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The Effect of Hoxa11-as on the Differentiation of Osteoblasts in Osteoporosis by Regulating Cellular Autophagy

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Abstract: Objective: To investigate the effect of long non-coding RNA (lncRNA) Hoxa11-as on the osteogenic differentiation in osteoporosis (OP) through the regulation of cellular autophagy. **Methods**: Establish a rat model of osteoporosis. Divide 12-week-old male rats into a blank group, a Hoxa11-as group, and a si-Hoxa11-as group, with 10 rats in each group. To establish glucocorticoid-induced osteoporosis, the required concentration is 1 ml/kg. The average body weight of this batch of rats is 500 g. The glucocorticoid is intraperitoneally injected at a dose of 0.5 ml/kg per day, and the intraperitoneal injection is continued for 8 weeks. After successfully establishing the rat model of osteoporosis, a bone rotary drill is used to create a 1-mm defect at the distal end of the femur of the model. At the same time, a hydrogel containing Hoxa11-as and si-Hoxa11-as plasmids is placed. After continuing the culture for 4 weeks, the samples are collected. Perform immunohistochemistry (IHC) and Real-time quantitative polymerase chain reaction (qPCR) experiments on the specimens to detect the protein expression levels of Hoxa11-as, LC3, Beclin1, and Runx2 factors. **Results**: The results of qPCR indicated that in the Hoxa11-as group, there were statistically significant differences in the mRNA expression levels of Hoxa11-as, LC3, Beclin1 and Runx2 ($P < 0.05$); in the si-Hoxa11-as group, there were also statistically significant differences in the mRNA expression levels of Hoxa11-as, LC3, Beclin1 and Runx2 ($P < 0.05$). **Conclusion**: Hoxa11-as has an inhibitory effect on cellular autophagy, while si-Hoxa11-as can activate cellular autophagy and enhance the differentiation level of osteoblasts.

Keywords: Hoxa11-as; Osteoporosis; Autophagy; Osteoblasts.

1. Introduction

Osteoporosis is one of the common orthopedic diseases. It is a systemic bone disease in which bone density and bone quality decrease due to various reasons, the bone microstructure is damaged, and bone brittleness increases, thus easily inducing fractures [1]. With the rapid aging of the global population, the prevalence of osteoporosis has increased significantly. This not only brings pain to patients but also imposes a heavy burden on society and families [2]. Therefore, understanding the pathogenesis of osteoporosis and formulating reasonable treatment plans are of great significance for reducing the burden on society and families [3]. Common causes of osteoporosis include aging, reduction of mechanical stimulation, and metabolic disorders of bones and hormones. Among them, the prevalence rate in women is significantly higher than that in men, which is related to the significant decrease in estrogen levels after menopause and the imbalance of endocrine metabolism [4]. According to relevant studies, the main cause of osteoporosis is the disorder of the balance directly between the reduced generation of osteoblasts and the continuous bone resorption of osteoclasts [5]. When osteoclasts are active and the activity of osteoblasts is weakened, bone formation will be affected, leading to the occurrence of osteoporosis [6].

There is evidence indicating that selective autophagy within cells plays an important role in regulating the proliferation, differentiation, and function of osteoblasts and osteoclasts [7]. Autophagy is a process in eukaryotic cells for proliferation, differentiation, self-degradation, and dynamic energy recycling. It can degrade damaged organelles in cells, while maintaining cellular homeostasis and providing energy and alkaline substances [8]-[9]. Autophagy is a normal physiological activity that occurs under healthy conditions, but it can also be accelerated under pathological conditions [10]-[11]. Abnormal autophagy will disrupt the balance of bone metabolism and play a crucial role in bone metabolic activities [12]. The homeostasis of bone metabolism mainly depends on the balance between the formation of osteoblasts and the bone resorption of osteoclasts. Bone marrow mesenchymal stem cells are the main source of osteoblasts and play a vital role in the development and maintenance of bones [13]-[14]. During the early process of osteogenic differentiation, the expression of the autophagosome marker LC3 in mesenchymal stem cells (MSCs) decreases, which indicates that autophagy can generate energy substrates to support the differentiation of MSCs [15][17]. The inhibition of autophagy in osteoblasts will lead to a decrease in bone mass, and the inhibition of autophagy in bone cells will lead to the aging of bone tissues[18]-[19]. These research findings fully demonstrate that autophagy plays an important role in the process of bone metabolism and is worthy of further exploration.

