

LINC00899 promotes osteogenic differentiation and alleviates osteoporosis via targeting miR-374a to regulate RUNX2

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Abstract

Objective: This study aims to illustrate the underlying molecular mechanisms of long noncoding RNAs (LncRNAs) LINC00899 in osteoporosis.

Methods: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to examine the levels of LINC00899, miR-374a and RUNX2 in clinical tissues or human bone mesenchymal stem cells (hBMSCs). The interaction between miR-374a and LINC00899 or RUNX2 was predicted by starBase and verified by luciferase reporter assay and RNA binding protein immunoprecipitation (RIP) assay. Alkaline phosphatase (ALP) activity and Alizarin Red S (ARS) staining were also used to evaluate the osteogenic ability of hBMSCs.

Results: The expression levels of LINC00899 were gradually increased, but miR-374a expression was decreased with the prolongation of osteogenic induction. In addition, the expression of LINC00899 was lowly expressed in osteoporotic patients' bone tissues and knockdown of LINC00899 decreased the expression of osteogenesis-related genes. Moreover, LINC00899 was confirmed to inhibit miR-374a expression by direct interaction. Finally, we demonstrated that RUNX2 was a target of miR-374a, and the silencing of miR-374a partially abolished the inhibitory effect of LINC00899 knockdown on the expression of RUNX2, OPN and OCN.

Conclusions: We demonstrated that LINC00899 facilitated the osteogenic differentiation of hBMSCs and prevented osteoporosis by sponging miR-374a and enhancing RUNX2 expression, which might provide a useful therapeutic strategy for osteoporosis patients.

Background

Osteoporosis is a common systemic bone disease in aged population and characterized by bone density and bone mass decrease, and bone microstructure destruction [1, 2]. Human bone mesenchymal stem cells (hBMSCs) are multipotent progenitor cells that have the potential to differentiate into osteoblasts, which play critical roles in bone formation [3]. Previous researches have revealed that recovering the osteogenic differentiation ability of hBMSCs inhibited bone loss in osteoporosis [4-6]. Therefore, the induction of directional differentiation of hBMSCs is a potential therapeutic strategy for osteoporosis.

Long non-coding RNAs (lncRNAs) are a class of non-protein-coding RNA with > 200 nucleotides (nt) in length [7, 8]. Increasing evidence indicated that lncRNA was involved in the occurrence and development of various diseases, including osteoporosis [9]. Jiang et al reported that SNHG1 inhibited osteogenic differentiation of BMSCs by negatively regulating p38 MAPK signal pathway[10]. Chen et al found that lncRNA Bmncr alleviated the progression of osteoporosis by inhibiting osteoclast differentiation [11]. It has reported that LINC00899 expression was upregulated in the serum and bone marrow of acute myeloid leukemia patients, which could be used as a potential biomarker for the diagnosis and prognosis in acute myeloid leukemia[12]. However, the underlying molecular mechanisms of LINC00899 in the occurrence and development of osteoporosis have not been explored.

MicroRNAs (miRNAs) are short non-coding RNAs with a length of ~22 nt, which act as vital regulators in biological processes, such as cell proliferation and differentiation [13]. Some miRNA has been reported to be involved in osteoporosis by binding the 3'-UTR of their target mRNA[14]. For example, Wang et al showed that miR-765 had a potential role in inhibiting osteogenic differentiation by targeting BMP6 [15]. Zhang et al newly identified that miR-664-5p promoted osteogenic differentiation by direct targeting HMGA2 [16]. Li et al reported that miR-291a-3p improved cell viability and promoted osteogenic differentiation by regulating DKK1 [17]. As RUNX2 was identified to be a downstream target of several miRNA, such as miR-21[18], miR-365a[19], and miR-217[20], we speculated that miR-374a might regulate osteoporosis progression by regulating RUNX2.

In the present study, we investigated the biological role of LINC00899 in osteoporosis. The results demonstrated that LINC00899 could sponge miR-374a to enhance RUNX2 expression, thus alleviating osteoporosis. These findings may provide a new therapeutic strategy for osteoporosis patients.

Materials And Methods

Clinical samples

Bone tissues were collected from osteoporosis patients (n=15) and healthy controls (n=15) at the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University between March 2016 and April 2018. Bone fragments extracted from the transcervical region of the femoral neck were dissected into smaller fragments, washed three times in PBS and stored at -80°C until further analysis. Written consents from all patients were collected before this work. This study was approved by the ethics committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University.

