LINC00899 Promotes Osteogenic Differentiation and Alleviates Osteoporosis via Targeting miR-374a to Regulate RUNX2

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Research article

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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) 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.

Results: 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. the expression levels of LINC00899 were gradually increased, but miR-374a expression was decreased with the prolongation of osteogenic induction. 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 osteogenic differentiation of hBMSCs and prevent 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 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]. 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 [12]. miRNA has been reported to be involved in osteoporosis by binding the 3'-'UTR of their target mRNA. For example, Wang et al showed

that miR-765 had a potential role in inhibiting osteogenic differentiation by targeting BMP6 [13]. Zhang et al newly identified that miR-664-5p promoted osteogenic differentiation by direct targeting HMGA2 [14]. Li et al reported that miR-291a-3p improved cell viability and promoted osteogenic differentiation by regulating DKK1 [15]. As RUNX2 was predicted as a potential target of miR-374a, 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% CO_2 at 37 °C. To induce osteogenic differentiation, 10 μmol/L dexamethasone, 200 μM 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). Cell transfection was carried out by Lipofectamine 2000 (Invitrogen).

Dual-luciferase reporter assay

Starbase (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.

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

Knockdown of LINC00899 promoted the progression of osteoporosis

First, RT-qPCR assay results showed that LINC00899 was highly expressed in non-osteoporotic tissues compared with that in osteoporotic tissues (Fig. 1A). To investigate the effect of LINC00899 on osteogenic differentiation, hBMSCs were transfected with shNC and shLINC00899. The transfection efficiency was confirmed by RT-qPCR (Fig. 1B). Moreover, it was revealed that knockdown of LINC00899

decreased the expression of osteogenesis-related genes (RUNX2, OPN and OCN) (Fig. 1C-E). 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 (Fig. 2A). 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 (Fig. 2B). RIP assay demonstrated that LINC00899 and miR-374a were significantly enriched in AGO2 compared with that in IgG (Fig. 2C). 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 (Fig. 2D). In addition, knockdown of LINC00899 decreased the expression of miR-374a (Fig. 2E). In sum, these data indicated that LINC00899 could inhibit miR-374a expression by direct interaction.

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

The dynamic expressions of LINC00899 and miR-374a were detected at 0 day, 7th days and 14th days during osteogenic differentiation. As shown in Fig. 3A and B, the expression levels of LINC00899 were gradually increased, but miR-374a expression was decreased with the prolongation of osteogenic induction. Moreover, the expression levels of RUNX2, OPN and OCN were gradually upregulated during the process of osteogenic differentiation (Fig. 3C-E).

LINC00899 regulated osteogenic differentiation through inhibiting miR-374a expression

Through starBase website, RUNX2 was predicted as a potential target of miR-374a (Fig. 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 (Fig. 4B). In addition, silencing of miR-374a increased the expression of miR-374a (Fig. 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 revealed that knockdown of LINC00899 decreased the expression of RUNX2, while the inhibition of miR-374 reversed the effect (Fig. 4D). Moreover, miR-374a inhibitor partially abolished the inhibitory effect of LINC00899 knockdown on the expression of OPN and OCN(Fig. 4E and F). 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[16]. Shen et al reported that lncRNA HOTAIR was highly expressed in osteoporosis patients, which prevented osteogenic differentiation by regulating Wnt/β-catenin pathway[17]. Regarding LINC00899, Zhou et al reported that LINC00899 suppressed breast cancer progression by regulating miR-425 expression [18]. Dong et al revealed that LINC00899 facilitated the progression of acute myeloid leukaemia [19]. 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, 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 KCNQ1OT1 promoted osteogenic differentiation through absorbing miR-374a to upregulate BMP2 [20]. Zhang et al identified NEAT1 promoted BMP1 expression to regulate osteogenic differentiation of hBMSCs by sponging miR-29b-3p [21]. In addition, miRNAs have been reported to be involved in the occurrence of bone metabolic diseases [22]. For instance, Fan et al identified that miR-532-3p attenuated osteogenic differentiation by downregulating ETS1 [23]. Gan et al reported that miR-19b-3p promoted osteogenic differentiation of BMSCs [24]. 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 [25]. Therefore, the regulatory mechanism of RUNX2 during osteogenic differentiation has attracted the attention of many researchers. Fu at al indicated that HOTAIRM1 promoted osteogenesis by modulating JNK and c-Jun activity to activate RUNX2 gene transcription [26]. Chen et al reported that lncRNA AWPPH contributed to non-traumatic osteonecrosis through upregulating RUNX2 [27]. 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. 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

