LncRNA MEG3 promotes proliferation and differentiation of osteoblasts through Wnt/β-catenin signaling pathway

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Abstract. – **OBJECTIVE:** We aimed at detecting the expression of long non-coding ribonucleic acid (lncRNA) maternally expressed 3 (MEG3) in the serum of fracture patients, and at investigating its impacts on the proliferation and differentiation of osteoblasts and the specific molecular mechanism of action.

PATIENTS AND METHODS: The serum samples of 48 fracture patients diagnosed in our hospital (Fracture group) and 30 healthy people receiving physical examination (Health group) were collected. The expression level of serum lncRNA MEG3 in Fracture group and Health group was measured via reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, a small interfering RNA (siRNA) was applied to construct mouse osteoblast cell line MC3T3-E1 with a stable knockout of MEG3. The growth status of the cell was observed, and the impacts of MEG3 knockout on the osteoblast proliferation were determined using cell counting kit-8 (CCK-8), a proliferation activity detection kit. Meanwhile, 5-ethynyl-2'-deoxyuridine (EdU) staining was applied to detect the proportion of EdU positive cells in the osteoblasts in Control group and MEG3 knockout group (MEG3 siRNA group). In addition, RT-PCR was performed to measure the messenger RNA (mRNA) levels of differentiation-related genes. Finally, RT-PCR and Western blotting assay were adopted to analyze the expression of the Wnt/β-catenin signaling pathway.

RESULTS: The expression of serum lncRNA MEG3 in fracture patients was increased markedly ($p<0.05$). Results of *in-vitro* cell experiment indicated that intervention with MEG3 siRNA could obviously promote the proliferation and differentiation of osteoblast cell line MC3T3-E1. The results of RT-PCR and Western blotting assay revealed that the role of MEG3 in promoting differentiation and proliferation might be mediated by the activation of the Wnt/β-catenin signaling pathway in osteoblasts.

CONCLUSIONS: LncRNA MEG3 can promote the proliferation and differentiation of osteoblasts by activating the Wnt/β-catenin signaling pathway, so it is expected to become a new target for accelerating the fracture healing.

Key Words:
lncRNA MEG3, Wnt/β-catenin signaling pathway, Osteoblasts, Proliferation, Differentiation.

Introduction

Fracture-induced trauma or bone regeneration after other diseases is a well-organized process involving multiple systems under the synergistic effects of immune cells and osteoclasts. The osteoclasts absorb organic and inorganic compounds released by the damaged bone, and the degraded

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compound substrates enter the blood circulation in the form of Ca^{2+} and so on[1]. In the meantime, cytokines after the damage process can initiate osteoblast differentiation, which will enable the fracture-induced trauma to develop in a favorable way and maintain benign homeostasis. Once such a good balance between bone formation of osteoblasts and their bone resorption is destroyed, it will lead to osteopenia and structural changes in bone tissues, thus increasing bone fragility and triggering fracture[2]. Therefore, both osteoblasts and osteoclasts play crucial roles in bone homeostasis and fracture healing[3]. During the callus reconstruction of fracture, the inhibitory effect of osteoclasts can interrupt the chondrolysis. Besides, the inhibition on the proliferation and differentiation of osteoblasts can result in slow new bone formation, further enlarging the fracture interspace and abnormally increasing the callus cartilage, ultimately leading to delayed fracture healing[4]. Moreover, the fracture healing is controlled by a variety of cells and cytokines, and the disturbances of osteoblasts (osteoclasts) or microenvironment will cause abnormality of fracture healing[5]. Studies showed that extracellular matrix proteins such as osteocalcin (OCN) generated by osteoblasts can be moderately controlled by the body to realize stable secretion, so they are the bases for maintaining bone homeostasis[6]. The damaged deposition of bone matrix will trigger a series of skeletal diseases, such as osteoporosis and osteogenesis imperfecta[7]. Hence, it is crucial to deeply understand the molecular regulatory networks of the proliferation and differentiation of osteoblasts in treating fractures and other bone diseases.

