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LINC00472 promotes osteogenic differentiation and alleviates osteoporosis by sponging miR-300 to upregulate the expression of FGFR2

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Abstract. – OBJECTIVE: To investigate the expression of LINC00472 in osteoporotic issues of patients, ovariectomized (OVX) mice and mice bone marrow mesenchymal stem cells (BMSCs), its effect on osteogenic differentiation of BMSCs and its mechanism.

PATIENTS AND METHODS: The expression of LINC00472 and miR-300 in osteoporosis patients (n=55), ovariectomized (OVX) mice (n=10) and mice BMSCs (n=3) was detected by RT-qPCR and the correlation between the expression of miR-300 and LINC00472 was analyzed. After transferring sh-LINC00472 and overexpression LINC00472 plasmids into mice BMSCs, the expression of ALP, Bglap, OPN, Runx2 was detected by RT-qPCR and Western blot, which were related with osteogenic differentiation. In addition, Luciferase activity was used to detect whether miR-300 combined with LINC00472 and FGFR2 in mice BMSCs directly. Finally, Western blot (WB) was used to detect the change of FGFR2 protein expression by miR-300 inhibitor and sh-LINC00472.

RESULTS: We found there was a negative correlation between the expression of miR-300 and LINC00472 in osteoporosis patients, bone tissues of OVX mice and mice BMSCs. The expression of LINC00472 in mice BMSCs was gradually increased with osteogenic differentiation. Transferring overexpression plasmid of LINC00472 into BMSCs, the expression of ALP, Bglap, OPN, Runx2 was increased both in mRNA and protein levels. Transferring sh-LINC00472 to BMSCs, the results were the opposite. Luciferase results showed that miR-300 could directly bind to LINC00472 and FGFR2 in mice BMSCs. What's more, RT-qPCR and WB results showed that transferring sh-LINC00472 could decrease the expression of FGFR2 mRNA and protein, while miR-300 inhibitor could recover this tendency.

CONCLUSIONS: According to these results, this study revealed the previously neglected LINC00472/miR-300/FGFR2 regulatory axis for the regulation of osteogenic differentiation in osteoporosis, which may be a potential target for the treatment of osteoporosis.

Key Words:
LINC00472, MiR-300, FGFR2, Osteogenic differentiation, Osteoporosis.

Abbreviations

OVX = ovariectomized; BMSCs = Bone marrow mesenchymal stem cells; WB = Western Blot; BSA = bovine serum albumin; MEM = Modified Eagle Medium; FBS = fetal bovine serum; RT-qPCR = reverse transcriptase-polymerase chain reaction; PBS = phosphate-buffered saline; miRNA = MicroRNA; d = Day.

Introduction

Osteoporosis is a common orthopedic disease, which is characterized by decreased bone mass and microstructure changes of bone tissue. The clinical symptoms of affected areas are joint pain with a gradual worsening[1]. Further, the population of osteoporosis is getting younger and younger. The repetition of the illness afflicted the patient. The high mouse of disability caused by osteoarthrosis has deprived many patients of their freedom of action. Because the traditional methods of diagnosis and treatment cannot fundamentally relieve the pain, many patients chose joint replacement to improve the quality of life[2]. With the development of modern molecular technology, the molecular research on joint disease continues to emerge, providing a new direction for the diagnosis and treatment of the disease[3,4].

Long non-coding RNAs (lncRNAs) have a full length longer than 200 nucleotides. The structures

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of lncRNAs are similar to mRNA molecules, but they lack the ability to translate into proteins. Long-chain non-coding RNA (long non-coding RNA, lncRNA) is associated with many diseases[5,6]. Recently, a novel mechanism has been found in which cross connection between lncRNAs and mRNAs exists by competing for shared microRNAs (MiRNAs) response elements. In this case, lncRNAs were a competitive endogenous RNAs (ceRNAs) to bind miRNAs, thereby modulating the depression of miRNA targets and increasing the level of post-transcriptional regulation[7]. In osteoporosis, Zhang et al[8] found that MSC-AS1 might promote the osteogenic differentiation of BMSCs through sponging microRNA-140-5p to up-regulate FGFR28. Wu et al[9] found that DGCR5 up-regulated the expression of Runx2 through miR-30d-5p to induce osteogenic differentiation of hMSCs.

LINC00472, is a novel long intergenic non-coding RNA (lncRNA), in close link to clinical and pathologic features of cancer. LINC00472 is highly expressed in a variety of tumors and involved in tumor progression[10]. It is reported that lncRNA LINC00472 can, through miR-196-5p, regulate the progression of lung cancer cell[11]. LINC00472 regulates hepatocellular carcinoma cell proliferation, migration and invasion by miR-93-5p/PDCD4[12]. What's more, the lncRNA LINC00472 also involved in the progress of breast cancer[13], diabetic kidney disease (DKD)[14] and colorectal cancer (CRC)[15]. But there are few researches on the role of LINC00472 in orthopaedical diseases, especially in osteoporosis.

In this study, we aimed to examine the expression and functions of LINC00472 in the pathogenesis of osteoporosis, as well as to disclose its molecular mechanisms. First, we measured the expression of LINC00472 in 55 osteoporosis patients' tissues. Then, OVX mice were analyzed to investigate the expression of LINC00472 in bone tissues. The effects of overexpression and knockdown of LINC00472 on the progress of osteogenic differentiation in mice BMSCs and the relationship between LINC00472 expression and miR-300 expression, were analyzed. Our results revealed a critical function of lncRNA LINC00472 in osteoporosis.

Patients and Methods

Patients

55 female patients with osteoporosis were treated in our Hospital from April 2013 to August 2017. The control group was of 50 normal female volunteers. There was no significant difference in age between the two groups. All subjects signed informed consent form. 5 mL of venous blood was collected from each subject during fasting in the morning. The blood samples were kept at 4°C for 10 min, and the upper serum (non-hemolytic state) was collected after the centrifugation of 3000 g/min for 10 min at 4°C. After centrifugation, 13500 g/min for another 15 min at 4°C, the supernatant was collected into Eppendorf (EP) tube, and placed in a -80°C refrigerator for future experiments. This investigation was approved by our Hospital Ethics Committee. A written notification of the signature was obtained from all participants prior to the study[16].

