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LncRNAp21 promotes osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the promoting effect of long non-coding ribonucleic acid p21 (lncRNAp21) on the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis (OP) through the Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS: A total of 30 healthy female rats were selected and randomly divided into three groups, including the lncRNAp21 group, OP model group (model group) and normal group. Rats in the lncRNAp21 group were given a certain quantity of lncRNAp21 inhibitors for gavage. Rats in the model group were regularly given 0.9% NaCl for gavage every day after the removal of bilateral ovaries. Meanwhile, rats in the normal group were fed normally without any changes. Bone mineral density (BMD) was measured after 12 weeks of modeling. The levels of procollagen type I N-terminal propeptide (PINP), serum estradiol (E2), osteocalcin (OC), bone alkaline phosphatase (BALP), C-terminal cross-linking telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRACP-5b) in the bone and serum of rats were measured by enzyme-linked immunosorbent assay (ELISA). Besides, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blotting were adopted to detect the mRNA and protein expressions of Wnt1 and β-catenin in bone tissues, respectively.

RESULTS: Compared with the normal group and lncRNAp21 group, the serum level of E2 in the model group decreased significantly ($p<0.05$). BMD and phosphorus (P) content in the model group were both markedly lower than those of the normal group and lncRNAp21 group. However, calcium (Ca) content was remarkably higher than that of the normal group and lncRNAp21 group ($p<0.05$). The serum levels of bone resorption markers (including TRACP-5b and CTX) in the model group were prominently higher than those of the normal group ($p<0.05$). However, the levels of the two markers in the lncRNAp21 group were significantly lower than the model group ($p<0.05$). Additionally, bone formation markers (including OC, PINP and BALP) in the serum of rats in the model group were notably higher than those in the normal group and lncRNAp21 group ($p<0.05$). QRT-PCR and Western blotting results revealed that the mRNA and protein expressions of Wnt1 and β-catenin in bone tissues of the model group were markedly lower than those of the normal group. However, the mRNA and protein expressions of Wnt1 and β-catenin in the lncRNAp21 group were remarkably higher than the model group ($p<0.05$).

CONCLUSIONS: Low expression of lncRNAp21 activates the Wnt/β-catenin signaling pathway by increasing E2 secretion, eventually stimulating bone formation and increasing osteogenic differentiation of mesenchymal stem cells in OP model rats.

Key Words:
Osteoporosis (OP), LncRNAp21, Wnt/β-catenin signaling pathway, Osteogenic differentiation.

Introduction

Osteoporosis (OP) refers to a metabolic imbalance between bone growth and bone resorption. Op may eventually result in decreased bone mass per unit structure and destroyed bone microstructure[1,2]. The regulation and differentiation of osteoclasts and osteoblasts are mainly involved in the dynamic process of bone metabolism imbalance in OP. During the process, signal pathways related to bone metabolism play irreplaceable regulatory roles[3]. Wnt proteins regulate osteoblast proliferation and differentiation through diffe-

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rent mechanisms. Meanwhile, its pathway plays a key role in the differentiation of varying bone tissues[4,5]. In addition, Wnt proteins induce osteoblast generation and modulate osteocyte differentiation. Ding et al[6] have demonstrated that these are closely associated with OP, arthritis, femoral head necrosis and other diseases. Human bone tissues are always in a dynamic balance between bone formation and bone resorption.

Research[7,8] has shown that long non-coding ribonucleic acids (lncRNAs), including lncRNAp2, lncRNA-micro RNA (lncRNA-miRNA), 1ncRNA-DNA and lncRNA-protein, play crucial and irreplaceable roles in various biological processes. Tang et al[9] has demonstrated that lncRNAs modulate the metabolism of bone cells. Meanwhile, lncRNAp21 promotes the proliferation of osteosarcoma cells. However, no research has elucidated whether it promotes the proliferation and differentiation of bone cells. In China, few studies have focused on the correlations of lncRNAp21 and Wnt/β-catenin with OP as well.

Therefore, a rat model of the postmenopausal OP was established through ovariectomy in this work. Furthermore, the effect of lncRNAp21 on the osteogenic differentiation of mesenchymal stem cells through the regulation of the Wnt/β-catenin signaling pathway was investigated. Our findings might provide a theoretical basis for biological researches of bone health.

