Original Paper

LncRNA LINC00311 Promotes the Proliferation and Differentiation of Osteoclasts in Osteoporotic Rats Through the Notch Signaling Pathway by Targeting DLL3

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Key Words

Linc00311 • DLL3 • Notch signaling pathway • Osteoporosis • Proliferation • Differentiation • Osteoclasts

Abstract

Background/Aims: Osteoporosis is a commonly occurring condition marked by a loss of bone density. Previous evidence has highlighted the roles played by microRNAs as potential treatment tools for the disease. At present, the influence of long non-coding RNAs (lncRNAs) on the progression of osteoporosis remains largely unclear. Thus, an investigation was conducted into the target relationship between LINC00311, which has been reported to be highly expressed in osteoporosis, and delta-like 3 (DLL3), which is involved in the Notch signaling pathway. In connection with a series of bioinformatic methods. An osteoporotic rat model was established by means of ovariectomy (OVX) to evaluate the influence exerted by DLL3 on LINC00311 on osteoclasts through the Notch signaling pathway. **Methods:** Osteoclasts were extracted from osteoporotic rats and transfected with the LINC00311-vector, sh-LINC00311, Notch activator, or a combination of the Notch activator and LINC00311-vector. Western blotting and RT-qPCR techniques were applied to determine the expression levels of LINC00311, DLL3, Notch1, Notch2, Jagged1, Hes-1 and TRAP in tissues and cells, while cell activity was detected by MTT assay. The cell cycle as well as the rate of apoptosis was detected by flow cytometry. The successfully established osteoporotic rats were designated into the OVX-siRNA, OVX-LINC00311 and OVX-control groups to observe the effects of LINC00311 on the proliferation and differentiation of osteoclasts. **Results:** Cells transfected with the LINC00311-vector exhibited increased expression levels of Notch2 and TRPA as well as increased cell activity, while decreased expression levels of DLL3, Notch1, Jagged1 and Hes-1, along with a decreased cell apoptosis rate, were observed. The opposite tendencies of these parameters were observed in the cells treated with shRNA-LINC00311. A key observation

was made when the Notch signaling pathway was activated, in that the cell activity was decreased while the rate of apoptosis increased. In comparison with the OVX-control group, the expression levels of LINC00311, Notch2 and TRAP as well as the positive expression rate of TRAP all exhibited reductions, while those of DLL3, Jagged1 and Notch1 were elevated in the OVX-siRNA group. Compared with those in the sham group, in the OVX-control and OVX-LINC00311 groups, LINC00311 and the expression levels of Notch2 and TRAP were increased; however, decreased levels of DLL3, Jagged1 and Notch1 were noted. **Conclusions:** Taken together, the key findings of the present study suggest that LINC00311 induces proliferation and inhibits apoptosis of osteoclasts via the regulation of the Notch signaling pathway by inhibiting DLL3 expression, ultimately demonstrating that LINC00311 and its target gene may serve as independent factors in cases of osteoporosis.

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Introduction

The hallmark feature of osteoporosis is represented by a systemic impairment of bone mass, microarchitecture and strength, with the impairment accompanied by an increased possibility of fragility fractures [1]. Over 200 million people worldwide suffer from osteoporosis [2]. Postmenopausal women represent the main risk group, compared with their male counterparts, to be diagnosed with the condition and a greater prevalence of the disorder strongly correlated with increasing age [3]. The most susceptible regions of fragility fracture, as a result of osteoporosis, include the hip, spine and wrist. Osteoporosis continues to place a significant burden not only on public health but also on the economy [4]. Present treatment approaches for osteoporosis include bisphosphonates, which are widely considered to be an effective form of treatment [5]. The bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) has been regarded as an essential factor in the regulation of a variety of physiological and pathological biological processes including that of osteoporosis [6]. Bone homeostasis is maintained by a high balance between osteoblast and osteoclast activity, with increased activity leading to bone loss and potentially causing osteoporosis [2]. Osteoblast differentiation is associated with the incidence of osteoporosis, with studies implicating long non-coding RNA (lncRNA) as a factor in the condition [7].

LncRNAs are involved in a variety of biological processes and are understood to be a heterogeneous group of non-coding transcripts longer than 200 nt [8]. LncRNA-DANCR has been reported to be a potential biomarker for osteoporosis [9]. Another lncRNA MEG3 has been demonstrated to suppress osteogenic differentiation; at the same time, lncRNA CCAT1/miR-148a and lncRNA LINC01133 have been shown to influence the process of proliferation in cases of osteoporosis [10, 11]. Studies have highlighted a correlation between the Notch signaling pathway and Hajdu-Cheney syndrome, a disorder that results in osteoporosis [12]. The proliferation and differentiation of bone cells from osteoporotic patients could increase due to an enhanced Notch signaling pathway; thus, that pathway deserves to be a potential target for osteoporosis treatment [13]. Delta-like 3 (DLL3), which at present has only been identified in mammals, is a divergent ligand and modulator located in the Notch signaling pathway, with variations in its expression linked to osteogenic induction [14, 15]. Nevertheless, lncRNA and DLL3 have not been investigated from an osteoporosis perspective. Thus, the purpose of the present study was to determine the role of lncRNA LINC00311 (LINC00311) and DLL3 in osteoporosis and their influence on the cellular processes of osteoclasts through the Notch pathway.

