

Insulin growth factor-1 promotes the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells through the Wnt/ β -catenin pathway

JING FENG¹ and ZHIQIANG MENG²

¹Jiaozuo Coal Industry (Group) Co. Ltd. Central Hospital, Jiaozuo, Henan 454000; ²Department of Orthopedics, The General Hospital of Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region 750004, P.R. China

Received October 31, 2020; Accepted May 24, 2021

DOI: 10.3892/etm.2021.10323

Abstract. Bone marrow mesenchymal stem cells (BMSCs) are stem cells that exist in bone marrow tissue and have osteogenic differentiation potential. Insulin growth factor-1 (IGF-1) plays a key role in the proliferation and osteogenic differentiation of BMSCs. However, the specific mechanism of IGF-1 in cell proliferation and osteogenic differentiation remains unclear. In the present study, BMSCs were transfected with lentivirus carrying the siRNA-Wnt3a gene, and the Wnt3a level in BMSCs was revealed to be reduced by western blotting, real-time quantitative polymerase chain reaction and immunofluorescence detection. Then, BMSCs were treated with 80 ng/ml IGF-1 in complete medium for 5 days. CCK-8 and cell cycle assays revealed that cell proliferation was significantly decreased in the siRNA-Wnt3a group than in the control group. The protein and mRNA levels of β -catenin and cyclin D1 were significantly downregulated in the siRNA-Wnt3a group compared with the control group. In addition, BMSCs were treated with IGF-1 in osteogenic differentiation medium for 7 and 21 days, and alkaline phosphatase staining and Alizarin Red staining demonstrated significantly reduced osteogenic differentiation ability in the siRNA-Wnt3a group compared with the control group. Furthermore, the protein and mRNA levels of β -catenin, RUNX2, and OPN were downregulated compared with the control group. Our findings revealed that IGF-1 promoted the proliferation and differentiation of BMSCs at least partially through the Wnt/ β -catenin pathway. These findings provided new insight into the clinical treatment of bone disease.

Introduction

Bone marrow mesenchymal stem cells (BMSCs), nonhematopoietic stem cells that exist in bone marrow tissue, were first reported by Friedenstein in the 1970s (1). BMSCs have the ability to differentiate into adipocytes, osteoblasts, skeletal muscle cells, chondrocytes, smooth muscle cells, tendon cells, hematopoietic support stroma cells and other mesodermal cells under certain conditions. The ability to differentiate into bone cells is important for bone metabolism, and there is evidence that abnormal differentiation of BMSCs is closely related to the occurrence of osteoporosis (2). After BMSCs differentiate into osteoblasts, their bone matrix synthesis, secretion and mineralization abilities are enhanced, thereby resulting in bone regeneration. Studies have demonstrated that transplanting cell culture media containing BMSCs into the bone defect site can promote bone defect repair (3-5). This osteogenic differentiation characteristic of BMSCs provides new treatment ideas for nonunion, bone defects, and osteolytic bone disease.

Insulin growth factor-I (IGF-1) is a single-chain basic polypeptide growth factor containing 70 amino acid residues that plays an important role in regulating cell proliferation, differentiation, and apoptosis (6). At present, IGF-1 is considered to be widely involved in bone growth and development. It has been reported that severe IGF-1 deficiency can cause short stature, and IGF-1 can significantly improve this condition (7). A previous study has confirmed that IGF-1 is positively correlated with bone density and that IGF-1 in the blood of patients with osteoporosis is significantly reduced (8). Studies have also confirmed that the development of IGF-1 knockout in mice is related to a decrease in the number of osteoblasts and a decrease in bone formation ability (9,10). Moreover, under pathological conditions, IGF-1 has been revealed to promote the mineralization of mesenchymal stem cells, thereby promoting fracture healing (11). However, the molecular mechanism by which IGF-1 promotes the proliferation and differentiation of BMSCs remains unclear.

The Wnt/ β -catenin pathway is currently widely investigated for its role in the pathogenesis of bone system-related diseases and bone metabolism. It has an important effect on

Correspondence to: Dr Zhiqiang Meng, Department of Orthopedics, The General Hospital of Ningxia Medical University, 804 Shengli South, Yinchuan, Ningxia Hui Autonomous Region 750004, P.R. China
E-mail: 243341001@qq.com

Key words: insulin growth factor-1, bone marrow mesenchymal stem cells, Wnt/ β -catenin, proliferation, osteogenic differentiation

bone remodeling and has the potential to control the differentiation direction of BMSCs (12). The Wnt family is composed of 19 highly conserved cysteine-rich secreted glycoproteins. In the Wnt/ β -catenin pathway, the Wnt protein first binds to the low-density lipoprotein receptor-related protein (LRP) 5/6 on the cell surface and to the frizzled protein Fzd complex to promote β -catenin polymerization and entry into the nucleus (13).

In the present study, the lentivirus carrying the siRNA-Wnt3a gene was transfected into BMSCs to cause low expression of the Wnt3a gene in BMSCs and then cells were treated with IGF-1. The aim of the present study was to explore the role of the Wnt/ β -catenin pathway in IGF-1 in promoting the proliferation and osteogenic differentiation of BMSCs.

Materials and methods

Cell culture. C57BL/6 mouse BMSCs were purchased from Cyagen Biosciences, Inc. BMSCs were placed in a complete medium, which consisted of basal medium (Cyagen Biosciences, Inc.), special 10% fetal bovine serum (FBS; Cyagen Biosciences, Inc.), 1% penicillin-streptomycin dual antibiotic solution and 1% glutamine. Cells were cultured at 37°C in an incubator containing 5% CO₂. When cell confluence reached 80-90%, the cells were digested and passaged at a ratio of 1:2. The cells were maintained in culture for no more than 10 passages.

Induction and identification of osteogenic differentiation. When the cells reached 80% confluence, they were incubated (37°C, 5% CO₂) with osteogenic induction medium containing 80 ng/ml IGF-1 (PeproTech, Inc.), Dulbecco's modified Eagle's medium [(DMEM)/F12 supplemented with 10% FBS, 0.25 mmol/l ascorbic acid, 10 mmol/l sodium β -glycerophosphate, 10⁻⁷ mol/l dexamethasone], and the medium was changed once every 2 days on average. Osteogenesis was monitored continuously, and 3 weeks after induction, the cells were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% Alizarin Red (Cyagen Biosciences, Inc.), and observed under a fluorescence microscope. Images were captured at 400 X magnification.