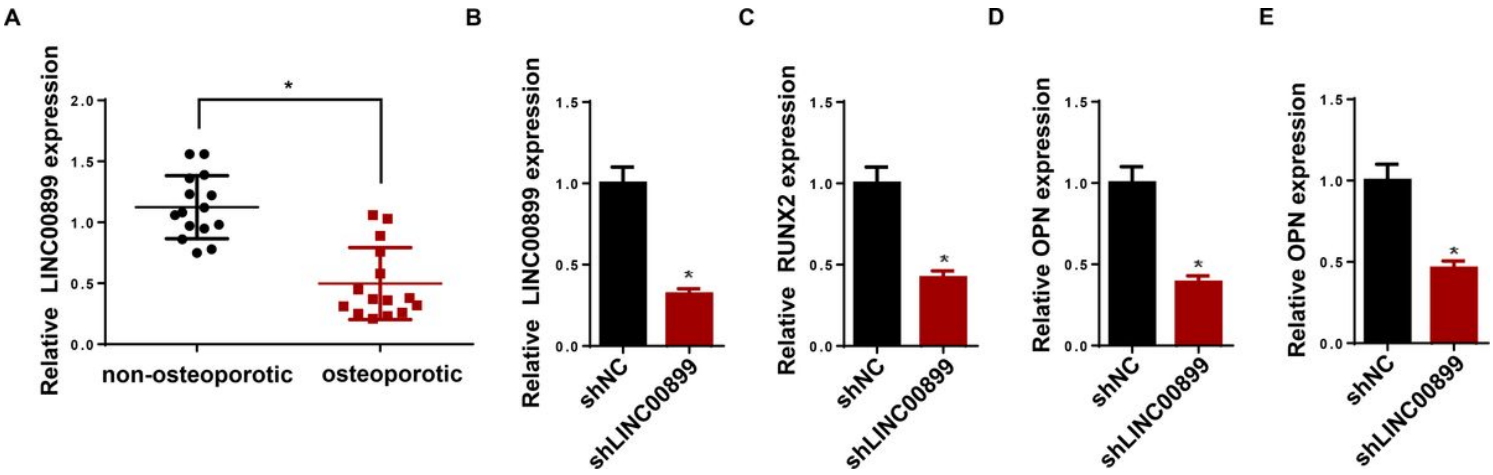

Figure 1

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. The data were presented as mean ± SD (*P < 0.05).