Materials and Methods

Bioinformatics prediction

The Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>) was explored in order to obtain gene expression data of osteoporosis (GSE7158 and GSE63009) as well as an annotation

file. Both microchips were detected by the Affymetrix Human Genome U133 Plus 2.0 Array (Shanghai Sangon Biotechnology Co. Ltd., Shanghai, China). Background correction and normalization of each data chip were processed using Affy package of R software [16]. The linear empirical Bayes model in the limma package was applied to conduct nonspecific filtration of the expression data, while the long non-coding RNA with differential expression were subsequently filtered [17]. MEM is a web-based tool that is largely used for co-expression queries for large collections of gene expression experiments. LncRNA with differential expression levels were predicted by Multi Experiment Matrix website (MEM, <http://biit.cs.ut.ee/mem/>). Access to several hundreds of publicly available gene expression datasets of different tissues, diseases and conditions was provided by MEM and arranged by microarray platform types and the species [18]. The target gene was analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using WebGestalt database (<http://www.webgestalt.org>) to confirm the major biochemical pathways of metabolism and signaling pathways of target gene [19].

Study subjects

Experiments were performed on healthy 3-month-old non-pregnant female Sprague-Dawley rats with no evidence of deformity, trauma or skin infection. The recruited rats weighed approximately 200 g, were provided by the animal experiment center of Tsinghua University Second Hospital and were housed in cages separately, and were granted free access to compound pellet feed, under a 12 h light/darkness cycle with controlled temperature conditions of $23 \pm 2^\circ\text{C}$. A series of intraperitoneal injections of sodium pentobarbital (0.35 mL/100 g) were administered to anesthetize rats, and after disinfection, an incision was made along the middle line of their respective abdomens. Rats from the ovariectomy (OVX) group were sutured by layer after complete removal of the bilateral ovaries, followed by postoperative fasting for 6 h. Surgical sutures were left in. All experiments in the present study were conducted under the approval of the Ethics Committee of Tsinghua University Second Hospital, according to the principles of animal protection and use. All efforts were made to minimize the suffering of animals in the study.

Cell culture and induction of differentiation

Rats with osteoporosis were selected and administered 3000 U of heparin (H104201, Aladdin, Shanghai, China) intraperitoneally. After 3 weeks of cervical dislocation after 30 min, and immersed in 75% ethanol for 10 min. The bilateral femur and tibia were removed under nonsterile conditions and washed 3 times with phosphate buffered saline (PBS) (D1040, Solarbio, Beijing, China). The marrow cavity of the rats was flushed with α -MEM medium (SH30253.01B, HyClone, South Logan, UT, USA) containing 15% fetal bovine serum (FBS) (16140063, Gibco, Grand Island, NY, USA) and 20 g/mL M-CSF (400-28, Peprotech, Rocky Hill, NJ, USA). The marrow surface was observed to have turned pale, while the cell suspension was prepared by repeated pipetting. The cell suspension was slowly spread on the lymphocyte separating solution (100-002, Ficoll, Sigma) according to the proportion of 1: 1, centrifuged at 3000 r/min for 5 min with a equal volume of serum-free α -MEM added to the middle layer of the mononuclear cells, and centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and the cells were suspended with 15% FBS α -MEM and 20 g/mL M-CSF. The cell density was adjusted to 1×10^4 cell/mL, followed by inoculation in a 25 cm² flask. The culture medium was 37°C in 5% CO₂ under saturated humidity conditions. Following 2 - 3 h of incubation, the adherent cells were collected. The cells were then suspended with α -MEM medium comprising 15% FBS, 20 g/mL M-CSF, 50 ng/mL recombinant human RANKL ligand (RANKL) (ab69517, Abcam, Cambridge, MA, USA), 10 mmol N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (15630080, Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (SV30010, HyClone). Cell density was adjusted to 1×10^6 cell/mL and the cells were then seeded into six-well plates (3 mL/well).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the tissues and cells using a Trizol kit (15596026, Life Technologies, Gaithersburg, MD, USA). The cDNA template was synthesized using a reverse transcription kit (18091050, Invitrogen, Carlsbad, CA, USA). After the reaction, the cDNA was either placed on ice or stored at -20°C . Real-time amplification was performed using an ABI7300 PCR instrument. The reaction system was composed of 1 μ L of upstream and downstream primer (concentration of 10 μ mol/L each), 1 μ L of cDNA template, 10 μ L of $2 \times$ SYBR Green qPCR Mix, and 7 μ L of ddH₂O. The reaction conditions were at 95°C for 3 min, at 95°C for 12 s and at 62°C for 40 s with 40 cycles. The $2^{-\Delta\Delta\text{Ct}}$ was used to highlight the relationship of the target gene

expression between the experiment and control groups. $\Delta\Delta Ct = \Delta Ct_{\text{ovariectomy}} - \Delta Ct_{\text{normal}}$, $\Delta Ct = Ct_{\text{RNA}} - Ct_{\text{internal reference RNA}}$. Each experiment was repeated three times in order to obtain the average value. β -actin was regarded as the internal reference [20] (Table 1).

