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Low-level laser irradiation promotes the differentiation of bone marrow stromal cells into osteoblasts through the APN/Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: The relationship between adiponectin (APN) pathway and Wnt pathway was explored through BMSCs, and the effect of low-level laser irradiation (LLLI) on bone marrow stromal cells (BMSCs) and its mechanism were further studied.

MATERIALS AND METHODS: 3-week-old Sprague-Dawley (SD) rats were selected, and mesenchymal stem cells were separately cultured and purified. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze cell proliferation. After osteogenic and adipogenic induction, cultures were conducted, respectively, cells were stained with alizarin red and oil red O. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the expressions of osteogenesis-related genes, runt-related transcription factor 2 (RUNX2), and osteocalcin (OC) and those of adipogenesis-related genes, peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (c/EBPα). Western blotting was used to detect the expressions of β-catenin in the cytoplasm and nucleus. The lentiviral expression vector of adiponectin receptors (APN-R) was constructed, and the expression of APN receptor genes was silenced. The expressions of β-catenin in APN receptors and the nucleus within cells were detected.

RESULTS: LLLI promoted the bone formation by inducing the differentiation direction of mesenchymal stem cells, increasing the number of osteoblasts in the bone marrow and inhibiting the reduction of the number of adipocytes. LLLI regulates the Wnt pathway, promotes the entry of β-catenin into the nucleus, activates the osteogenic effect of the Wnt pathway so as to promote the bone formation of osteoblasts and inhibit bone resorption of osteoclasts. LLLI promotes the entry of β-catenin into the nucleus and the osteogenic differentiation of BMSCs through the APN pathway.

CONCLUSIONS: In summary, LLLI can promote osteogenesis and inhibit adipocytes formation, thus attenuating bone resorption of osteoclasts. The mechanism of LLLI is that it promotes the entry of β-catenin into the nucleus and regulates the Wnt pathway and the differentiation direction of mesenchymal stem cells through the APN signal pathway, thus promoting bone formation.

Key Words:
LLLI, BMMSCs, Osteoblasts, APN, Wnt/β-catenin.

Introduction

Osteoporosis (OP) is a metabolic bone disease characterized by bone loss, abnormal bone tissue microstructures, and increased bone fragility, which can deteriorate bone tissues[1]. It can lead to fractures triggered by minor trauma or low energy trauma, thus resulting in disability or even death. The bone reconstruction is achieved through the balance between the continued accumulation of old bones and the formation of new bones so as to maintain their own structural integrity. The lack of bone formation and excess bone resorption will lead to OP[2,3]. Mesenchymal stem cells in membranes and cartilages are differentiated into osteoblasts and then ossified, which are two ways of bone formation. In these two osteogenesis processes, the differentiation

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mechanisms of osteoblasts are basically the same, that is, the differentiation of bone marrow stromal cells (BMSCs)[4,5]. BMSCs have a far-ranging differentiation potential. In addition to being differentiated into osteoblasts, BMSCs also have abilities of adipogenic and chondrogenic differentiation. Several studies[6-8] have shown that the osteogenic differentiation capacity of BMSCs in patients with OP is decreased and shifts to adipogenic differentiation. Therefore, inhibiting the adipogenic differentiation of BMSCs so as to promote osteogenesis is one of the possible mechanisms of the treatment of OP.

Although a variety of drugs have been applied for OP treatment, the desired effect is still not achieved. Low-level laser irradiation (LLLI) therapy is a physical therapy of applying coherent or non-coherent low-intensity lasers to the biological reactions with no losses and non-thermal mechanisms caused by lesion tissues or monolayer cells so as to achieve therapeutic purposes[9,10]. Studies[11,12] have shown that LLLI therapy can activate osteoblasts, promote bone repair, enhance bone structures in the peri-menopausal rat model and increase bone density, so it is one of the effective intervention measures for OP in middle-aged women, but its mechanism is not yet clear.

Adiponectin (APN) is an adipokine secreted by adipocytes. Osteoblasts express APN receptors and are regulated by APN. APN increases bone density by inhibiting osteoclast formation and promoting osteoblast formation. In the study of bone disease in diabetic patients[13], researchers found that APN is involved in tissue repair and regeneration by regulating BMSCs, thus improving bone remodeling. The function of osteoblasts is regulated by multiple cell signal transduction processes. Wnt signaling pathway is a key pathway in cell development and regulation of growth, which plays a key role in osteoblast orientation, differentiation, development, and proliferation, so it is a very important regulatory pathway for bone metabolism[14,15]. In this study, the relationship between APN pathway and Wnt pathway was explored through BMSCs, and the effect of LLLI on BMSCs and its mechanism were further studied.