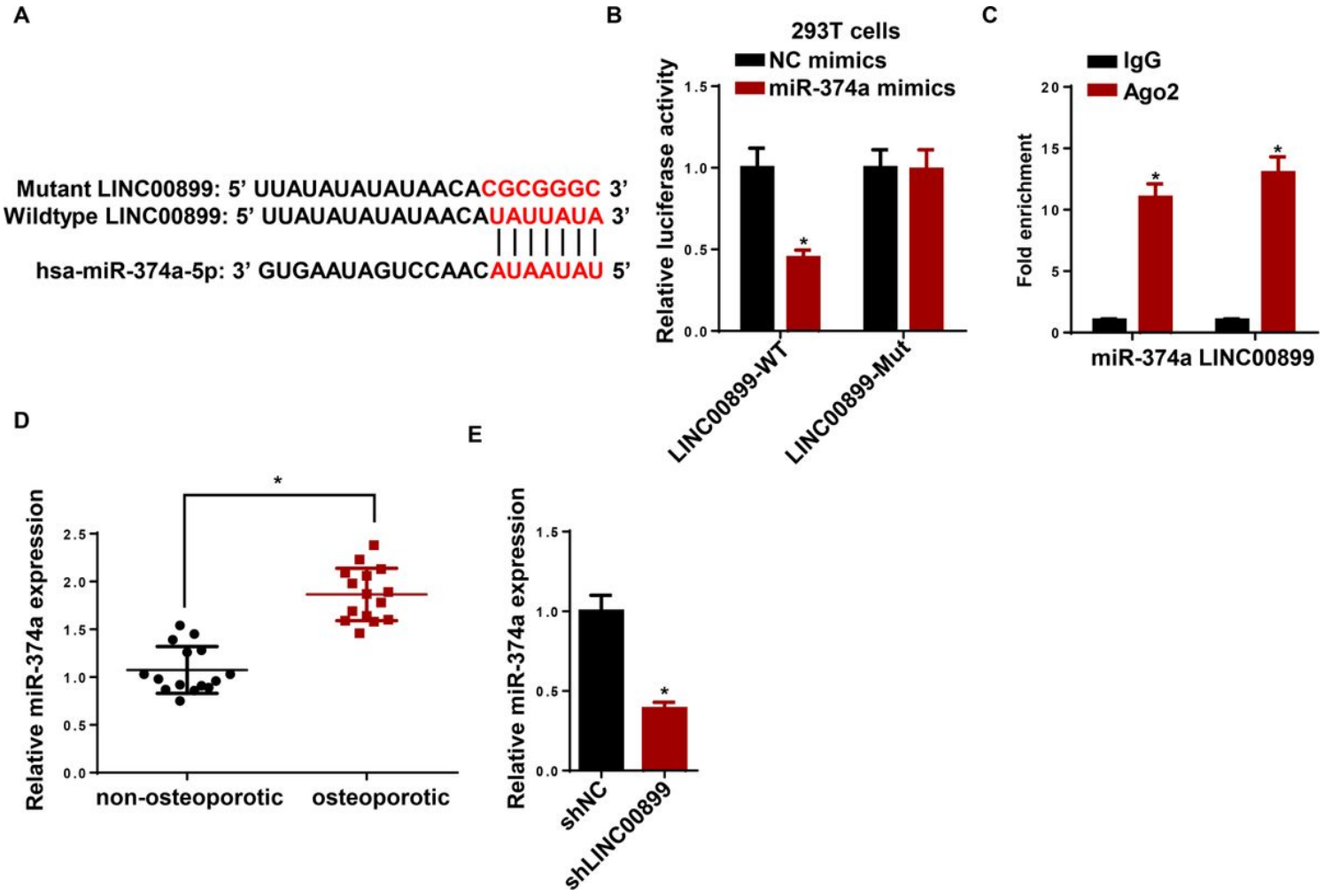

Figure 2

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 and shLINC00899 was detected by RT-qPCR. The data were presented as mean ± SD (*P < 0.05).

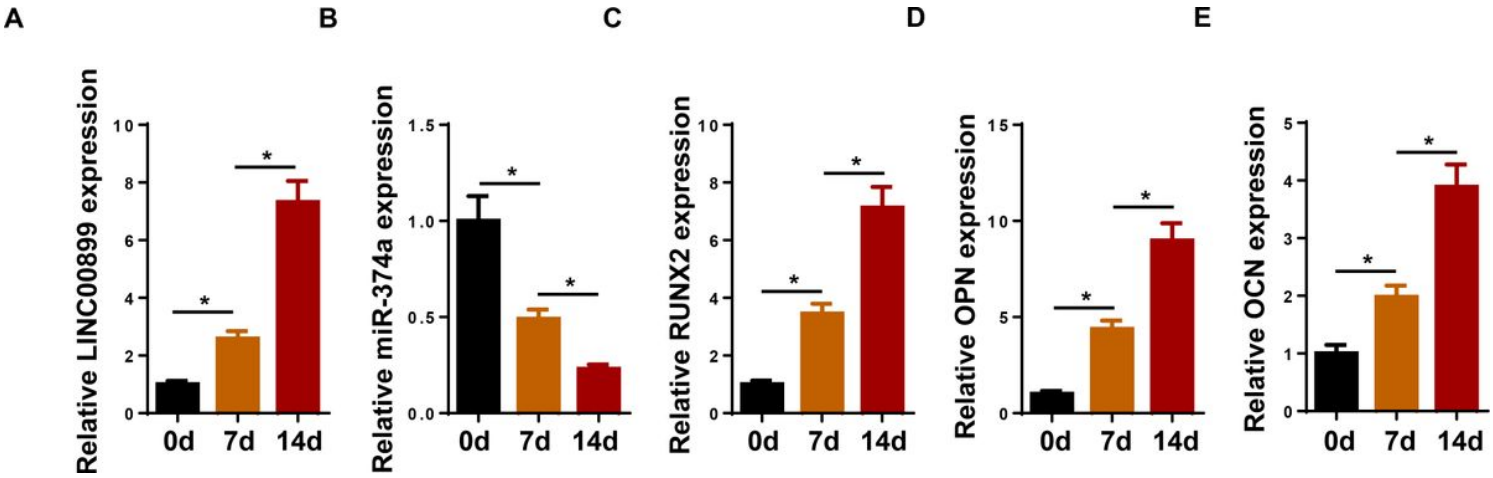

Figure 3

The expression levels of LINC00899 and miR-374a during osteogenic differentiation (A-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-E) RT-qPCR was used to detect the expression levels of RUNX2, OPN and OCN during osteoblast differentiation of hBMSCs. The data were presented as mean ± SD (*P < 0.05).

