

Exosomes from bone marrow mesenchymal stem cells promoted osteogenic differentiation by delivering miR-196a that targeted Dkk1 to activate Wnt/ β -catenin pathway

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Research

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Abstract

Background:

Osteoporosis is the most common bone metabolic disease. Emerging evidence suggests that exosomes are secreted by diverse cells such as bone marrow mesenchymal stem cells (BMSCs), and play important role in cell-to-cell communication and tissue homeostasis. Recently, the discovery of exosomes has attracted attention in the field of bone remodeling.

Methods:

The exosomes were extracted from BMSCs and labeled by PKH-67, and then incubated with hFOB1.19 cells to investigate the miR-196a function on the osteoblast differentiation of hFOB1.19. The osteoblast differentiation was detected via alizarin red staining and the expression of osteoblast genes were detected by western blot. The cell apoptosis was detected by flow cytometer. The target relationship of miR-196a and Dickkopf-1 (Dkk1) were verified by luciferase assay and western blot.

Results:

Here we demonstrated that exosomes extracted from BMSCs (BMSC-exo) significantly promoted hFOB1.19 differentiation to osteoblasts. We found that BMSC-exo were enriched with miR-196a and delivered miR-196a to hFOB1.19 cells to inhibit its target Dkk1, which is a negative regulator of Wnt/ β -catenin pathway.

Conclusion:

BMSC-exo activated Wnt/ β -catenin pathway to promote osteogenic differentiation, while BMSC-exo failed to exert the effects when miR-196a was deprived. In conclusion, miR-196a delivered by exosomes from BMSCs plays an essential role in enhancing osteoblastic differentiation by targeting Dkk1 to activate Wnt/ β -catenin pathway.

1. Introduction

Osteoporosis (OP) is a systematic bone disease characterized by bone loss, imbalance of bone metabolism and destruction of trabecular microstructure, and is the most common geriatric disease, especially in postmenopausal women ^[1]. Several studies have confirmed that bone healing in osteoporotic women and osteoporotic animals is remarkably delayed ^[2, 3]. In the past few years, a variety of strategies have been developed to treat osteoporotic defects, such as autologous bone transplantation,

allogeneic bone transplantation, and the combination of scaffold materials with growth factors or cells, but the efficacy is not satisfactory.

Bone remodeling consists of two processes, osteoblast mediated bone formation and osteoclast mediated bone resorption. Once the homeostasis of bone remodeling is broken, bone diseases such as osteoporosis will develop. Bone marrow mesenchymal stem cells (BMSCs) are regarded as promising seed cells in tissue engineering due to easy accessibility and multipotent ability to differentiate into adipocyte, osteoblast, cardiomyocytes, and neurons. Recently, emerging evidence has demonstrated that the cross-talk between monocyte-macrophage-osteoclasts and osteoblasts plays a vital role in the pathology of osteoporosis [4, 5]. Osteoblasts could be differentiated from BMSCs, and such a process is finely regulated by several transcription factors [6]. A series of bone-derived regulators responsible for the cross-talk have already been identified, such as transforming growth factor- β (TGF- β), bone morphogenetic protein-9 (BMP-9), runt-related transcription factor - 2 (Runx2), Osterix (Osx), and alkaline phosphatase (ALP) [7, 8]. Therefore, further understanding of the mechanism underlying osteogenic differentiation is crucial for the development of therapeutic approaches for osteoporosis.

Interestingly, bone remodeling is regulated by the factors packaged in lipid bilayered membrane vesicles called exosomes [9, 10]. Exosome, as an important part of the microenvironment, is a membrane vesicle secreted by numerous types of cells with 40–150 nm in diameter, such as dendritic cells, reticulocytes, tumor cells, B cells, T cells, mast cells, epithelial cells, and BMSCs [11, 12]. Prevailing evidences revealed that exosomes play pivotal role in cell communication to regulate the function and differentiation of homogeneous and heterogeneous recipient cells by transferring biologically active molecules, such as proteins, lipids, mRNA, microRNAs (miRNAs) [5, 13]. Earlier studies based on animal models have suggested that local transplantation of BMSCs promoted bone regeneration [14]. BMSCs actively produce exosomes, and BMSC-conditioned medium potently stimulates bone regeneration [15]. However, the potential role and underlying mechanisms of BMSC-derived exosomes (BMSC-exo) in bone regeneration have not been fully elucidated.

Qin et al. [13] compared the miRNAs in BMSCs and its exosomes by RNA sequencing, and found that three osteogenic-related miRNAs including miR-196a, miR-27a and miR-206 were highly enriched in BMSC-exo. Notably, miR-196a has been identified to promote pancreatic cancer cell proliferation [16]. However, there are few reports about the role of miR-196a in the occurrence and development of osteoporosis.

In this study, we presented in vitro evidence to demonstrate that BMSC-exo could enter osteoblasts to promote osteoblastic differentiation. Moreover, we found that miR-196a was a key exosomal component to promote osteoblastic differentiation via targeting Dickkopf-1 (Dkk1) which is a known negative regulator of Wnt/ β -catenin pathway.

2. Materials And Methods

2.1 Cell culture and osteogenic differentiation induction

Human BMSCs and hFOB1.19 cells were obtained from Boyan Biotechnology (Shanghai, China). BMSCs were cultured in the Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, China) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 1% L-glutamic acid and 1% double antibiotics (Sigma-Aldrich, St. Louis, MO). BMSCs were maintained in 5% CO₂ incubator at 37 °C. BMSCs were digested with trypsin for passage when the cell confluence was up to 80%. Second-passage BMSCs were inoculated into 6-well plates (3 × 10⁴ cells/ml) and 24-well plates (7 × 10⁴ cells/ml), respectively.

hFOB1.19 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, CN) containing 10% FBS, and were maintained in 5% CO₂ incubator at 37 °C. Cell passage was performed when the cell confluence was up to 80% and the cells were seeded into 6-well plates (2 × 10⁵ cells/ml). Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the instructions.