Materials and Methods

Isolated Culture and Purification of Mesenchymal Stem Cells

3-week-old Sprague-Dawley (SD) rats were sacrificed and disinfected using 75% alcohol. The bilateral femurs and tibias were taken, and alcohol infiltrated gauzes were used to remove soft tissues and connective tissues. In the ultra-clean workbench, epiphyses in both ends of the femur and tibia were cut so that the bone marrow cavity was exposed. Alpha-modified minimum essential medium (α-MEM) was extracted using a 1 mL syringe to wash the bone marrow cavity repeatedly until the bone marrow contents were blown into the centrifuge tube. Then, cells were blown to prepare the cell suspension until it was even. After the centrifugation at 1000 r/min for 10 min, the supernatant was discarded, and 2 mL α-MEM containing serum and double antibodies was added, followed by repeated blowing until it became an uniform cell suspension. Cell suspension was inoculated in a culture flask, and cultured in an incubator containing 5% CO_2 at 37°C for 3 days; the solution was replaced once every three days until the incubator was filled with cells; 0.25% trypsin was used to digest cells and passage them to the third generation. This investigation was approved by the Animal Ethics Committee of the 89th Hospital of People's Liberation Army Animal Center.

Identification of Surface Antigens of Mesenchymal Stem Cells

The second- and third-generation mesenchymal stem cells were digested with 0.25% trypsin into the cell suspension. The cell suspension was grouped, and each group was added with positive antibodies, CD29 and CD44 and negative antibodies, CD34 and CD45, respectively. Flow cytometry was used for detection.

Immunofluorescence Detection of Cells

The third-generation mesenchymal stem cells were prepared into the cell suspension. After being counted, they were inoculated on coverslips and cultured in an incubator containing 5% CO_2 at 37°C for 3 days. Then, the culture was terminated, and the medium was discarded. Pre-cooled 4% paraformaldehyde was added for fixation for 30 min; 0.1% Triton was used for permeation for 30 min; and the blocking solution was added for incubation at 4°C overnight. Fluorescence-labeled APN receptor antibodies and Hoechst mixture were added and incubated at room temperature for 2 h in a dark place. After cells were sealed, the confocal laser scanning observation was conducted for antibody fluorescence.

Detection of the Proliferation of BMSCs

The cell suspension was seeded in 96-well culture plates at 10^4 cells/well with a volume of 100 μL each. At 3 days after culture, 10 Ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well (5 mg/mL), and 150 μL dimethyl sulfoxide (DMSO) was added to each well after a 4-day incubation. The optical density was measured at the wavelength of 490 nm using the enzyme-linked immunosorbent assay.

Alkaline Phosphatase (AP) Stain

Mesenchymal stem cells were made into single cell suspension, and 10^6 cells were seeded in 6-well plates. At 3 days after culture, the solution was replaced. Then, the culture was continued until each cell was filled with cells, and 4% paraformaldehyde was added for fixation for 30 min. AP staining kit included Solution A and B. In the preparation of the staining fluid, a drop of Solution A and a drop of Solution B were added into 1 mL deionized water, and then the mixture was preserved in the dark. Each well was added with the staining fluid covering the bottom of the well, and after the incubation at 37°C for 2 h, the results were observed.

Alizarin Red Stain

Cell culture methods were the same as those of AP stain. Pre-cooled 70% alcohol was used to fix cells for 1 h. 1 g alizarin red S powder was added to 100 mL 0.1 M Tris hydrochloride (Tris-HCl) (pH 8.3). 40 mM alizarin red S stain was conducted at room temperature for 10 min, which was observed under a microscope.

Oil Red O Stain

Cell culture methods were the same as those of AP stain. 4% paraformaldehyde was used for fixation for 45-60 min, and 60% isopropanol was used for dehydration for 3-5 min. Oil red O working fluid was added to the well, and cells were incubated at room temperature for 30 min, followed by counterstaining with hematoxylin for 30 s. Cells were sealed using glycerol gelatin, which was observed under a microscope.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cells were collected, and the total RNA was extracted and quantified for reverse transcription. PCR amplification and agarose gel electrophoresis were conducted. After the electrophoresis, cells were observed and analyzed on a gel imager.