There are various factors involved in the process of regulating autophagy, and these factors directly or indirectly affect the occurrence of autophagy. Beclin1 is the core part of the autophagy initiation complex. It can interact with the complexes of PI3K, such as proteins like Vps34 and Vps15, to form phosphatidylinositol-3-phosphate (PI3P). The bound complex initiates the nucleation process of autophagosomes, which is the initial step in the formation of autophagosomes[20]-[21]. Beclin1 participates in the

occurrence and development of autophagy through a variety of signal transduction effects[22]-[23]. For example, the mTOR signaling pathway is an important intracellular nutrient and energy sensor. When nutrients are sufficient, mTOR can phosphorylate Beclin1, inhibiting its activity and thus suppressing autophagy; when nutrients are deficient, the activity of mTOR is inhibited, Beclin1 is dephosphorylated, and its activity is enhanced, thereby promoting the occurrence of autophagy[24]-[26]. LC3 is a homologue of the autophagosome of yeast Atg8, and its lipid form LC3-II is considered a marker of autophagosomes. It is an essential autophagic protein in autophagosomes and is widely used as a marker for experimentally detecting autophagy. Runx2 is considered a key factor in initiating the differentiation of MSCs into osteoblasts. It can activate the expression of specific genes of osteoblasts, enabling MSCs to differentiate into osteoblasts in a directed manner. During the process of promoting the maturation of osteoblasts, Runx2 can regulate the expression of osteocalcin and osteopontin. These factors are important indicators for measuring the maturation of osteoblasts[27]-[28].

Osteoblasts are also regulated by lncRNA during the differentiation process. During osteoblast differentiation, lncRNA participates in controlling the processes of mRNA transcription and translation[29]-[30]. Moreover, lncRNA can generate smaller non - coding molecules, such as miRNA, which increases osteoblast differentiation by activating Runx2 and SP7[31]. In recent years, the role of lncRNA in related fields such as bone metabolism and cancer diagnosis has attracted much attention. Among them, lncRNA Hoxa11 - as can participate in scar formation through the Wnt pathway and is an essential initiator and promoter in the proliferation and metastasis of malignant tumors[32]-[33]. According to relevant reports, lncRNA Hoxa11 - as can promote the activation of the PI3K - AKT pathway in cells. The activation of the PI3K - AKT - mTOR signaling pathway can increase the expression of the downstream mTOR factor, thereby reducing osteoblast autophagy[34]. Can increasing cellular autophagy promote the formation of osteoblasts? This makes the treatment of osteoporosis by regulating cellular autophagy a new option.

The above studies show that lncRNA has the potential to regulate cellular biological functions by regulating the PI3K - AKT - mTOR signaling pathway. In osteoporosis, whether Hoxa11 - as can also affect the bone remodeling process by regulating cellular autophagy function has not been reported yet. Therefore, in - depth research on the relationship between Hoxa11 - as and cellular autophagy is of great significance for revealing the pathogenesis of osteoporosis and finding new therapeutic targets.

2. Materials

2.1. Source of Sprague-Dawley (SD) rats

Thirty 12-week-old male Sprague-Dawley (SD) rats (with a body weight between 410 and 500 g) were selected. The SD rats were provided by Guangdong Vital River Laboratory Animal Technology Co., Ltd. The experimental animals were tested by a third-party experimental animal quality supervision and testing institution, and the corresponding experimental animal use license was obtained. This study was approved by the Experimental Animal Ethics Committee of Youjiang Medical University for Nationalities. All experimental operations were carried out in strict accordance with the relevant management regulations and usage specifications of experimental animals.