Animal Model

The 10-week female mice (weight = 20 ± 1.8 g) were provided by the Animal Experimental Center of Guangdong Academy of Medical Sciences (Guangdong, China). All mice were reared under standard pathogen-free conditions with a light cycle of 12 hours, a temperature of 25°C and 60% air humidity. Mice had free access to food and water. All the animals were domesticated for a week before the start of the experiment. The procedure was conducted in accordance with the Orthopedic Ethics Committee of our hospital. The mice were divided into ovariectomized (OVX) group (n=10) and sham operation control group (n=10) randomly. They were anesthetized with ketamine (40 mg/kg) and rompun (10 mg/kg) and then fixed and disinfected. The skin and muscles of mice were cut on both sides of the midline of the dorsal spinal cord. In the OVX experimental group, bilateral ovaries and surrounding adipose tissue were removed, while in the sham operation group, only peri-ovarian adipose tissue was removed. After that, the muscles and skin were stitched together. The mice were euthanized 8 weeks after operation. Blood samples were collected. The femur was taken from mice and the attached soft tissue was removed. All of samples were frozen in liquid nitrogen immediately and stored at -80°C refrigerator for further investigations[17].

Bone Mineral Density (BMD) Assessment

Micro-CT scanning using Latheta LCT-200 was utilized to measure the weight of bone mineral density of mouse femur. The VGStudio MAX V2.2 3D reconstruction software was used to analyze the measured data.

ELISA Assay of β-CTX

The concentration of serum β-CTX level was examined using β-CTX enzyme-linked immunosorbent assay kit (R&D, Minneapolis, MN, USA), according to the manufacturer's instructions.

ELISA Assay of ALP

The concentration of bone-specific ALP levels in serum was examined using bone-specific alkaline phosphatase ELISA kit (Cusabio, Beijing, China), according to the manufacturer's instructions.

Extraction and Culture of BMSCs

Primary bone marrow mesenchymal stem cells were obtained from 8-week normal mice. After the mice were executed by the neck, they were immediately immersed 10 min in 75% alcohol. The soft tissue was removed, and both ends of the femur and tibia were resected. Culture medium was used (10% FBS of high sugar DMEM/F12) and the bone marrow cavity was washed, the evenly blown rinse was filtered through a 200-mesh aseptic filter, and the supernatant was centrifuged at 1000 r/min for 10 min at room temperature. The cells were suspended in α-MEM (HyClone, South-Logan, UT, USA) added with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 2 mM glutamine, 100 U/mL penicillin (HyClone, South-Logan, UT, USA) and 100 mg/mL streptomycin (HyClone, South-Logan, UT, USA) at 37°C with 5% CO_2 atmosphere. The medium was changed every two days. The third generation of well-growing BMSCs was inoculated into 6-well plate at a density of 5.0×10^4/mL for osteogenic differentiation. After BMSCs grew to 70% and 80% fusion, α-MEM containing 10 mmol/L dexamethasone, 50 mmol/L vitamin C and 10 mmol/L β-glycerophosphate was added for inducing osteogenesis. Osteogenic differentiation was induced by culturing for 14 days and cell culture medium was changed every 3 days[17].

RNA Extraction and Real-time Quantitative PCR Assays

Total RNAs from tissues, BMSCs, tissues and serum were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription polymerase chain reaction (RT-qPCR) was performed using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's protocol. The levels of mRNA expression were quantified by standard Real-time PCR protocol with SYBR Premix Ex Taq (TaKaRa, Dalian, China). Reactions and signal detection were measured using a real time PCR system (Bio-Rad, Hercules, CA, USA). β-actin/U6 was used as a reference gene[18,19]. The gene specific primers were reported in Table I.

Construction of Plasmid, shRNA and Cell Transfection

We insert the full length of human LINC00472 into pcDNA (Invitrogen, Carlsbad, CA, USA) vector to get LINC00472-pcDNA for overexpression. The plasmid complementary deoxyribonucleic acid LINC00472 lentivirus shRNA vector

Table I. Primers for selected genes.

Gene name	Primers	
	Sense	Antisense
LINC00472	CAACACAACACAGGAGGGGA	CCAAATAACGGGGGCTACCA
miRNA-300	CCGGTTCAAGAGGAGACCAG	GGAGGGTAATTGAGGAAGGA
ALP	ACTGGCTGTGCTCTCCCTAC	GACCTCTCCCTTGAGTGTGG
Bglap	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG
Opn	AGCAAGAAACTCTTCCAAGCAA	GTGAGATTCGTCAGATTCATCCG
Runx2	AGAGTCAGATTACAGATCCCAGG	TGGCTCTTCTTACTGAGAGAGG
FGFR2	AGCACCATACTGGACCAACAC	GGCAGCGAAACTTGACAGTG
miR-206	CAGATCCGATTGGAATGTAAGG	TATGCTTGTTGTCGTCTCTGTGTC
miR-128	GGTCACAGTGAACCGGTC	GTGCAGGGTCCGAGG
miR-217	TACTGCATCAGGAACTGACTGGA	GTGCAGG GTCCGAGGT
miR-214	TGCCTGTCTACACTTGCTGTGC	GCGAGCACAGAATTAATACGAC
miR-215	TGGATTTGGACGCATTGGTC	TTTGCACTGGTACGTGTTGATA
miR-2861	ACACTCCAGCTGGGGGGGCCTGGCGGT	TGGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC
β-actin	CTCTTTTCCAGCCTTCCTTCT	TGGAAGGTGGACAGTGAGG

and the control shRNA targeting GFP were designed and synthesized by the reagent Company GeneChem Technologies (Shanghai, China). The third generation of well-grown BMSCs was selected to overexpress lentiviral transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. At indicated time point post the transfection, cells were harvested for further analysis.

Luciferase Activity

The sequences of wt-LINC00472/mut-LINC00472 (wt-FGFR2/mut-FGFR2) were inserted into the firefly Luciferase in basic pGL3 control vector (Promega, Madison, WI, USA)[20]. Briefly, the third generation of well-grown BMSCs was cultured overnight after being seeded into a 24-well plate, co-transfected with the wt-LINC00472/mut-LINC00472(wt-FGFR2/mut-FGFR2) reporter gene plasmid and miR-300 mimics or miR-inhibitor. *Renilla* expression vector was transfected into each group to serve as a normalized control. After transfected for 48 h, the Dual-Luciferase reporter assay system kit (Promega, Madison, WI, USA) was used to measure both firefly and *Renilla* Luciferase activities. Data were normalized against the activity of the *Renilla* Luciferase.

Western Blot

The total cell lysate was prepared by destroying cells with RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged with 12000 rpm at 4°C for 10 mins. BCA protein analysis reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the concentration of protein. The same amount of different protein samples was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF film was sealed with 0.1% Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk powder for 1 h. Then, the different protein blots were incubated with the primary antibodies overnight at 4°C and subsequently with secondary antibodies at room temperature over 2 h. The primary antibodies were as follows: Anti-FGFR2 (Abcam, Cambridge, MA, USA) diluted at 1:1000, anti-Bglap (Abcam, Cambridge, MA, USA) diluted at 1:1000, anti-Opn (Abcam, Cambridge, MA, USA) diluted at 1:1000, anti-Runx2 (R&D System, Minneapolis, MN, USA) diluted at 1:1000, anti-β-actin (Abcam, Cambridge, MA, USA) diluted at 1:2000. Horseradish peroxidase (HRP) coupled with secondary antibody (Abcam, Cambridge, MA, USA, dilution at 1:10000) was used for the detection of primary antibody. Enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize binding antibodies[9].