In recent years, attention has been paid to long non-coding ribonucleic acids (lncRNAs): long RNA molecules with a transcript length of over 200 nucleotides, by researchers due to their capability of regulating various biological functions. Previous studies[8] revealed that lncRNAs themselves in cells cannot encode and express corresponding proteins. However, a majority of lncRNAs can not only regulate organisms at many levels, including (post-) transcriptional level, but also actively participate in the occurrence and development of diseases[9,10]. As one of the lncRNAs, maternally expressed 3 (MEG3) is widely expressed in various normal tissues, which often serves as a cancer suppressive factor for multiple cancers, including liver cancer and glioma[11]. Studies showed that MEG3 has potential effects in angiogenesis and pathological processes of vascular function in diabetes mellitus[12]. According to the latest investigations[13], MEG3 can be involved in the osteogenic differentiation of bone marrow mesenchymal stem cells. However, the function and action of MEG3 in osteoblast differentiation still remains unclear, and the specific mechanism needs to be further explored. In this research, the difference in serum lncRNA MEG3 between healthy people and fracture patients was detected first. Subsequently, the impacts of lncRNA MEG3 knockout on the proliferation and differentiation of osteoblast cell line MC3T3-E1 were determined. Finally, the specific molecular mechanism of action leading to the difference was explored, so as to reveal the role of MEG3 in osteoblast differentiation.

Patients and Methods

Serum Specimens

A total of 48 fracture patients aged (50.55±6.78) years old and diagnosed in our hospital from January 2018 to December 2018, were selected. 30 other healthy people aged (48.42±5.89) years old and receiving physical examination, were enrolled into Control group. 4 mL of venous blood was collected from every participant, added with sodium citrate for anticoagulation and frozen in a refrigerator at -20°C for standby use. Signed written informed consents were obtained from all participants before the study. This research was approved by the Ethics Committee of Affiliated Stomatological Hospital of Jiamusi University.

Cell Culture

The mouse osteoblast cell line MC3T3-E1 (purchased from Bioleaf, Shanghai, China) was added into a medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% triple antibody and then cultured in an incubator. The medium was changed every 48 hours.

MEG3 Knockout

Cells in logarithmic phase were fetched for digestion and inoculation. When the fusion reached 65-85%, cells were subjected to starvation to realize synchronous growth. Next, MEG3 small interfering RNA (siRNA) was dissolved to achieve a final concentration of 20 μmol/L. Cells were divided into 2 groups, namely, Control group and MEG3 knockout group (MEG3 siRNA group). Next, transfection solution was added to continue culturing.

Detection of Expressions of Differentiation- and Pathway-Related Genes via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA in the cells was extracted through TRIzol reagent method (Invitrogen, Carlsbad, CA, USA). (2) The messenger RNA (mRNA) was synthesized into cDNA by means of reverse transcription. (3) The primer sequences of target genes and internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to the sequences on GenBank, and the expression levels of target genes were detected *via* RT-PCR. The primer sequences of MEG3, runt-related transcription factor 2 (RUNX2), Collagen α1, osteocalcin (OCN), Wnt, β-catenin and Osterix (Osx) are shown in Table I.

5-Ethynyl-2′-Deoxyuridine (EdU) Staining

24 h after MEG3 knockout in MC3T3-E1 cells with siRNA, Click-iT EdU staining kit (Invitrogen, Carlsbad, CA, USA) was applied to stain the MC3T3-E1 cells according to the specific procedures in the kit instructions. Finally, the cells were photographed and counted.

Cell Proliferation Assay via Cell Counting Kit-8 (CCK-8)

The cells in logarithmic growth phase were cultured for 0, 24, 48 and 96 h. After that, 120 μL developing reagent was added into each well, followed by incubation in the incubator at 37°C for 3 h and measurement of absorbance at 540 nm in each group by virtue of an ultraviolet spectrophotometer.

Western Blotting Assay

(1) The medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) 3 times. (2) The lysis buffer prepared at an appropriate proportion was added to sufficiently lyse the cells and release the proteins, followed by collection of supernatants *via* centrifugation. Protein' s concentration was detected and calculated in accordance with the instructions of bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). (3) The total protein extracted from the osteoblasts was subjected to water bath for 8 min and centrifugation at 1,000 g for 5 min. After that, the Western blotting assay was performed in the sequence of preparation of 10% separation gel and 5% spacer gel, loading for electrophoresis, membrane transfer through semi-dry process, sealing, incubation with primary antibody overnight and incubation with secondary antibody. Then, protein bands were scanned using an Odyssey membrane scanner and quantified: the level of proteins to be detected was corrected *via* that of GAPDH.