Cell culture and osteogenic differentiation induction

Human bone mesenchymal stem cells (hBMSCs) were obtained from the BeNa Culture Collection (BNCC, Beijing, China). The hBMSCs cultured in α -MEM supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin and 100 U/ml streptomycin in an incubator with 5% CO₂ at 37°C. To induce osteogenic differentiation, 10 μ mol/L dexamethasone, 200 μ M ascorbic acid and 10 mmol/L β -glycerophosphate were added, and the induction medium was changed every 3 days.

Cell transfection

The small short hair RNA (shRNA) targeting LINC00899 (shLINC00899) with its negative control (shNC), miR-374a mimics with its negative control (NC mimics), and miR-374a inhibitor with its negative control (NC inhibitor) were purchased from GenePharma (Shanghai, China). To overexpress LINC00899, the full-length LINC00899 sequence was inserted into the pcDNA3.1 vector (GenePharma, Shanghai). Cell transfection was carried out by Lipofectamine 2000 (Invitrogen). The subsequent experiments were performed following 48 h of transfection.

Bioinformatic prediction and dual-luciferase reporter assay

Starbase 2.0 (<http://starbase.sysu.edu.cn/>) was used to predict the binding sites between miR-374a and LINC00899 or RUNX2. The pmirGLO-LINC00899-WT/Mut and pmirGLO-RUNX2-WT/Mut reporters were obtained from GenePharma (Shanghai, China). Then miR-374a mimics or NC mimics was co-transfected with these above reporters into 293T cells. 48 h after transfection, the relative luciferase activity was detected using dual-luciferase reporter assay system (Promega).

RT-qPCR

Total RNAs were extracted from tissues and hBMSCs using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNAs were reverse-transcribed to cDNAs through reverse transcriptase kit (Takara, Otsu, Japan) or the TaqMan® miRNA reverse transcription kit (Thermo Fisher Scientific). RT-qPCR was performed on the ABI 7900 Detection System (Applied Biosystems, USA) by using the SYBR-Green PCR Master Mix kit (Takara, Dalian, China). The primer sequences were as follows: LINC00899 forward, 5'-CAGTCAGCCTCAGTTTCCAA-3' and reverse, 5'-AGGCAGGGCTGTGCTGAT-3'; miR-374a forward, 5'-GGTCACAGTGAACCGGTC-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; RUNX2 forward, 5'-CTTATACAATGTCAACAGCC-3' and reverse, 5'-TCCTTATGCTCTTTCTTCC-3'; GAPDH forward, 5'-CCACTCCTCCACCTTTGAC-3' and reverse, 5'-ACCCTGTTGCTGTAGCCA-3'; and U6 forward, 5'-CTTCGGCAGCACATATACT-3' and reverse, 5'-AAAATATGGAACGCTTCACG-3'.

RNA immunoprecipitation (RIP) assay

RIP assay was carried out using Magna RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Briefly, cell lysate was incubated in RIP buffer containing magnetic beads conjugating with the Ago2 antibody (Anti-Ago2, Abcam) or IgG antibody (Anti-IgG, Abcam). Subsequently, the enrichment of LINC00899 and miR-374a was determined by RT-qPCR.

Alkaline phosphatase (ALP) activity

ALP activity was detected at 0, 7 and 14 days after cell culture, using the Alkaline Phosphatase Assay Kit (Abcam). Briefly, after collected and rinsed with PBS, hBMSCs were incubated and lysed with RIPA buffer (Beyotime, Shanghai, China). The cell lysate was centrifuged with 10,000 rpm/min for 5 min, and the supernatant was used to measure ALP activity.

Alizarin Red S (ARS) staining

After osteogenic differentiation, treated hBMSCs were washed twice with PBS and then fixed with 4% paraformaldehyde for 20 min. Then the cells were washed twice with PBS. Subsequently, cells were stained with 1 ml ARS at 37°C for 10 min. After being washed with distilled water, the cells were captured with an optical microscope.

Western Blot

Total proteins were extracted from hBMSCs using RIPA buffer (Beyotime, Shanghai, China). Protein samples were separated by 10% SDS-PAGE and transferred onto the polyvinylidene difluoride (PVDF) membranes. After being blocked for 2 hr with 5% non-fat dry milk at room temperature, the membranes were incubated at 4°C overnight with antibodies against RUNX2, OPN and OCN, and GAPDH at 4°C overnight. After that, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37°C. Finally, the protein blots were measured using the Pierce™ ECL A and assessed by Quantity One software (Bio-Rad Laboratories, CA, USA).