Statistical Analysis

Values were expressed as the mean ± SD. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (a Jolla, CA, USA) were used for statistical assay. The significance between groups was analyzed by Student's *t*-test. p-value <0.05 was considered as statistically significant.

Results

Expression of LINC00472 and MiR-300 in Serum of Patients With Osteoporosis

First, we detected the expression of LINC00472 in peripheral blood of osteoporosis group (n = 55) and in healthy group (n = 50) by RT-qPCR. The expression of LINC00472 in osteoporosis patients was significantly lower than that in healthy group (Figure 1A). Considering miRNA can regulate the expression of lncRNA, we predicted potential target miRNAs through online databases (microRNA, Starbase, and RegRNA2.0), and detected the expression of some potential target miRNAs in patients' peripheral blood (Figure 1B). We found that the expression of miR-300 was significantly decreased (Figure 1C) and there was a significant negative correlation between the expression of LINC00472 and miR-300 in patients with osteoporosis (Figure 1D).

The Expression LINC00472 and MiR-300 In Bone Tissue of OVX Mice

To further study the mechanism of LINC00472 and miR-300, we used OVX mice as osteoporosis model. Eight weeks after OVX operation, Micro-CT scanning results showed marked decrease of tibia bone mineral density in OVX mice (Figure 2A). The content of β-CTX and the activity of ALP in serum of mice in sham group and OVX group were detected. The results showed that the content of β-CTX and activity of ALP in serum of mice in OVX group were significantly increased (Figure 2B,2C). The to-

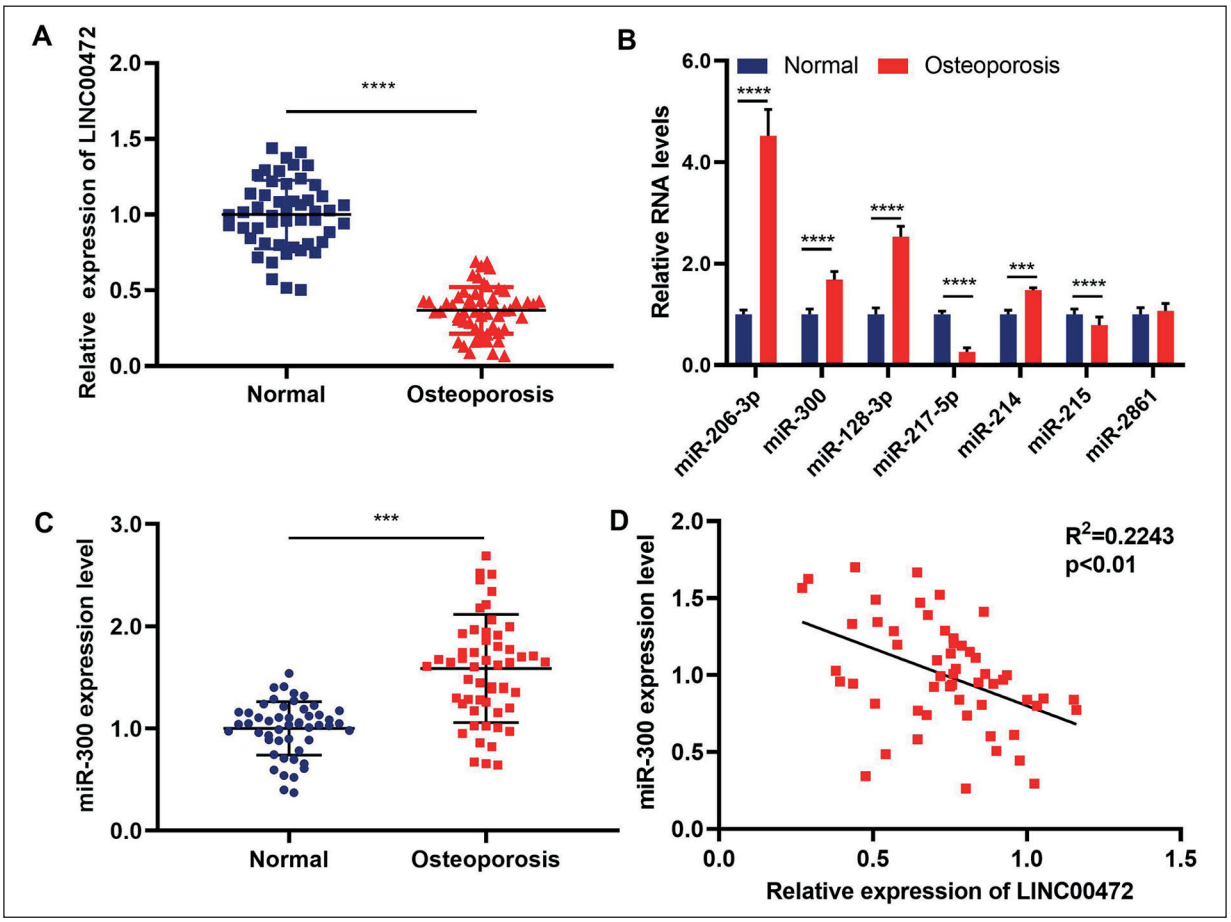

Figure 1. LINC00472 was reduced and miR-300 was increased in serum samples of patients with osteoporosis. **A**, The relative expression of LINC00472 in peripheral blood of osteoporosis group (n = 55) and in healthy group (n = 50). The relative expression levels were normalized to the mean value of normal volunteers. **** $p<0.0001$. **B**, The relative expression of some miRNA in peripheral blood of osteoporosis group (n = 55) and in healthy group (n = 50). The relative expression levels were normalized to the mean value of normal volunteers. **** $p<0.0001$, *** $p<0.001$ **C**, The relative expression of miR-300 in peripheral blood of osteoporosis group (n = 55) and in healthy group (n = 50). The relative expression levels were normalized to the mean value of normal volunteers. *** $p<0.001$. **D**, The negative correlation between the miR-300 and the LINC00472.

tal RNAs were extracted from the bone tissues of sham group and OVX mice. The expression of LINC00472 and miR-300 was detected by RT-qPCR. It was found that the expression of LINC00472 in OVX group was significantly lower than that in sham group, while the expression of miR-300 was significantly increased in OVX group (Figure 2D).