Materials and Methods

Materials and Reagents

Serum bone alkaline phosphatase (BALP), tartrate-resistant acid phosphatase 5b (TRACP-5b), procollagen type I N-terminal propeptide (PINP), estradiol (E2) and c-terminal cross-linking telopeptide of type I collagen (CTX) detection kits and osteocalcin (OC) were purchased from the Beijing Institute of Bioengineering (Beijing, China); Reverse Transcription (RT) reagent tissue kit and total RNA extraction kit from Promega (Madison, WI, USA); primers were synthesized by Promega (Madison, WI, USA); Wntl, β-catenin and β-actin primary antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA); corresponding horseradish-labeled secondary antibodies from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China) and enhanced chemiluminescence (ECL) kit from Millipore (Billerica, MA, USA).

Experimental Instruments

Prodigy-BX IL dual-energy X-ray absorptiometry (DXA) apparatus for bone mineral density (BMD) was bought from Lunar (San Francisco, CA, USA); MK3 full-automatic micropore reader from Thermo Fisher Scientific (Waltham, MA, USA); ALCYON 300i full-automatic biochemical meter from Abbott Laboratories (Abbott Park, IL, USA); ultraviolet spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA); 7300 Real Time-fluorescence quantitative Polymerase Chain Reaction (PCR) instrument from Applied Biosystems (Foster City, CA, USA) and protein vertical electrophoresis system and semi-membrane transfer instrument from Bio-Rad (Hercules, CA, USA).

Animal Grouping

A total of 30 rats were selected in this study. Among them, 20 female rats were randomly ovariectomized to prepare the rat model of OP. BMD in the whole body of rats decreased significantly after 8 weeks. One week later, the administration was performed. OP model rats were randomly divided into three groups, including the lncRNAp21 group and OP model group (model group). Rats in the model group were given 0.9% NaCl for gavage every day, while rats in the lncRNAp21 group received gavage with lncRNAp21 inhibitors for 10 weeks. Meanwhile, 10 rats were randomly selected as a normal group without treatment. This study was approved by the Animal Ethics Committee of Shanxian County Central Hospital Animal Center.

OP Rat Modeling

At 12 h after the last administration, indexes were observed. Rats underwent solid fasting for 24 h, with free access to water. Meanwhile, they were anesthetized through the injection of chloral hydrate (3.6%). After anesthesia, rats in each group were fixed on an experimental table in the prone position. After the spinal column was straightened, BMD was detected by DXA. After the determination of BMD, blood samples were taken from rats and centrifuged at 5000 g/min for 15 min. After standing for 1 h, the serum samples were preserved. Subsequently, the serum levels of E2, OC, BALP, CTX, TRACP-5b were determined in strict accordance with the kit instructions. The contents of serum phosphorus (P) and calcium (Ca) were detected using an automatic biochemical analyzer. Finally, the bone tissues of the right lower limb of rats were collected after blood collection.

Detection of the mRNA Expression of the Wnt/β-Catenin Signal in Rats

0.1 g bone tissues were first taken from the right lower limb of rats after repeated grinding and quick freezing using liquid nitrogen. Total RNA in tissues was extracted through ultrasonic grinding using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After quantitative determination, extracted total RNAs were reversely transcribed into complementary deoxyribonucleic acid (cDNA). Fluorescent dye and primers were then added for amplification. Specific PCR conditions were: denaturation at 95°C for 120 s and at 96°C for 10s and annealing at 72°C for 30 s and at 75°C for 35 s, for a total of 40 cycles, followed by reaction at 72°C for 300 s. The relative expressions of genes were calculated using the $2^{-\Delta Ct}$ method. QRT-PCR relative amplification method was adopted for statistical analysis[9]. Each experiment was repeated 3 times. Designed specific primers were shown in Table I.

Determination of the Protein Expressions of β-Catenin and Wnt1 in Bone Tissues by Western Blotting

Phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China) homogenate, radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) tissue lysate and grinding fluid were added into 1 g bone tissue with good condition. After protein quantification, the proteins were subjected to a water bath at 100°C for 900 s. Subsequently, extracted proteins were separated by electrophoresis with 45 g polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Roche, Basel, Switzerland) by the semi-dry method. After sealing with skim milk powder for 2 h, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with corresponding secondary antibodies for 2 h at room temperature. Enhanced chemiluminescence (ECL; Millipore, Billerica, MA, USA) reagent was then added for color observation and exposure. β-catenin was used as an internal reference. The images were scanned, and the absorbance value of wavebands was analyzed using ImageJ software (NIH, Bethesda, MD, USA). Each experiment was repeated at least three times.