Statistical Analysis

All experiments were repeated at least three times. The data were analyzed using Prism 6.0 (GraphPad Software, USA) and represented as mean ± standard deviation (SD). Student's t-test was used to analyze the difference between the two groups. One-way ANOVA and Tukey's test were used to analyze the difference among multiple groups. $P < 0.05$ was considered statistically different.

Results

The expression levels of LINC00899 and miR-374a during osteogenic differentiation

The dynamic expressions of LINC00899 and miR-374a were detected on 0 day, 7th days and 14th days during osteogenic differentiation. As shown in Figure 1A and B, the expression levels of LINC00899 were gradually increased, but miR-374a expression was decreased with the prolongation of osteogenic induction. Moreover, RT-qPCR and western blot indicated that the mRNA and protein expression levels of RUNX2, OPN and OCN were gradually increased during the process of osteogenic differentiation (Figure 1C-F). In addition, the ALP assay and ARS also showed that ALP activity and mineralized nodules area were significantly increased during osteogenic induction, respectively (Figure 1E). The above results indicated that LINC00899 was upregulated and miR-374a was downregulated during osteogenic differentiation.

Knockdown of LINC00899 promoted the progression of osteoporosis

RT-qPCR assay results showed that LINC00899 was highly expressed in non-osteoporotic tissues compared with that in osteoporotic tissues (Figure 2A). To investigate the effect of LINC00899 on osteogenic differentiation, LINC00899 was knocked down in hBMSCs on 14 days of osteogenic induction. The transfection efficiency was confirmed by RT-qPCR (Figure 2B). Moreover, it was revealed that knockdown of LINC00899 decreased the expression of osteogenesis-related genes (RUNX2, OPN and OCN) at mRNA and protein levels (Figure 2C-F). In addition, ALP assay indicated that LINC00899 knockdown decreased ALP activity (Figure 2G), and ARS showed the number of mineralized nodules was decreased following transfection with shLINC00899 (Figure 2H). Taken together, our results demonstrated that LINC00899 knockdown inhibited osteogenic differentiation of hBMSCs.

miR-374a is a target of LINC00899

The binding site of miR-374a to LINC00899 was predicted by starBase (Figure 3A). Dual-luciferase reporter assay revealed that miR-374a overexpression reduced the luciferase activity of wild-type LINC00899, but had no effect on the mutant LINC00899 (Figure 3B). RIP assay demonstrated that LINC00899 and miR-374a were significantly enriched in AGO2 compared with that in IgG (Figure 3C). Furthermore, the expression of miR-374a was examined in non-osteoporotic tissues and osteoporotic tissues and the results showed that miR-374a was lowly expressed in non-osteoporotic tissues (Figure 3D). In addition, knockdown of LINC00899 increased the expression of miR-374a and the upregulation of LINC00899 decreased miR-374a expression (Figure 3E). In sum, these data indicated that LINC00899 could inhibit miR-374a expression by direct interaction.

LINC00899 regulated osteogenic differentiation through inhibiting miR-374a expression

Through starBase website, RUNX2 was predicted as a potential target of miR-374a (Figure 4A). Next, dual-luciferase reporter assay indicated that miR-374a mimics weakened the luciferase activities of wild-type of RUNX2 but no significant change in mutant-type RUNX2 (Figure 4B). In addition, silencing of miR-374a increased the expression of RUNX2 (Figure 4C). To further investigate whether LINC00899 regulated RUNX2 expression via miR-374a, hBMSCs were transfected with shNC, shLINC00899, and shLINC00899 + miR-374a inhibitor. RT-qPCR western blot revealed that knockdown of LINC00899 decreased the expression of RUNX2, while the inhibition of miR-374 reversed the effect (Figure 4D and E). Moreover, miR-374a inhibitor partially abolished the inhibitory effect of LINC00899 knockdown on the expression of OPN and OCN (Figure 4F-H). The ALP activity assay further validated that miR-374a inhibitor reversed the regulatory impact of LINC00899 knockdown on ALP activity (Figure 4I). ARS staining showed that miR-374a inhibitor significantly recovered the osteogenic capacity of hBMSCs transfected with shLINC00899 (Figure 4J). These results indicated that LINC00899 upregulated RUNX2 expression by sponging miR-374a to alleviate osteoporosis.

Discussion

In the present study, we demonstrated that LINC00899 facilitated osteogenic differentiation and prevented osteoporosis through regulating miR-374a/RUNX2 axis. Specifically, LINC00899 downregulated miR-374a expression, which then targeting 3'-UTR of RUNX2 and regulating its expression. Consistently, the upregulation of RUNX2 inhibited the progression of osteoporosis.