The Expression of LINC00472 and MiR-300 During the Differentiation of Mice BMSCs

The primary culture of mouse BMSCs for 24 hours was followed by changing the liquid, and then changing the liquid every 2 days. The third generation of well-grown MSC was induced to differentiate with dexamethasone, vitamin C and b-glycerophosphate. After 7 days of culture in the induction medium, the activity of ALP in the supernatant of the cells was detected (Figure 3A). Compared with the group without dexamethasone, the content of ALP in the induction medium was significantly higher than that in the group without dexamethasone. BMSCs were cultured for 0 d, 1 d, 7 d, 7 d, 14 d, then the total RNA was extracted. RT-qPCR was used to analyze the mRNA expression of ALP, Bglap, Opn and Runx2, which related to differentiation. The expression of these mRNA was significantly increased with the increasing of the inductive time (Figure 3B). In addition, the expression of LINC00472 gradually increased in the process of

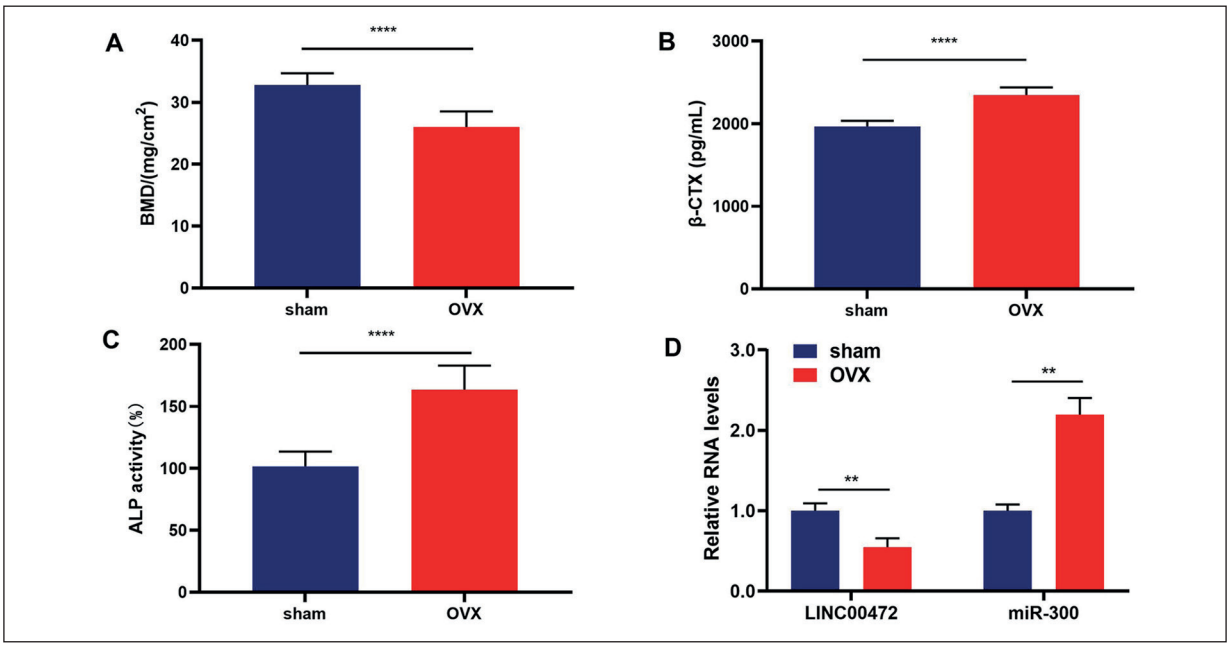

Figure 2. The expressions of LINC00472 and miR-300 in bone tissue of OVX mice. The osteoporosis model of mouse was established by OVX operation. Mice were divided into sham control (n=10) and OVX groups (n=10). **A**, BMD of mice spine was assessed by Micro-CT scanning 8 weeks after OVX operation. **** p<0.0001. **B**, The serum level of bone conversion marker β-CTX. **** p<0.0001. **C**, The serum level of relative ALP activity. **** p<0.0001. **D**, The relative expression of lncRNA-LINC00472 and miR-300 in bone tissues of sham control (n=10) and OVX groups (n=10). **** p<0.0001.

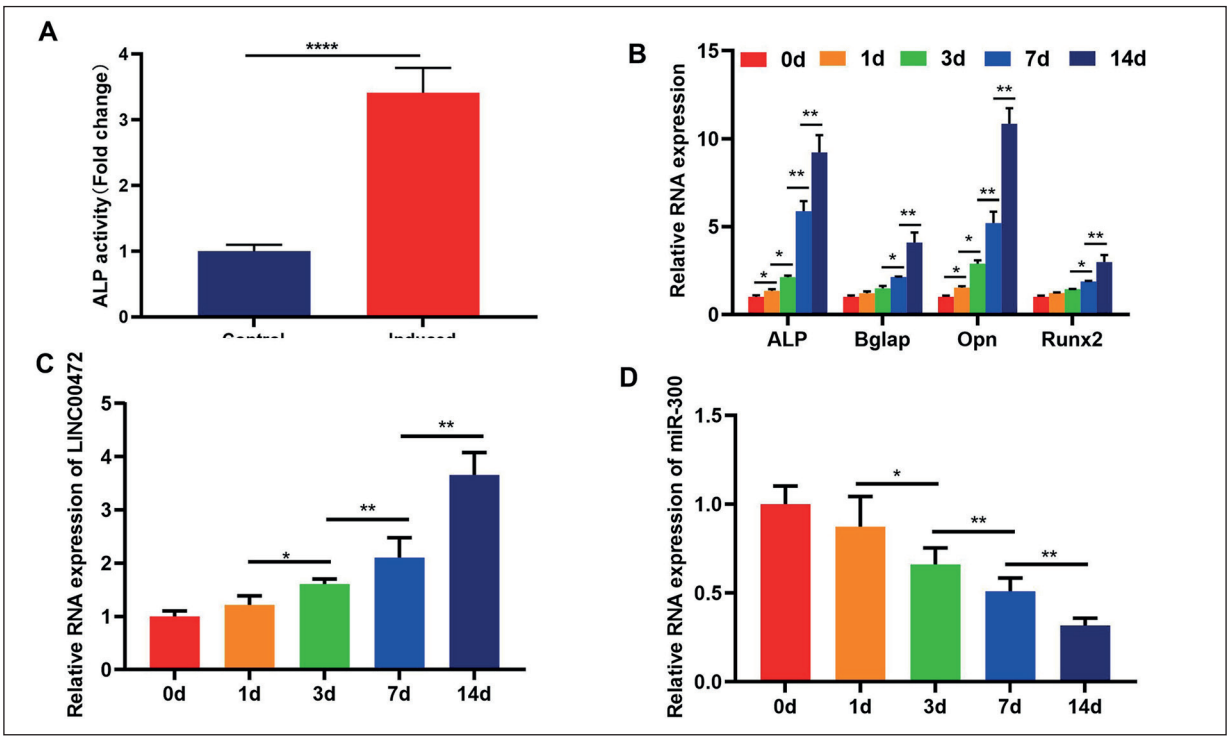

Figure 3. The expressions of LINC00472 and miR-300 during the differentiation of mice BMSCs. **A**, The relative activity of ALP in BMSCs was measured on the 7th day of induction. **B**, The mRNA expression levels of osteoblast marker gene such as ALP, Bglap, Opn and Runx2 in the process of differentiation of BMSCs. **C**, The relative expression of LINC00472 became increased gradually as the process of induction detecting by RT-qPCR (* p<0.05, ** p<0.01). **D**, The relative expression of miR-300 became decreased gradually as the process of induction detecting by RT-qPCR. (* p<0.05, ** p<0.01).

differentiation (Figure 3C), while the expression of miR-300 gradually decreased in the process of differentiation (Figure 3D).