Table I. Designed primer sequences.

Protein	Gene	Primer sequence
Wnt1	Forward	CAAGATCGTCAACCGAGGCT
	Reverse	TCACACGTGCAGGATTCGAT
β-catenin	Forward	GCGCCATTTTAAGCCTCTCG
	Reverse	CTGAAGCTGCTCCTCAGACC
β-actin	Forward	GAGCTGTCTGCCTTGGTAGT
	Reverse	GCAGTCCTTCTGGCCCATAC

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. E2 content, BMD, bone resorption markers, bone formation markers and Wnt1 and β-catenin mRNAs in the normal group, model group and lncRNAp21 group were compared by *t*-test. Univariate analysis was performed to compare the differences among different groups. Count data were expressed as ($\bar{x}\pm s$). $p<0.05$ was considered statistically significant.

Results

Serum Content of E2 in Rats

In this experiment, the ovariectomized female rat model was used to simulate postmenopausal OP. The results showed that, compared with the normal group and lncRNAp21 group, the serum content of E2 in rats of the model group was significantly reduced ($p<0.05$; Figure 1).

BMD in Rats

The results manifested that BMD and P contents in the model group were significantly lower than those of the normal group. However, Ca content was markedly higher than that of the normal group ($p<0.05$). In comparison with the model

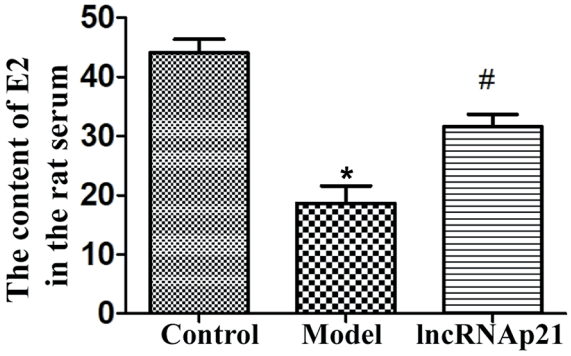

Figure 1. Comparison of serum E2 content among three groups of rats. Note: $^{*}p<0.05$ *vs.* normal group, and $^{\#}p<0.05$ *vs.* model group.

Table II. Detection results of serum Ca, P and BMD in the three groups ($\bar{x}$±s).

Group	Number of rats (No.)	Ca (mmol/L)	P (mmol/L)	BMD (g/cm²)
Normal group	10	3.12±0.21	4.65±0.31	0.197 ± 0.008
Model group	10	4.22 ± 0.11*	2.84 ± 0. 17*	0.147 ± 0.003*
LncRNAp21 group	10	3.48 ± 0. 23#	3.35 ± 0. 27#	0.165±0.006#

Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

group, BMD and P content in the lncRNAp21 group increased significantly, while Ca content decreased remarkably (p<0.05; Table II).

Serum Bone Resorption Markers in Rats

In comparison with the model group, serum bone resorption markers (including TRACP-5b and CTX) in the normal group increased significantly (p<0.05). However, those in the lncRNAp21 group were remarkably reduced (p<0.05; Table III).

Content of Serum Bone Formation Markers in Rats

Bone formation markers (including OC, PINP and BALP) in the serum of rats in the model group were notably higher than those of the normal group and lncRNAp21 group (p<0.05; Table VI).

MRNA Expressions of Wnt1 and β-Catenin in Bone Tissues of Rats in Each Group

QRT-PCR results indicated that compared with the normal group, the mRNA expression levels of Wnt1 and β-catenin in bone tissues of rats in the model group were significantly down-regulated. Compared with the model group, the mRNA expression levels of Wnt1 and β-catenin in the lncRNAp21 group was remarkably up-regulated (p<0.05; Figure 2).