Extractions of Nucleoproteins and Cytoplasmic Proteins

Cells were collected, and 0.2 mL cytosol extraction buffer A mix (CEB-A mix) was added to the cell precipitates. The mixture was vigorously shaken for 15 s, incubated on ice for 10 min, and added with 11 μL pre-cooled CEB-B, followed by further shaking for 5 s and incubation on ice for 1 min. After being shaken for 5 s and centrifugation at 16,000×g for 5 min, the supernatant was transferred to pre-cooled shell tube to obtain cytoplasmic protein. Precipitates were added with 100 μL pre-cooled nuclear extraction buffer mix (NEB mix) to be prepared as the suspension. The above procedures were repeatedly conducted once every 10 min for 4 times, and then centrifugation was performed at 16,000 × g for 10 min. After that, the supernatant was transferred to a new shell tube to obtain nucleoproteins.

Western Blotting

The third-generation mesenchymal stem cells were collected, the cells were lysed, and the total cell protein was extracted and quantified. Polyacrylamide gel electrophoresis was performed for cytoplasmic proteins, nucleoproteins and the total protein, and after the membrane transfer, the proteins were sealed using the defatted milk. The primary antibodies of target proteins were incubated and sealed at 4°C overnight. On the next day, after membrane washing, the secondary antibodies were incubated at room temperature for 2 h, and the luminous liquid was prepared by the isometric mixture of Solution A and B. Chemiluminescence apparatus was used to detect the expression of proteins.

Construction of Gene Silencing Cells Using Lentiviral Vectors

The optimal transfection titer was determined by pre-experiments, 0.25% trypsin was applied to digest cells, which were then prepared into the cell suspension. After being counted, cells were inoculated at a cell density of 5×10^4/mL in 96-well plates to determine the multiplicity of infection (MOI) value for transfecting lentiviruses. After transfection for 12 h, the virus culture medium was discarded, and the culture was continued in the normal culture medium until the plate wells were filled with cells.

Statistical Analysis

Measurement data were expressed as mean ± standard deviation. All the data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY USA). The *t*-test was used for intergroup comparisons, the chi-square test was used for comparisons among multiple samples, and the Student-Newman-Keuls (SNK)-q test was used for pairwise comparisons. $p<0.05$ represented that the difference was statistically significant.

Results

Identification of Mesenchymal Stem Cells by a Flow Cytometer

Mesenchymal stem cells were identified according to cell surface antigens. Flow cytometry results showed that the number of cells carrying the positive antigen CD29 accounted for 99.1% of the total cells in the positive test. In the negative test, the number of cells carrying the negative antigen CD45 accounted for 3.2% of the total cells, indicating that mesenchymal stem cells are purified, and the cells are mesenchymal stem cells (Figure 1A).

Osteogenic Differentiation and Adipogenic Differentiation of BMSCs

Normal cultured cells were evenly distributed, whose morphology showed long shuttle type. At 14 days after the osteogenic induction of mesenchymal stem cells, AP stain showed that a large number of AP precipitates were formed between cells, indicating that cells are differentiated into osteoblasts. At 21 days after cell osteogenic induction, alizarin red S stain revealed that a large number of mineralized nodules appeared between cells, suggesting that cells are differentiated into osteocytes. At 17 days after the adipogenic induction of mesenchymal stem cells, oil red O stain showed that a large number of lipid droplets were generated and stained red in the cells, manifesting that the adipogenic inducing liquid induces the differentiation of mesenchymal stem cells into adipocytes (Figure 1B).

LLLI Promoted the Proliferation of BMSCs, Facilitated Osteogenic Differentiation and Inhibited Adipogenic Differentiation

The results of cell survival rate test showed that LLLI could promote the proliferation of BMSCs. Alizarin red S stain revealed that the number of mineralized nodules in the osteogenic induction group was less than that in the osteogenic induction + LLLI group, and the difference was statistically significant. Oil red O stain showed that the number of lipid droplets in the adipogenic induction group was higher than that in the adipogenic induction + LLLI group, and the difference was statistically significant. Osteogenic induction detection demonstrated that the expression levels of osteogenesis-related genes, runt-related tran-