2.2 Luciferase assay

Target genes of miR-196a was predicted by MicroRNA.org. The 3'-UTR of Dkk1 gene was commercially synthesized (Sino Biological Inc., Beijing, China) and cloned into pmirGLO vector (Promega, Madison, WI, USA). Luciferase activity assay was performed using Dual-Luciferase Reporter System with the miR-control set at 1.0. miR-196a mimics, inhibitor and negative control oligonucleotides were obtained from GenePharma (Shanghai, China), and transfected into hFOB1.19 cells in 24-well plates (5 × 10⁴ cells per well) together with luciferase vector. Luciferase activities were measured 48 hours after transfection.

2.3 Osteoblast differentiation

hFOB1.19 cells were transfected with miR-196a mimics, miR-196a inhibitor and si-Dkk1 (all from Sino Biological Inc. Beijing, China). At 48 h after transfection, osteogenic differentiation was induced by culture in DMEM medium containing 10% FBS, 1% L-glutamamide, 1% double antibiotics, 0.25 mM ascorbic acid, 10 mM β-phosphoglycerol and 10 nM dexamethasone.

2.4 Isolation of BMSCs exosomes

Exosomes isolation was performed using an Exoquick reagent (System Biosciences, Pu Mai Technology, Beijing, China) according to the manufacture's protocol with minor modifications. Briefly, BMSCs culture medium was centrifuged at 8,000 g for 30 min. Then the supernatants containing exosomes were concentrated using a 100 kDa ultrafiltration Vivaflow 200 module to 10–15 ml, and to a final volume of between 0.5 and 1 ml by a 100 kDa ultracentrifuge tube (3,000 g × 30 min). Next, exosomes were precipitated by adding Exoquick reagent (at 1:4 ratio), and incubated overnight at 4 °C. Then the exosome precipitation was obtained by centrifugation at 1,500 g for 30 min, and resuspended in PBS. Exosome protein content was measured using BCA protein assay kit (Thermo Fisher Scientific, China).

2.5 Exosomes labeling and uptake

To label BMSCs exosomes, 60 µg of isolated samples were incubated with 1 ml PKH-67 green fluorescent dye (1×10^{-3} mM, Sigma Aldrich) for 5 min, and washed using 100 kDa filter to remove excess dye. The labeled exosomes were incubated with hFOB1.19 cells for 48 h, and then hFOB1.19 cells were stained with DAPI and photographed using laser-scanning confocal microscope.

2.6 Flow cytometry

Cells were fixed with pre-cooled 70% alcohol for 24 h, centrifuged at 1,000 r/min for 5 min, followed by 2 washes with PBS. Next, cells were incubated with propidium iodide (PI) (Dongren Chemical Technology, Shanghai, China) containing RNase in the dark at 4 °C. Next, cells were incubated with Annexin V-FITC (Partec GmbH, CyFlow Space) and PI at room temperature for 15 min and washed twice with phosphate buffer saline (PBS). Cell apoptosis was analyzed using a flow cytometer at an excitation wavelength of 488 nm.

2.7 Alizarin red staining

After osteoblast induction for 21 days, cells were fixed in 60% isopropanol for 1 min, followed by washing with PBS for 2 min. Subsequently, cells were stained with 10% alizarin red dyestuff (ScienCell, USA) for 10 min, followed by washing with PBS for 3 times. The cells were observed under optical microscope (Olympus, Tokyo, Japan).

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

After 7 days, 14 days and 21 days of osteoblast induction, total RNA samples were extracted using TRIzol reagent (Lifetech, USA). The RNA was reversely transcribed to cDNA according to the instructions of First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). PCR was performed with the following primers: miR-196a forward, CGTCAGAAGGAATGATGCACAG; miR-196a reverse, ACCTGCGTAGGTAGTTTCATGT; U6 forward, CTCGCTTCGGCAGCACA; U6 reverse, AACGCTTCACGAATTTGCGT.

2.9 Western blot analysis

Proteins of nuclear fraction and cytoplasmic fraction were extracted from hFOB1.19 cells with the Nuclear and Cytoplasmic Protein Extraction Kit (Thermo Fisher Scientific, USA), and subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, membrane was incubated overnight using antibodies against RUNX2 (1:500, abcam, UK), OCN (1:500, abcam, UK), ALP (1:1,000, abcam, UK), OPN (1:1000, abcam, UK), Dvl (1:500, abcam, UK), GSK3β (1:500, abcam, UK), caspase-3 (1:500, abcam, UK), caspase-6 (1:1,000, abcam, UK), Alix (1:1,000, abcam, UK), TSG101 (1:1,000, abcam, UK), CD91 (Rabbit Anti-LRP1 antibody, 1:20,000, abcam, UK), CD63 (1:1,000, abcam, UK), CD9 (1:2000, abcam, UK), β-catenin (1:1,000, Santa Cruz, CA, USA), Dkk1 (1:1,000, Proteintech, Chicago, IL, USA), GAPDH (1:2,000, Proteintech, Chicago, IL, USA). Then the membranes were incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG (abcam, UK) for

2 h at room temperature. The blots were developed with an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) and the bands were quantified using Bio-Rad imaging system (Hercules, CA, USA).

