migrated cells were quantified under an inverted light microscope (Olympus, Japan).

2.10 Osteogenic differentiation assay

5×10^5 BMSCs were seeded into a 24-well plate per well. When cells reached 100% confluence, the basal medium was changed into osteogenic induction medium: α -MEM containing 10% FBS, 1% penicillin/streptomycin, 2 mM β -glycerophosphate (D301908, Aladdin, Shanghai, China), 100 μM ascorbic acid 2-phosphate (A103534, Aladdin), and 10 nM dexamethasone (D137736, Aladdin). BMSCs were treated with PBS, 2 h ABs, 4 h ABs, 6 h ABs, and 12 h ABs. To detect mineral nodule formation, after culturing in the osteogenic medium for 21 days, BMSCs were fixed with 4% PFA for 30 minutes at room temperature, and then Alizarin red staining was performed. Alizarin red-positive rate was analyzed by detecting absorbance (OD at 562 nm) after extracting by 10% Cetylpyridinium chloride (A600106-0100, Sangon Biotech, Shanghai, China).

2.11 qRT-PCR and Western blot

After BMSCs were cultured for 2 days in an osteogenic medium, the osteogenic differentiation-related genes alkaline phosphatase (ALP), OPN, markers runt-related transcription factor 2 (Runx2) were evaluated by qRT-PCR as previously described. Gapdh was used as the internal control. All the primer sequences used in this experiment can be seen in Table S1.

Purified ABs and BMSCs were dissolved in RIPA Lysis Buffer (KGP702-100, KeyGEN, China). After being quantified by BCA assay, the proteins were loaded on sodium dodecyl sulfate-polyacrylamide (SDS) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, the membranes were blocked with 5% BSA for 2 h at room temperature and incubated with primary antibodies overnight at 4°C . Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were detected by the imaging system and quantified by ImageJ software.

2.12 Statistical analysis

All data are representative for at least three experiments of similar results performed in triplicate unless otherwise indicated. Data are expressed as mean $\pm$ SD. Comparisons between two groups were analyzed using independent unpaired two-tailed Student's t-tests, and comparisons between more than two groups were analyzed using one-way ANOVA with the Bonferroni adjustment. P values less than 0.05 were considered statistically significant.

3. Results

3.1 STS stimulated the generation of BMSCs-derived ABs

We used STS (500 nM), a widely used apoptosis inducer,[19] to stimulate BMSCs apoptosis. TEM showed shrinkage of the cell, breakage of the nucleus and cytoplasmic organelles, and cell membrane detachment and blebbing (Fig. 1A), which are typical features of apoptotic cells. Light microscopic analyses showed obvious morphological changes with the prolong of STS treatment (Fig. 1B). FCM analyses (Fig. 1C-D) of Annexin V/ PI - stained BMSCs showed the percentage of apoptotic cells increased gradually with the prolongation of STS treatment from 49.8% at 2 h to 75.4% at 12 h, suggesting STS induced BMSCs apoptosis in a time-dependent manner. Besides, the most cells after STS treated for 4 h were undergoing early apoptosis compared with other groups. According to the percentage of positive cells, we defined four stages of apoptosis: the early (STS treatment for 2 h), the middle (STS treatment for 4 h), the middle-late (STS treatment for 6 h), and late (STS treatment for 12 h) stage.

Then we used ultracentrifugation to extracted ABs from apoptotic BMSCs for identification as previously described (Fig. 2A)[18]. TEM analysis confirmed the presence of round-shaped vesicles surrounded by a bilayer membrane (Fig. 2B). Ultrathin sections of ABs imaged by TEM showed nucleus fragments, cytoplasmic organelles, and autophagosomes, which were also presented in apoptotic cells. Western blot analysis showed that ABs contained specific markers including histone 3 (H3), and Complement C3b, and lack the exosome markers (CD63) (Fig. 2C). Then, we detect the expression of Annexin V in ABs. The ratio of Annexin V⁺ in ABs was up to 81.3% (Fig. 2D). The protein content showed that the longer the STS treatment time was, the higher ABs protein was (Fig. 2I). The results showed the ABs we used in this study were vesicles as approximately 1–4 μ m in size, with Annexin V positive and H3, C3b expression.