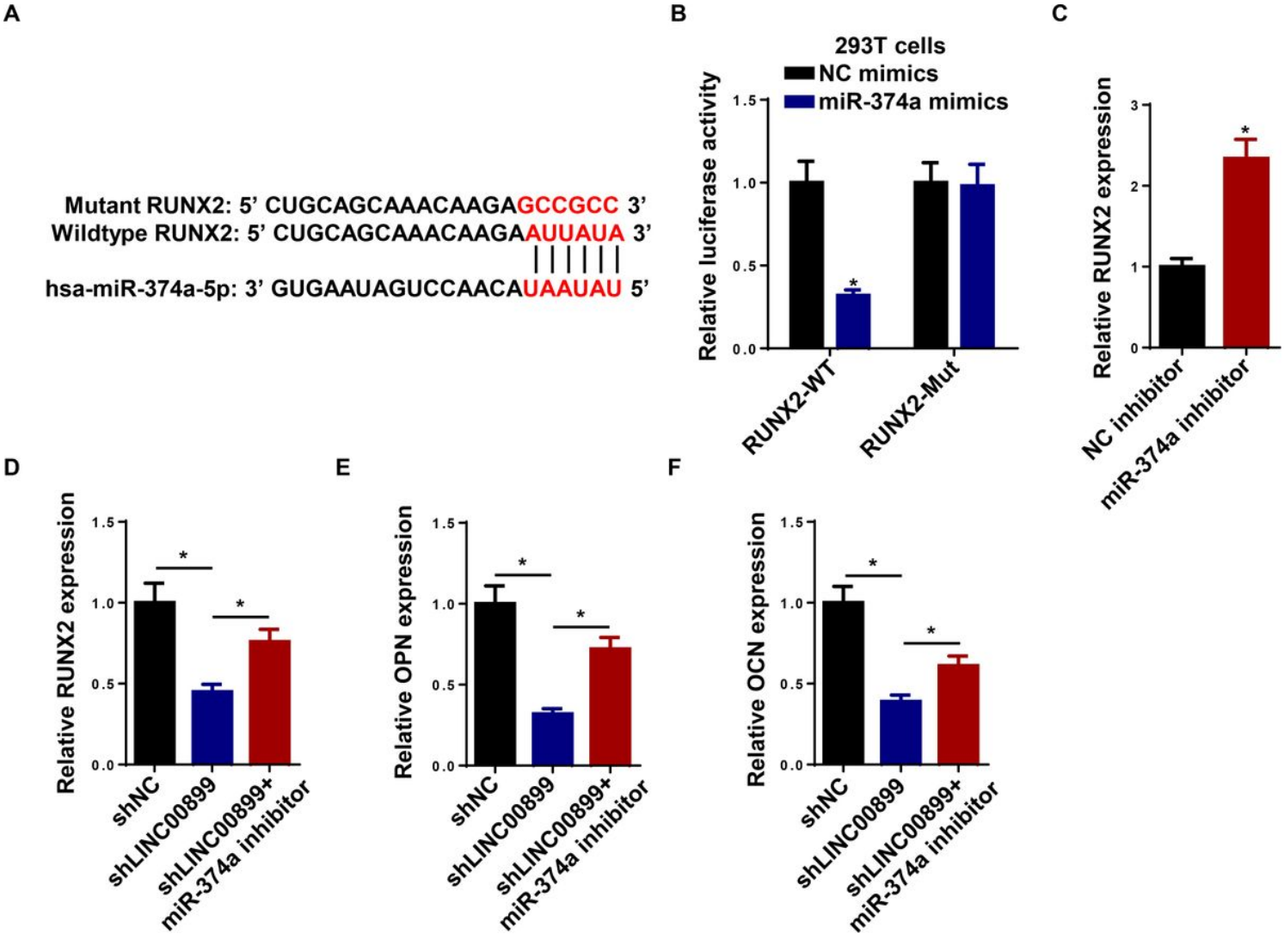

Figure 4

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-F) The expressions of RUNX2, OPN and OCN were determined in hBMSCs transfected with shNC, shLINC00899, shLINC00899+miR-374a inhibitor by RT-qPCR. The data were presented as mean ± SD (*P < 0.05).