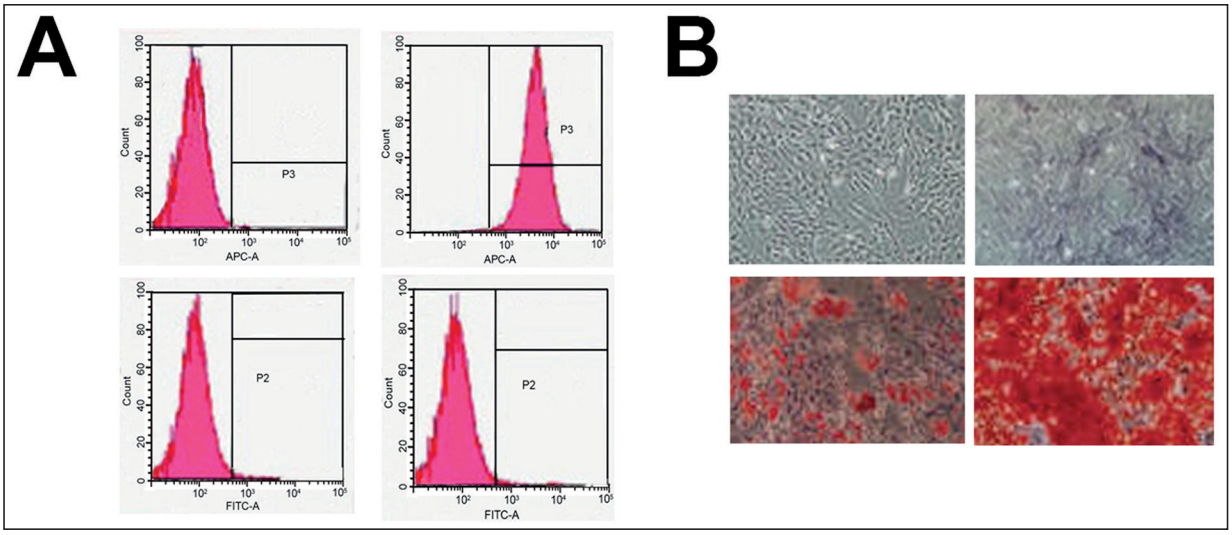

Figure 1. Identification of mesenchymal stem cells and osteogenic and adipogenic differentiation of BMSCs. ***(A)*** Phenotype of bone marrow mesenchymal stem cells identified by flow cytometry. ***(B)*** Osteogenic induction and adipogenic induction of bone marrow mesenchymal stem cells.

scription factor 2 (RUNX2) and osteocalcin (OC) in the groups receiving LLLI were significantly higher than those in the induction groups. Results of adipogenic induction detection revealed that the expression levels of adipogenesis-related genes, peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) were significantly lower than those in the control group (Figure 2).

LLLI Activated the Wnt Signaling Pathway

The detection results showed that the expression level of the nucleoprotein β-catenin was increased after LLLI treatment, indicating that the transfer of β-catenin to the nucleus under the action of LLLI was promoted. The detection results of cytoplasmic proteins showed that the stable β-catenin in the cytoplasm was also increased after LLLI treatment. The experimental results illustrated that LLLI regulated the differentiation of cells by promoting the entry of stable non-phosphorylated β-catenin into the nucleus and regulating the Wnt pathway (Figure 3).

APN Receptors Were Expressed in Mesenchymal Stem Cells

PCR was used to detect the expression of receptor genes in cells, including mesenchymal stem cells, osteoblasts, and APN receptor-positive cells. Results showed that APN receptor genes and proteins were negative in osteoblasts. Immunofluorescence results demonstrated that the surfaces of mesenchymal stem cells were combined with green fluorescent APN receptor antibodies, and the nucleus was stained blue through Hoechst stain, indicating that APN receptors exist in cell membranes of mesenchymal stem cells. These results suggested that APN receptors are not expressed in osteoblasts, but expressed in BMSCs (Figure 4).

LLLI Activated the Wnt Signaling Pathway and Regulated the Differentiation Direction of Cells Through the APN Signaling Pathway

Western blotting results revealed that the expression of APN in cells increased significantly and the amount of β-catenin in the nucleus of BMSCs treated with LLLI was higher than that of the blank control group, and the difference was statistically significant. The amount of β-catenin in the nucleus of BMSCs with APN-R gene silencing was not significantly increased compared with that of the blank control group, and the difference was not statistically significant (Figure 5).