Drug toxicity test. BMSCs in the logarithmic growth phase were digested and resuspended, and 100 μ l of cell suspension (1x10⁴/ml) was added to a 96-well plate. The cells were incubated at 5% CO₂, 37°C for 12 h, and then treated with different concentrations of IGF-1 (0, 40, 60, 80 and 100 ng/ml) with 5 replicate wells for each concentration, after which the cells were cultured for 5 days. Then, a total of 10 μ l of Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc.) was added to each well on days 1, 2, 3, 4, and 5. After incubation for 2 h, a microplate reader was used to obtain the OD value for each group of cells at a wavelength of 450 nm.

Lentiviral transfection of BMSCs and cell grouping. BMSCs were transfected with a multiplicity of infection (MOI) of 25, 50, 100, and 200 respectively, and 200 was found to be the best MOI. BMSCs in good condition were selected and inoculated on 24-well plates at a density of 1x10⁴ cells/well and incubated

overnight at 37°C. After 24 h, recombinant LV-small interfering (si)RNA-Wnt3a-mus (forward: AGTGCCTCGGAG ATGGTGGTAG; reverse: GGGTTAGGTTTCGAGAAG TTGGG) lentivirus (GenePharma Co., Ltd.) or empty vector (GenePharma Co., Ltd.) lentivirus was added to the medium at a multiplicity of infection of 200 (MOI=200). The normal group was cultured with complete medium without adding lentivirus. After 96 h of infection, green fluorescent protein (GFP) was observed at x400 under an inverted fluorescence microscope (Olympus Corporation). Two groups were used: IGF-1 + EV (empty vector) and IGF-1 + siRNA-Wnt3a.

CCK-8 assay for detection of cell proliferation. A total of 1x10⁴ cells/ml in good condition were inoculated on a 96-well plate, after which 100 μ l of complete medium containing 80 ng/ml IGF-1 was added to 5 replicate wells per group, and the cells were incubated in 5% CO₂ at 37°C. Then, a total of 10 μ l of CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well on days 1, 2, 3, 4, and 5. After incubation for 2 h, a microplate reader was used to obtain the OD value at a wavelength of 450 nm for each group of cells.

Cell cycle detection. Cells in logarithmic growth phase were digested and centrifuged at 500 x g for 5 min at room temperature. Approximately 1x10⁶ cells in each group were collected and washed twice with PBS. Then, the supernatant was discarded, and the cells were fixed with precooled 75% ethanol overnight at 4°C. Next, the cells were washed 2 times with PBS, after which the supernatant was discarded, and the cells were incubated with 500 μ l of PI/RNase (1:9) Staining Buffer (Nanjing KeyGen Biotech Co., Ltd.) for 60 min at room temperature. The proportions of cells in the cell cycle (G1, S and G2 phases) were detected by flow cytometry (cat. no. A00-1-1102; Beckman Coulter, Inc.).

Western blotting (WB). Each group of cells was collected, total protein was extracted with RIPA lysis buffer (KeyGen Biotech Co., Ltd.), and the protein concentration was measured by the BCA method. The proteins (50 μ g/well) were used for 10% SDS-PAGE, then transferred onto a PVDF membrane. Primary antibodies against runt-related transcription factor 2 (RUNX2; 1:1,000; product code ab23981), Wnt3a (1:1,000; product code ab219412), β -catenin (1:1,000; product code ab32572; all from Abcam), GAPDH (1:2,000; cat. no. bsm-52262R; BIOSS), cyclin D1 (1:10,000; product code ab134175) and osteopontin (OPN; 1:1,000; product code ab63856; both from Abcam) were incubated overnight at 4°C. The membrane was washed 3 times with TBST (1%), incubated at room temperature for 2 h with an HRP-labeled secondary antibody (1:5,000; cat. no. bs-40295G-HRP; BIOSS) and washed again with TBST. The ECL (KeyGen Biotech Co., Ltd.) signal was detected after exposure in a darkroom for approximately 3 min. Imaging was performed using a Bio-Rad imaging system (Bio-Rad Laboratories, Inc.), and the ratio of the target protein level to GAPDH was calculated. The experiment was repeated 3 times.

RNA extraction and real-time quantitative polymerase chain reaction (qPCR). A total RNA extraction kit (Omega Bio-Tek, Inc.) was used to extract total cell RNA according

Table I. Primers used for qPCR.

Gene	Primer Sequence (5'–3')
<i>Wnt3a</i>	F: AGTGCCTCGGAGATGGTGGTAG R: GGGTTAGGTTTCGAGAAGTTGGG
<i>β-catenin</i>	F: GCTCTTCGTCATCTGACCAGC R: GAGCAAGTTCACAGAGGACC
<i>Cyclin D1</i>	F: CGTATCTTACTTCAAGTGC GTG R: ATGGTCTCCTTCATCTTAGAGG
<i>RUNX2</i>	F: TTTAGGGCGCATTTCCTCATC R: TGTCTTGTGGATTA AAAAGGACTTG
<i>OPN</i>	F: GCAGACACTTTCCTCAATCG R: GGGACTCCTTAGACTCACCGC
<i>GAPDH</i>	F: TGTGTCCGTCGTGGATCTGA R: TTGCTGTTGAAGTCGCAGGAG

qPCR, quantitative polymerase chain reaction; F, forward; R, reverse; RUNX2, runt-related transcription factor 2; OPN, osteopontin.

to the manufacturer's instructions. RNA concentration was measured and reverse transcribed, according to the Omega kit instructions. The detection primers for *Wnt3a*, β -catenin, cyclin D1, RUNX2, OPN, and GAPDH were designed and synthesized by Sangon Biotech, Co., Ltd. (Table I). The qPCR amplification process was: Denaturation at 94°C for 30 sec, annealing at 60°C for 15 sec and extension at 72°C for 10 sec, for 45 cycles. qPCR was performed using green PCR SuperMix (TransGen, Biotech Co., Ltd.) on an Analytik Jena GmbH instrument (Qtower3G). GAPDH was used as the internal reference gene, and the target gene expression level obtained using the $2^{-\Delta\Delta CT}$ method (14).