2.10 Statistical analysis

Data were expressed as mean \pm standard deviation (SD), and analyzed with Graph-Pad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical analyses were performed using one-way ANOVA, followed by Turkey's post-test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1 BMSC-exo can enter hFOB1.19 cells

Exosomes were extracted from BMSCs, and exosome related proteins were detected to verify the section of exosomes. The expression of CD63, CD81, CD9, ALIX and TSG101 was increased in extracted exosomes ($p < 0.05$) (Fig. 1A, B). As expected, compared to hFOB1.19 cells, the exosomes extracted from BMSCs showed higher miR-196a expression, while the expression of miR-196a further increased in the exosomes extracted from BMSCs transfected with miR-196a, but decreased in those transfected with miR-196a inhibitor ($p < 0.05$) (Fig. 1C). Flow cytometry revealed that the positive rate of CD9 and CD63 were 95.06% and 99.82%, respectively (Fig. 1D). Using PKH-67 to label BMSC-exo, we observed that BMSC-exo was taken by recipient hFOB1.19 cells ($p < 0.05$) (Fig. 1E).

3.2 miR-196a enriched exosomes promoted osteoblast differentiation

After exosomes were taken, alizarin red staining was performed to examine the effect of miR-196 on osteoblast differentiation. The results indicated a large number of calcified nodules in hFOB1.19 cells (NC group, Fig. 2A). Compared with control group, calcified nodules were significantly increased when miR-196a enriched exosomes were applied ($p < 0.05$), but decreased when miR-196a inhibitor exosomes were taken ($p < 0.05$). As shown in Fig. 2B, compared with NC group, the expression of RUNX2, ALP, OCN and OPN significantly increased in hFOB1.19 cells treated with the exosomes extracted from miR-196a mimic transfected BMSCs ($p < 0.05$), but markedly reduced in cells treated with the exosomes extracted from miR-196a inhibitor transfected BMSCs ($p < 0.05$). When BMSCs-exosome was taken, the percentage of hFOB1.19 cell apoptosis significantly decreased compared to the control group, the take-up of miR-196a enriched exosomes further decreased the percentage of cell apoptosis. When the expression of miR-196a in the exosomes was decreased, the percentage of cell apoptosis increased (Fig. 3A, B). The expression of apoptosis related proteins caspase-3 and caspase-6 showed the same trend ($p < 0.05$) (Fig. 3C, D).

3.3 Dkk1 is a direct target of miR-196a

Next, we used three publicly available algorithms, Target Scan, miRDB and microRNA to identify putative targets of miR-196a. Among potential targets we focused on Dkk1 because previous study revealed that Dkk1 was a target of miR-196^[17]. To confirm whether miR-196a targets Dkk1, we examined the effects of miR-196a mimic and inhibitor on luciferase activity in HFOB1.19 cells transfected with luciferase reporters containing wild type (WT) or mutant (Mut) 3' UTR of Dkk1. The results showed that luciferase activity of WT was significantly decreased by miR-196a mimic but increased by miR-196a inhibitor ($p < 0.05$) (Fig. 4A). Meanwhile, miR-196a mimics had no significant effect on luciferase activity of mutant construct. Moreover, we examined the effect of miR-196a mimic and inhibitor on miR-196a expression and Dkk1 protein expression in HFOB1.19 cells. The results demonstrated that miR-196a mimic decreased Dkk1 expression, while miR-196a inhibitor increased Dkk1 expression ($p < 0.05$) (Fig. 4B, C). In addition, miR-196a mimic increased miR-196a expression, while miR-196a inhibitor decreased miR-196a expression ($p < 0.05$) (Fig. 4D). These results confirmed that Dkk1 is a target of miR-196a.

3.4 Dkk1 knockdown promoted osteoblast differentiation

To investigate the role of Dkk1 in osteoblast differentiation, si-Dkk1 was transfected into BMSCs and the exosomes were extracted. The expression of Dkk1 was detected by Western blot analysis (Fig. 5A, B). In the exosomes extracted from BMSCs transfected by si-Dkk1, Dkk1 expression was significantly suppressed ($p < 0.05$). Alizarin red staining showed that when Dkk1 suppressed exosomes were taken by HFOB1.19 cells, osteoblast differentiation increased compared with cells treated by BMSCs-exosomes (Fig. 5C). The expression of osteoblast induction related proteins such as RUNX2, ALP, OCN and OPN showed the same trend ($p < 0.05$) (Fig. 5D).

3.5 BMSCs-exo targeted Dkk1 to activate Wnt/ β -catenin pathway in hFOB1.19 cells

Wnt/ β -catenin pathway play a key role in osteoblast differentiation, Dkk1 as a negative regulator of this pathway has been confirmed to be a direct target of miR-196a. Therefore, we detected the levels of Dkk1 and other components of Wnt/ β -catenin pathways in hFOB1.19 cells by Western blot analysis (Fig. 6A). Densitometry analysis showed that Dkk1 levels decreased significantly in cells treated with BMSCs-exosomes compared to control cells, and further decreased in cells treated with miR196a mimic (Fig. 6B). Consistently, Wnt, Dvl, β -catenin levels increased significantly while p-GSK3 β levels decreased significantly in cells treated with BMSCs-exosomes and miR196a mimic compared to control cells (Fig. 6C). Collectively, these results indicated that BMSCs-exo targeted Dkk1 to activate Wnt/ β -catenin pathway.