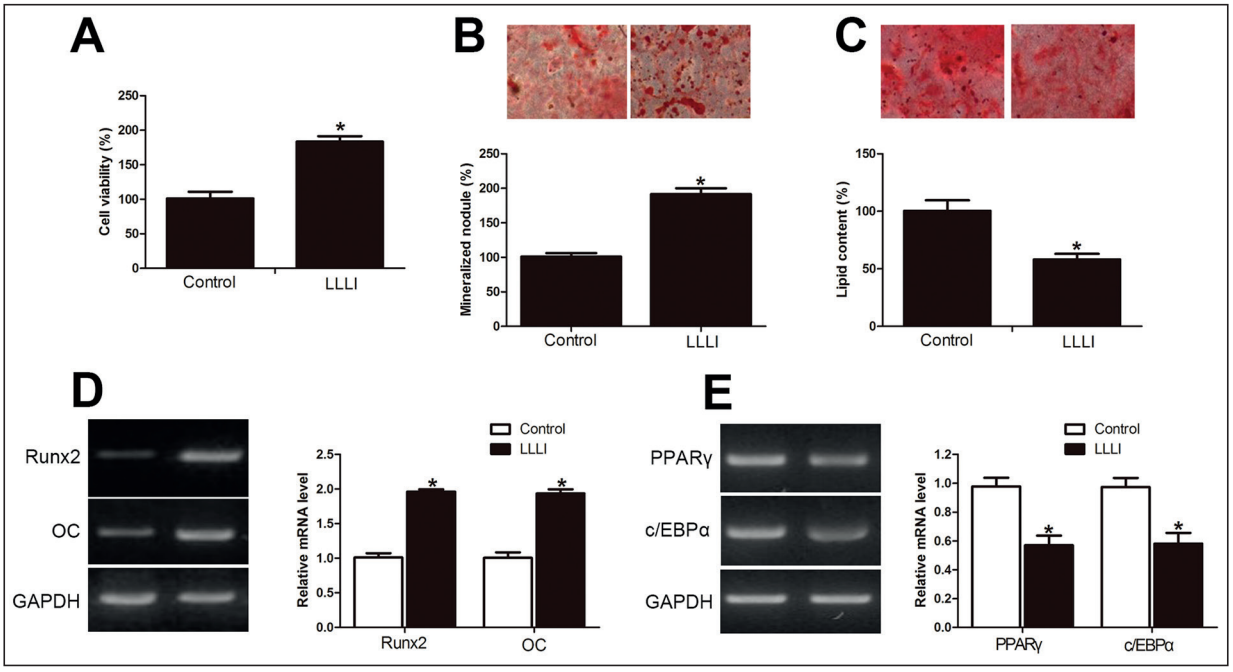

Figure 2. LLLI promoted the proliferation of BMSCs, facilitated osteogenic differentiation and inhibited adipogenic differentiation. ***(A)*** Cell viability detected in BMSCs by MTT. ***(B)*** LLLI promotes osteogenesis by Alizarin Red staining. **(C)** LLLI inhibits adipogenesis by Oil Red O staining. ***(D)*** LLLI promotes the expression of osteogenic genes. ***(E)*** LLLI inhibits the expression of adipogenic genes. *$p < 0.05$ *vs.* Control.