Immunofluorescence (IF). After BMSCs were cultured on a 24-well plate at 37°C for one week, *Wnt3a*, β -catenin, cyclin D1, RUNX2, and OPN were detected by IF. The culture medium was discarded, and the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, rinsed 3 times with PBS, permeabilized with 1% Triton X-100 for 15 min and blocked with 1% bovine serum albumin for 30 min at room temperature. Then, the samples were washed and incubated overnight at 4°C with primary antibodies against *Wnt3a* (1:100), β -catenin (1:250), cyclin D1 (1:50), RUNX2 (1:400) and OPN (1:500). Subsequently, the cells were incubated with a fluorescence-conjugated secondary antibody (1:800; cat. no. SA00007-2; Proteintech, Inc.) for 1.5 h at room temperature. Nuclei were stained with DAPI (OriGene Technologies, Inc.) for 3 min at room temperature. The presence of the proteins was examined under a fluorescence microscope (Olympus Corporation) at x400.

Alkaline phosphatase (ALP) staining. Cells in good condition were selected, plated on 24-well plates, and cultured (37°C, 5% CO₂) in osteogenic differentiation medium containing 80 ng/ml IGF-1 for 7 days. The solution was discarded, after which the cells were rinsed twice with PBS, incubated with ALP fixative solution for 3 min at room temperature, and

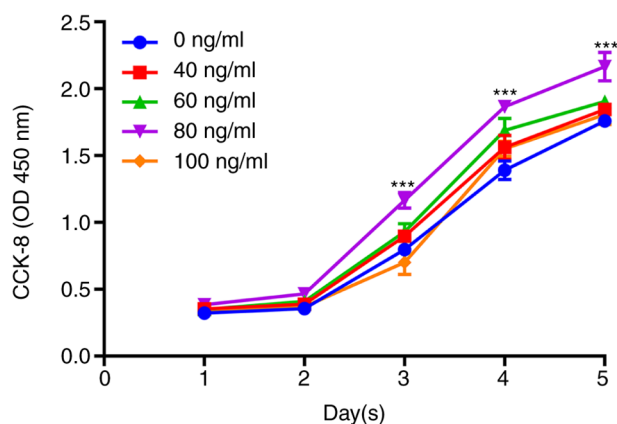


Figure 1. CCK-8 detects the cytotoxicity of BMSCs at various concentrations of IGF-1. A concentration 80 ng/ml was the optimal IGF-1 concentration. ***P<0.001 vs. 0 ng/ml. CCK-8, Cell Counting Kit-8; BMSCs, bone marrow mesenchymal stem cells; IGF, insulin growth factor; OD, optical density.

rinsed again with PBS. Then, the cells were incubated with the prepared 3% ALP incubation solution (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min in the dark at 37°C, rinsed with PBS, counterstained with nuclear fast red staining solution (Beijing Solarbio Science & Technology Co., Ltd.) for 5 min at room temperature, rinsed with PBS again, and observed under a fluorescence microscope (Olympus Corporation) at x400.

Alizarin Red staining (ARS). Cells in good condition were selected, plated on 6-well plates, and cultured (37°C, 5% CO₂) in osteogenic differentiation medium containing 80 ng/ml IGF-1 for 3 weeks. After the culture medium was discarded, the cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 30 min, rinsed twice with PBS, incubated with a 0.2% Alizarin Red (Cyagen Biosciences, Inc.) solution for 5 min at room temperature and rinsed again with PBS. The cells were placed under an inverted fluorescence microscope (Olympus Corporation) for observation at x400.

Statistical analysis. Quantitative data are expressed as the means \pm standard deviation. The experiment was repeated 3 times, and GraphPad Prism 8 software (GraphPad Software Inc.) was used for data analysis. Comparisons of the mean differences between groups were performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test, and P<0.05 was considered to indicate a statistically significant difference.

Results

Optimal drug concentration of IGF-1. Drug toxicity was detected by CCK-8 assay. It was revealed that cell proliferation was increased with increasing IGF-1 concentration. When the concentration was 80 ng/ml, cell proliferation was the highest, and the difference was statistically significant (P<0.001); a concentration of 100 ng/ml resulted in decreased cell proliferation (Fig. 1). Therefore, 80 ng/ml was the optimal drug concentration of IGF-1 used in subsequent experiments.

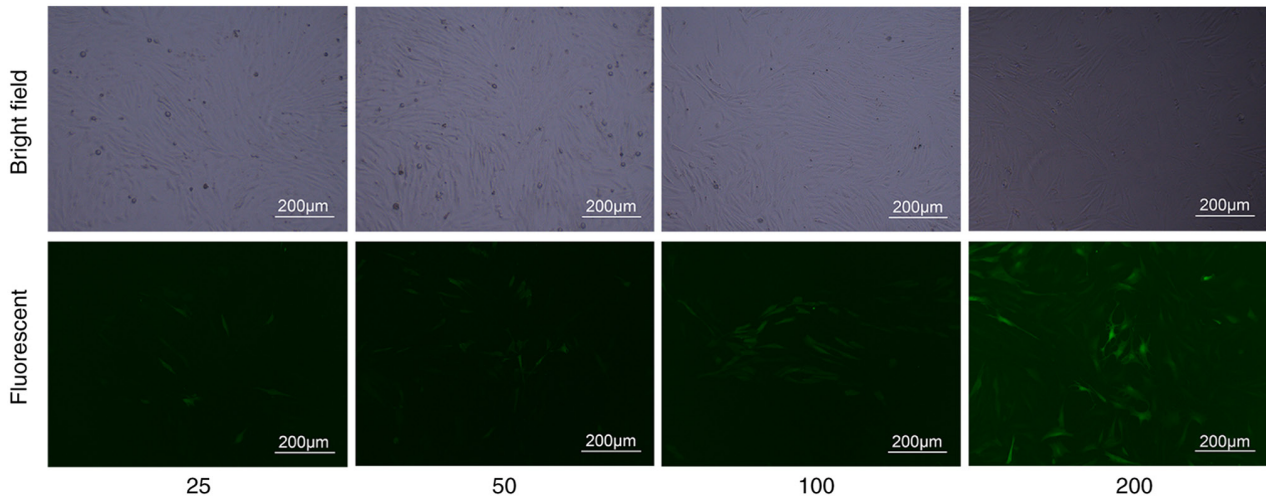


Figure 2. MOI value determination during lentivirus transfection. When the MOI value was 200, the fluorescence area reached 80-90%. MOI, multiplicity of infection.