Table I. Primer sequences of indexes for RT-PCR.

Target gene		Primer sequence (5'-3')*
GAPDH	Forward	
	Reverse	GACATGCCGCCTGGAGAAAC
		AGCCCAGGATGCCCTTTAGT
MEG3	Forward	
	Reverse	GAGTGTTTCCCTCCCCAAGG
		GCGTGCCTTTGGTGATTCAG
Runx2	Forward	
	Reverse	GTCCAACCCGTAAGGT
		CGCTGCTGAGTCGATGCTAGCT
Collagen1 α1	Forward	
	Reverse	ACGTAGCTAGCTAGTCGGTATG
		AAAACGTGGCTAGTCGATCG
OCN	Forward	
	Reverse	ATCGTAGCTAGCTAGTCGAGCA
		CCCCCTGTGCTAGCTAGCTAGC
OSX	Forward	
	Reverse	GTGCTGATGTTAGCTAGCTAGCT
		AGCTAGTCGTAGCTAGCTGATCG
β-catenin	Forward	GCCAAGTGGGTGGTATAGAG
	Reverse	CTGGGTATCCTGATG TGC
Wnt	Forward	TGCCGGACTCTCATGAAC
	Reverse	GTGTGGTCCAGCACGTCTTG

Statistical Analysis

All the data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), the measurement data were presented as mean ± standard deviation, and *t*-test was performed for comparison of data between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of Serum lncRNA MEG3 in Healthy People and Fracture Patients

Firstly, the level of lncRNA MEG3 in the serum of the two groups of participants was detected *via* RT-PCR. Results (Figure 1) indicated that the expression level of serum lncRNA MEG3 in fracture patients was remarkably higher than that in healthy people, which was about 8.96 times that of healthy people ($p<0.05$). As shown in Figure 2, the MC3T3-E1 cells just adhering to the wall were round, or they turned spindle-shaped and polygonal (Figure 2A). After culture for 96 h, the size of cells was increased constantly, cells were fused and arranged in a cobblestone-like pattern (Figure 2B).

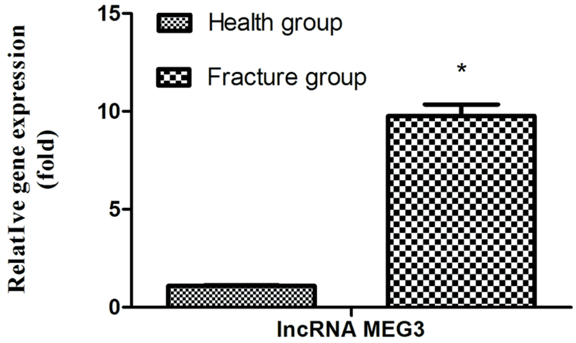

Figure 1. Expression of serum lncRNA MEG3 in fracture patients. Health: healthy controls, Fracture: fracture patients, *$p<0.05$ *vs.* Health group, with a statistical difference.

Construction of Cell Lines with lncRNA MEG3 Knockout

To deeply investigate the role of MEG3 in the fracture healing, MEG3 in the MC3T3-E1 cells was knocked out using siRNA. Later, RT-PCR was adopted to detect the knockout efficiency of MEG3 in the MC3T3-E1 cells, and it was manifested that the expression level of MEG3 was decreased by 84.50% in MEG3 siRNA group compared with that in Control group ($p<0.05$) (Figure 3).

EdU Staining Results in Two Groups of Cells

To further determine the impacts of MEG3 knockout on the proliferative capacity of the two groups of cells, EdU staining was utilized to assess the proliferative capacity of each group of cells. The results (Figure 4) displayed that the number of EdU positive cells in MEG3 siRNA group was notably greater than those in Control group ($p<0.05$), illustrating that silencing lncRNA MEG3 can effectively inhibit the proliferation of osteoblasts.