The Expression of LINC00472 can Promote the Differentiation of Mice BMSCs

To further explore the role of LINC00472 in osteoporosis, we studied the function of LINC00472 in osteogenic differentiation. When mice BMSCs were transfected with sh-LINC00472-1 or sh-LINC00472-2, both sh-LINC00472-1 and sh-LINC00472-2 could inhibit the expression of LINC00472. But the efficiency of transfecting sh-LINC00472-1 was better. Therefore, sh-LINC00472-1 was chosen for subsequent experiments (Figure 4A). The overexpression plasmid was transfected into BMSCs. Compared with the control group, the expression level of LINC00472 in BMSCs transfected overexpression plasmid was significantly increased (Figure 4B).

Fibroblast growth factor receptor (FGFR) participates in many osteogenesis-related signal pathways, such as Wnt, ERK, p38, PLCγ and PKC. By regulating these signal pathways, FGFR can indirectly regulate the expression of osteogenesis-related genes[16,20], so we detected the expression of FGFR2 in mice BMSCs cells which transfected with sh-LINC00472 and overexpression plasmid. What's more, the expressions of osteogenesis-related genes (ALP, Bglap, OPN and Runx2) were also detected in BMSCs cells which transfected with sh-LINC00472 and overexpression plasmid. We found that, when BMSCs transfected with sh-LINC00472, both mRNA and protein expression of FGFR2, ALP, Bglap, OPN and Runx2 were reduced (Figure 4C,4E). However, the opposed result was showed in BMSCs transfected with pcDNA-LINC00472 (Figure 4D,4F). The LINC00472 could regulate the expression of FGFR2, which plays an important role in osteogenic differentiation.

Targeting Association Between LINC00472 and MiR-300 In Mice BMSCs

The expression of miR-300 was elevated in BMSCs treated with sh-LINC00472 (Figure 5A) and its expression was decreased in BMSCs treated with pcDNA-LINC00472(Figure 5B). Those results imply that the expression of LINC00472 was related with miR-300. Furthermore, an online target prediction algorithm TargetScan bioinformatics analysis was performed to explore whether miR-300 can bind to LINC00472, and the result showed that miR-300 and LINC00472 had complementary binding sites (Figure 5C). So, we conducted a Luciferase reporter assay. Compared with other groups, co-transfection with miR-300 mimic and wt-LINC00472 significantly decreased the Luciferase activity in BMSCs (Figure 5D). The data revealed that miR-300 could directly bind to LINC00472 binding sites.

The MiR-300 Could Regulate the Expression of FGFR2 In Mice BMSCs

We had proved that LINC00472 could affect the expression of FGFR2, and that the expression of LINC00472 was related to miR-300. Of note, we need to verify whether miR-300 can regulate the expression of FGFR2. A bioinformatics analysis showed that miR-300 and FGFR2 have 7-bp hypothetical complementary binding sites (Figure 6A) and the result of Luciferase reporter showed that co-transformation of miR-300 mimic and FGFR2-wt could significantly reduce the Luciferase activity (Figure 6B). The third generation of BMSCs with good growth was divided into non-treatment group, miR-300 inhibitor group and miR-300 mimic group. After 24 hours, the supernatant was taken and the cells were collected. Total RNA and total protein were extracted from three groups of cells. The content of ALP in the supernatants was detected by ALP assay kit. Compared with the untreated group, it was found that the content of ALP in the supernatant of the miR-300 inhibitor group significantly increased while the content of ALP significantly decreased in the miR-300 mimic group (Figure 6C). When BMSCs were treated with miR-300 inhibitor, the mRNA (Figure 6D) and protein levels of FGFR2 (Figure 6E) were significantly increased, and its expression in BMSCs treated with miR-300 mimic was decreased. These results suggest that miR-300 can affect the expression of FGFR2.

The LINC00472 Regulated BMSCs Differentiation by MiR-300/FGFR2 Axis

To further clarify the regulatory relationship between miR-300 and FGFR2 by LINC00472, we transfected BMSCs with sh-LINC00472 and then added miR-300 inhibitor to observe the effect of miR-300 inhibitor and non-miR-300 inhibitor on the expression of FGFR2. When BMSCs transfected with sh-LINC00472, the protein expression of FGFR2 decreased, but then the expression of FGFR2 could be restored after the addition of miR-300 (Figure 7A). The mRNA expression level of FGFR2 was consistent with the mRNA level of FGFR2 (Figure 7B). The content of ALP in the