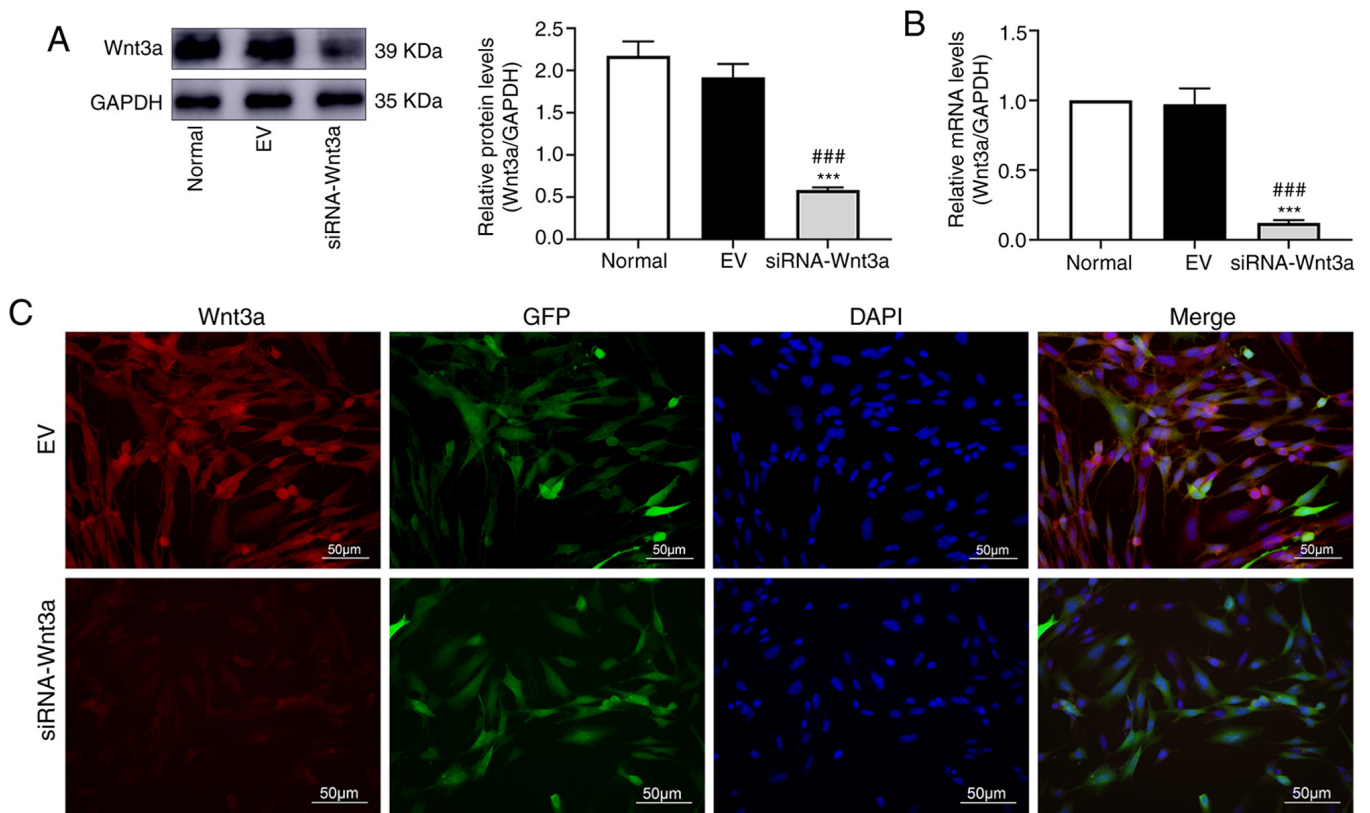


Figure 3. BMSCs are transfected with lentivirus carrying siRNA-Wnt3a. (A) Western blotting of the protein expression level of Wnt3a. (B) Quantitative polymerase chain reaction was used to detect the RNA expression level of Wnt3a. (C) Immunofluorescence was used to detect the expression level of Wnt3a. ***P<0.001 vs. the EV group; ###P<0.001 vs. the normal group. BMSCs, bone marrow mesenchymal stem cells; si, small interfering; EV, empty vector.

Lentiviral transfection. BMSCs were transfected with an MOI of 25, 50, 100, and 200 for 96 h. When the MOI was 200, the cells with green fluorescence reached 90%. (Fig. 2). In the subsequent experiment, transfection was performed with an MOI of 200.

BMSCs are transfected with lentivirus carrying siRNA-Wnt3a. BMSCs were transfected with lentivirus

carrying siRNA-Wnt3a or empty vector. WB revealed that in the siRNA-Wnt3a group, the Wnt3a protein level was significantly lower than that in the empty vector group and the normal group (P<0.001 vs. both groups), but the difference between the empty vector group and the normal group was not significant (Fig. 3A). It was revealed by qPCR that the RNA level of Wnt3a in the siRNA-Wnt3a group was significantly lower than that in the empty vector group and the