2.2. Drugs

HOXA11-AS and si-HOXA11-AS were purchased from Wuhan Miaoling Biotechnology Co., Ltd. The LC3B antibody, Bcelin1 antibody, and Runx2 antibody were purchased from Chengdu Zhengneng Biotechnology Co., Ltd.

3. Method

3.1. Grouping of Sprague-Dawley (SD) Rats and Establishment of the Osteoporosis Model

Twelve-week-old male rats were divided into a blank group, a Hoxa11-as group, and a si-Hoxa11-as group, with 10 rats in each group. To establish glucocorticoid-induced osteoporosis, the required concentration was 1 ml/kg. The average body weight of this batch of rats was 500 g. Glucocorticoid was intraperitoneally injected at a dose of 0.5 ml/kg per day, and the intraperitoneal injection was continued for 8 weeks. After successfully establishing the rat model of osteoporosis, a bone rotary drill was used to create a 1-mm defect at the distal end of the femur of the model. Meanwhile, a hydrogel containing Hoxa11-as and si-Hoxa11-as plasmids was placed. After continuing the culture for 4 weeks, the samples were collected.

3.2. Experimental Methods

3.2.1. Decalcification and Embedding Treatment of Specimens

The harvested specimens were decalcified using the EDTA decalcification method. They were first soaked in a formaldehyde solution for 24 hours for fixation. Then, the specimens were placed in an EDTA decalcification solution and soaked. The decalcification solution was replaced every four days, and the soaking lasted for four weeks. When it was found through inspection that the surface of the specimens had softened, it indicated successful decalcification.

3.2.2. Verification of the Establishment of the Osteoporosis Rat Model by HE Staining

Perform HE staining on the cut specimen tissue slides. Cover the sample with hematoxylin staining solution and stain for 5 minutes. After washing off the hematoxylin staining solution, add 1% hydrochloric acid ethanol for differentiation. The differentiation time is 7 seconds, and then remove the differentiation solution. Add an appropriate amount of hematoxylin bluing solution and wash it thoroughly. Drop eosin solution to cover the sample and stain for 10 seconds. Dehydrate and make it transparent in absolute ethanol, and then the slide can be sealed.

3.2.3. Immunohistochemistry (IHC) of Tissues

Place the cut specimen tissue slides into a pot containing EDTA antigen retrieval solution and distilled water and bring it to a boil. Take out the slides after 10 minutes and let them cool down at room temperature. Drop 3% hydrogen peroxide in the center of the specimens, and incubate them with shaking for 10 minutes. Block the specimens with 5% BSA solution for 1 hour. Add the antibodies against Hoxa11-as, LC3, Beclin1 and Runx2, and incubate them overnight in a refrigerator at 4°C. After the incubation of the primary antibodies is completed, add the HRP-labeled mouse/rabbit secondary antibody reagent and incubate at room temperature for 30 minutes. Then add the chromogenic solution for color

development.

3.2.4. Quantitative Real-time Polymerase Chain Reaction (qPCR) of Tissues to Measure Protein Expression Levels

The harvested rat bone tissues were thoroughly ground in a grinder using TRIzol and grinding beads. The obtained suspension was transferred into a 1.5 ml EP tube, and a chloroform substitute was added. Then, the RNA was extracted by centrifugation. After reverse transcription and amplification, the relative mRNA expression levels of various factors were calculated and analyzed using the 2-△△Ct method, with GAPDH as the internal reference. The data were exported for statistical analysis. The primer table is shown in Table 1.