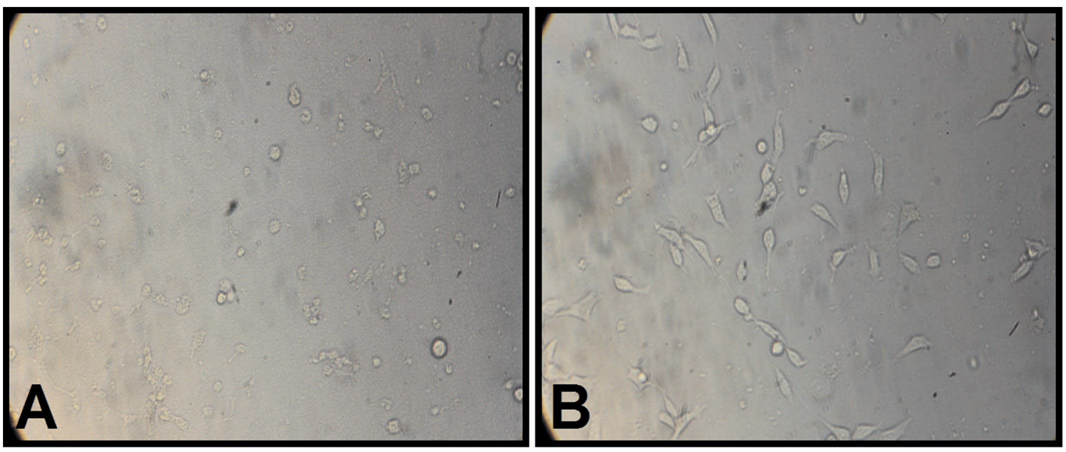

Figure 2. Morphological change in MC3T3-E1 cells just after subculture (***A***×10), with initial morphology of cells in round or oval shape. After culture for approximately 4 d, the morphology is changed into a cobblestone-like pattern (***B***×20).

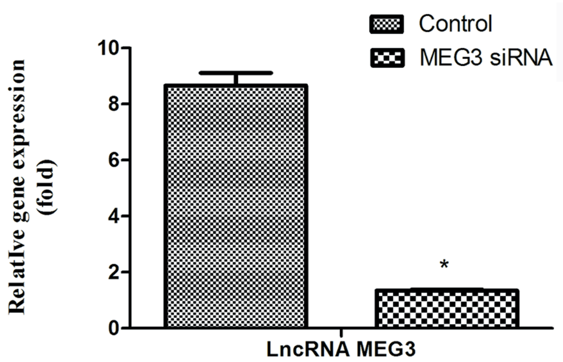

Figure 3. Detection of lncRNA MEG3 knockout. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

Impacts of lncRNA MEG3 Knockout on CCK-8 Proliferation Assay of Osteoblasts

Meanwhile, CCK-8 proliferation assay was conducted to detect the OD480 in the two groups of cells at 0, 24, 48 and 96 h, so as to reflect the cell proliferative capacity. According to the results in Figure 5, MEG3 siRNA group had evidently enhanced proliferative capacity of osteoblasts at 24, 48 and 96 h in comparison with Control group ($p<0.05$).

Impacts of MEG3 Knockout on Osteoblast Differentiation

The results of detecting mRNA of osteoblast differentiation-related genes (Figure 6) manifested that the mRNA levels of differentiation-related genes, RUNX2, Collagen α1, OCN and Osx, in MEG3 siRNA group were higher than those in Control group ($p<0.05$), revealing that cell differentiation capacity in MEG3 siRNA group is enhanced prominently.

Impacts of MEG3 Knockout on Genes of Wnt/β-Catenin Signaling Pathway in Osteoblasts

Considering the vital role of Wnt/β-catenin signaling pathway, RT-PCR was applied to measure the expression levels of Wnt and β-catenin genes, and it was revealed that the Wnt/β-catenin signaling pathway in osteoblasts was activated significantly after MEG3 knockout ($p<0.05$) (Figure 7).