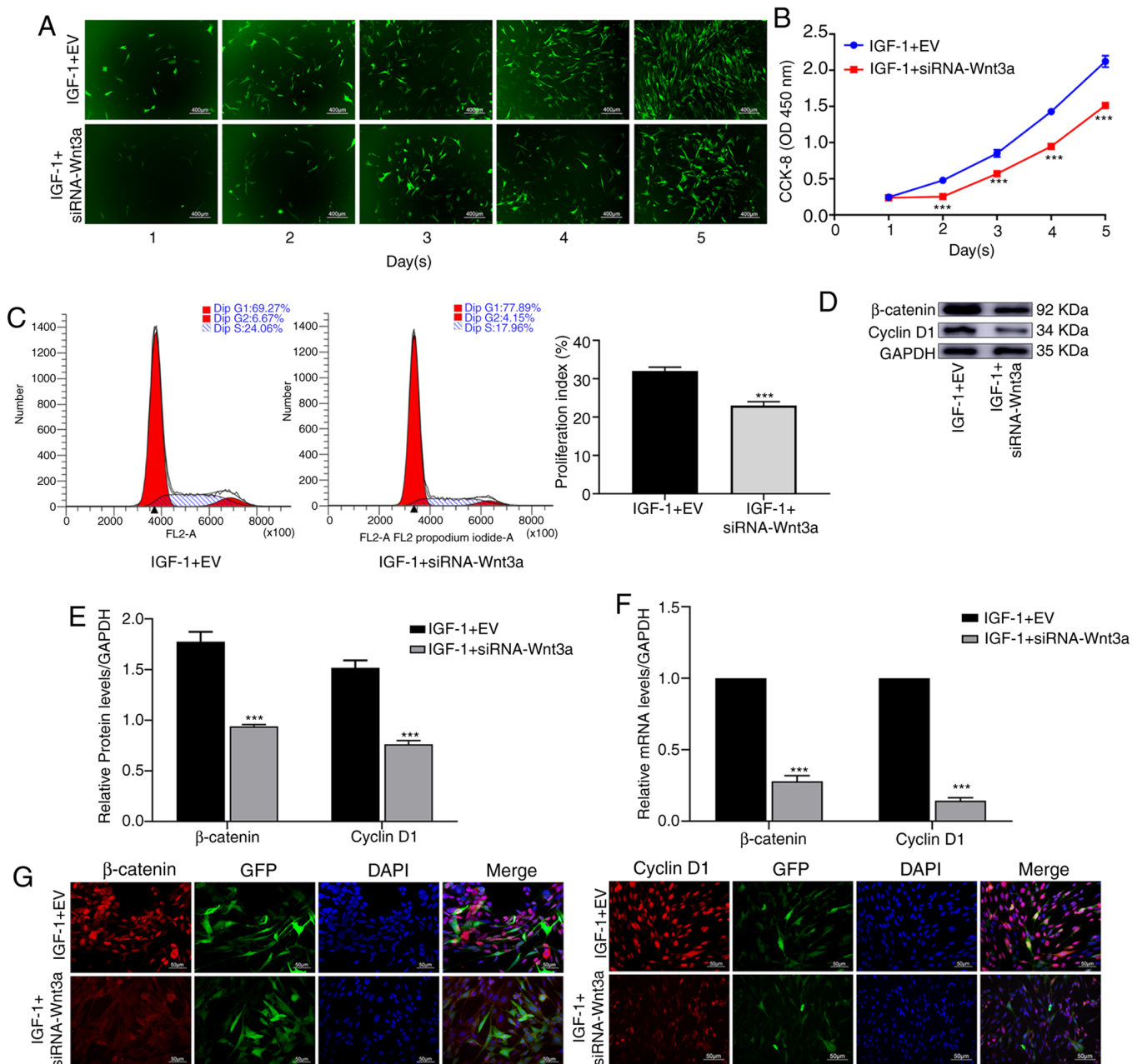


Figure 4. By inhibiting the Wnt/ β -catenin pathway, IGF-1 reduces the proliferation of BMSCs. BMSCs were transfected with empty vector or the siRNA-Wnt3a gene. Then, the cells were treated with IGF-1 (80 ng/ml). (A) The proliferation of the cells for 5 consecutive days using a fluorescence microscope. (B) CCK-8 was used to detect the proliferation of BMSCs. (C) Cell cycle detection assays were used to detect the proliferation of BMSCs. (D and E) β -Catenin and cyclin D1 protein expression was determined by western blotting. (F) The relative mRNA expression of β -catenin and cyclin D1 was evaluated by quantitative polymerase chain reaction. (G) Immunofluorescence was used to detect the expression of β -catenin and cyclin D1 in BMSCs. *** $P < 0.001$ vs. IGF-1 + EV. IGF, insulin growth factor; BMSCs, bone marrow mesenchymal stem cells; CCK-8, Cell Counting Kit-8; si, small interfering; EV, empty vector.

normal group ($P < 0.001$ vs. both groups), with no significant difference between the empty vector group and the normal group (Fig. 3B). In subsequent experiments, the empty vector group served as the control group. IF also revealed that the Wnt3a protein expression level in the siRNA-Wnt3a group was significantly lower than that in the empty vector group (Fig. 3C).

After blocking Wnt3a, IGF-1 decreases the proliferation of BMSCs. Cells in each group were treated with IGF-1. It was revealed that in the IGF-1 + siRNA-Wnt3a group, cell proliferation was significantly slower than that in the IGF-1 + EV group

(Fig. 4A). The CCK-8 assay verified that cell proliferation in the IGF-1 + siRNA-Wnt3a group was significantly decreased ($P < 0.001$) (Fig. 4B). Flow cytometry was used to investigate the cell cycle, and the proliferation index was revealed to be significantly different between the two groups ($P < 0.001$) (Fig. 4C). WB revealed that the protein levels of β -catenin and cyclin D1 in the IGF-1 + siRNA-Wnt3a group were significantly lower than those in the control group ($P < 0.001$) (Fig. 4D and E). Based on qPCR, RNA levels of β -catenin and cyclin D1 were significantly lower in the IGF-1 + siRNA-Wnt3a group than in the control group ($P < 0.001$) (Fig. 4F). IF also confirmed that the fluorescence intensity of β -catenin and cyclin D1 in the