Table 1. Primer Sequences

Primer Information	Primer Name	Primer sequence (5'-3')
GAPDH	R-GAPDH-S	CTGGAGAAACCTGCCAAGTATG
	R-GAPDH-A	GGTGGAAGAATGGGAGTTGCT
Hoxa11-as	R-Hoxa11-as-S	CGAACCTTGGGCTGCTTTA
	R-Hoxa11-as-A	AGGACTGGAACTCGGACTTGG
LC3	R- LC3-S	ACAGCATGGTGAGTGTGTCC
	R- LC3-A	AAGGTTTCCTGGGAGGCGTA
Runx2	R- Runx2-S	ATGCTTCATTCGCCTCACAAA
	R- Runx2-A	GCACTCACTGACTCGGTTGG
Beclin1	R- Beclin1-S	AGGAGTTGCCGTTGTACTGTTCT
	R- Beclin1-A	GTGTCTTCAATCTTGCCTTTCTCC

3.2.5. Statistical Methods

Statistical analysis was performed using GraphPad Prime10. Measurement data were expressed as mean ± standard deviation. When the data conformed to a normal distribution and had homogeneous variances, one-way analysis of variance (ANOVA) was used, and the least significant difference (LSD) method was applied for multiple comparisons among groups. If the data did not conform to a normal distribution, a non-parametric test for multiple independent samples was adopted. Enumeration data were expressed as rates, and the chi-square test was used. A P value less than 0.05 was considered to indicate a statistically significant difference. (*P＜0.05, ** P＜0.01, *** P＜0.001)

4. Results

4.1. Results of HE Staining of the Osteoporosis Rat Model

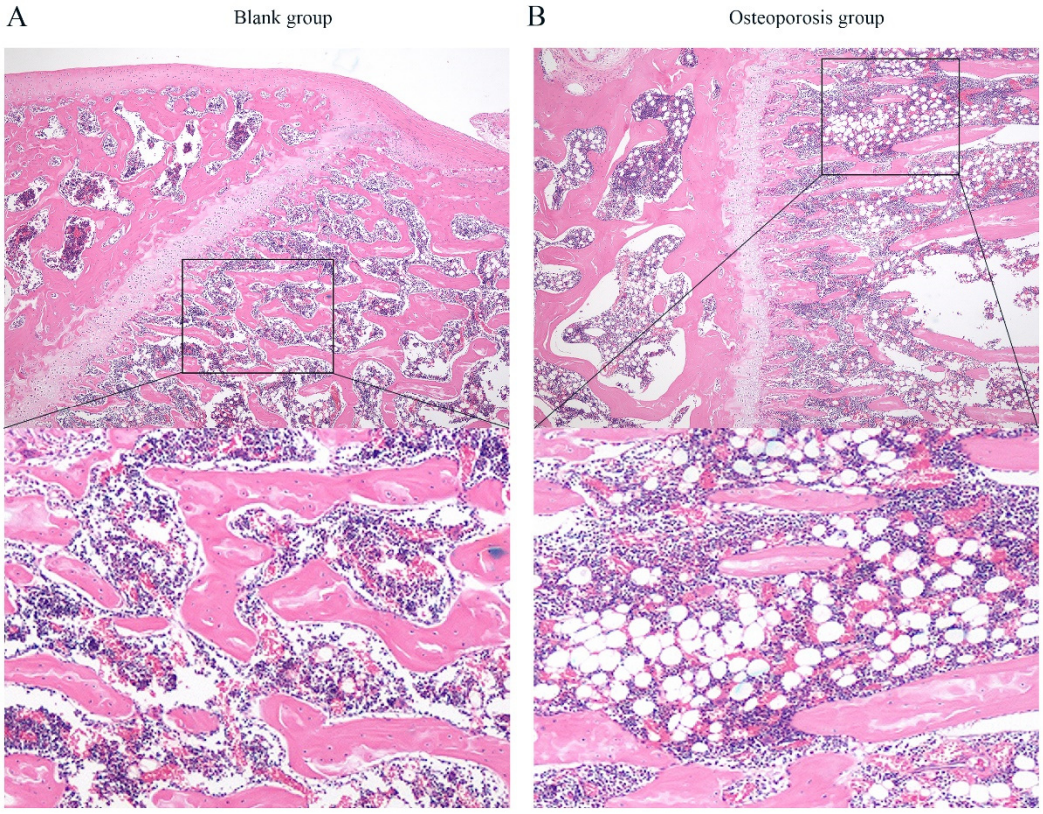

Figure 1. Results of HE staining. A represents the Blank group, and B represents the Osteoporosis group