3.2 ABs from the middle stage of apoptosis are the most potent to induce proliferation of BMSCs

Next, we determined the effect of ABs on the recipient BMSCs. Firstly, we examined whether recipient BMSCs could engulf ABs in the micro-environment. After BMSCs co-cultured with ABs for 24 h, immunofluorescent staining showed that BMSCs could successfully uptake exogenous PKH67-labeled ABs (Fig. 3).

To compare the proliferation potency of ABs from different stages of apoptosis on BMSCs, the growth curves determined by the CCK-8 assay (Fig. 4A) showed that ABs from the middle stage of apoptosis promoted mouse BMSCs (m-BMSCs) proliferation, while ABs from other stages of apoptosis has no obvious effect. To confirm that the effect of ABs is not species-specific, we also repeated the above

experiment using BMSCs and ABs derived from rats. Although slightly different compared with m-BMSCs, the CCK-8 assay on rat-BMSCs (r-BMSCs) (Fig. 4B) showed that ABs from all stages of apoptosis promoted the growth and proliferation of r-BMSCs. But it should be noted that ABs from the middle stage of apoptosis had the strongest ability to promote the proliferation of r-BMSCs. In brief, ABs from the middle stage of apoptosis had the highest ability to promote the proliferation of BMSCs.

3.3 ABs from the middle stage of apoptosis are the most potent to induce migration of BMSCs

We used the scratch healing assay and Boyden chamber assay to compare the effect of ABs from different stages of apoptosis on the migration of BMSCs. Scratch healing assay showed that ABs accelerated the movement of m-BMSCs to the scratch area. Surprisingly, ABs from the middle stages of apoptosis had the highest ability to promote wound healing (Fig. 4C). Similar results that ABs from the middle stage of apoptosis have the highest ability to promote wound closure were found in r-BMSCs (Fig. 4D). The Boyden chamber assay showed that ABs accelerated the migration of m-BMSCs from the upper chamber to the lower chamber, but the number of migrating cells was highest in the STS-treated 4 h group (Fig. 4E). These results were consistent with the effect of ABs on r-BMSCs (Fig. 4F). Taken together, ABs promoted migration of BMSCs, and ABs from the middle stages of apoptosis had the strongest ability to promote migration.

3.4 ABs from the middle stage of apoptosis are the most potent to induce osteogenesis of BMSCs

To compare the osteogenic potency of ABs from different stages of apoptosis on BMSCs, m-BMSCs were co-cultured with ABs from different stages of apoptosis during osteogenic induction. Alizarin red staining (Fig. 5A-B) showed that ABs enhanced the capacities of m-BMSCs for osteogenic differentiation. Interestingly, ABs from the middle stage of apoptosis showed significantly higher osteogenic potency compared to other stages of apoptosis. We confirmed that the level of osteogenic Runx2 and ALP in recipient BMSCs were upregulated after exposure to ABs derived from the middle stage of apoptosis (Fig. 5C). We also confirmed this observation in r-BMSCs. The osteogenic differentiation assay with r-BMSCs showed that ABs enhanced the osteogenic differentiation of r-BMSCs. Treatment with ABs from the middle stage of apoptosis resulted in the highest degree of mineralization and the most of calcium nodules (Fig. 5D-E). Alizarin red staining and qRT-PCR (Fig. 5F) showed consistent results suggesting the highest osteogenic potency of r-BMSCs after co-cultured with ABs from the middle stage of apoptosis. Taken together, ABs can promote the osteogenic differentiation of BMSCs, and ABs from the middle stage of apoptosis have the highest ability to promote osteogenic differentiation.