Western blotting

The total protein of both tissues and cells was extracted by means of radioimmunoprecipitation (RIPA) assay buffer containing phenylmethane sulfonyl fluoride (PMSF). The Bradford method was applied in order to determine the concentration of total protein. After 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the separated protein was transferred onto polyvinylidene fluoride (PVDF) membranes (LC2005, Invitrogen, CA, USA), sealed by Tris-buffered saline and Tris-buffered saline with Tween-20 (TBST) composed of 0.5% fat milk powder for 1 h. β -Actin, which was regarded as the internal reference, was incubated overnight with the following: rabbit anti-DLL3 (#2483, CST, Beverly, MA, USA) at 1:10000 dilution, Notch1 (#4147, CST, Beverly, MA, USA) at 1:10000 dilution, Notch2 (#4530, CST, Beverly, MA, USA) at 1:10000 dilution, Jagged1 (MA5-15012, Invitrogen) at 1:1000 dilution, Hes-1 (PA5-23283, Invitrogen) at 1:1000 dilution, tartrate-resistant acid phosphatase (TRAP) (ab126775, Abcam) at 1:1000 dilution, β -actin (PA1-183, Invitrogen, Carlsbad, CA, USA) antibody at 1:2000 dilution. Next, horseradish peroxidase-labeled sheep-anti-rabbit secondary antibody (ab150077, Abcam) was used for hybridization for a 2 h period, followed by autoradiograph recording. After scanning, the Gel-Pro Analyzer 4.0 (Media Cybernetics, Silver Springs, MD, USA) was used to analyze the gray values of protein bands. The experiment was repeated 3 times.

Cell transfection

The osteoclasts were inoculated in a 96-well plate 24 h prior to cell transfection. Upon reaching 50% confluence, the cells were transfected in accordance with the instructions of the Lipofectamine 2000 (11668019, Thermo Fisher Scientific, Waltham, MA, USA). Following transfection, the medium was changed after 6 h had elapsed. The cells were then collected for subsequent experiments after 48 h. The cells were assigned into the LINC00311-vector group (transfected with LINC00311 plasmid), the blank group (cells without transfection), the negative control (NC) group (transfected with empty plasmid), the shRNA-LINC00311 group (transfected with the LINC00311 silencing sequence, the most efficient silent sequences were screened from shRNA-LINC00311-1, shRNA-LINC00311-2, and shRNA-LINC00311-3), the Notch activator group (Notch signaling pathway activator rh-NF- κ B at 1 gsu/mL was added to the medium without transfection), and the Notch activator + LINC00311-vector group (Notch signaling pathway activator rh-NF- κ B at 1 gsu/mL was added to the medium for the transfection of the LINC00311 plasmid) (Table 2).

TRAP staining

The cells were inoculated in a 48-well plate and cultured in accordance with the aforementioned designated grouping above, followed by discarding of the culture medium. The cells were then washed twice with PBS and fixed in 4% paraformaldehyde (p1110-100, Tideradar Beijing Technology Co., LTD, Beijing, China) for 10 min twice, washed again with PBS twice, stained with TRAP under 37°C (387A, Sigma) for 30 min, and then washed with double-distilled water three times. The number of osteoclasts was observed and counted under a microscope (DM1000, Leica, Wetzlar, Germany), and cells with 3 nuclei or more were considered to be osteoclasts, followed by TRAP-positive staining. Two slices were obtained from each rat and observed under a 10 $\times$ 20-fold light microscope. Five microscopic visual fields ($\times$ 100) were randomly sampled from each slice. The number of osteoclasts in each field was counted, and the mean number of osteoclasts was subsequently calculated.

Table 1. Prime sequence for RT-qPCR. Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, forward; R, reverse; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase

Gene	Sequence
LINC00311	F: 5'-TCGCTGCGTCCTAACCAAAT-3' R: 5'-AGCAGACCAAGATGCTCAG-3'
Notch1	F: 5'-CTGAGGCAAGGATTGGAGTC-3' R: 5'-GAATGGAGGTAGGTCCGAAG-3'
Notch2	F: 5'-TGTGCCGTGTGTGCTTAA-3' R: 5'-TGCTGTGGCTCTGCTG-3'
Jagged1	F: 5'-GGCGAACTGAAAGG-3' R: 5'-GCTTCGGCTTAGGCTC-3'
Hes-1	F: 5'-GCCAGTGTCTGAC-3' R: 5'-TCACCTCGTCTACTCG-3'
TRAP	F: 5'-GCAAGGTCCGCTATC-3' R: 5'-GCCTCAGCCGCTATC-3'
β -actin	F: 5'-GTGGGCGCTAGG-3' R: 5'-CTCTTTCACGCACGACGATTTC-3'

Table 2. Transfection sequence

Name	Sequence
shRNA-LINC00311-1	5'-TGCTGTTGACAGTGAGCGCCCTCGCTGCTCTCAACCAATAGTGAAGCCACAGATGTATTTGGTTAGGACGAGCGAGGATGCCTACTGCCTCGGA-3'
shRNA-LINC00311-2	5'-TGCTGTTGACAGTGAGCGACCTAATCAGGCAGAGTAGTTAGTGAAGCCACAGATGTAACCTACTCTGCCTGAGTTAGGCTGCCTACTGCCTCGGA-3'
shRNA-LINC00311-3	5'-CCGGGTCTCAACCAATGCTGGACTCTCGAGAGTCCAGCATTTGGTTAGGACTTTTGG-3'