LncRNA has been confirmed to play a vital role in bone metabolism diseases. Han et al revealed that downregulation of lncRNA TUG1 effectively inhibited osteoclast proliferation and might serve as a potential target for the treatment of osteoporosis[21]. Shen et al reported that lncRNA HOTAIR was highly expressed in osteoporosis patients, which prevented osteogenic differentiation by regulating Wnt/ β -catenin pathway[22]. Regarding LINC00899, Zhou et al reported that LINC00899 suppressed breast cancer progression by regulating miR-425 expression [23]. Dong et al revealed that LINC00899 facilitated the progression of acute myeloid leukaemia [24]. However, the biological function of LINC00899 in osteogenic differentiation and osteoporosis is unknown. In our study, we for the first time demonstrated

that LINC00899 was lowly expressed in osteoporotic patients' bone tissues. Moreover, knockdown of LINC00899 decreased the expression of osteogenesis-related genes and inhibited ALP activity and Alizarin red S accumulation, which resulted in inhibition of osteogenic differentiation and promoted osteoporosis progression.

Increasing evidence has indicated that lncRNA can act as a miRNA sponge to regulate osteogenic differentiation and osteoporosis progression. For example, Wang et al reported that lncRNA KCNQ10T1 promoted osteogenic differentiation through absorbing miR-374a to upregulate BMP2 [25]. Zhang et al identified NEAT1 promoted BMP1 expression to regulate osteogenic differentiation of hBMSCs by sponging miR-29b-3p [26]. In addition, miRNAs have been reported to be involved in the occurrence of bone metabolic diseases [27]. For instance, Fan et al identified that miR-532-3p attenuated osteogenic differentiation by downregulating ETS1 [28]. Gan et al reported that miR-19b-3p promoted osteogenic differentiation of BMSCs [29]. In our study, miR-374a was predicted as a potential target of LINC00899, and dual-luciferase reporter and RIP assays verified the interaction. Moreover, we explored the dynamic expressions of LINC00899 and miR-374a during osteogenic differentiation and the results indicated LINC00899 expression gradually increased but miR-374a decreased during osteogenic differentiation. The above results indicated that miR-374a might be associated with the osteoporosis progression by regulating osteogenic differentiation.

RUNX2 is widely recognized as an important transcription factor for osteogenic differentiation [30]. Therefore, the regulatory mechanism of RUNX2 during osteogenic differentiation has attracted the attention of many researchers. Fu et al indicated that HOTAIRM1 promoted osteogenesis by modulating JNK and c-Jun activity to activate RUNX2 gene transcription [31]. Chen et al reported that lncRNA AWPPH contributed to non-traumatic osteonecrosis through upregulating RUNX2 [32]. However, the mechanisms associated with RUNX2 in osteoporosis have not been fully explored. Herein, we actively explored new mechanisms by which RUNX2 regulates osteogenic differentiation. In this study, we identified that RUNX2 was the target of miR-374a. RT-qPCR assay showed that RUNX2 expression was increased during osteogenic differentiation, and the expression of RUNX2 significantly increased in hBMSCs transfected with miR-374a inhibitor. LINC00899 depletion inhibited osteogenesis-related markers Runx2, OPN and OCN, which was counteracted by inhibition of miR-374a. Moreover, the miR-374a inhibitor also neutralized the variation of ALP and Alizarin red S accumulation induced by shLINC00899. In addition, we found that LINC00899 regulated RUNX2 expression by targeting miR-374a, which revealed the regulatory roles of LINC00899/miR-374a/RUNX2 axis during the progress of osteogenic differentiation.

Conclusion

In summary, our study uncovered that LINC00899 promoted osteogenic differentiation through miR-374a/RUNX2 axis. These findings may provide a novel theoretical basis for the treatment of osteoporosis.

Abbreviations

long noncoding RNAs: LncRNAs; human bone mesenchymal stem cells: hBMSCs; Real Time-quantitative Polymerase Chain Reaction: RT-qPCR; RNA binding protein immunoprecipitation: RIP; MicroRNAs: miRNAs; nucleotides: nt;

Declarations

Acknowledgments

Not applicable.