4. Discussion

EVs have been considered as novel targets for the development of diagnostic, prognostic, and therapeutic agents. EVs potentially act as a carrier to transport and exchange molecular information among different types of cells, tissues, and organs[20, 21]. Whereas intensive studies have focused on the role of EVs (exosome or microvesicles), the relationship between the formation and function of ABs from apoptotic BMSCs remains largely unexplored. Furthermore, it is also unclear whether these ABs from different

stages of apoptosis have different effects on BMSCs properties. Our studies show that after STS stimulation, apoptotic BMSCs-derived ABs regulate the growth and differentiation of the recipient BMSCs. Surprisingly, we found the function of ABs is related to the process of apoptosis since ABs from the middle stage of apoptosis had the strongest ability to promote proliferation, migration, and differentiation. To our best knowledge, this is the first study exploring the relation between ABs and the apoptotic process. Our study potentially provides novel insights into ABs from different stages of apoptosis on regulating the BMSCs vitality.

More diverse contents are encapsulated in the ABs, including but not limited to proteins, lipids, RNA, and DNA molecules[18]. In this study, we observed the contents (such as the broken nucleus, autophagosome) of apoptotic BMSCs were also presented in ABs, and ABs were engulfed by BMSCs, suggesting that ABs can be used as a natural carrier to encapsulate and transport bioactive contents to promote the communication between survival and dead BMSCs. However, given that confocal microscopy cannot be used to examine 3-dimensional subjects, it is unclear whether the ABs were engulfed partially or entirely into the epithelial cells or if they were mainly interacting with the cell-surface molecules. Besides, we found ABs can promote the proliferation, migration, and differentiation of BMSCs. Like other EVs, ABs are found closely related to cell survival, proliferation, and differentiation. Endothelial cells-derived ABs promote the proliferation of human endothelial progenitor cells in vitro.[22] ABs from cardiomyocytes enhance the proliferation and differentiation of resident stem cells by transporting specific miRNAs.[23] ABs activate the Wnt/ β -catenin pathway to promote the proliferation and osteogenic, and lipogenic differentiation of BMSCs.[24] Apoptotic BMSCs can still regulate recipient cells by continuously releasing ABs, implying ABs a new mechanism for communicating cell death and survival.

Interestingly, we found ABs from the middle stage of apoptosis have the highest ability to promote proliferation, migration, and osteogenic differentiation of BMSCs. Coincidentally, four hours after STS induction, most BMSCs were positive for Annexin V and negative for PI (characteristics of early apoptosis). We suspect that the function of ABs may be related to the process of apoptosis. During the early stages of apoptosis, autoantigens such as H2B and DNA, RNA were distributed into small ABs from lymphoblasts, which were subsequently engulfed by monocyte-derived phagocytes[25]. Zhu, Z., et al. found that ABs from the early-middle stage of LPS-induced apoptosis had the strongest ability to promote proliferation compared with LPS-induced for 12 h[26]. After inducing apoptosis for 4 h, ABs could promote the incorporation of Sca-1 + progenitor cells and limited atherosclerosis[27]. In addition, during the process of apoptosis, the substance of apoptotic cells undergoes a series of changes, such as condensation of the nucleus and chromatin, breakage of the nucleus, and cytoplasmic organelles. ABs formed at different stages of apoptosis may encapsulate different substances. These results implied the phases of apoptosis may play a critical role in regulating the function of ABs. But how the process of apoptosis regulates the function of ABs needs further explored.

5. Conclusions

In summary, we demonstrated that the STS induced BMSCs apoptosis at four stages and generated abundant ABs. These ABs can be engulfed by normal BMSCs and promoted the proliferation, migration, and osteogenic differentiation of BMSCs. Besides, ABs from the middle stage of apoptosis had the strongest ability to maintain the properties of the receipt BMSCs. These results imply the potential relation of ABs and the apoptotic process on regulating the vitality of BMSCs.

Abbreviations

BMSCs: bone marrow mesenchymal stem cells; ABs: apoptotic bodies; AiP: apoptosis-induced compensatory proliferation; EVs: extracellular vesicles; STS: staurosporine; PI: propidium iodide; TEM: Transmission Electron Microscopy; FCM: Flow Cytometry; CCK8: cell counting kit-8; ALP: alkaline phosphatase; OPN: osteopontin; Runx2: markers runt-related transcription factor 2; SDS: sodium dodecyl sulfate-polyacrylamide; PVDF: sodium dodecyl sulfate-polyacrylamide; m-BMSCs: mouse BMSCs; r-BMSCs: rat-BMSCs.