MTT assay

After transfection, the density of single cell suspension was adjusted to 5×10^6 cell/mL, and the cells were seeded into 96-well plates in each group after cell adherence. Three wells were randomly selected from each group; at the 12th, 24th and 48th h marks, 20 μ L of 5 mg/mL MTT (M2128, Sigma) was added to those wells for an additional 4 h of culturing, followed by removal of the culture medium. Dimethyl sulfoxide (DMSO, 150 μ L; D5879-100ML, Sigma) was then added to each well. The plate was placed in a dark condition void of light and vortexed for 10 min with the absorbance value measured at 570 nm. The experiment was repeated 3 times.

5-ethynyl-2'-deoxyuridine (EdU) cell proliferation assay

EdU was used to measure cell viability, which consisted of a chemical method for the fast and sensitive detection of DNA synthesis *in vivo*. Cells in the logarithmic growth phase were collected and adjusted to a density of 5×10^6 cells/mL and seeded into a 12-well plate. Upon reaching confluence, the cells were transfected and then cultured for 72 h. Next, 300 μ L of EdU medium (50 μ M) was added to each well for incubation for 2 h, with the medium discarded. The cells were then washed twice with PBS (twice per washing). Afterward, 50 μ L of fixation fluid (PBS + 4% polyoxymethylene) was added to the cells in each well and incubated at room temperature for 30 min, with the fixation allowed to run. After the addition of 50 μ L of glycine (2 mg/mL) in each well, the cells were discolored, placed on the shaking table and incubated for 5 min, followed by the removal of the glycine. Next, 100 μ L of 1X Apollo® was added to the cells, discolored, placed on the shaking table and incubated at room temperature for 30 min under dark conditions, with the reaction allowed to dip off. Finally, the cells were added with 100 μ L of penetrant (10% TritonX-100), discolored, placed on the shaking table and rinsed 2–3 times (10% TritonX-100), with the penetrant discarded. Flow cytometry was used to detect the fluorescence intensity.

Flow cytometry

After 48 h of transfection, the cells were washed three times with PBS, centrifuged for supernatant removal, and suspended with PBS. The concentration of the cells was adjusted to approximately 1×10^5 cell/mL, and 1 mL of precooled 70% ethanol was added to fix the cells for 1 h at 4°C, after which the cells were centrifuged, the ethanol was discarded, and the cells were washed with PBS twice in order to discard the supernatant; next, 1 mL of RNase A was added while avoiding light exposure, and the cells were heated in a water bath for 30 min. Then, 400 μ L of propidium iodide (PI) (P4170, Sigma) was added for staining and mixing. After avoiding light for 30 min, flow cytometry (CytoFLEX Beckman, Coulter, Fullerton, CA, USA) was applied to record the fluorescence at a wavelength of 488 nm for cell cycle access purposes.

After 48 h of transfection, the cells were treated with trypsin-free ethylenediaminetetraacetic acid (EDTA) (EDTA-10107, BOSTER Biological Technology CO, LTD, Wuhan, China), collected in a flow tube, and centrifuged; the supernatant was then removed, followed by three cold PBS washes. Based on the instructions, an annexin-V-FITC apoptosis detection kit (K201-100, Biovision, Milpitas, CA, USA), annexin-V-FITC, PI, and HEPES buffer were mixed at a proportion of 1: 2: 50 for the annexin-V-FITC/PI dye. Cells adjusted to 1×10^6 were suspended with 100 μ L each of the dye, vibrated and mixed, followed by incubation at room temperature for 15 min. Subsequently, 1 mL of HEPES buffer was added, vibrated and mixed. FITC and PI fluorescence were detected by 525 nm and 620 nm band filters at 488 nm wavelength, and apoptosis was detected accordingly.

In vivo identification experiment

Twenty-four rats were randomly selected as the sham group, while seventy-two rats were placed into the OVX group. All rats were anesthetized by means of intraperitoneal injection with 0.8% sodium pentobarbital (0.35 mL/100 g). Following disinfection, an incision was made along the middle line of the abdomen. The rats in the OVX group were sutured by layer after complete removal of bilateral ovaries and undergoing a 6 h period of postoperative fasting. Six hours later, the rats were granted free access

to water. The surgical sutures were left in. The sham group underwent a sham surgery without ovary removal. The rats were then fed in a well-ventilated environment at room temperature with natural light or the specific pathogen-free (SPF) condition. Then, 24 rats were randomly selected from the OVX group and intraperitoneally injected with siRNA or LINC00311 separately and served as the OVX-siRNA group and the OVX-LINC00311 group, respectively, with the remaining rats as the OVX-control group. Dual energy X-ray absorptiometry (DEXA, Hologic QDR24500A, Waltham, MA, USA) was applied to access the bone mass density (BMD) after 3 months had elapsed.

Hematoxylin-eosin (HE) staining

Decalcification of bone tissue process was conducted according to the following: the right femurs were subsequently immersed in a mixture of 330 mL of formic acid, 660 mL of 10% formalin, and 330 mL of hydrochloric acid for decalcification, with the mixture changed at frequent intervals every day. The bones were extracted and washed with flowing water for 12 h until they could be easily punctured by disposable syringes.