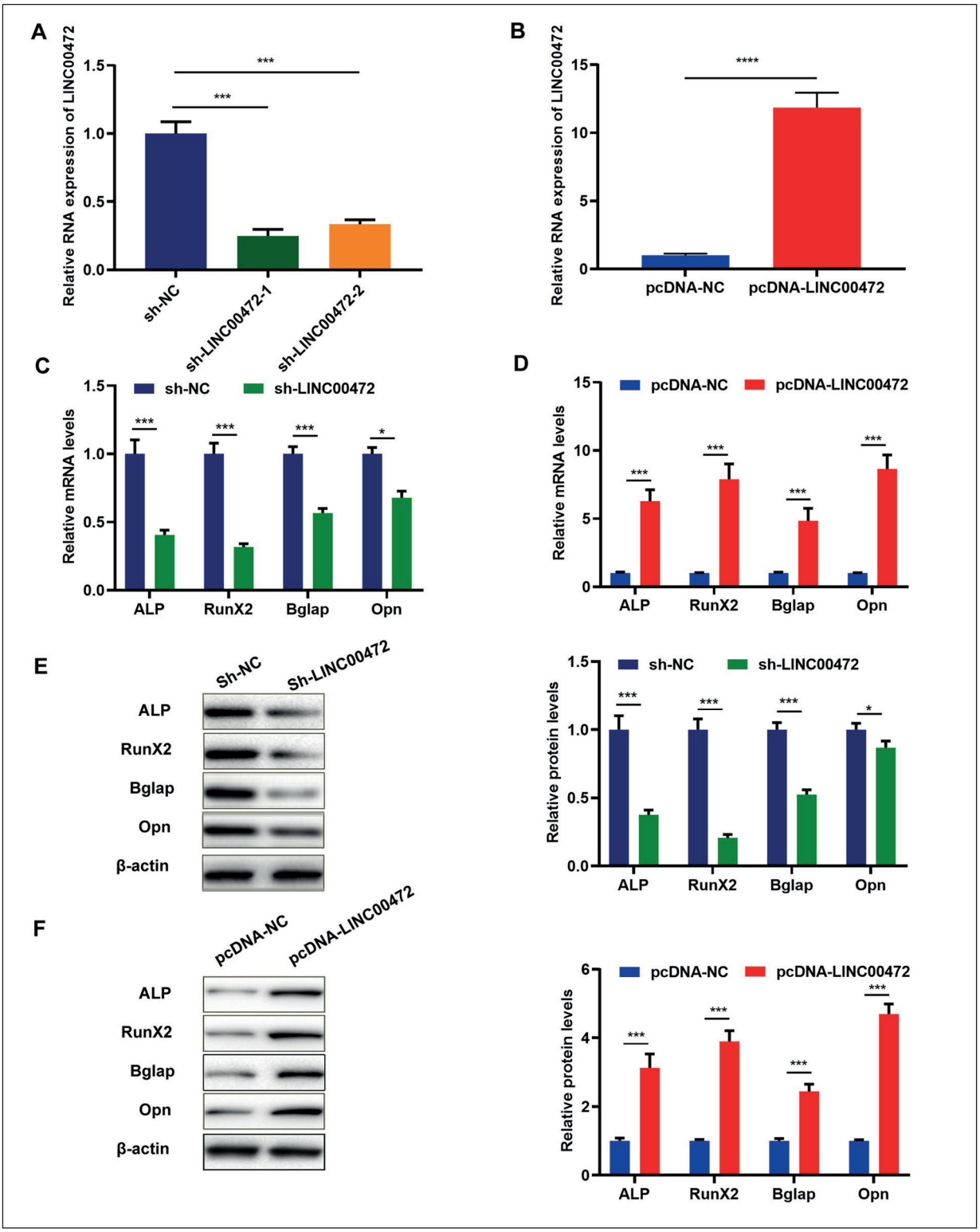

Figure 4. The LINC00472 can affect the differentiation of BMSCs. **A**, **B**, The expression of lncRNA-LINC00472 in BMSCs cells transfected with shRNA-LINC00472 and overexpression plasmids. *** $p<0.001$, **** $p<0.0001$. **C**, **D**, The mRNA expression levels of osteoblast marker gene such as ALP, Bglap, Opn and Runx2 and crucial factors for the osteogenic differentiation FGFR2 in BMSCs transfected with sh-LINC00472 and overexpressed. * $p<0.05$, *** $p<0.001$. **E**, **F**, The protein expression levels of osteoblast marker gene ALP, Bglap, Opn and Runx2 and crucial factors for the osteogenic differentiation FGFR2 in BMSCs transfected with sh-LINC00472 and overexpressed, * $p<0.05$, *** $p<0.001$.

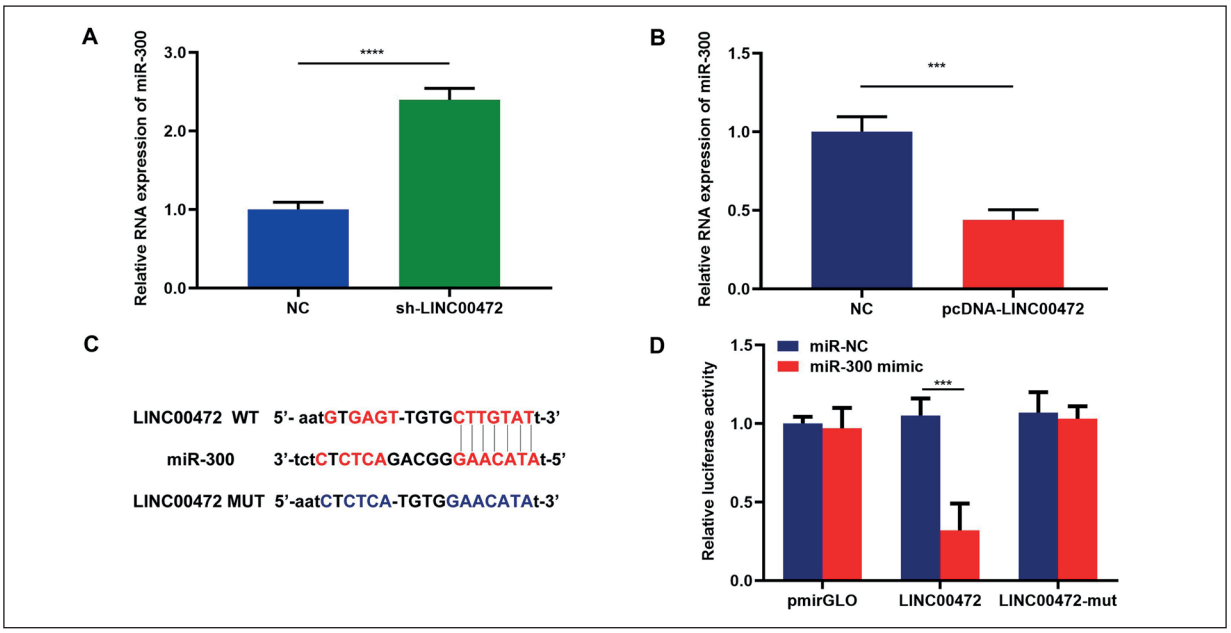

Figure 5. lncRNA-LINC00472 can directly binding to miR-300 in BMSCs. **A**, The relative expression of miR-300 in BMSCs cells transfected with shRNA-LINC00472 and overexpression plasmids. **** $p<0.0001$. **B**, The relative expression of miR-300 in BMSCs cells transfected with overexpression plasmids. **** $p<0.0001$. **C**, The predicted binding sites between LINC00472 and miR-300. **D**, The luciferase reporter assay. Co-transfection with miR-300 mimic and LINC00472-Wt significantly decreased the Luciferase activity of BMSCs cells compared with others group. *** $p<0.001$.

supernatant of different groups cells was detected. Compared with the control group, the activity of ALP decreased in transfected sh-LINC00472 groups, but miR-300 inhibitor could counteract the inhibitory effect (Figure 7C). In addition, the expressions of ALP, Bglap, Opn and Runx2, differentiated related gene, were detected by RT-qPCR (Figure 7D). After transfection of sh-LINC00472, the expression of differentiation-related gene mRNA decreased significantly, but miR-300 inhibitor could counteract this inhibitory effect. So far, we have proved LINC00472 can regulate the process of osteogenic differentiation through miR-300/FGFR2 axis in BMSCs.