Observe the results of HE staining, as shown in Figure 1: Figure A is from the blank group, which is a HE-stained image of the femoral section of a normal 20-week-old rat; Figure B is a HE-stained image of the femoral section of a 20-week-old osteoporosis model rat. Both groups were observed under a 40-fold microscope. The pink part is the bone tissue, and the purple part is the bone marrow tissue. Compared with the blank group, the trabeculae in the osteoporosis group are arranged sparsely, with obvious fractures. The area of the trabeculae is significantly reduced, and the bone marrow cavity is enlarged. It can be concluded that the osteoporosis rat model was successfully established.

4.2. Results of Tissue Immunohistochemistry (IHC)

The results of IHC are shown in Figure 2. When observing the images under a microscope at a magnification of X200, the brown part represents the cell nucleus, and the positive staining appears brown. Hoxa11-as often exerts its function in the cell nucleus, but it can also be expressed in the cytoplasm. Therefore, positive expressions are often observed in both the cell nucleus and the cytoplasm in the images. LC3 and Beclin1 are mainly expressed in the cytoplasm and are basically not expressed in the cell nucleus. Runx2 is expressed in the cell nucleus.

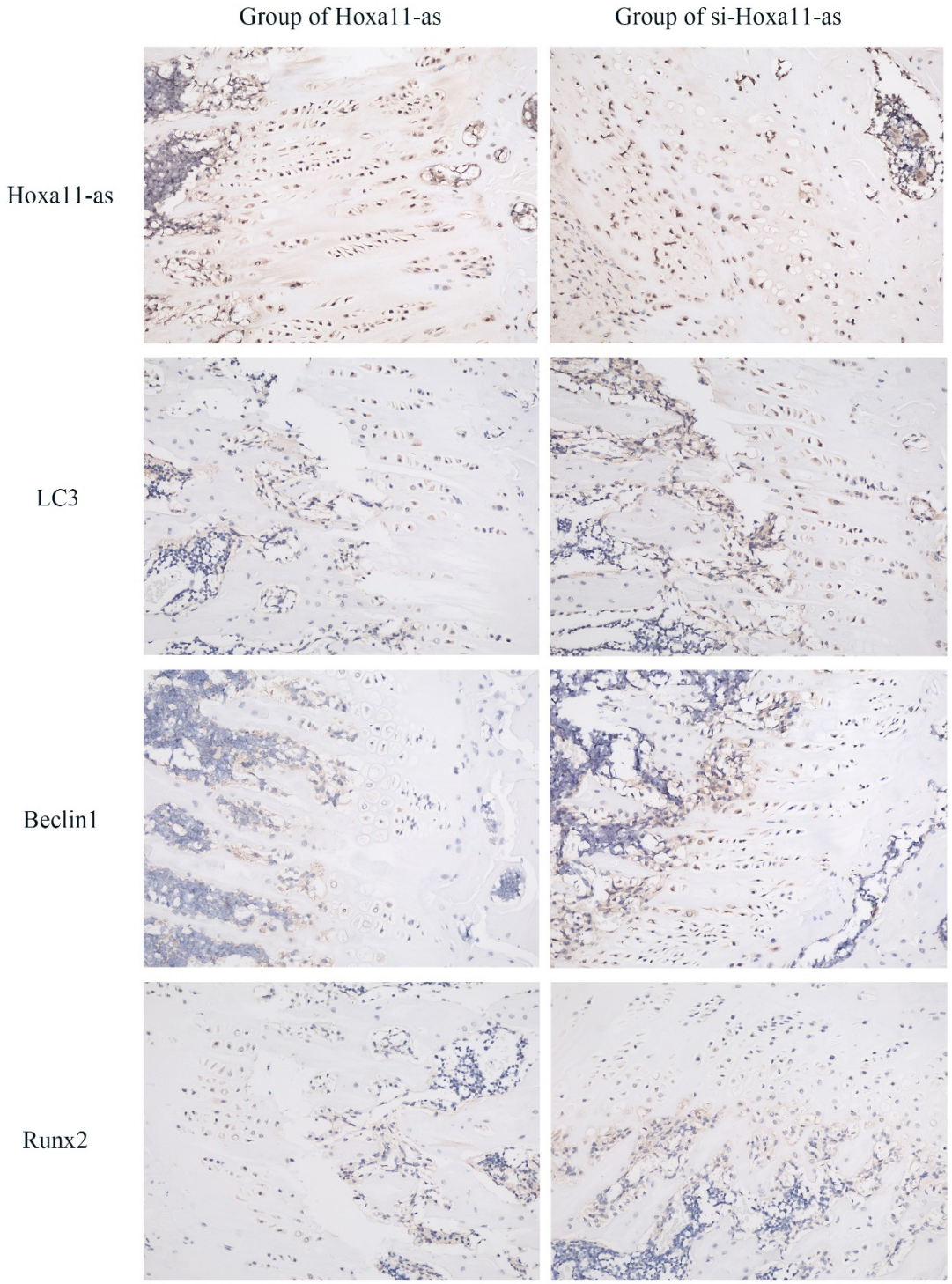

Figure 2. Results of immunohistochemistry (IHC) staining

4.3. Results of Tissue Quantitative Polymerase Chain Reaction (qPCR)

The results of qPCR are shown in Figure 3: Figure A shows the mRNA expressions after transfection in the blank group, the si-Hoxa11-as group, and the Hoxa11-as group. The mRNA expression in the Hoxa11-as group was significantly different ($P < 0.05$). The qPCR results indicated that in the Hoxa11-as group, there were statistically significant differences in the mRNA expression levels of Hoxa11-as, LC3, Beclin1, and Runx2 ($P < 0.05$); in the si-Hoxa11-as group, there were also statistically significant differences in the mRNA expression levels of Hoxa11-as, LC3, Beclin1, and Runx2.

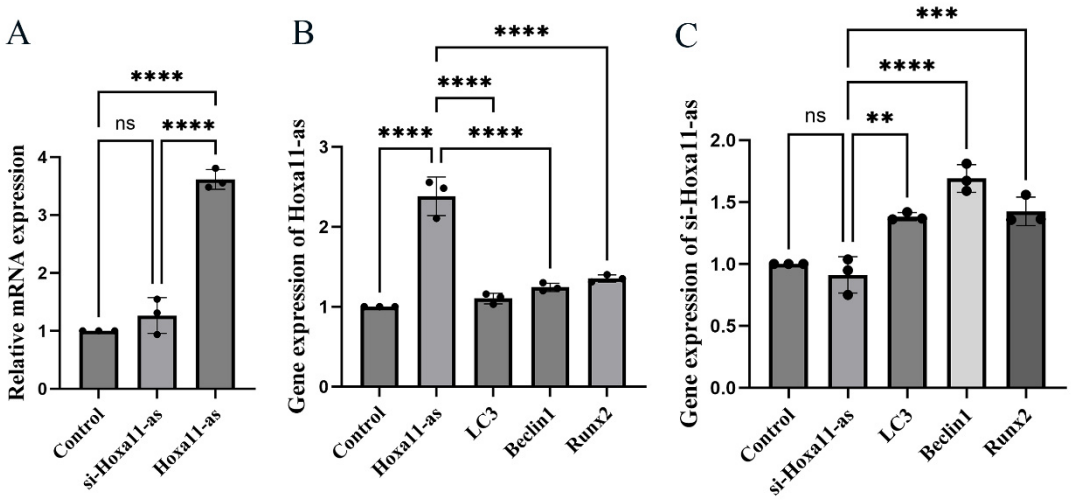

Figure 3. Results of qPCR. A shows the mRNA expression levels after transfection; B shows the mRNA expression levels of Hoxa11-as, LC3, Beclin1 and Runx2 in the Hoxa11-as group; C shows the mRNA expression levels of Hoxa11-as, LC3, Beclin1 and Runx2 in the si-Hoxa11-as group