To slice bone tissue, a rotary microtome (RM2245, Leica) was used. The steps were performed as follows: the femurs were extracted, washed using running water for 30 min, immersed in 95% ethanol I and 95% ethanol II for 30 min each, and immersed in absolute ethanol I for 1 h, absolute ethanol II for 1 h and absolute ethanol III for 1 h. The slices were then sequentially immersed in cedar wax II, cedar wax I, I and III, (X820585-500 mL, HongZhe Biological Technology Limited Company, Anhui, China) for 0.5 h, 1 h and 0.5 h, respectively. Next, the samples were treated in paraffin I, II and III for 0.5 h, 1 h and 0.5 h, respectively, embedded with embedding machines and sliced by ultra-thin semi-automatic microtome. The distal femurs were sliced into 4 μ m sequential slices and attached to an anti-slip slide for roasting at 60°C.

Statistical analysis

SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis. Measurement data are presented as the mean $\pm$ standard deviation (SD). Comparisons between the two groups were examined using the *t*-test, while comparisons among more than two groups were analyzed by one-way analysis of variance (ANOVA). The results were considered to be significant when *p* was less than 0.05.

Results

LINC00311 is selected for this study

The analysis of GSE7158, in regard to osteoporosis, revealed that LINC00311 was highly expressed in osteoporosis (*p* < 0.05) (Fig. 1A). The analysis of chip GSE63009 showed that after being treated with bisphosphonate, the expression of LINC00311 in osteoclasts was reduced more so than in those that were not treated (Fig. 1B), further confirming the high expression of LINC00311 in osteoclasts. Hence, LINC00311 was selected for further study.

DLL3 is a target gene of LINC00311

The MEM website was employed for the prediction of the target gene of lncRNA, the results of which revealed that DLL3 was

Fig. 1. LINC00311 expresses at a high level in osteoclasts during osteoporosis (A: GSE7158; B: GSE63009) Note: N = 3.

mRNA expression levels of Notch2 and DLL3 or those of the related genes in the Notch signaling pathway (all $p < 0.05$). Compared with those of the blank and NC groups, increased expression levels of LINC00311, Notch2 and TRAP were detected in the LINC00311-vector group (both $p < 0.05$), while the other mRNA expression levels exhibited marked decreases (all $p < 0.05$). The expression levels of LINC00311 and TRAP were decreased in the shRNA-LINC00311 group, while the expression levels of the other genes all exhibited distinct increases (all $p < 0.05$). In the Notch activator group, the mRNA expression levels of Notch1, Notch2, Jagged1 and Hes-1 were elevated, although no significant difference in relation to the expression levels of LINC00311 and DLL3 was observed, while the mRNA expression of TRAP decreased (all $p < 0.05$). In the LINC00311-vector + Notch activator group, expression levels of LINC00311 and Notch2 were notably increased, while the expression of DLL3 was significantly decreased ($p < 0.05$). No significant difference was observed in regard to the expression levels of TRAP, Notch1, Notch2, Jagged1 and Hes-1 (all $p > 0.05$) (Fig. 5). In osteoclasts, LINC00311 overexpression was shown to increase Notch2 and TRAP mRNA expression levels while lowering the mRNA expression levels of DLL3, Notch1, Notch2, Jagged1, and Hes-1.

LINC00311 elevates Notch2 and TRAP protein expression levels while lowering DLL3, Notch1, Jagged1, and Hes-1 in osteoporotic rats

Western blotting methods were conducted in order to detect the protein expression levels of LINC00311, DLL3, Notch1, Notch2, Jagged1, and Hes-1 in osteoclasts, no significant difference between the blank group and the NC group was detected in relation to the protein expression levels of LINC00311 and DLL3 or the related genes in the Notch signaling pathway (all $p > 0.05$). Compared with those of the blank and NC groups, expression levels of the TRAP protein in the LINC00311-vector group were significantly increased, while those of the other proteins were notably decreased (all $p < 0.05$) (Fig. 6). The expression of Notch2 and TRAP protein in the shRNA-LINC00311 group was significantly decreased while the expression levels of the other proteins were significantly increased (all $p < 0.05$). No significant difference was observed in regard to the expression of DLL3 in the Notch activator group ($p > 0.05$), while the expression levels of Notch1, Notch2, Jagged1 and Hes-1 proteins were significantly increased, accompanied by notably decreased expression levels of TRAP protein (all $p < 0.05$). Notch2 expression levels were increased and DLL3 expression levels significantly decreased in the LINC00311-vector + Notch activator group ($p < 0.05$), while Notch1, Notch2, Jagged1, Hes-1 and TRAP expression levels had no remarkable differences (all $p > 0.05$) (Fig. 6). Based on the obtained results, we concluded that in osteoclasts,

Fig. 3. LINC00311-shRNA1 is selected for further experiments. Note: N = 3; the comparison was analyzed using an ANOVA.

4. TRAP staining results indicate that LINC00311 increases TRAP-positive osteoclasts in osteoporotic rats ($\times 200$). Note: N = 5; the comparison was analyzed using an ANOVA; A, TRAP staining of bone tissues in each group; B, diagram of TRAP-positive cell number in each group; *, $p < 0.05$ compared with the NC and blank groups; ANOVA, one-way analysis of variance.