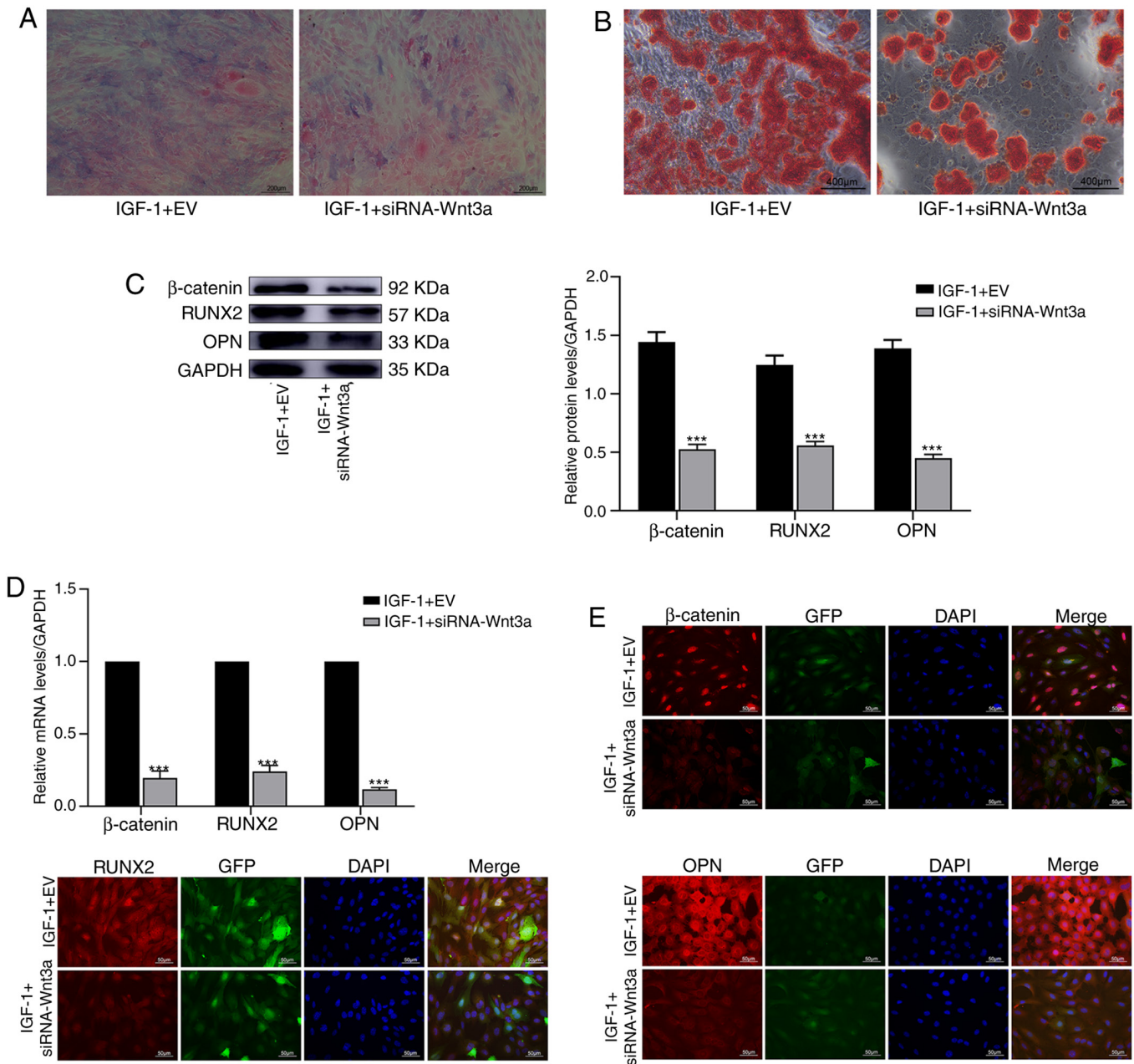


Figure 5. By inhibiting the Wnt/ β -catenin pathway, IGF-1 reduces the osteogenic differentiation of BMSCs. BMSCs were transfected with empty vector or the siRNA-Wnt3a gene. Then, the cells were treated with IGF-1 (80 ng/ml). (A) Alkaline phosphatase staining of BMSCs for 1 week. (B) Alizarin Red staining of BMSCs for 3 weeks. (C) β -catenin, RUNX2 and OPN protein expression was determined by western blotting for 1 week. (D) The relative mRNA expression of β -catenin, RUNX2, and OPN was evaluated by quantitative polymerase chain reaction for 1 week. (E) Immunofluorescence was used to detect the expression of β -catenin, RUNX2 and OPN in BMSCs for 1 week. *** $P < 0.001$ vs. IGF-1 + EV. IGF, insulin growth factor; BMSCs, bone marrow mesenchymal stem cells; RUNX2, runt-related transcription factor 2; OPN, osteopontin; si, small interfering; EV, empty vector.

IGF-1 + siRNA-Wnt3a group was significantly lower than that in the control group (Fig. 4G).

After blocking Wnt3a, IGF-1 decreases the osteogenic differentiation of BMSCs. Cells of each group were stained with ALP for 7 days after osteogenic differentiation and staining in the IGF-1 + siRNA-Wnt3a group was weaker than that in the control group (Fig. 5A). Cells were also stained with ARS for 21 days after osteogenic differentiation, which revealed that the number and area of reddish-brown mineralized nodules in the IGF-1 + siRNA-Wnt3a group were markedly lower than those in the control group (Fig. 5B). WB and qPCR revealed that

the protein and RNA levels of β -catenin, RUNX2 and OPN were significantly different between the two groups ($P < 0.001$) (Fig. 5C and D). IF indicated that the fluorescence intensity of β -catenin, RUNX2 and OPN was markedly weaker in the IGF-1 + siRNA-Wnt3a group than in the IGF-1 + EV group (Fig. 5E).

Discussion

In the present study, it was revealed that IGF-1 enhanced the proliferation and osteogenic differentiation of BMSCs. When Wnt3a gene expression in BMSCs was inhibited, the ability of

IGF-1 to promote the proliferation and osteogenic differentiation of BMSCs was reduced. A study has confirmed that the Wnt/ β -catenin pathway plays an important role in the process by which IGF-1 promotes the proliferation and osteogenic differentiation of BMSCs (15).