4. Discussion

Osteoporosis is a metabolic bone disease characterized by decreased bone mass and microstructural destruction of bone tissue, resulting in increased bone fragility and fracture. ^[18] The proliferation and

differentiation of BMSCs are closely related to bone metabolism. One aspect of osteoporosis is low potential of osteoblast differentiation^[19].

The growing evidence has revealed that miRNAs play important role in osteoblast differentiation. Kim et al.^[20] found that miR-196a positively regulated osteoblast differentiation but the mechanism remained unclear. Exosomes can directly transfer various bioactive molecules including mRNAs, microRNAs and proteins from donate cells to recipient cells. In this study we found that BMSC-exo can be taken by recipient hFOB1.19 cells, and osteoblast differentiation of hFOB1.19 was promoted by BMSC-exo. Moreover, osteoblast differentiation was enhanced when miR-196a enriched BSMCs-exo was taken up, but was antagonized when miR-196a was deprived. Therefore, miR-196a as one of the osteoblast-related miRNAs found enriched in BMSC-exo^[13], may play an indispensable role in osteoblast differentiation. Moreover, we found that miR-196a directly inhibited the expression of its target Dkk1, a known negative regulator of Wnt/ β -catenin pathway^[21, 22].

Osteoblast differentiation involves multiple signaling pathways. Wnt signaling participates in cell proliferation, differentiation, migration, apoptosis to maintain the dynamic balance of cells^[23, 24]. We found that Dkk1 was downregulated while Wnt/ β -catenin signaling was activated when BMSCs-exo was delivered to hFOB1.19 osteoblast cells. Taken together, these results suggest that BMSCs-exo promotes osteoblast differentiation by delivering miR-196a which activates Wnt/ β -catenin signaling via targeting Dkk1. BMSCs-exo could be considered as a new therapeutic approach to alleviate osteoporosis.

In conclusion, miR-196a is an essential exosome component of BMSCs-exo. The delivery of miR-196a by BMSCs-exo could target Dkk1 and activate Wnt/ β -catenin signaling, leading to enhanced osteoblast differentiation.

Abbreviation

OP	Osteoporosis
BMSCs	Bone marrow mesenchymal stem cells
TGF- β	transforming growth factor- β
BMP-9	bone morphogenetic protein-9
Runx2	runt-related transcription factor -2
Osx	Osterix
ALP	alkaline phosphatase
miRNAs	microRNAs
BMSC-exo	BMSC-derived exosomes
Dkk1	Dickkopf-1
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
PI	propidium iodide
PBS	phosphate buffer saline
PVDF	polyvinylidene fluoride
SD	standard deviation
WT	wild type
Mut	mutant

Declarations

Ethics approval and consent to participate

Applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

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Author Contributions

Conceptualization, Zhi Peng; Data curation, Zhi Peng, Kaishun Yang and Sheng Lu; Investigation, Zhi Peng, Zhenkai Lou, Zhongjie Li, Shaobo Li, Kaishun Yang and Chao Li; Methodology, Zhi Peng, Zhenkai Lou and Sheng Lu; Project administration, Zhenkai Lou; Resources, Zhongjie Li; Writing–original draft, Chao Li; Writing–review & editing, Chao Li.