5. Discussion

In this experiment, we established a rat osteoporosis model and studied the effect of lncRNA on osteoblasts in osteoporosis through its regulatory effect on cellular autophagy. According to the qPCR results, the mRNA expression levels of LC3, Beclin1, and Runx2 were affected by Hoxa11-as. When Hoxa11-as was highly expressed, LC3, Beclin1, and Runx2 showed low expression to varying degrees; when si-Hoxa11-as was expressed, LC3, Beclin1, and Runx2 showed a trend of high expression. This demonstrated the influence of Hoxa11-as on cellular autophagy and osteoblast differentiation. The IHC results also support this view. In the Hoxa11-as group, the positive cell staining of LC3, Beclin1, and Runx2 was significantly lower than that in the si-Hoxa11-as group, which also indicated that after the expression of si-Hoxa11-as, the protein expression levels of LC3, Beclin1, and Runx2 showed an increasing trend. Through this experiment, it was found that si-Hoxa11-as can effectively increase the level of cellular autophagy and, at the same time, affect the formation and differentiation of osteoblasts in osteoporosis. In the future, the mechanism of its action will be further explored to find new targets for the treatment of osteoporosis.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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References

[1] Srivastava M, Deal C. Osteoporosis in elderly: prevention and treatment. Clin Geriatr Med. 2002 Aug;18(3):529-55.

[2] Xiao PL, Cui AY, Hsu CJ, et al. Global, regional prevalence, and risk factors of osteoporosis according to the World Health Organization diagnostic criteria: a systematic review and meta - analysis [J]. Osteoporos Int, 2022, 33 (10): 2137 - 2153.

[3] Yong EL, Logan S. Menopausal osteoporosis: screening, prevention and treatment. Singapore Med J. 2021 Apr;62 (4): 159-166.

[4] Li J, Chen X, Lu L, et al. The relationship between bone marrow adipose tissue and bone metabolism in postmenopausal osteoporosis [J]. Cytokine Growth Factor Rev, 2020, 52: 88 - 98.

[5] Deng P, Yuan Q, Cheng Y, et al. Loss of KDM4B exacerbates bone - fat imbalance and mesenchymal stromal cell exhaustion in skeletal aging [J]. Cell Stem Cell, 2021, 28 (6): 1057 - 1073.e7.

[6] Chen C, Huang L, Chen Y, et al. Hydrolyzed egg yolk peptide prevented osteoporosis by regulating Wnt/β - catenin signaling pathway in ovariectomized rats [J]. Sci Rep, 2024, 14 (1): 10227.

[7] Wang S, Deng Z, Ma Y, et al. The Role of Autophagy and Mitophagy in Bone Metabolic Disorders [J]. Int J Biol Sci, 2020, 16 (14): 2675 - 2691.

[8] Yoshida GJ. Therapeutic strategies of drug repositioning targeting autophagy to induce cancer cell death: from pathophysiology to treatment [J]. J Hematol Oncol, 2017, 10 (1): 67.

[9] Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms [J]. J Pathol, 2010, 221 (1): 3 - 12.

[10] Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis [J]. Biol Chem, 2012, 393 (7): 547 - 564.

[11] Wang Y, Liu N, Lu B. Mechanisms and roles of mitophagy in neurodegenerative diseases [J]. CNS Neurosci Ther, 2019, 25 (7): 859 - 875.

[12] Wang Z, Liu N, Liu K, et al. Autophagy mediated CoCrMo particle - induced peri - implant osteolysis by promoting osteoblast apoptosis [J]. Autophagy, 2015, 11 (12): 2358 - 2369.

[13] Yang X, Yang J, Lei P, et al. LncRNA MALAT1 shuttled by bone marrow - derived mesenchymal stem cells - secreted exosomes alleviates osteoporosis through mediating microRNA - 34c/SATB2 axis [J]. Aging (Albany NY), 2019, 11 (20): 8777 - 8791.

[14] Zaidi M. Skeletal remodeling in health and disease [J]. Nat Med, 2007, 13 (7): 791 - 801.