Author's contribution

XG designed and supervised the study, analyzed the data, written and edited the manuscript. YX, KY collected and analyzed the data, edited the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee. Written informed consent was obtained from all individual participants included in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

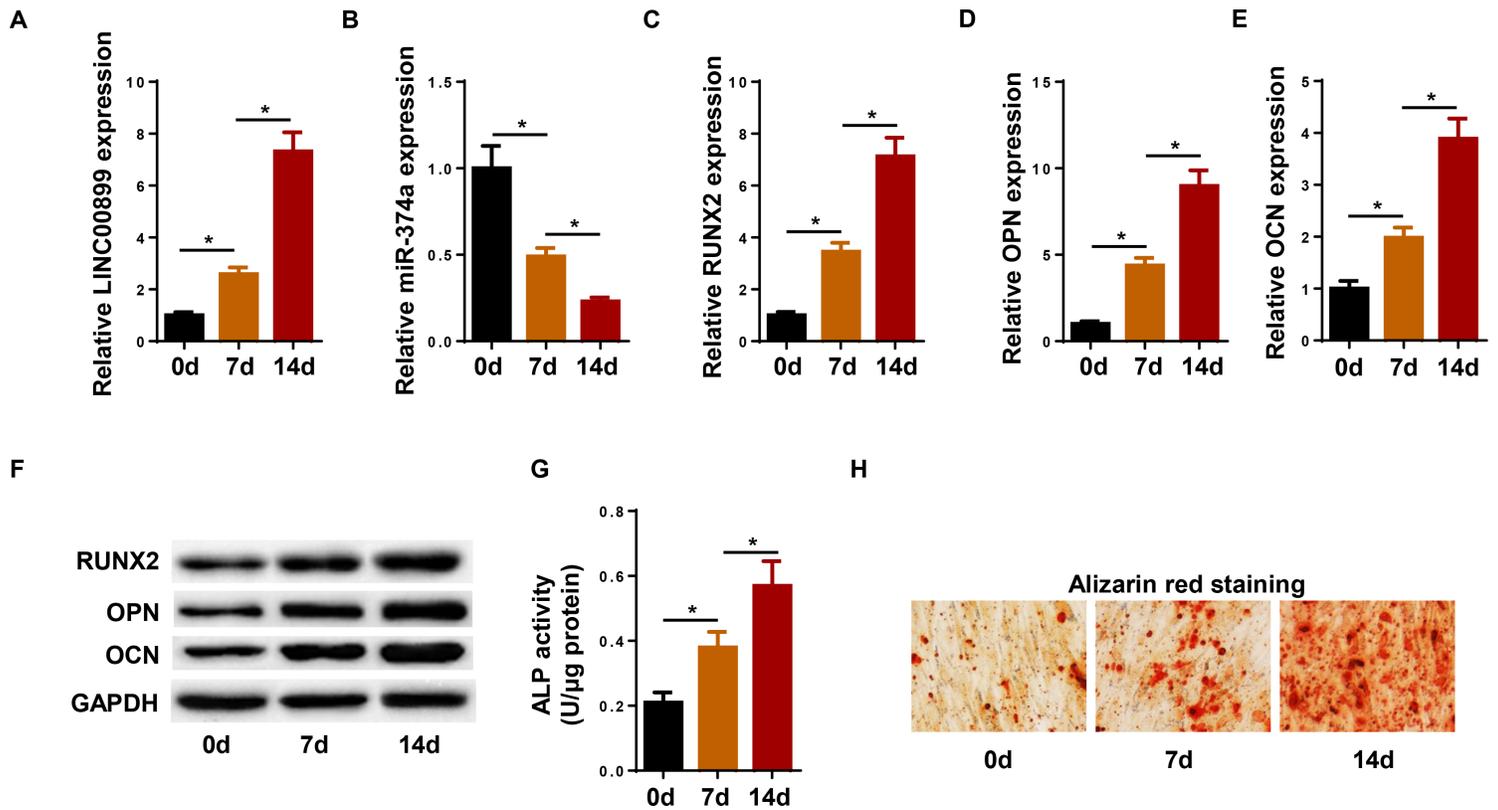


Figure 1

The expression levels of LINC00899 and miR-374a during osteogenic differentiation (A and B) The expression levels of LINC00899 and miR-374a in hBMSCs treated with osteogenic differentiation medium for 0, 7 and 14 days were detected by RT-qPCR. (C-F) RT-qPCR and western blot were used to detect the mRNA and protein levels of RUNX2, OPN and OCN during osteoblast differentiation of hBMSCs. (G and H) ALP activity and Alizarin red S staining were detected on 0, 7 and 14 days after osteogenic induction The data were presented as mean \pm SD (*P < 0.05).