Fig. 5. RT-qPCR suggests that LINC00311 elevates Notch2 and TRAP mRNA expression levels while descending DLL3, Notch1, Jagged1, and Hes-1 in osteoporotic rats. Note: N = 3; the comparison was analyzed using an ANOVA; *, $p < 0.05$ compared with the NC and blank groups; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase.

Fig. 6. Western blot analysis indicates that LINC00311 increases Notch2 and TRAP protein expression levels while lowering DLL3, Notch1, Jagged1, and Hes-1 in osteoporotic rats. Note: N = 3; the comparison was analyzed using an ANOVA; A, protein bands in each group; B, protein expression levels in each group; *, $p < 0.05$ compared with the NC group and the blank group; ANOVA, one-way analysis of variance; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase; NC, negative control.

LINC00311 overexpression could increase Notch2 and TRAP protein expression levels while reducing the protein expression levels of DLL3, Notch1, Jagged1, and Hes-1.

LINC00311 promotes the proliferation of osteoclasts in osteoporotic rats

The MTT assay was employed to measure osteoclast proliferation, the results of which demonstrated that there was no significant difference in regard to the proliferation activity between the NC and blank groups ($p > 0.05$). Compared with that of the blank and NC groups, the cell proliferation activity in the shRNA-LINC00311 group and the Notch activator group was significantly reduced, while the proliferation activity in the LINC00311-vector group was markedly increased ($p < 0.05$). No significant difference regarding cell proliferation activity in the shRNA-LINC00311 + Notch activator group was observed ($p > 0.05$) (Fig. 7). The aforementioned results demonstrated that LINC00311 overexpression could be attributed to the proliferation of osteoclasts.

LINC00311 promotes the proliferation of osteoclasts in osteoporotic rats

The EdU assay was applied in order to measure osteoclast proliferation, the results of which are illustrated in Fig. 8. No significant difference was observed in regard to proliferation between the NC and blank group ($p > 0.05$). Compared with those of the blank and NC groups, the fluorescence intensity and proliferation in the shRNA-LINC00311 and Notch activator groups were significantly reduced (both $p < 0.05$); the fluorescence intensity and proliferation in the LINC00311-vector group were significantly elevated (both $p < 0.05$); and cell proliferation in the LINC00311-vector + Notch activator group showed no notable difference ($p > 0.05$). The above results indicated that LINC00311 overexpression might be attributed to the proliferation of osteoclasts.

LINC00311 accelerates osteoclast growth and inhibits apoptosis in osteoporotic rats

Flow cytometry was performed to determine cell cycle and apoptosis. PI staining results revealed that there was no significant difference regarding cell cycle distribution between the blank and NC groups ($p > 0.05$). Compared with that of the blank and NC groups, the proportion of cells at the G1 phase was markedly increased, while the proportion of cells

Fig. 7. LINC00311 promotes the osteoclast proliferation in osteoporotic rats detected by MTT assay. Note: N = 3; the comparison was analyzed using an ANOVA; *, $p < 0.05$ compared with the NC group and the blank group; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; OD, optical density; NC: negative control; DLL3: delta-like 3; Hes-1: hairy and enhancer of split-1; TRAP: tartrate-resistant acid phosphatase.

at the S and G2 phases was significantly decreased in the shRNA-LINC00311 group and Notch activator group (all $p < 0.05$). The proportion of cells at the G1 phase in the LINC00311-vector group was notably reduced, while the proportion of cells at the S1 and G2 phases were significantly increased (all $p < 0.05$). No significant difference regarding the percentage of cells at the G1, S and G2 phases in the LINC00311-vector + Notch activator group was detected (all $p > 0.05$) (Fig. 9).

Annexin V/PI double-staining results indicated that compared with that of the blank group and the NC group, the apoptosis rate of the LINC00311-vector group was significantly decreased, while the rate of apoptosis in the shRNA-LINC00311 group and the Notch activator group was notably increased (all $p < 0.05$). There was no significant difference detected in regard to the rate of apoptosis between the LINC00311-vector + shRNA-LINC00311 group ($p > 0.05$) (Fig. 10). The above findings indicated that LINC00311 overexpression might promote the proliferation of osteoclasts while hindering the process of apoptosis.

Fig. 8. LINC00311 promotes the osteoclast proliferation in osteoporotic rats detected by EdU assay. Note: NC: negative control; DAPI, diamidino-2-phenylindole; EdU, 5-ethynyl-2'-deoxyuridine.

Osteoporotic rat model is successfully established

Compared with those of the sham group, the BMDs of the 4, 5 lumbar, femur and tibia were significantly decreased in the OVX group (all $p < 0.05$), which was largely indicative of successful osteoporotic rat modeling (Fig. 11). On the other hand, in comparison with those of the sham group, the BMDs of the 4, 5 lumbar, femur and tibia were significantly decreased in the OVX-LINC00311 group, while no notable reductions were detected in the OVX-siRNA group.