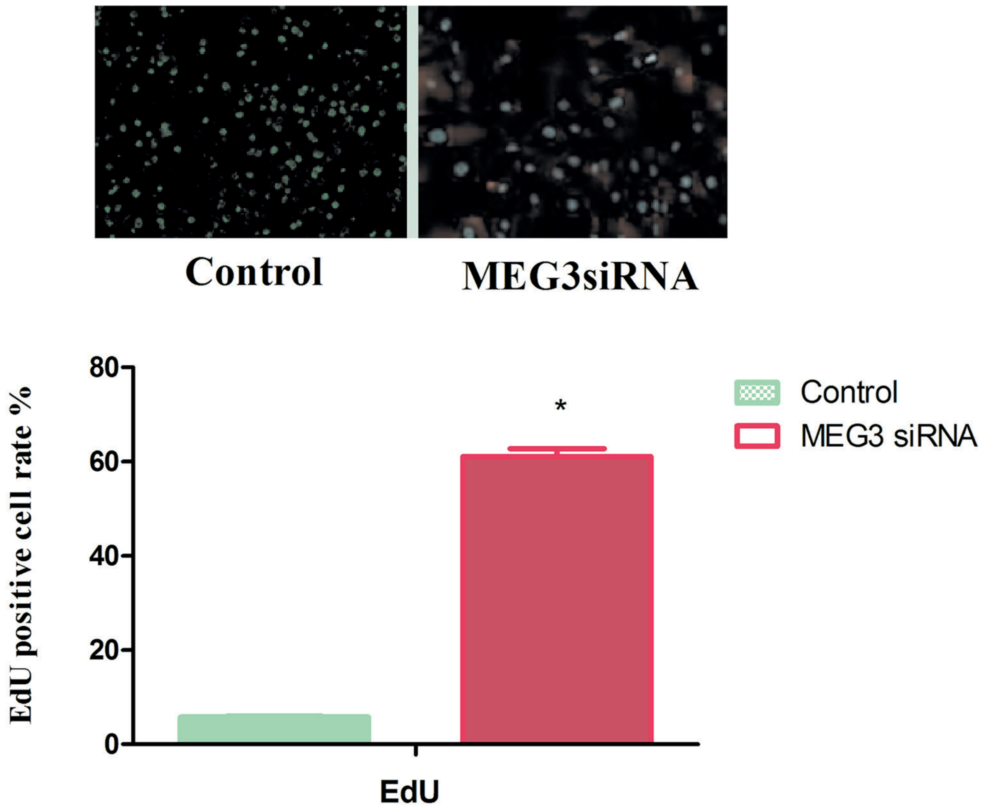

Figure 4. EdU staining results in each group of cells. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

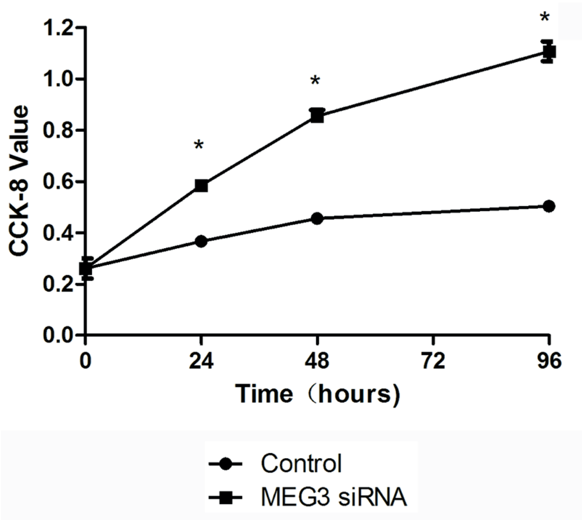

Figure 5. Detection of cell proliferative capacity *via* CCK-8. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

Impacts of MEG3 Knockout on Proteins of Wnt/β-Catenin Signaling Pathway in Osteoblasts

In order to observe the impacts of MEG3 knockout on the proteins of Wnt/β-catenin signaling pathway in osteoblasts, the expression levels of Wnt and β-catenin proteins were detected, and it was shown that the Wnt/β-catenin proteins in osteoblasts were increased remarkably ($p<0.05$) (Figure 8).

Discussion

Fracture and osteoporosis, frequently occurring in the elderly, are bone metabolic diseases that seriously affect the life of middle-aged and elderly people and have numerous causes and regulatory factors[14]. Currently, osteoporosis and other bone diseases have become important public health problems threatening the health of the elderly around the world[15]. Therefore, how to treat such bone diseases more favorably and effectively are the problems to be urgently solved. This involves the impacts of a variety of genes and other regulatory factors on the skeletal healing which is a complex and dynamic process regulated by multiple cell components and cytokines. Increasing the generation of osteoclasts during the fracture healing can accelerate cartilage reabsorption and promote formation of synostosis at the same time, while repressing the differentiation of osteoblasts or osteoclasts will inhibit the skeletal healing[16,17]. Scholars[18-20]