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Figures

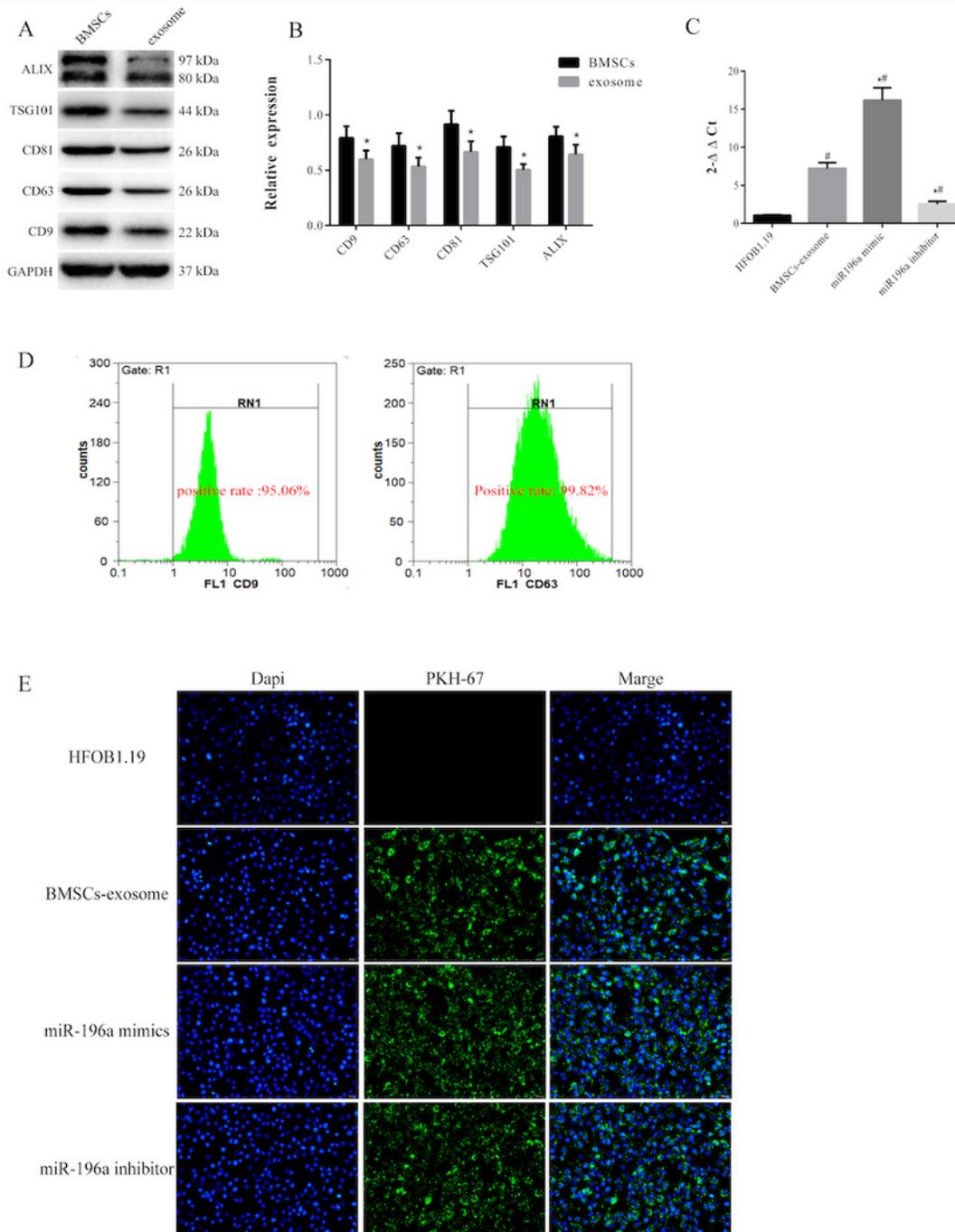


Figure 1

The identification of BMSCs exosomes. (A-B) The expression of exosomes section associated protein was detected. (C) The relative expression of miR-196a was detected in different exosomes. (D) The positive rat of exosomes section associated protein CD9 and CD63. Data were mean \pm SD. n = 6. * P < 0.05, compared to sham group. (E) BMSC-exo was isolated and labeled by PKH-67 to observe the intake of exosomes. (40*) Blue represented Dapi staining, green represented PKH-67 staining. n = 6.

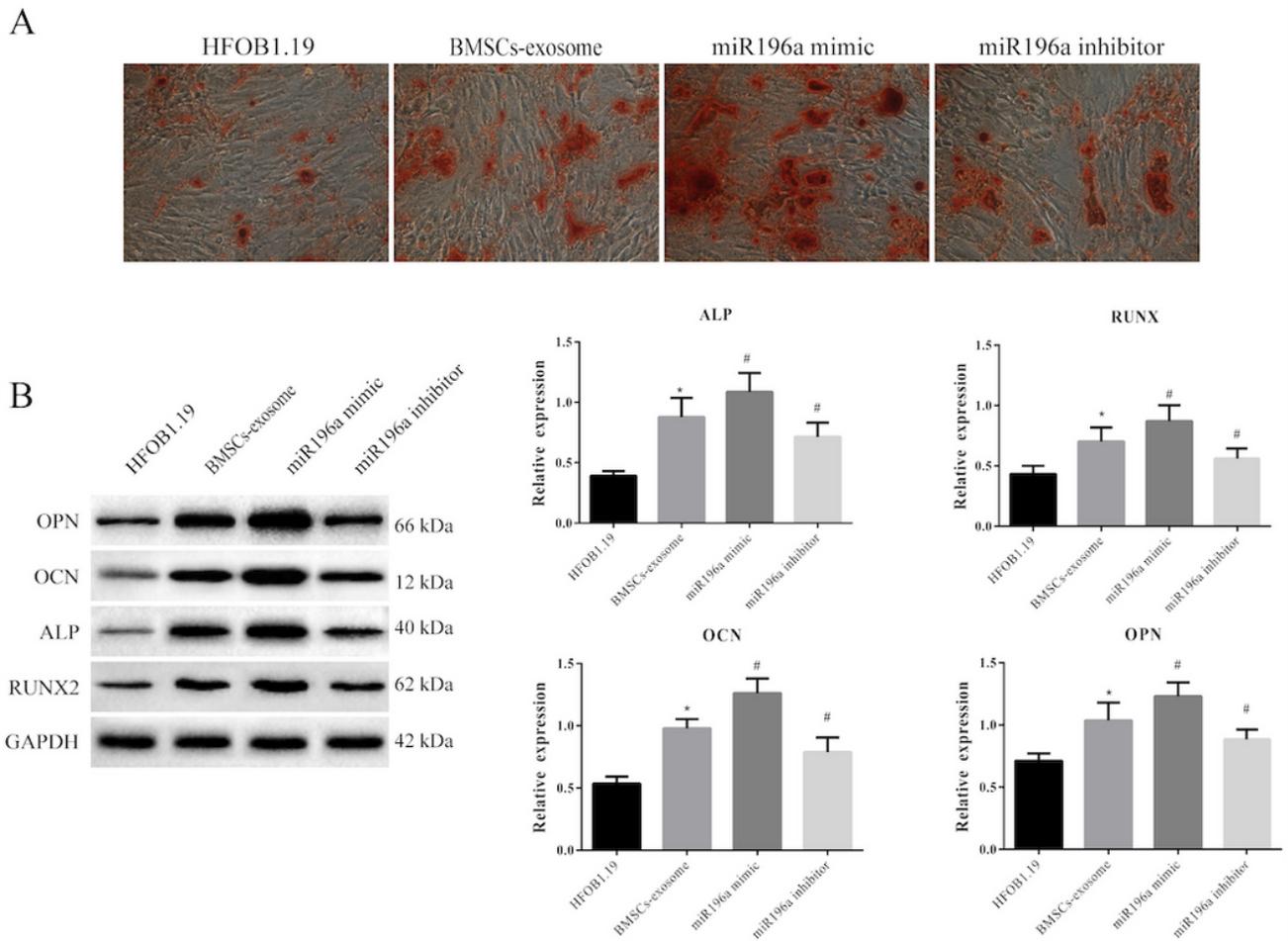


Figure 2

miR-196a enriched exosomes promoted osteoblast differentiation. (A) Osteoblast differentiation of hFOB1.19 cells was detected by alizarin red staining. (B) The expression of osteogenic related genes RUNX2, ALP, OCN, and OPN in hFOB1.19 cells treated with or without BMSC-exo. Data were mean \pm SD. n = 6. * P < 0.05, compared to sham group.