Discussion

Osteoporosis is mainly caused by the weakening of osteogenic differentiation and the enhancement of osteoclast differentiation[21,22]. Bone marrow mesenchymal stem cells are non-hematopoietic stem cells in bone marrow tissue, which have multi-directional differentiation potential and high ability of self-renewal and proliferation. Under certain conditions, bone marrow mesenchymal stem cells can differentiate into osteoblasts, chondrocytes and other cells. Osteogenic inducers containing dexamethasone can induce BMSCs to differentiate into osteoblasts. BMSCs are an important model for inducing osteoblast differentiation *in vitro*[23].

LncRNA is a non-coding RNA, with a length of more than 200 nt that accounts for more than 80% of the total ncRNAs. Many functional lncRNA have stable secondary structure, unique subcellular localization and tissue-specific expression pattern. Increasing studies have shown that although lncRNA is not directly involved in protein coding, it can be methylated by participating in DNA methylation[10]. It is involved in the activation or silencing of regulatory genes and as the precursor of small molecule RNA, regulates the process of cell life[24]. In recent years, more and more studies have found that lncRNA is involved in the regulation of osteogenic differentiation of BMSC. Zhu et al[25] found that in the process of BMSC osteogenic differentiation, the expression of LMCD1 is up-regulated. LMCD1 can protect Runx2 and Smad1 proteins from Smurf1-induced ubiquitin degradation, thus regulating BMP signal transduction to promote osteogenic differentiation. Zhang et al[8] found that MSC-AS1 might pro-

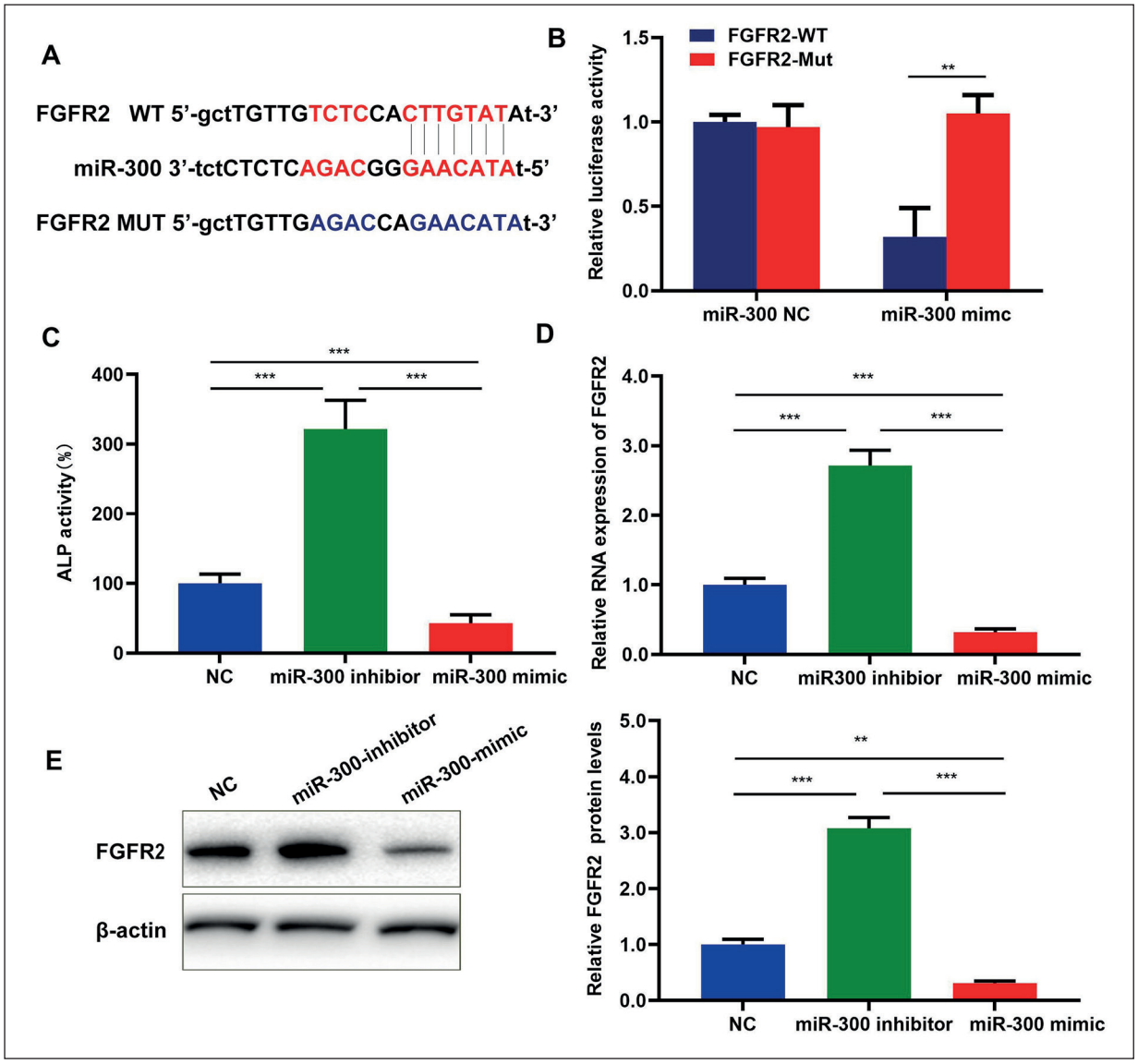

Figure 6. The miR-300 can directly binding to FGFR2 in BMSCs. **A**, The predicted binding sites between FGFR2 and miRNA-300. **B**, The Luciferase reporter assay. Co-transfection with miR-300 mimic and FGFR2-Wt significantly increased the Luciferase activity of BMSCs cells compared with others group. *** $p<0.001$. **C**, The relative activity of ALP in BMSCs cells, treated with miR-300 inhibitor and miR-300 mimic. **D**, The relative RNA expression of FGFR2 in the BMSCs cells, treated with miR-300 inhibitor and miR-300 mimic. *** $p<0.001$. **E**, The relative protein expression of FGFR2 in the BMSCs cells, treated with miR-300 inhibitor and miR-300 mimic. *** $p<0.001$.

mote the osteogenic differentiation of BMSCs through sponging microRNA-140-5p to up-regulate FGFR2.