BMSCs are nonhematopoietic stem cells with multilineage differentiation potential that exist in a variety of tissues (16). In particular, their ability to differentiate into bone cells has become a research focus in recent years. IGF-1 is a type of growth factor that plays an important role in regulating the proliferation, differentiation and apoptosis of tissue cells (17). Previous studies have also reported that IGF-1 could regulate the proliferation, differentiation and apoptosis of BMSCs through the STAT3/Akt, ERK1/2, BMP2, PI3K/AKT signaling pathways (18,19). However, there are few reports on the role of Wnt/ β -catenin pathway in these processes. In the present study, it was mainly investigated whether IGF-1 regulates the proliferation and differentiation of BMSCs through the Wnt/ β -catenin pathway.

A lentivirus carrying siRNA-Wnt3a was transfected into BMSCs to silence Wnt3a gene expression. The lentiviral vector is a gene therapy vector, which is developed based on human immunodeficiency type I virus (HIV-I). It can infect nondividing cells, integrate large fragments of exogenous genes into chromosomes and support long-term stable expression (20). In the present study, WB, qPCR and IF confirmed that Wnt3a protein and RNA levels were significantly lower in the siRNA-Wnt3a group than in the control group. This model provides a strong background to study the role of the Wnt/ β -catenin pathway in IGF-1-mediated promotion of BMSC proliferation and differentiation.

CCK-8 and cell cycle assays were used to assess cell proliferation. The CCK-8 assay is a fast, highly sensitive, nonradioactive colorimetric method widely used in cell proliferation and toxicity (21). Cell cycle detection includes G1, G2, and S phases, and the proliferation index (G2 + S/G1 + G2 + S) is used to assess cell proliferation (22). In our experiments, CCK-8 binding and the proliferation index were significantly reduced after the Wnt/ β -catenin pathway was inhibited. WB, qPCR, and IF confirmed that β -catenin and cyclin D1 were significantly downregulated at the protein and mRNA levels. β -catenin is a key regulator in the Wnt/ β -catenin pathway. When Wnt3a is inhibited, β -catenin level in the cytoplasm is decreased, inhibiting downstream pathways (23). Cyclin D1 plays an important role in cell proliferation and is a downstream target gene in the Wnt/ β -catenin pathway (24). The present study revealed that IGF-1 can promote the proliferation of BMSCs, which is consistent with previously reported results (18). However, when the Wnt/ β -catenin pathway was inhibited, the proliferation of BMSCs induced by IGF-1 was decreased. Therefore, it was concluded that IGF-1 promoted the proliferation of BMSCs at least partially through the Wnt/ β -catenin pathway.

In addition, ALP staining and ARS were used to detect osteogenic differentiation. ALP is a phosphatase that is widely present in mammals and is often used as an indicator of early osteogenic differentiation (25). Alizarin Red reacts with calcium to produce a deep red color and is often used as an indicator of late osteogenic differentiation (26). In the

present study, the intensity and area of ALP staining and ARS were significantly reduced after the Wnt/ β -catenin pathway was inhibited. WB, qPCR and IF confirmed that β -catenin, RUNX2 and OPN were significantly downregulated at the protein and mRNA levels. RUNX2 is a member of the RUNX2 family of transcription factors, which plays an important role in the formation and reconstruction of bone tissue (27). OPN is a glycosylated protein that is widely present in the extracellular matrix. It is closely related to bone formation and development (28). In the present study, IGF-1 promoted the osteogenic differentiation of BMSCs, which is consistent with the results of recent related study (29). However, after the Wnt/ β -catenin pathway was inhibited, the ability of IGF-1 to promote osteogenic differentiation was decreased. Therefore, it was inferred that IGF-1 promoted the differentiation of BMSCs at least partially through the Wnt/ β -catenin pathway.

In the present study, the role of the Wnt/ β -catenin pathway in the mechanism by which IGF-1 promotes the proliferation and differentiation of BMSCs was verified through *in vitro* experiments. However, further *in vivo* experiments are required to support our conclusions and provide new strategies and directions for the treatment of nonunion, bone defects and other diseases. Upon inhibition of the Wnt/ β -catenin pathway, IGF-1 promoted the proliferation of BMSCs and decreased their differentiation ability. In summary, IGF-1 at least partially promoted the proliferation and osteogenic differentiation of BMSCs through the Wnt/ β -catenin pathway.

Acknowledgements

Not applicable.

Funding

The present study was supported by the General Hospital of Ningxia Medical University (Clinical Medicine Research Center of Autonomous Region) Open Project (grant no. 020007004127).