Pathological changes of bone tissues in the sham and OVX groups are observed

HE staining was used to observe pathological changes of bone tissues in the sham and OVX groups. As shown in Fig. 12, the rats in the sham group were discovered to have uniform, robust, plump, reticulate, continuous, dense, regular, rich, brown and well-formed trabecular bone of the cancellous bone area. The bone marrow cavity was small with abundant marrow cells within the cavity with a scarce amount of fat droplets. Compared with that of the sham group, the trabecular bone in the cancellous bone area of the rats in the OVX group was

Fig. 9. PI staining results indicates that LINC00311 accelerates osteoclast cell cycle progression in osteoporotic rats. Note: N = 3; the comparison was analyzed using an ANOVA; A, flow cytometry results for cell cycle; B, comparison of cell cycle distribution; *, $p < 0.05$ compared with the NC group and the blank group; NC, negative control; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase.

Fig. 10. Annexin V/PI double staining reveals that LINC00311 inhibits osteoclast apoptosis in osteoporotic rats. Note: N = 3; comparison analyzed using an ANOVA; A, flow cytometry results for cell apoptosis; B, comparison of cell apoptosis rate; *, $p < 0.05$ compared with the NC group and the blank group; NC, negative control; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase.

Fig. 11. Osteoporotic rat model successfully established. Note: N = 24; the comparison was analyzed using an ANOVA; *, $p < 0.05$ compared with the sham group; L4, the fourth lumbar; L5, the fifth lumbar; BMD, bone mineral density; OVX, ovariectomy.

significantly increased, sparse, irregular, and partial fractured; the connectivity between the trabecular bones was poor, composed of a large number of cecum, enlarged bone marrow cavity, decreased hematopoietic cells and increased fat droplets. Compared to that in the sham group, in the OVX-siRNA group, the trabecular bone in the cancellous bone area of the rat exhibited a slight decrease and was sparse, while the bone marrow cavity was partially enlarged with a decreased amount of fat droplets.

LINC00311 increases TRAP positive osteoclasts in osteoporotic rats

To determine the number of positive cells in the osteoporotic rats, TRAP staining was employed. The cells were regarded as osteoclasts in the event that the number of nuclei was greater than 3, as subsequently determined to be positive by TRAP staining. The TRAP staining results indicated that the number of positive cells in the osteoporotic bone tissue in the OVX group (5.36 ± 0.93) and the OVX-LINC00311 group (8.62 ± 0.87) was much higher than that in the sham group (2.35 ± 0.79) ($p < 0.05$). The number of positive cells in osteoporotic

bone tissue in the OVX-siRNA group was 2.58 ± 1.12 , which showed no significant difference when compared with that of the sham group (Fig. 13). The results suggested that LINC00311 might increase the amount of TRAP-positive bone tissues in osteoporotic rats.

LINC00311 elevates Notch2 and TRAP expression levels while lowering those of DLL3, Notch1, Jagged1, and Hes-1 in osteoporotic rats

RT-qPCR and Western blotting methods were applied in order to detect the mRNA and protein expression levels of LINC00311, DLL3, Notch1, Notch2, Jagged1, and Hes-1. As illustrated in Fig. 14, compared with those of the sham group, the mRNA and protein expression levels of LINC00311, Notch2 and TRAP in the bone tissues of the OVX group were significantly increased, while the mRNA and protein expression levels of DLL3, Notch1, Jagged1 and Hes-1 were decreased (all $p < 0.05$); the TRAP protein expression levels were significantly increased in the bone tissues of the OVX group ($p < 0.05$). The mRNA and protein expression levels of LINC00311, Notch2 and TRAP in the bone tissues of the OVX-LINC00311 group were increased significantly compared with those of the OVX group, while reductions in the mRNA and protein expression levels of DLL3, Notch1, Jagged1 and Hes-1 were recorded (all $p < 0.05$). Compared with those of the OVX group, the mRNA and protein expression levels of LINC00311, Notch2 and TRAP in the OVX-siRNA group were significantly decreased, while the mRNA and protein expression levels of DLL3, Notch1, Jagged1 and Hes-1 were elevated (all $p < 0.05$). The results suggested that in osteoclasts LINC00311 overexpression might increase the Notch2 and TRAP mRNA expression levels while lowering those of DLL3, Notch1, Jagged1, and Hes-1.

Discussion

The prevalence of osteoporosis has experienced progressive increases, with many studies highlighting the demand for more effective management and treatment protocols for patients

Fig. 12. HE staining shows the pathological changes of bone tissues of osteoporotic rats (×200). Note: HE, hematoxylin-eosin; OVX, ovariectomy.

Fig. 13. TRAP staining results implies that LINC00311 increases TRAP-positive osteoclasts in bone tissues during osteoporosis (×200). Note: N = 3; one comparison was analyzed using t test; A, TRAP staining results of bone tissues; B, comparison of TRAP positive cells between the Sham group and OVX groups; *, $p < 0.05$ compared with Sham group; OVX, ovariectomy.