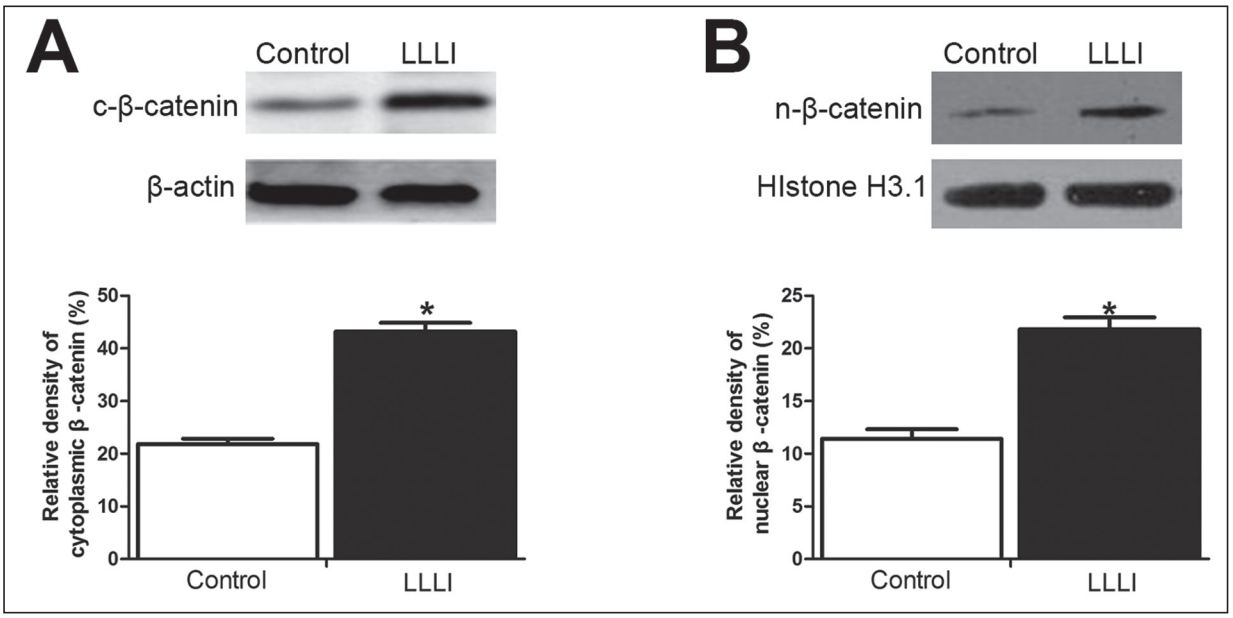

Figure 3. LLLI activated the Wnt signaling pathway. ***(A)*** Western Blot analysis of cytoplasmic β-catenin (c-β-catenin) protein levels of BMSCs. ***(B)*** Western Blot analysis of nuclear β-catenin (n-β-catenin) protein levels of BMSCs. *$p < 0.05$ *vs.* Control.

Discussion

Mesenchymal stem cells are a class of cells that can be differentiated into various cells, including hematopoietic parenchymal cells, stromal cells and cells other than hematopoietic cells, especially mesoderm and neuronal ectodermal cells, such as osteocytes, chondrocytes, and adipocytes[16]. We isolated and purified mesenchymal stem cells by the adherence method. The identification of mes-

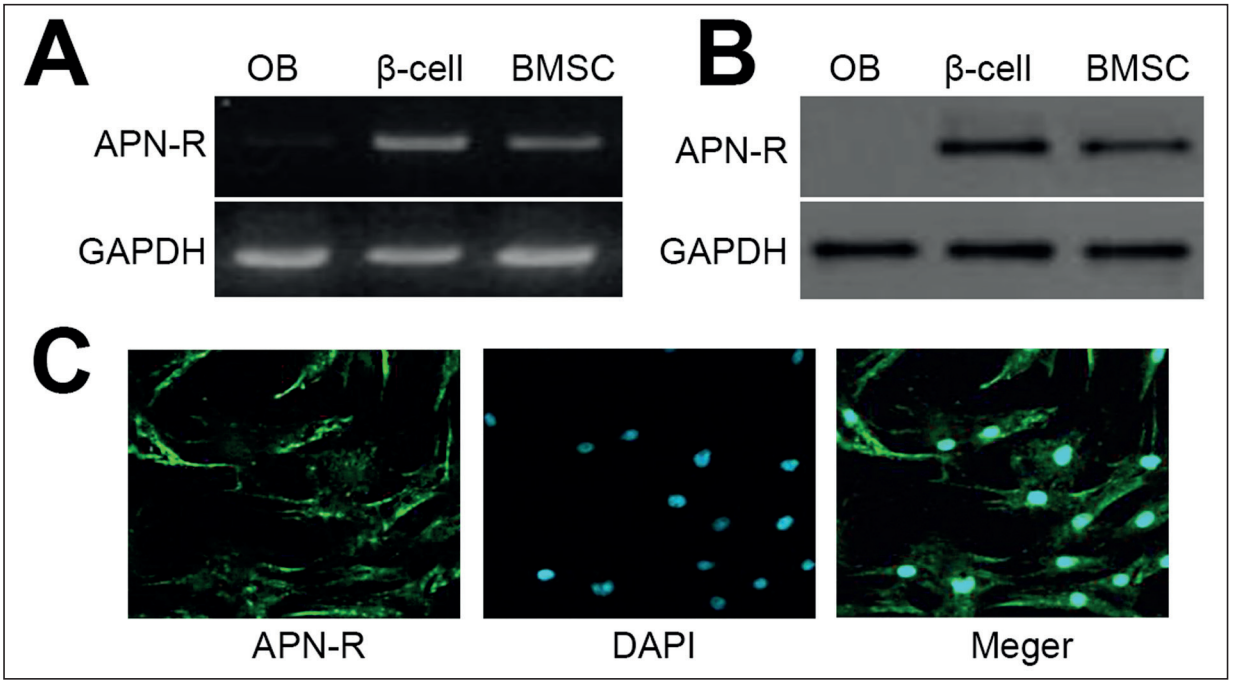

Figure 4. APN receptors were expressed in mesenchymal stem cells. ***(A-C)*** APN-R was detected by Real-time PCR, Western Blot analysis and immunofluorescence in BMSCs.