Availability of data and materials

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

Authors' contributions

JF performed the experiments, collected the results and wrote the manuscript. ZM designed the experiments, analyzed the data and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Friedenstein AJ, Latzinik NV, Gorskaya YuF, Luria EA and Moskvina IL: Bone marrow stromal colony formation requires stimulation by haemopoietic cells. *Bone Miner* 18: 199-213, 1992.
- Yang A, Yu C, You F, He C and Li Z: Mechanisms of zuogui pill in treating osteoporosis: Perspective from bone marrow mesenchymal stem cells. *Evid Based Complement Alternat Med* 2018: 3717391, 2018.
- Yin N, Wang Y, Ding L, Yuan J, Du L, Zhu Z, Pan M, Xue F and Xiao H: Platelet-rich plasma enhances the repair capacity of muscle-derived mesenchymal stem cells to large humeral bone defect in rabbits. *Sci Rep* 10: 6771, 2020.
- Yanagihara K, Uchida S, Ohba S, Kataoka K and Itaka K: Treatment of bone defects by transplantation of genetically modified mesenchymal stem cell spheroids. *Mol Ther Methods Clin Dev* 9: 358-366, 2018.
- Jiang XR, Yang HY, Zhang XX, Lin GD, Meng YC, Zhang PX, Jiang S, Zhang CL, Huang F and Xu L: Repair of bone defects with prefabricated vascularized bone grafts and double-labeled bone marrow-derived mesenchymal stem cells in a rat model. *Sci Rep* 7: 39431, 2017.
- Křížková K, Chrudinová M, Povalová A, Selicharová I, Collinsová M, Vaněk V, Brzozowski AM, Jiráček J and Žáková L: Insulin-insulin-like growth factors hybrids as molecular probes of hormone: Receptor binding specificity. *Biochemistry* 55: 2903-2913, 2016.
- Backeljauw P: Therapy with recombinant human IGF-1 for children with primary insulin-like growth factor-I deficiency. *Growth Horm IGF Res* 51: 22-26, 2020.
- Gillberg P, Olofsson H, Mallmin H, Blum WF, Ljunghall S and Nilsson AG: Bone mineral density in femoral neck is positively correlated to circulating insulin-like growth factor (IGF)-I and IGF-binding protein (IGFBP)-3 in Swedish men. *Calcif Tissue Int* 70: 22-29, 2002.
- Halmos T and Suba I: The physiological role of growth hormone and insulin-like growth factors. *Orv Hetil* 160: 1774-1783, 2019 (In Hu).
- Salih DA, Mohan S, Kasukawa Y, Tripathi G, Lovett FA, Anderson NF, Carter EJ, Wergedal JE, Baylink DJ and Pell JM: Insulin-like growth factor-binding protein-5 induces a gender-related decrease in bone mineral density in transgenic mice. *Endocrinology* 146: 931-940, 2005.
- Aboalola D and Han VKM: Insulin-like growth factor binding protein-6 promotes the differentiation of placental mesenchymal stem cells into skeletal muscle independent of insulin-like growth factor receptor-1 and insulin receptor. *Stem Cells Int* 2019: 9245938, 2019.
- Du JH, Lin SX, Wu XL, Yang SM, Cao LY, Zheng A, Wu JN and Jiang XQ: The function of Wnt ligands on osteocyte and bone remodeling. *J Dent Res* 98: 930-938, 2019.
- Saad FA: Novel insights into the complex architecture of osteoporosis molecular genetics. *Ann N Y Acad Sci* 1462: 37-52, 2020.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Gugjoo MB, Amarpal, Abdelbaset-Ismail A, Aithal HP, Kinjavdekar P, Pawde AM, Kumar GS and Sharma GT: Mesenchymal stem cells with IGF-1 and TGF-β1 in laminin gel for osteochondral defects in rabbits. *Biomed Pharmacother* 93: 1165-1174, 2017.
- Fu X, Liu G, Halim A, Ju Y, Luo Q and Song AG: Mesenchymal stem cell migration and tissue repair. *Cells* 8: 784, 2019.
- Ahmad S, Ahmad K, Lee EJ, Lee YH and Choi I: Implications of insulin-like growth factor-1 in skeletal muscle and various diseases. *Cells* 9: 1773, 2020.
- Wang XH, Wu HY, Gao J, Wang XH, Gao TH and Zhang SF: IGF1R facilitates epithelial-mesenchymal transition and cancer stem cell properties in neuroblastoma via the STAT3/AKT axis. *Cancer Manag Res* 11: 5459-5472, 2019.
- Zhang C, Hong FF, Wang CC, Li L, Chen JL, Liu F, Quan RF and Wang JF: TRIB3 inhibits proliferation and promotes osteogenesis in hBMSCs by regulating the ERK1/2 signaling pathway. *Sci Rep* 7: 10342, 2017.
- Song L, He J, Gao Y, Fang Y, Zhang L, Wang J, Sun F, Zhang F, Zeng Y, Zeng F and Zhang J: Improved biosafety of a lentiviral vector by reducing cellular gene activation. *J Gene Med* 21: e3087, 2019.
- Cai L, Qin X, Xu Z, Song Y, Jiang H, Wu Y, Ruan H and Chen J: Comparison of cytotoxicity evaluation of anticancer drugs between real-time cell analysis and CCK-8 method. *ACS Omega* 4: 12036-12042, 2019.
- Sosnowska M, Kutwin M, Jaworski S, Strojny B, Wierzbicki M, Szczepaniak J, Łojkowski M, Świączkowski W, Bałaban J, Chwalibog A and Sawosz E: Mechano-signalling, induced by fullerene C₆₀ nanofilms, arrests the cell cycle in the G2/M phase and decreases proliferation of liver cancer cells. *Int J Nanomedicine* 14: 6197-6215, 2019.
- Annavarapu SR, Cialfi S, Dominici C, Kokai GK, Uccini S, Ceccarelli S, McDowell HP and Helliwell TR: Characterization of Wnt/β-catenin signaling in rhabdomyosarcoma. *Lab Invest* 93: 1090-1099, 2013.
- Zheng W, Lin P, Ma Y, Shao X, Chen H, Chen D, Liu X, Li X and Ye H: Psoralen promotes the expression of cyclin D1 in chondrocytes via the Wnt/β-catenin signaling pathway. *Int J Mol Med* 40: 1377-1384, 2017.
- Posa F, Di Benedetto A, Cavalcanti-Adam EA, Colaianni G, Porro C, Trotta T, Brunetti G, Lo Muzio L, Grano M and Mori G: Vitamin D promotes MSC osteogenic differentiation stimulating cell adhesion and αvβ3 expression. *Stem Cells Int* 2018: 6958713, 2018.
- Schürmann M, Wolff A, Widera D, Hauser S, Heimann P, Hütten A, Kaltschmidt C and Kaltschmidt B: Interaction of adult human neural crest-derived stem cells with a nanoporous titanium surface is sufficient to induce their osteogenic differentiation. *Stem Cell Res* 13: 98-110, 2014.
- Tang J, Xie J, Chen W, Tang C, Wu J, Wang Y, Zhou XD, Zhou HD and Li YP: Runt-related transcription factor 1 is required for murine osteoblast differentiation and bone formation. *J Biol Chem* 295: 11669-11681, 2020.
- Luukkonen J, Hilli M, Nakamura M, Ritamo I, Valmu L, Kauppinen K, Tuukkanen J and Lehenkari P: Osteoclasts secrete osteopontin into resorption lacunae during bone resorption. *Histochem Cell Biol* 151: 475-487, 2019.
- Reible B, Schmidmaier G, Moghaddam A and Westhauser F: Insulin-like growth factor-1 as a possible alternative to bone morphogenetic protein-7 to induce osteogenic differentiation of human mesenchymal stem cells in vitro. *Int J Mol Sci* 19: 1674, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.