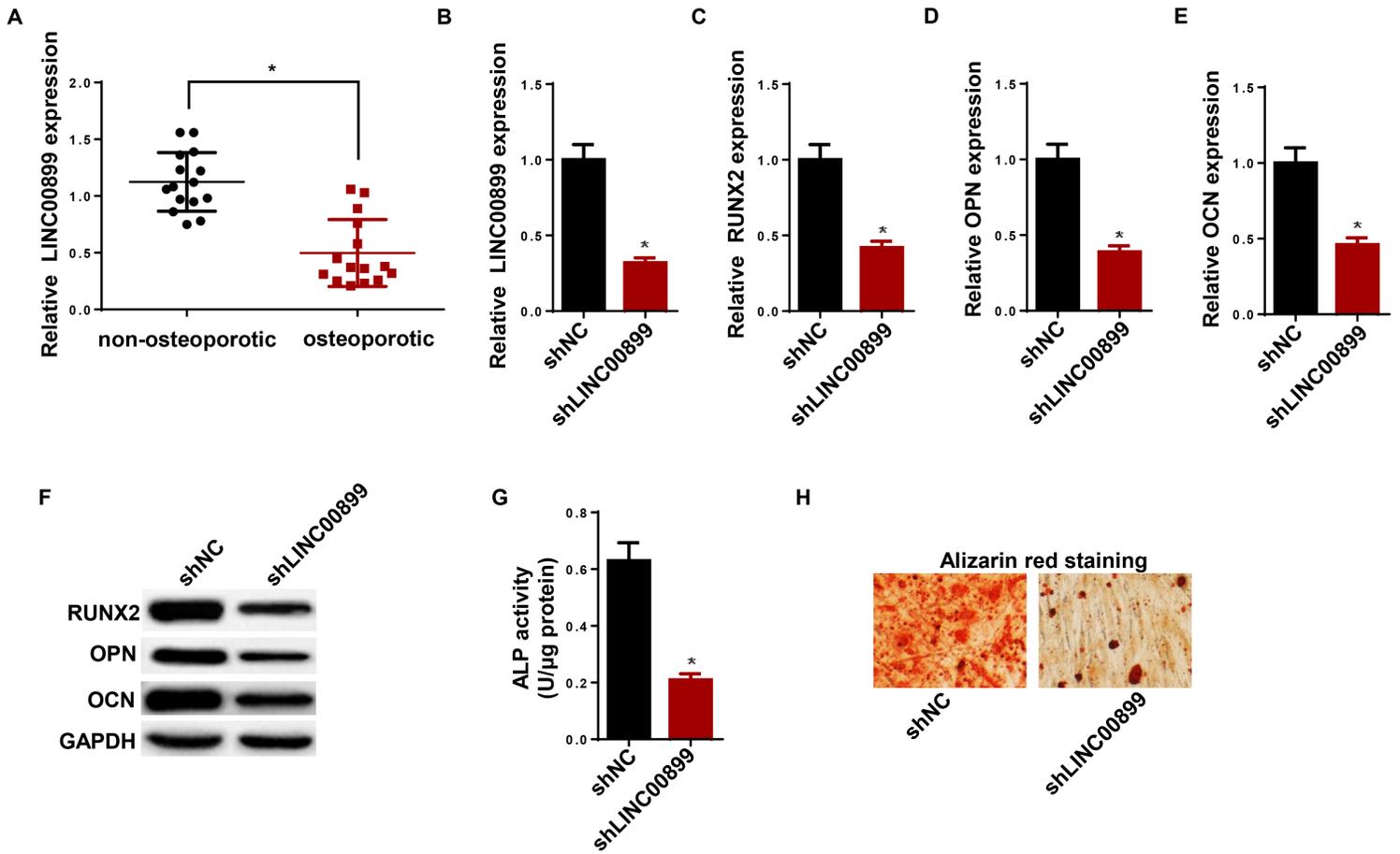


Figure 2

Knockdown of LINC00899 promoted the progression of osteoporosis (A) RT-qPCR was used to detect the expression of LINC00899 in osteoporotic tissues and non-osteoporotic tissues. (B) The expression of LINC00899 in hBMSCs transfected with shNC and shLINC00899 was detected by RT-qPCR. (C-E) The expression of osteogenesis-related genes (RUNX2, OPN and OCN) in hBMSCs transfected with shNC and shLINC00899 was detected by RT-qPCR. (F) Western blot was utilized to detect the protein level of RUNX2, OPN and OCN in hBMSCs transfected with shNC and shLINC00899. (G) ALP activity was detected in hBMSCs transfected with shNC and shLINC00899. (H) Alizarin red S staining was measured in hBMSCs transfected with shNC and shLINC00899. The data were presented as mean \pm SD (* $P < 0.05$).