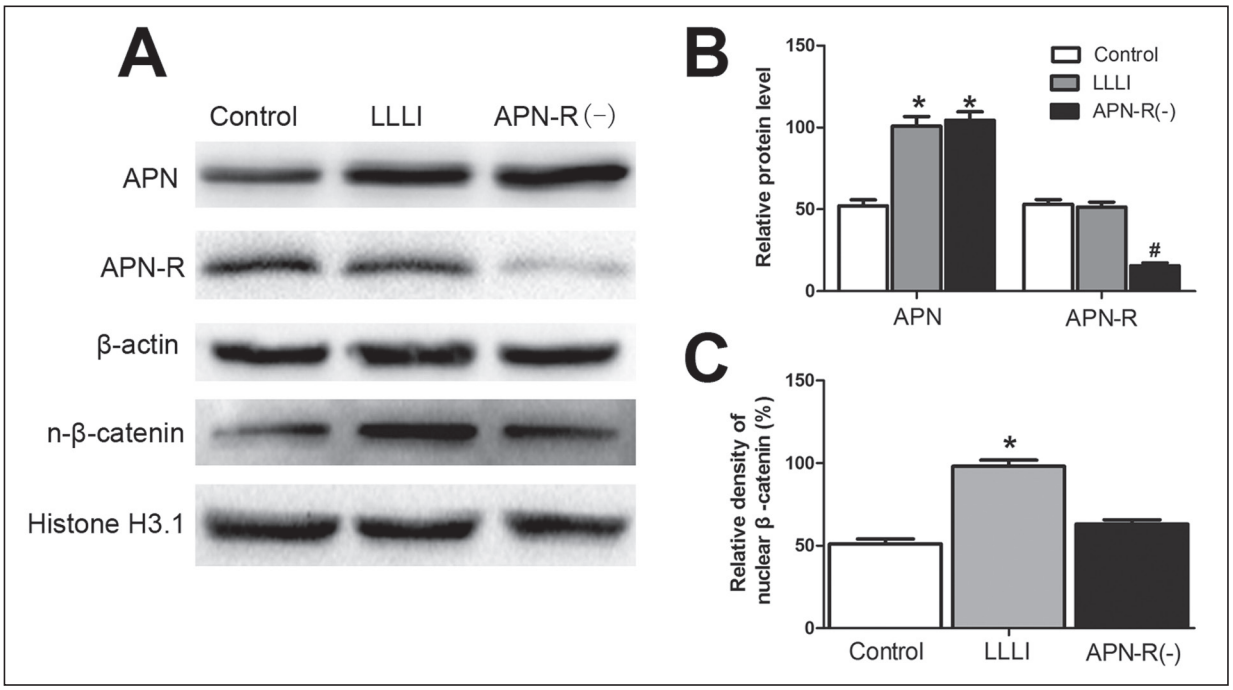

Figure 5. LLLI activated the Wnt signaling pathway and regulated the differentiation direction of cells through the APN signaling pathway. ***(A)*** Western Blot analysis reveals the expression of APN, APN-R, n-β-catenin. ***(B)*** Semiquantitative analysis of APN, APN-R. ***(C)*** Semiquantitative analysis of n-β-catenin. $p < 0.05$ *vs.* Control. $^{\#}p < 0.05$ *vs.* LLLI.

enchymal stem cells is divided into surface antigen identification, cell-induced multi-directional differentiation identification, and morphological characterization. We measured the expression of the positive antigen CD29, whose percentage of positive cells was 99.1%, and the percentage of negative cells was 3.2%, indicating that the cultured cells are purified as mesenchymal stem cells. The multi-directional differentiation ability of mesenchymal stem cells is also one of the gold standards for identifying BMSCs. At 14 days after BMSCs received osteogenic induction and culture, AP stain results showed that the activity of AP was enhanced, and the cells were stained blue, suggesting that BMSCs are induced to be differentiated into osteoblasts. At 21 days after the osteogenic induction, alizarin red stain results manifested that a large number of mineralized nodules were deposited in intercellular substance and stained into red, indicating that BMSCs are induced to be differentiated into osteocytes. At 17 days after adipogenic-induced differentiation, oil red O stain results showed that a large amount of lipid droplets were generated in cells, suggesting that BMSCs are induced to be differentiated into adipocytes. The above results revealed that BMSCs were differentiated into osteoblasts, osteocytes, and adipocytes under different induction conditions and at different induction times.