Fig. 14. RT-qPCR and Western blot analysis demonstrates that LINC00311 elevates Notch2 and TRAP expression levels while lowering those of DLL3, Notch1, Notch2, Jagged1, and Hes-1 in bone tissues during osteoporosis. Note: N = 3; comparison was analyzed using an ANOVA; A, mRNA expression levels of relative genes in each group; B, protein bands of relative genes in each group; C, protein expression levels of relative genes in each group; *, $p < 0.05$ compared with the sham group; OVX, ovariectomy; DLL3, delta-like 3; Hes-1, hairy and enhancer of split-1; TRAP, tartrate-resistant acid phosphatase.

suffering from osteoporosis [21]. Largely due to the influence of multiple osteogenetic signals, which are specialized for the proliferation and differentiation of osteoblasts, an increase in the number of osteoblasts results from the mesenchymal progenitor cells [2]. The objective of the current study was to elucidate the correlation between LINC00311 and DLL3 from an osteoporosis perspective. The main conclusion of our study provided verification demonstrating that the upregulation of LINC00311 could inhibit the expression of DLL3 and regulate the Notch signaling pathway to promote the proliferation and differentiation of osteoblasts.

Compared with that in the sham group, in the OVX group, the expression of LINC00311 among the osteoclasts was higher, while the expression of DLL3 and the related genes in the Notch signaling pathway was lower. The findings of a recent study regarding dosage compensation, imprinting, and homeotic gene expression revealed that a wide range of intervening noncoding RNAs (lncRNAs) could function as the interface between specific chromatin remodeling activities and DNA, suggesting they are pervasively expressed in the genome [22]. LncRNAs have been employed as biomarkers and therapeutic targets in various skeletal diseases including osteoarthritis and osteoporosis, as well as in cancers related to the skeletal system [23], among which the lncRNA H19 has been observed to act as a regulatory element in patients suffering from osteoporosis [19]. Elevated expression of the lncRNA MyoD has been detected in skeletal muscle cells and lncRNA HOTAIR expression has been observed in osteosarcoma tissues [24]. DLL3 is a spondylocostal dysplasia/pudgy gene whose mutation may lead to axial skeletal defects and spondylocostal dysostosis [26-28]. Similarly, DLL3 has been reported to be a candidate gene that has been implicated in vertebral malformations, while its homolog to Notch represents a significant factor in somitogenesis, capable of promoting osteoclastogenesis through Notch2 [29, 30]. A key finding of the present study provided evidence suggesting that it is reasonable to estimate that LINC00311 is upregulated in osteoporosis and that DLL3 is a factor affecting osteoporosis largely due to the increased expression levels of a great number of lncRNAs in orthopedic diseases, with many orthopedic diseases shown to be influenced by DLL3.

Furthermore, we found that compared with those in the sham group, the mRNA and protein expression levels of Notch1, Notch2, Jagged1 and Hes-1 were significantly increased in the OVX group. In comparison with those in the NC and blank groups, the expression levels of DLL3, Notch1, Jagged1 and Hes-1 were notably decreased in the LINC00311 vector group. Jagged1 is a ligand of Notch1 and Notch2, and the differential expression levels of the two depend on the thymic cell populations and ontogenetic stages [31, 32]. Osteoclastogenesis is enhanced by the Notch2/DLL1 axis but suppressed by the Notch1/Jagged1 axis [30]. Hes-1 is a major transcriptional downstream target regulated by the Notch signaling pathway, a pathway that is critical for osteoblast differentiation [33, 34]. Osteoblasts are implicated in the incidence and progression of osteoporosis [35]. Many lncRNAs are regulated by osteoblasts, including the lncRNA repertoire and lncRNA-p21 [36, 37]. DLL3 is an upregulated gene of the Notch signaling pathway, which is also regulated by osteoblast activity [15, 38, 39].

In addition, when compared with that in the sham group, the ratio of TRAP-positive osteoclasts in the OVX group increased. In comparison to that in the NC and blank groups, the proliferation in the LINC00311-vector group was increased, while the ratio of apoptosis and the percentage of TRAP-positive osteoclasts decreased. A previous report indicated that lncRNA TUG1 promotes the proliferation while acting to inhibit the apoptosis of osteosarcoma cells by binding to PRC2 [40]. Linc RNA NONHSAT009968, H19 and HIF 1 α -anti-sense 1 have been identified to promote osteoblastic differentiation as a result of their potential cis-regulated mRNA targeting, which represents a central osteoblast differentiation regulator [41-43]. DLL3 restoration in methylation-silenced HuH2 cells results in the promotion of apoptosis owing to the activity of Huwel, while DLL3 may inhibit proliferation [44, 45]. The upregulation of the Notch signaling pathway may inhibit proliferation while promoting the apoptosis of osteosarcoma cells because doxorubicin treatment mediates a decrease in proliferation and increase in apoptosis of osteosarcoma, effects that are facilitated by that signaling pathway [46].

In conclusion, the key findings of this study suggest that LINC00311 could well possess the attributes required for osteoclast differentiation via the downregulation of DLL3 and LINC00311. Downregulated DLL3 can promote proliferation and differentiation while inhibiting the apoptosis of osteoclasts by activating the Notch signaling pathway. Therefore, the identification of LINC00311 and the Notch signaling pathway may aid in facilitating the existing understanding of the mechanisms of osteoporosis, with the potential of serving as a prognostic marker for the treatment of osteoporosis in the future. However, due to the sample size and funding limitations, further studies are required to fully understand the specific mechanisms of LINC00311 combined with the Notch signaling pathway in osteoporosis treatment.

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