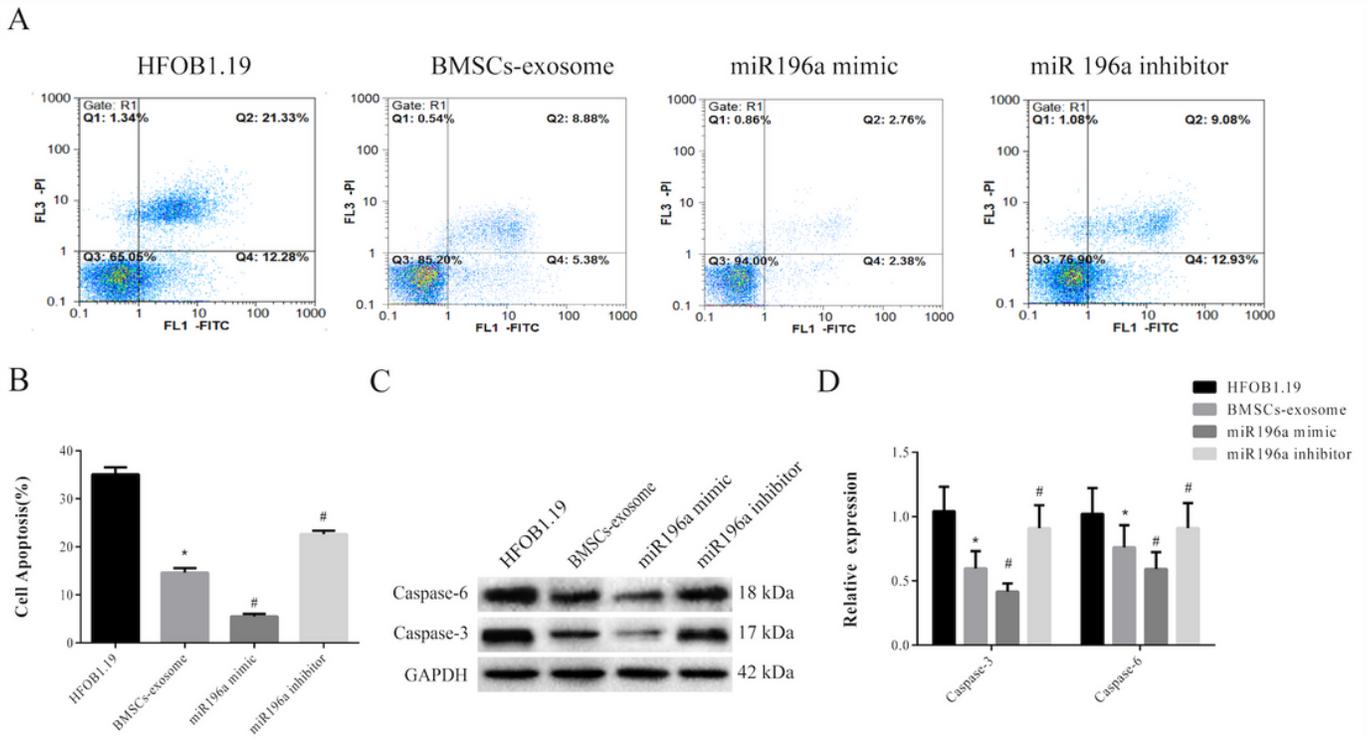


Figure 3

miR-196a enriched exosomes suppressed the apoptosis of hFOB1.19 cells. (A-B) Cell apoptosis was detected by flow cytometry. (C-D) The expression of apoptosis associated proteins caspase-3 and caspase-6. Data were mean \pm SD. n = 6. * P < 0.05, compared to sham group.