MTT is an experiment to detect cell proliferation and cell viability. The experimental results showed that LLLI could promote the proliferation of mesenchymal stem cells. LLLI was treated on the basis of osteogenic and adipogenic inductions. Among osteogenic induction groups, the results of alizarin red staining showed that the number of mineralized nodules produced by LLLI-treated cells was more than that of the osteogenic induction group. Among adipogenic induction groups, the results of oil red O stain revealed that the number of lipid droplets in the group receiving LLLI was lower than that of the adipogenic group. The above results demonstrated that LLLI promoted the differentiation of BMSCs into osteoblasts, but inhibited the differentiation to adipocytes. To further elucidate the role of LLLI, the expressions of genes that promote osteogenesis and inhibit adipogenesis were detected. In the signaling molecule pathway of osteogenic differentiation, osteogenic differentiation genes included OC, osteoprotegerin (OPG), RUNX2, and Osterix[17,18], while adipogenic differentiation genes included PPARγ and c/EBPα[19,20]. The results of gel electrophoresis showed that the

expression levels of OC and RUNX2 in the group receiving LLLI were higher than those in the osteogenic induction group. Among adipogenic induction groups, the expression levels of PPARγ and c/EBPα in the group undergoing LLLI were lower than those in the adipogenic group. The results revealed that LLLI promoted the expression of osteogenic differentiation genes, but inhibited the expression of adipogenic differentiation genes, that is, it promoted the differentiation of mesenchymal stem cells into osteoblasts, but inhibited the differentiation into adipocytes, so it played a role in bone formation.

The bone steady state is regulated by two Wnt-mediated major molecular pathways. These two Wnt pathways are the classical β-catenin-dependent Wnt pathway and the non-classical β-catenin-independent Wnt pathway[21]. The classical Wnt pathway and non-classical Wnt pathway can increase the bone formation and promote the differentiation of pre-osteoblasts. The β-catenin signaling pathway is needed not only for osteogenesis, but also for inhibition of bone formation. The classical Wnt pathway can promote the differentiation of mesenchymal stem cells into osteoblasts by activating the expression of osteogenesis-related genes. The Wnt/β-catenin pathway has been reported in many publications involved in the intermodulation of many channels[22]. We speculated that LLLI could promote the osteogenic differentiation of cells by regulating the Wnt/β-catenin pathway. In the experiment, after mesenchymal stem cells were treated with LLLI, the contents of β-catenin in the cytoplasm and nucleus were detected. Our results indicated that LLLI promoted the transfer of β-catenin from cytoplasm to the nucleus and also enhanced the differentiation of mesenchymal stem cells.

To further study the molecular mechanism of LLLI in promoting osteogenic differentiation and inhibiting adipogenic differentiation, the role of APN pathway in LLLI was investigated. The results of immunofluorescence showed that APN receptors with green fluorescence were combined with the surface of cell membranes, indicating that APN receptors were expressed in BMSCs. RT-PCR and Western blotting revealed the same results. The above results further confirmed that APN receptors were expressed in BMSCs. In this study, gene silencing BMSCs were constructed, and APN gene silencing cells were obtained using lentivirus transfection method. By constructing gene silencing cells, the difference in the protein expression between gene silencing genes and normal gene was detected after both of them were treated with LLLI so as to determine the role of the APN pathway. The experimental cells were divided into the blank control group, LLLI group, and BMSCs + LLLI group with APN receptor gene silencing. The change of β-catenin in the nucleus was detected. The results showed that the β-catenin expression in the nucleus in groups not treated with LLLI was lower than that in the LLLI treatment groups, which manifested that LLLI could activate β-catenin in the Wnt pathway, suggesting that LLLI is involved in β-catenin by APN, and increase the entry of β-catenin into the nucleus, thus promoting the osteogenic differentiation of mesenchymal stem cells. Therefore, LLLI increases the entry of β-catenin into the nucleus and the osteogenic differentiation of BMSCs through the APN pathway and Wnt pathway.

Conclusions

We showed that, LLLI promoted osteogenesis and attenuated adipocytes differentiation, thus inhibiting bone resorption of osteoclasts. The mechanism of LLLI is that it promotes the entry of β-catenin into the nucleus and regulates the Wnt pathway and the differentiation direction of mesenchymal stem cells through the APN signal pathway, thus promoting bone formation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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