Protein Expressions of Wnt1 and β–Catenin in Bone Tissues of Rats Detected via Western Blotting

Western blotting was adopted to detect the protein expressions of Wnt1 and β-catenin in bone tissues of rats in the three groups. It was found

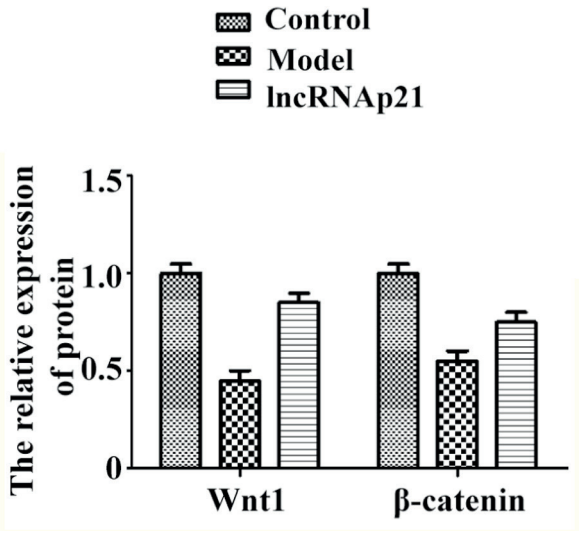

Figure 2. MRNA expression levels of β-catenin and Wnt1 in the three groups of rats detected via qRT-PCR. Note: *p<0.05 vs. normal group, and #p<0.05 *vs.* model group.

that compared with the normal group, the protein expression levels of Wnt1 and β-catenin in the model group were remarkably reduced. Moreover, compared with the model group, the protein expression levels of Wnt1 and β-catenin in the lncRNAp21 group were markedly elevated (p<0.05; Figure 3-5).

Discussion

OP is a systemic metabolic bone disease, which is related to the increase of age to a certain degree. As the population aging proceeds worldwide, OP has become an important hot issue

Table III. Results of serum bone resorption markers in the three groups ($\bar{x}$±s).

Group	Number of rats (No.)	CTX (μg/L)	TRACP-5b (ng/L)
Normal group	10	183 ± 15	1256 ± 105
Model group	10	356 ± 25*	2542 ± 156*
LncRNAp21 group	10	289±22#	1876±111#

Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

Table IV. Comparisons of serum bone formation markers among different groups of rats ($\bar{x}$±s).

Group	Number of rats (No.)	OC (ng/L)	PINP (μg/L)	BALP (U/L)
Normal group	10	1673±155	14.23 ± 1.02	114±11
Model group	10	1977 ± 126*	18.46 ± 1.36*	145 ± 13*
LncRNAp21 group	10	1773± 202#	16.11±2.03#	128±14#

Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

globally[10]. During the prevention and treatment of OP, it has been found that the β-catenin/Wnt signaling pathway plays an irreplaceable role in the occurrence and development of OP[11]. Postmenopausal OP in middle-aged and elderly women is a crucial factor in increasing fracture risk[12-14]. OP is characterized by decreased bone tissue per unit volume and increased bone fragility caused by the decrease of estrogen secretion1[15,16]. In this experiment, the ovariectomized female rat model was successfully established to simulate female postmenopausal OP. The results showed that, compared with the normal group and lncRNAp21 group, the serum content of E2 in rats of the model group was significantly reduced (p<0.05). This suggested that low expression of lncRNAp21 could increase the expression of serum E2 in rats and relieve osteoporosis symptoms.

In this study, BMD and P content in model group were significantly lower than those of the normal group and lncRNAp21 group, whereas Ca content was markedly higher than the normal group and lncRNAp21 group (p<0.05). Serum bone resorption markers (including TRACP-5b and CTX) in the model group increased remarkably when compared with those in the normal group (p<0.05). Meanwhile, those in the lncRNAp21 group significantly decreased in comparison with the model group (p<0.05). Additionally, bone formation markers (including OC, PINP and BALP) in the serum of rats in the model group were notably higher than those of the normal group and lncRNAp21 group (p<0.05). Liu et al[17] and Kemi et al[18] have proved that Ca and P are biochemical markers of bone metabolism in serum, which are commonly applied. Elevated serum Ca often indicates accelerated dissolution of bones, representing bone loss. There is a proportion