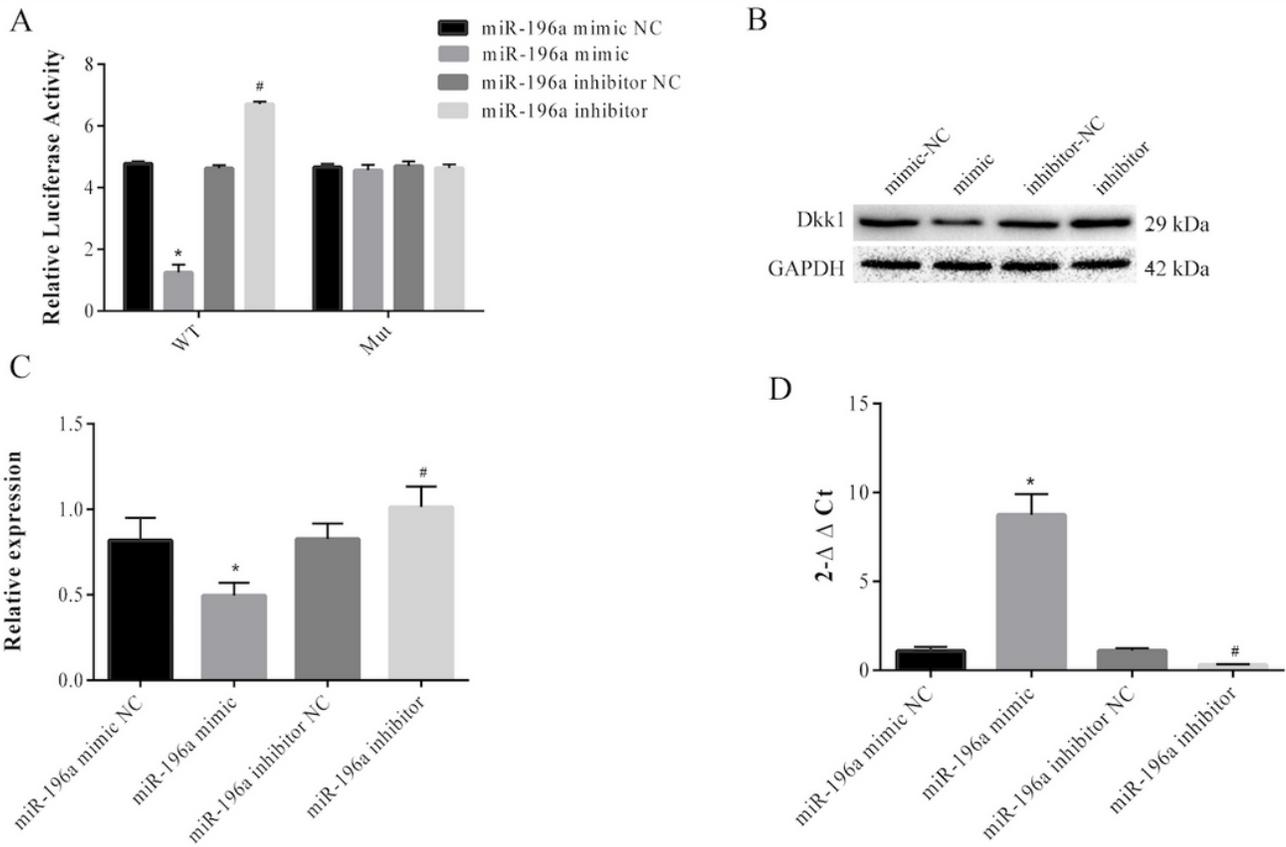


Figure 4

miR-196a directly targeted Dkk1. (A) The effects of miR-196a mimics, inhibitor and NC on relative luciferase activity in hFOB1.19 cells transfected with wild type (WT) or mutant (MUT) Dkk1 3'-UTR. (B, C) Effects of miR-196a mimics, inhibitor and NC on Dkk1 expression detected by Western blot analysis. (D) Effects of miR-196a mimics, inhibitor and NC on miR-196a level detected by qRT-PCR. Data were mean \pm SD. n = 6. * P < 0.05, compared to sham group.