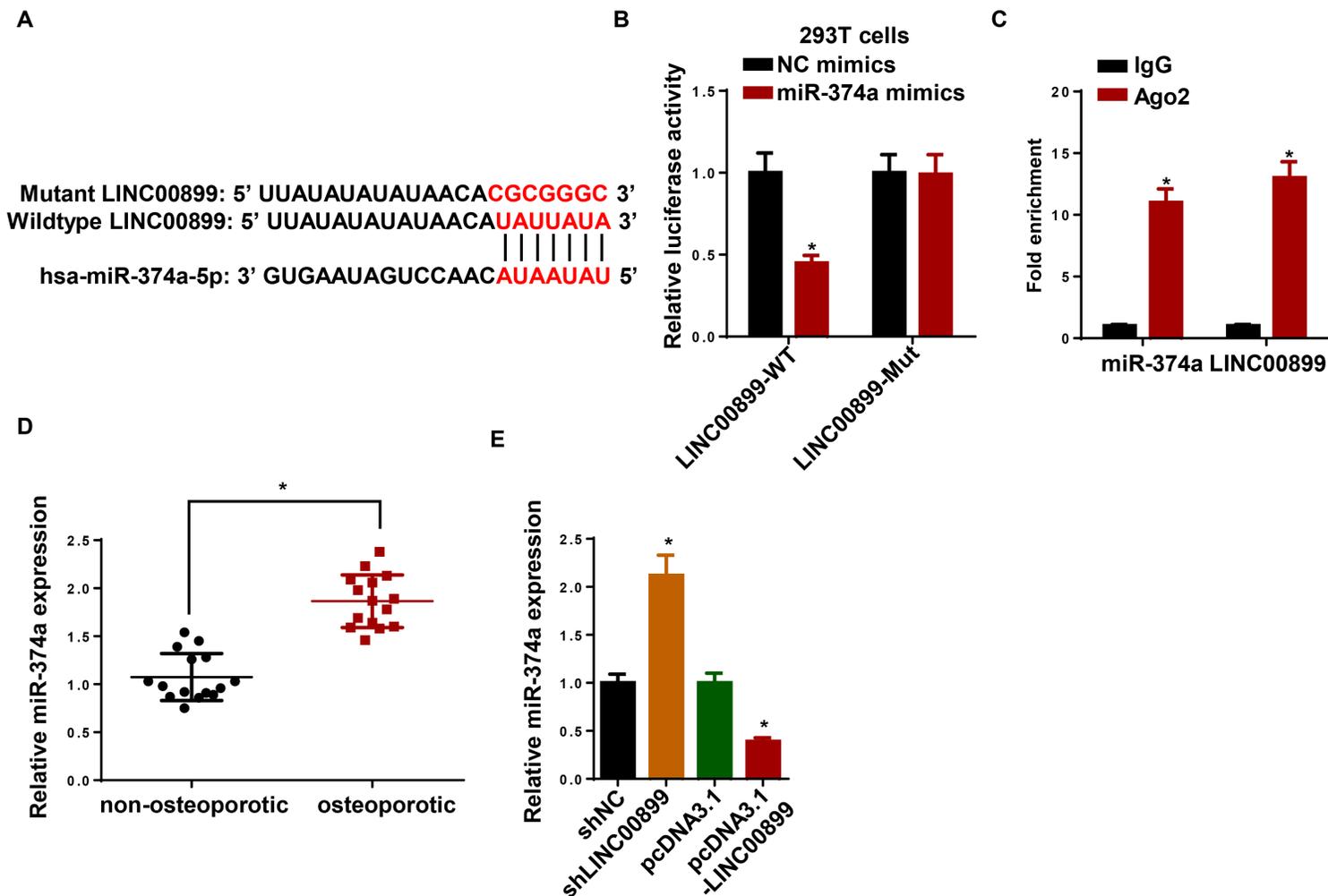


Figure 3

miR-374a was a target of LINC00899 (A) The potential binding site for miR-374a to LINC00899 was predicted by the starBase. (B) Dual-luciferase reporter assay was used to determine the luciferase activity of wild-type LINC00899 or mutant-type LINC00899 in 293T cells. (C) The RIP assay was utilized to analyze the interaction between miR-374 and LINC00899. (D) RT-qPCR used to detect the expression of miR-374a in osteoporotic tissues and non-osteoporotic tissues. (E) The expression of miR-374a in hBMSCs transfected with shNC, shLINC00899, pcDNA3.1, and pcDNA3.1-LINC00899 was detected by RT-qPCR. The data were presented as mean \pm SD (* $P < 0.05$).