[15] Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing [J]. EMBO J, 2000, 19 (21): 5720 - 5728.

[16] Pierrefite - Carle V, Santucci - Darmanin S, Breuil V, et al. Autophagy in bone: Self - eating to stay in balance [J]. Ageing Res Rev, 2015, 24 (Pt B): 206 - 217.

[17] Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy [J]. Int J Biochem Cell Biol, 2004, 36 (12): 2503 - 2518.

[18] Li H, Li D, Ma Z, et al. Defective autophagy in osteoblasts induces endoplasmic reticulum stress and causes remarkable bone loss [J]. Autophagy, 2018, 14 (10): 1726 - 1741.

[19] Yin X, Zhou C, Li J, et al. Autophagy in bone homeostasis and the onset of osteoporosis [J]. Bone Res, 2019, 7: 28.

[20] Tran S, Fairlie WD, Lee EF. BECLIN1: Protein Structure, Function and Regulation [J]. Cells, 2021, 10 (6): 1522.

[21] Funderburk SF, Wang QJ, Yue Z. The Beclin 1 - VPS34 complex--at the crossroads of autophagy and beyond [J]. Trends Cell Biol, 2010, 20 (6): 355 - 362.

[22] Nishimura T, Tooze SA. Emerging roles of ATG proteins and membrane lipids in autophagosome formation [J]. Cell Discov, 2020, 6 (1): 32.

[23] Robinson JS, Klionsky DJ, Banta LM, et al. Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases [J]. Mol Cell Biol, 1988, 8 (11): 4936 - 4948.

[24] Mei Y, Ramanathan A, Glover K, et al. Conformational Flexibility Enables the Function of a BECN1 Region Essential for Starvation - Mediated Autophagy [J]. Biochemistry, 2016, 55 (13): 1945 - 1958.

[25] Kim J, Kundu M, Viollet B, et al. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1 [J]. Nat Cell Biol, 2011, 13 (2): 132 - 141.

[26] Ma X, Liu H, Murphy JT, et al. Regulation of the transcription factor EB - PGC1α axis by beclin - 1 controls mitochondrial quality and cardiomyocyte death under stress [J]. Mol Cell Biol, 2015, 35 (6): 956 - 976.

[27] Komori T. Whole Aspect of Runx2 Functions in Skeletal Development. Int J Mol Sci. 2022 May 21;23(10):5776.

[28] Lin TC. RUNX2 and Cancer. Int J Mol Sci. 2023 Apr 10;24 (8): 7001.

[29] Zhi F, Ding Y, Wang R, et al. Exosomal hsa_circ_0006859 is a potential biomarker for postmenopausal osteoporosis and enhances adipogenic versus osteogenic differentiation in human bone marrow mesenchymal stem cells by sponging miR - 431 - 5p [J]. Stem Cell Res Ther, 2021, 12 (1): 157.

[30] Cui Y, Fu S, Sun D, et al. EPC - derived exosomes promote osteoclastogenesis through LncRNA - MALAT1 [J]. J Cell Mol Med, 2019, 23 (6): 3843 - 3854.

[31] Cao G, Meng X, Han X, et al. Exosomes derived from circRNA Rtn4 - modified BMSCs attenuate TNF - α - induced cytotoxicity and apoptosis in murine MC3T3 - E1 cells by sponging miR - 146a [J]. Biosci Rep, 2020, 40 (5): BSR 20193436.

[32] Sun XJ, Wang Q, Guo B, et al. Identification of skin - related lncRNAs as potential biomarkers that involved in Wnt pathways in keloids [J]. Oncotarget, 2017, 8 (21): 34236 - 34244.

[33] Xue JY, Huang C, Wang W, et al. HOXA11 - AS: a novel regulator in human cancer proliferation and metastasis [J]. Onco Targets Ther, 2018, 11: 4387 - 4393.

[34] Lyu Y, Bai L, Qin C, et al. Long noncoding RNAs in neurodevelopment and Parkinson's disease [J]. Animal Model Exp Med, 2019, 2 (4): 239 - 251.