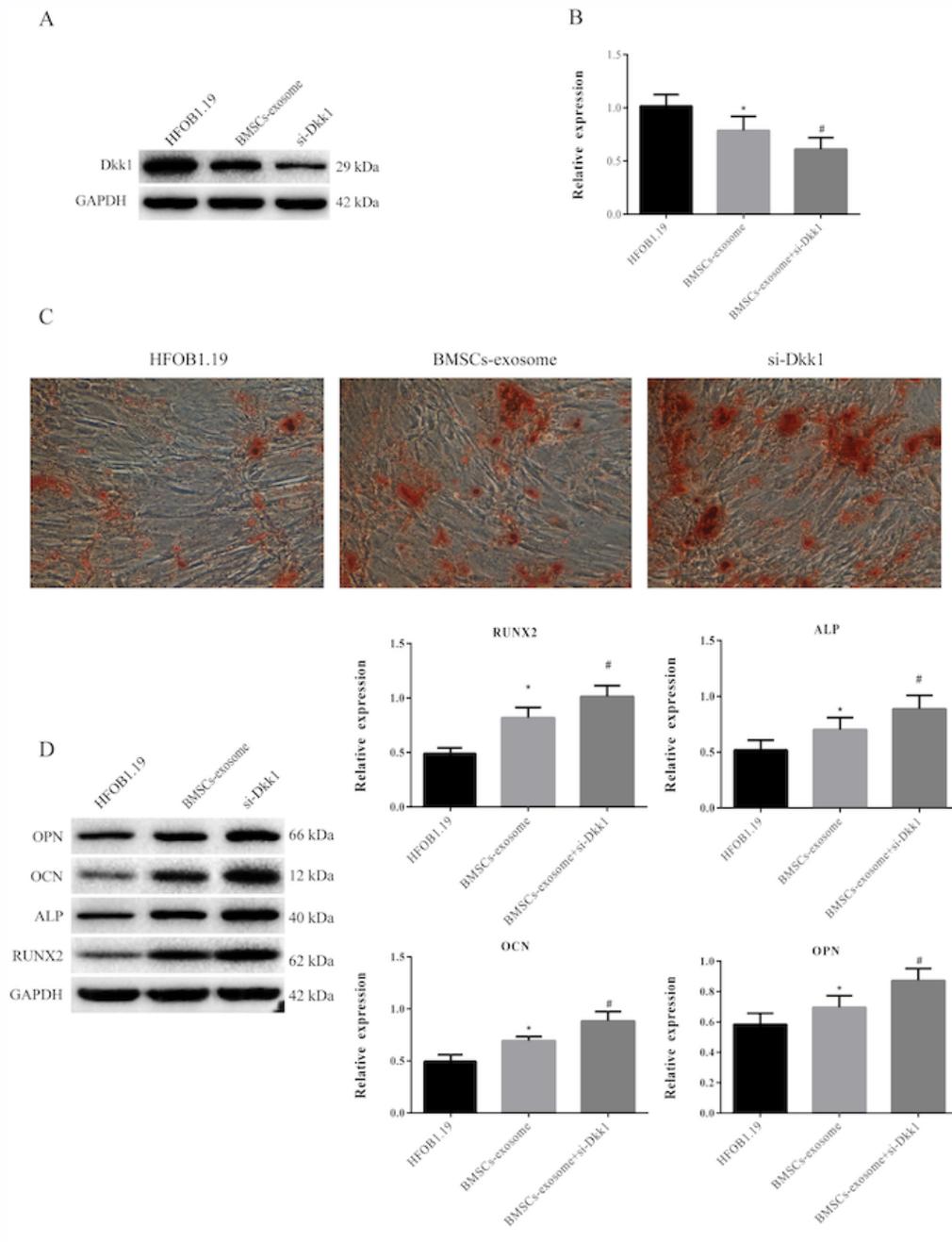


Figure 5

Dkk1 knockdown promoted osteoblast differentiation. (A-B) Effect of si-Dkk1 on Dkk1 protein expression. (C) Osteoblast differentiation was detected by alizarin red staining. (D) Effect of si-Dkk1 on osteogenic related genes RUNX2, ALP, OCN, and OPN in hFOB1.19 detected by Western blot analysis. Data were mean \pm SD. n = 6. * P < 0.05, compared to sham group.

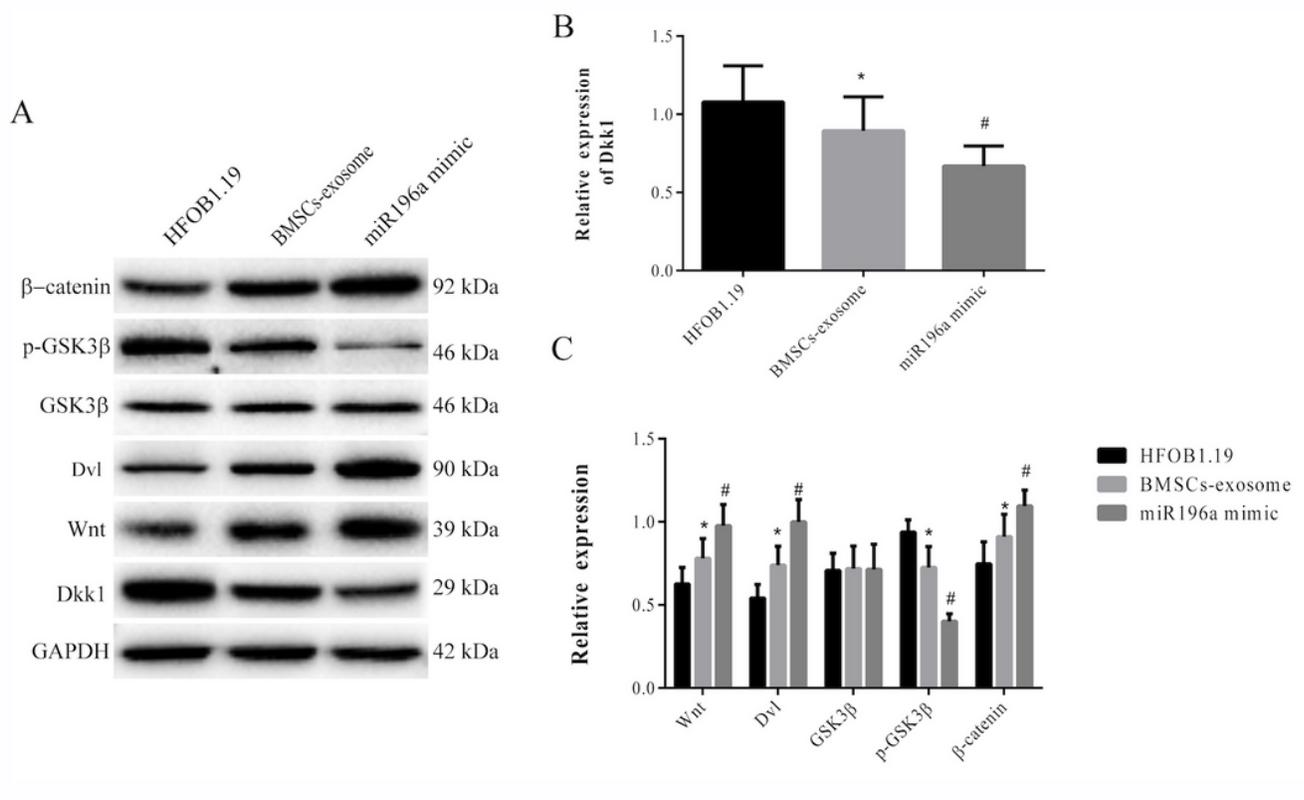


Figure 6

BMSCs-exo inhibited Dkk1 to activate Wnt/ β -catenin pathway in hFOB1.19 cells. (A) Western blot analysis of protein levels in hFOB1.19 cells. (B) Densitometry analysis of Dkk1 levels. (C) Densitometry analysis of Wnt, Dvl, GSK3 β , p-GSK3 β and β -catenin levels. Data were mean \pm SD. n = 6. *, P < 0.05, compared to sham group