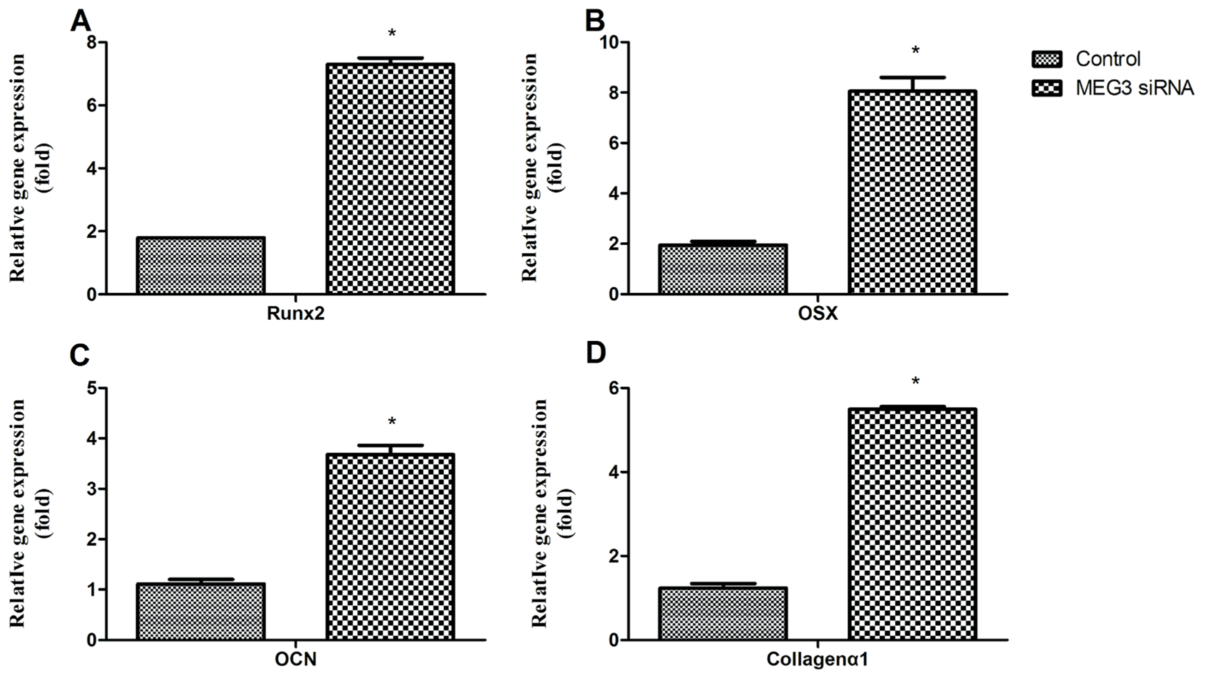

Figure 6. Impacts of MEG3 knockout on osteoblast differentiation. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

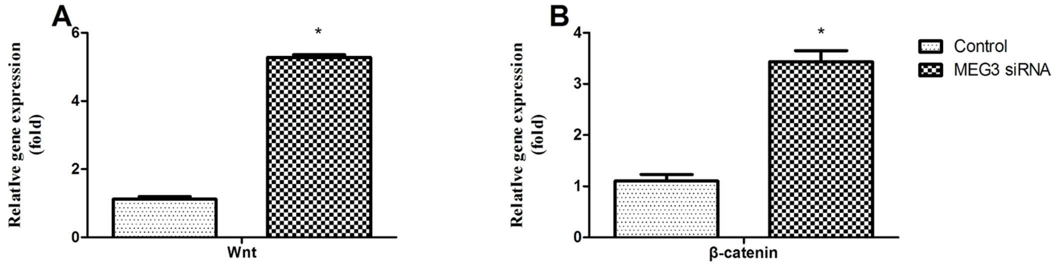

Figure 7. Impacts of MEG3 knockout on genes of Wnt/β-catenin signaling pathway in osteoblasts. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