Declarations

Acknowledgements

Not applicable

Ethics approval and consent to participate

The care and use of the laboratory animals followed the guidelines of the Institutional Animal Care and Use Committee of West China School of Stomatology, Sichuan University.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no Competing interests.

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Authors' contributions

M.L., L.L., and W.T. designed the study. M.L. performed the experiments. M.L., X.X., and L.L. supervised the study. H.H., C.L., and Q.T. analyzed and interpreted the data. X.G. and J.Y. performed the statistical analysis. M.L. and L.L. wrote the manuscript. L.L., and W.T. revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

STS induced BMSCs apoptosis. (A) The morphology of apoptotic BMSCs was observed by TEM analysis. The yellow arrows pointed out the breakage of the nucleus, and the blue arrows showed the cell membrane detachment and blebbing. (B) Light microscopy showed the morphologic change of BMSCs with STS treated at different times (0 h, 2 h, 4 h, 6 h, and 12 h). (C) Flow cytometric analyses of Annexin V/ PI-stained BMSCs showed apoptotic rate after STS treated at different times. (D) Quantitative statistics used for the rate of total apoptotic cells, early apoptotic cells, and late apoptotic cells. Scale bar,

5 μm (A); 100 μm (B). $n = 3$ per group. Error bars represent S.D. from the mean values. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$, * $P < 0.05$.

Figure 2

Characterization of ABs. (A) The schematic graph shows the protocol of isolation of BMSC-ABs. (B) The round-shaped of ABs surrounded by a bilayer membrane was observed by TEM. The blue arrows pointed out the ABs. Ultrathin sections of ABs imaged by TEM showed the internal structure of ABs. The black arrows pointed out nucleus fragments, and the yellow arrows pointed out the autophagosomes. (C) The expressions of GAPDH, H3, C3b, and CD63 in cell debris and ABs were detected by Western blot. (D) The ABs were detected by flow cytometry. 1 and 4 μm - size calibration beads were used to gate ABs. The blue box pointed out ABs. Flow cytometry identified the rate of Annexin V+ ABs. (E) Quantitatively analyzed the protein content of ABs from different stages of apoptosis. Scale bar, 0.5 μm (A); 1 μm (B). $n = 3$ per group. Error bars represent the S. D. from the mean values. **** $P < 0.0001$; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; N.S., no significance.

Figure 3

ABs Labeling and Cellular Uptake. Immunofluorescent staining showed that PKH67-labeled ABs derived from m-BMSCs were engulfed by normal m-BMSCs at 24 h co-cultured. Scale bar, 20 μm.

Figure 4

The effect of ABs on the proliferation and migration of BMSCs. (A-B) The effect of ABs on the proliferation ability of m-BMSCs and r-BMSCs was measured by the CCK-8 assay respectively. (C-D) The effect of ABs on the wound closure ability of m-BMSCs and r-BMSCs were measured by scratch healing assay respectively. (E-F) The effect of ABs on the migration and invasion ability of m-BMSCs and r-BMSCs was measured by transwell assay respectively. Scale bar, 200 μ m (C-D); 100 μ m (E-F). n = 3 per

group. Error bars represent the S. D. from the mean values. ****P < 0.0001; ***P < 0.001, **P < 0.01, *P < 0.05; N.S., no significance.

Figure 5

The effect of ABs on the osteogenic differentiation of BMSCs. (A-B) ABs increased the mineralized nodule formation of m-BMSCs during osteogenic induction, as assessed by alizarin red staining. (C) ABs from m-BMSCs promote the expression of Runx2 and ALP, as assessed by qRT-PCR. (D-E) ABs increased the

mineralized nodule formation of r-BMSCs during osteogenic induction, as assessed by alizarin red staining. (F) ABs from r-BMSCs promote the expression of Runx2 and OPN, as assessed by qRT-PCR. Scale bar, 200 μ m (A), (D). n = 3 per group. Error bars represent the S. D. from the mean values. ****P < 0.0001; ***P < 0.001, **P < 0.01, *P < 0.05; N.S., no significance.

Supplementary Files

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