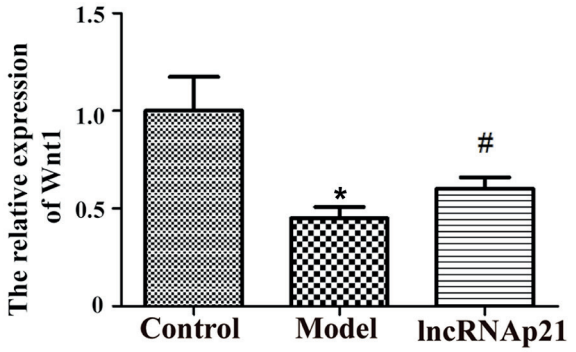

Figure 4. Protein expression level of Wnt1 in the three groups of rats. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

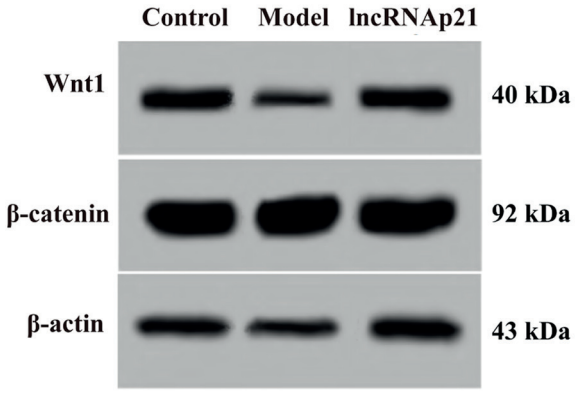

Figure 3. Protein expression levels of Wnt1 and β-catenin in the three groups of rats detected via Western blotting. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

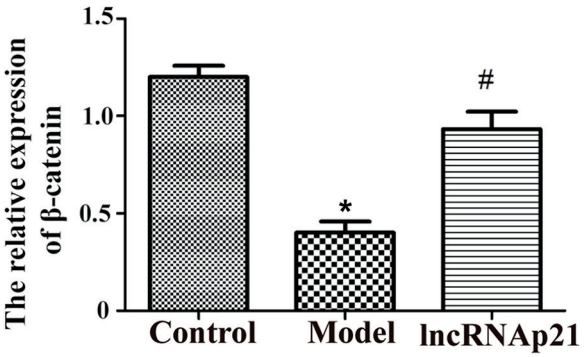

Figure 5. Protein expression level of β-catenin in the three groups of rats. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

of serum Ca to P. Meanwhile, the concentrations of serum Ca and P can reflect the degree of bone resorption to a certain extent. As biochemical markers of bone formation, OC, PINP and BALP can effectively reflect the activity of osteoblasts. Some studies[19,20] have also indicated that Akt promotes the proliferation and differentiation of osteoblasts. Therefore, lncRNAp21 reduces the differentiation of osteoblasts by suppressing Akt signals, which is similar to the results of this study.

RT-PCR and Western blotting results revealed that, compared with the normal group, the protein expression levels of Wnt1 and β-catenin in the model group were remarkably reduced ($p<0.05$). Meanwhile, the protein expression levels of Wnt1 and β-catenin in the lncRNAp21 group were markedly higher than the model group ($p<0.05$). The Wnt/β-catenin signaling pathway, one of the most classical signaling pathways, plays a vital role in the differentiation of varying bone tissues[21,22]. Acting as the most pivotal nuclear transcription factors in bone tissues, the Wnt/β-catenin signaling pathway modulates cell cycle, regulates osteoblast proliferation and stimulates bone formation. Evidence[23,24] has found that lncRNAp21 inhibits the mRNA expression levels of Wntl and β-catenin. Meanwhile, it suppresses the secretion of basic fibroblast growth factors, insulin-like growth factors and vascular endothelial growth factors by BMMSCs. This may eventually lead to the differentiation of BMMSCs into osteoblasts by regulating the Wnt1/β-catenin signal. The prevention and treatment mechanisms of OP are primarily achieved by the interaction and regulation of a series of signal molecules and cytokines and signaling pathways, thereby inhibiting bone resorption and promote bone formation. Therefore, the Wnt/β-catenin pathway is presumed to play a vital role in the treatment and diagnosis of OP[23,25,26].

Conclusions

We observed that lncRNAp21 activates the Wnt/β-catenin signaling pathway by increasing E2 secretion, thus stimulating bone formation and increasing the osteogenic differentiation of mesenchymal stem cells in an OP model. Our findings provide novel insights into the clinical prevention and treatment of OP.

Conflict of interest

The authors declare no conflicts of interest.

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