In this study, we found the decreased expression of LINC00472 and increased expression of miR-300 in patients with osteoporosis and in bone tissues of OVX mice. What's more, the expression of LINC00472 was increased and the expression of miR-300 was decreased in the process of osteogenic differentiation of mice BMSC. These data meant there was a negative correlation between the expression of miR-300 and LINC00472 in osteoporosis. When BMSCs were transfected with LINC00472 overexpression plasmid, the expression of osteogenic differentiation related gene (ALP, Bglap, Opn and Runx2) was significantly increased, and the osteogenic differentiation gene was significantly decreased after sh-LINC00472 transfection, indicating that LINC00472 can affect the process of osteogenic differentiation.

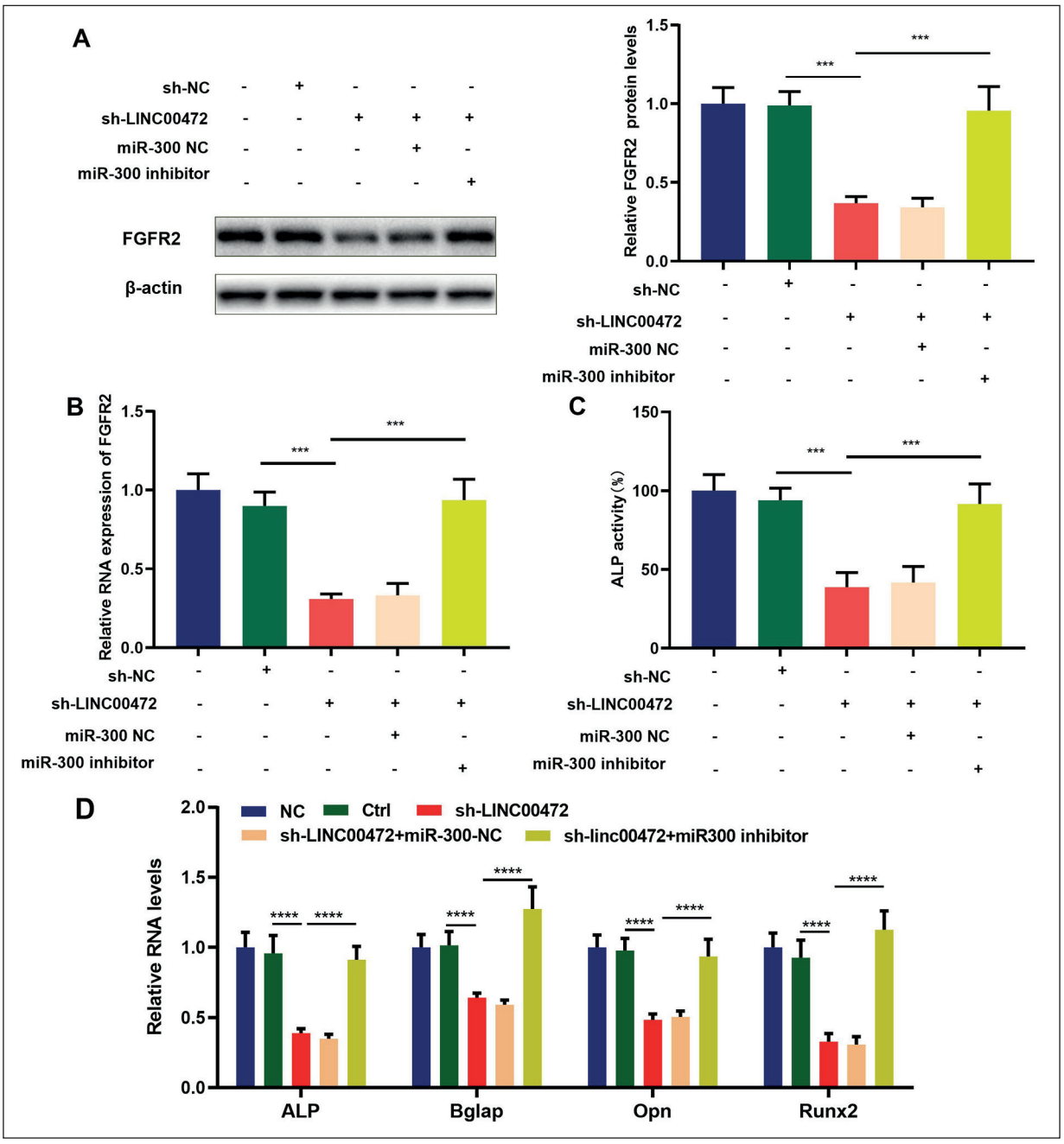

Figure 7. The LINC00472 can regulated BMSCs differentiation by miR-300/FGFR2 axis. **A**, The relative expression of FGFR2 protein, detected by WB, was significantly decreased treated with sh-LINC00472 and the tendency could be recovered by miR-300-inhibitor in BMSCs. *** $p<0.001$. **B**, The relative expression of FGFR2 mRNA, detected by RT-PCR, was significantly decreased treated with sh-LINC00472 and the tendency could be recovered by miR-300-inhibitor in BMSCs. *** $p<0.001$. **C**, The relative activity of ALP in BMSCs cells was significantly decreased treated with sh-LINC00472 and the tendency could be recovered by miR-300-inhibitor. *** $p<0.001$. **D**, The mRNA expression levels of osteoblast marker gene such as ALP, Bglap, Opn and Runx2 were significantly decreased treated with sh-LINC00472 and the tendency could be recovered by miR-300-inhibitor in BMSCs cells. **** $p<0.0001$.

FGFs can bind to FGFR, cause FGFR dimerization and phosphorylate its own tyrosine residues, activate multiple signal transduction pathways, and regulate a variety of growth-related processes, including endochondral ossification and intramembranous ossification. The expression of Runx2 in human osteoblasts activated by FGFR2 was significantly increased[26,27]. We detected the expres-

sion of BMSCs in FGFR2 after transfection of sh-LINC00472 and pcDNA-LINC00472. It was found that LINC00472 could regulate the expression of FGFR2. In addition, the analysis of online targetscan database suggested that miR-300 may bind to LINC00472 and FGFR2, which was confirmed by the result of Dual-Luciferase report. Subsequently, we treated the BMSCs, transfected with sh-LINC00472 with miR-300 inhibitor and found that miR-300 inhibitor could restore the inhibitory effect of sh-LINC00472 on the expression of FGFR2.

So far, we have proved that the expression of LINC00472 decreased in osteoporosis, and LINC00472 can regulate the process of osteogenic differentiation through miR-300/FGFR2 axis in BMSCs.

Conclusions

Our results first revealed that the expression of LINC00472 decreased in patients with osteoporosis, while the increased expression of miR-300 led to the decreased expression of FGFR2. What's more, our findings indicated a novelty mechanism that LINC00472 affects bone differentiation by sponging miR-300 to regulate FGFR2 expression, suggesting that LINC00472 can be used as a potential target for treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Foundation

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