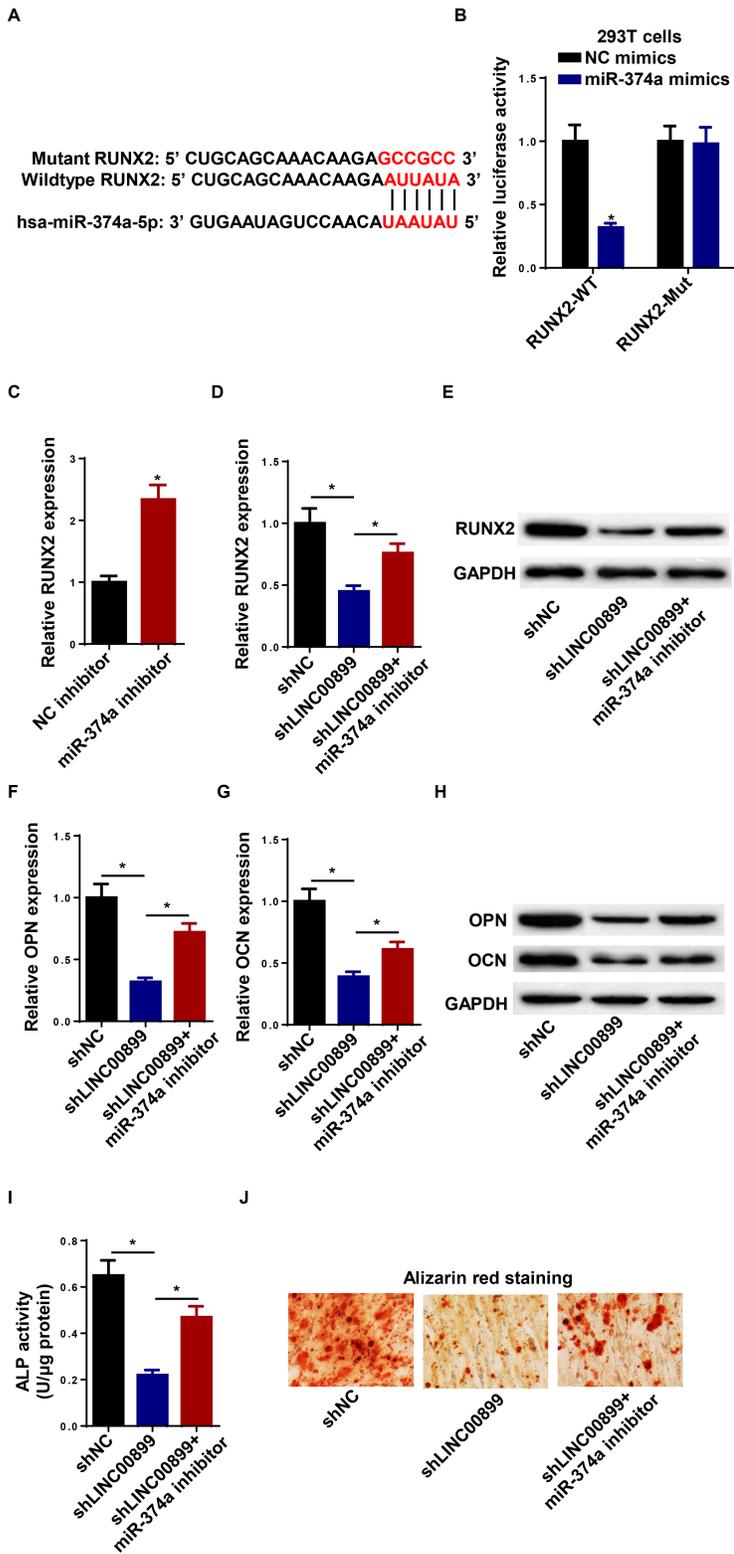


Figure 4

LINC00899 regulated osteogenic differentiation through inhibiting miR-374a expression (A) The predicted binding sites of RUNX2 and miR-374a. (B) Dual-luciferase reporter assay was used to determine the luciferase activity of RUNX2-WT or RUNX2-Mut in 293T cells. (C) The expression of RUNX2 in hBMSCs transfected with NC inhibitor and miR-374a inhibitor was detected by RT-qPCR. (D-H) The mRNA and protein levels of RUNX2, OPN and OCN were determined in hBMSCs transfected with shNC, shLINC00899,

shLINC00899+miR-374a inhibitor by RT-qPCR and Western blot. (I) ALP activity was detected in hBMSCs transfected with shNC, shLINC00899, and shLINC00899+miR-374a inhibitor. (J) Alizarin red S staining was detected in hBMSCs transfected with shNC, shLINC00899, and shLINC00899+miR-374a inhibitor. The data were presented as mean \pm SD (*P < 0.05).

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