revealed that many lncRNAs play crucial roles in various diseases, including tumors, cardiovascular diseases, endocrine disorders and bone diseases. In this research, it was indicated that the expression level of serum lncRNA MEG3 in fracture patients was notably higher than that in healthy people ($p<0.05$), suggesting that lncRNA MEG3 has important effects on the fracture healing. To further investigate the role of MEG3 in the fracture healing, MEG3 in the MC3T3-E1 cells was knocked out using siRNA. Next, the knockout efficiency of MEG3 in the MC3T3-E1 cells was detected and the expression level of MEG3 was decreased markedly inMEG3 siRNA group compared with that in Control group. The existing findings in this work are consistent with those in previous reports. Besides, some studies have revealed that the generation of osteoblasts and osteoclasts is regulated by multiple genes or proteins. Thus, seeking the key genes and proteins inducing bone diseases and designing drugs with these genes or proteins as the targets, will provide new ideas for accelerating the fracture healing[21]. It has been proved that the Wnt/β-catenin signaling pathway is responsible for various biological processes, including tissue homeostasis and cancer. Recently, much importance has been attached to the role of Wnt/β-catenin signaling pathway in bone formation and homeostasis, a vital signaling cascade in osteoblast differentiation[22]. According to Li et al[23] findings, the Wnt/β-catenin signaling pathway exerts indispensable and important effects in regulating osteoblast differentiation. The proliferation and differentiation of osteoblasts can be accelerated efficiently by up-regulating the Wnt/β-catenin signal in osteoblasts, while lncRNA MEG3 is proven to be able to regulate the Wnt/β-catenin signaling pathway in such disease models as fracture[24]. Several previous studies have been conducted to demonstrate the mechanism of Wnt/β-catenin signaling pathway in inducing differentiation in the bone, but the results are far from satisfactory. The proliferation and differentiation levels of osteoblasts were measured in this research, and the results indicated that the proportion of EdU positive cells and the number of positive cells detected *via* CCK-8 proliferation activity assay were increased evidently, which are consistent with the findings of Sun et al[25]. Meanwhile, the mRNA levels of differentiation-related markers such as Osx, RUNX2, Collagen α1 and OCN were detected, and they were upregulated significantly in MEG3 siRNA group. Li et al[26] also

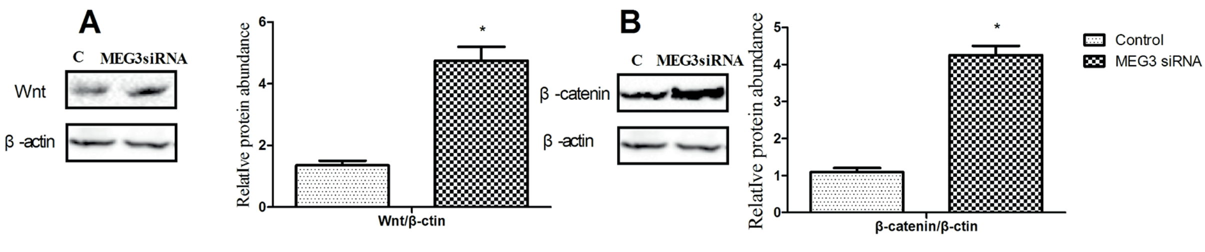

Figure 8. Impacts of MEG3 knockout on proteins of Wnt/β-catenin signaling pathway in osteoblasts. Control: blank control group, MEG3 siRNA: MEG3 knockout group, *$p<0.05$ *vs.* Control group, with a statistical difference.

revealed that the mRNA levels of RUNX2 and OCN were elevated remarkably, which are in line with the results in this experiment. Finally, the expression levels of related genes and proteins in the classical Wnt/β-catenin signaling pathway were analyzed, and it was discovered that the content of Wnt/β-catenin genes and proteins were increased notably, which is identical with the findings of Chen et al[27]. Hence, it was conjectured that lncRNA MEG3 is capable of influencing the proliferation and differentiation of osteoblasts through the up-regulation of the Wnt/β-catenin signaling pathway. Although such an effect had been testified in this research, there were still certain limitations in our experiment. In subsequent complementary experiments in model animals, diverse cell lines can be introduced, and more genes and proteins in the Wnt/β-catenin signaling pathway can be measured, so as to further verify the effect.

Conclusions

Proliferation and differentiation levels of osteoblasts were elevated prominently after silencing lncRNA MEG3, which were mainly manifested as the significant increases in the number and proportion of positive cells. In the meantime, the mRNA levels of differentiation-related markers were markedly upregulated. The levels of Wnt/β-catenin genes and proteins were significantly raised. Therefore, we demonstrated that lncRNA MEG3 has important regulatory effects on the proliferation and differentiation of osteoblasts, which is probably mediated by the Wnt/β-catenin signaling pathway. In subsequent studies, animal experiments and more cell lines can be added, and multiple techniques including flow cytometry can be adopted to verify and explore other possible mechanisms of action.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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