

Downregulation of lncRNA ANRIL Inhibits Osteogenic Differentiation of Periodontal Ligament Cells via Sponging miR-7 through NF-κB Pathway

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Research Article

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Abstract

Background Long non-coding RNAs (lncRNAs) are dysregulated in periodontitis development and involved in osteogenesis. The current study aimed was to investigate the function of lncRNA ANRIL in periodontal ligament cells (PDLCs) and potential molecular mechanisms.

Methods Firstly, the level of ANRIL was tested by qPCR. Then PDLCs were treated with a mineralizing solution to induce the osteogenic differentiation. ALP activity was measured and protein levels of BMP2, Osterix, and OCN were measured by western blot. A target of ANRIL was verified using dual-luciferase reporter assay. MiR-7 level was measured by qPCR and the signalings of NF- κ B pathway were tested by western blot.

Results ANRIL expression was downregulated in PDL tissues. Next, ALP activity and protein levels of BMP2, Osterix, and OCN were reduced to show PDLCs were differentiated. ANRIL level was increased in differential PDLCs, and which knockdown inhibited osteogenic differentiation. Then, miR-7 was found as a target of ANRIL. The miR-7 level was upregulated in PDL tissues and reduced in differential PDLCs. Inhibition of miR-7 suppressed ALP activity and BMP2, Osterix, and OCN expression. Moreover, inhibition of miR-7 reversed the effects on the osteogenic differentiation induced by knockdown of ANRIL. Besides, the levels of p-P65 and p-I κ B α were elevated by ANRIL downregulation and were rescued by suppressing miR-7.

Conclusions Knockdown of ANRIL inhibited osteogenic differentiation via sponging miR-7 through the NF- κ B pathway, suggesting that ANRIL might be a therapeutic target for periodontitis.

Background

Periodontitis is a chronic, nonspecific, multifactorial inflammatory disease associated with periodontal support tissue. It will cause pathological loss of the periodontal ligament and alveolar bone, leading to teeth loss ultimately [1]. Diabetes, obesity, and aging are associated with the pathogenic factors of periodontitis [2–4]. Periodontitis may be a potential risk factor for other human diseases like Alzheimer's disease, stroke, which affect human's systemic health [5, 6]. The periodontal ligament (PDL), made up of collagen fiber bundles and cells, has many functions, including tooth-supporting, tooth nutrition, alveolar bone remodeling, damage tissue repairing [7]. Periodontal ligament cells (PDLCs) play essential roles in maintaining homeostasis of periodontal tissue and repairing periodontal ligament. Unfortunately, periodontitis injures the osteogenic differentiation of PDLCs [8]. The treatment of periodontitis is complex, and there is still a lack of early screening biomarkers and therapeutic targets.

Long non-coding RNAs (lncRNAs) are a class of more than 200 nt, non-coding transcripts. Recently, with the gradual deepening of lncRNA's biological and functional roles of lncRNA, we found lncRNA acts as miRNAs sponges and then regulate mRNA effects [9]. An increasing body of evidence has suggested that lncRNAs are involved in human diseases, such as cardiovascular diseases [10], malignant tumors [11], and inflammation-related diseases [12]. As important inflammatory regulators, lncRNAs are often

abnormally expressed in the progression of periodontitis [13] and associated with osteogenesis (14). An antisense lncRNA, antisense non-coding RNA in the INK4 locus (ANRIL), is located at the CDKN2A/B genomic locus, consisting of at least 21 exons and a large number of reported isoforms [15]. ANRIL is reported as an immune response-related lncRNA, which expression is reduced in peripheral blood of patients with periodontitis [16]. However, biological functions are still largely unknown.

In the present study, the effects of ANRIL on the osteogenic differentiation of PDCLs were explored. Moreover, ANRIL was found to be sponging to miR-7. Notably, the mechanism of ANRIL sponged to miR-7 to regulate the osteogenic differentiation of PDLCs was investigated.

Methods

PDL tissues collection

A total of 30 patients with periodontitis and 30 healthy control participated in the study. This study protocol was approved by the Ethics Committee of Beihua University Affiliated Hospital. Written informed consent was provided prior to the study. All participants were diagnosed with periodontitis or not by Beihua University Affiliated Hospital. None of them had infectious diseases, a history of smoking, and orthognathic surgery. At routine premolar or third molar extractions, the PDL tissues were separated from the middle 1/3 of the dental roots. Partial tissues were stored at -80°C until further use. All methods in the study were carried out in accordance with the Helsinki guidelines and declaration.

Cell culture and osteogenic induction

Other tissues were cut into 1 mm³ pieces and digested by 3mg/ml of collagenase type I (Sigma-Aldrich, USA) and 4 mg/ml of dispase (Corning, USA) at 37°C. The cell suspension was maintained in DMEM (Hyclone, USA) supplemented with 10% FBS (Solarbio, China) and 1% penicillin/streptomycin (Solarbio, China) at 37°C with 5% CO₂. The medium was changed every 2-3 days until cell passage to the fifth generation. For osteogenic induction, PDLCs were seeded into 6-well plates until the confluence researched exceed 70%. DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma-Aldrich, USA), 50 µg/ml vitamin C (Aladdin, China), and 10 nM dexamethasone (Aladdin, China) was used as an osteogenic-induced medium. Two weeks post-incubation, PDLCs were harvested for testing osteogenic differentiation.

Alkaline phosphatase (ALP) activity analysis

ALP activity was assessed by the ALP Assay Kit (Beyotime, China). Briefly, PDLCs were lysed by lysis buffer and seeded into 96-well plates. The test buffer was added and incubated with cells at 37°C for 10 min. After stopping the reaction, the absorbance was measured at 405nm.

Dual-luciferase reporter assay.

The sequences of ANRIL containing the miR-7 potential binding sites were amplified and inserted into pGL3 vectors (Promega, USA) as ANRIL-WT group. The ANRIL-MUT group was obtained by targeted mutation. MiR-7 mimic and mimic negative control (NC) were purchased from GenePharma (Shanghai, China). HEK-293T cells were seeded into 24-well plates, and co-transfected with ANRIL-WT or ANRIL-MUT and miR-7 mimic or mimic NC using Lipofectamine 2000 (Invitrogen, USA). After 24 h, the relative luciferase activity (firefly activity normalized to Renilla activity) was measured by Dual-luciferase Reporter Assay Kit (Promega, USA).

Cell transfection

shRNA-NC, shRNA-ANRIL, miR-7 inhibitor, and inhibitor-NC were all acquired from GenePharma (Shanghai, China). PDLCs in the logarithmic growth phase were seeded into 6-well plates and transfection process was used Lipofectamine 2000 (Invitrogen, USA). After 48 h, transfection efficiency was detected.

qPCR

Total RNA was isolated from PDLCs by TRIzol reagent (Sigma-Aldrich, USA). After concentration and purity testing, RNA was reverse transcribed into cDNA using INRcute IncRNA cDNA First Chain Synthetic Kit (Tiangen, China), and miRNA reverse transcription was conducted by miScript II RT Kit (Qiagen, Germany). INRcute IncRNA qPCR Detection Kit (SYBR Green) (Tiangen, China) was performed for qPCR of IncRNA with the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 sec and 60°C for 15 sec. qPCR was used to measure miR-7 level by MicroRNAs qPCR Kit (SYBR Green Method) (Sango, China) with the conditions as 95°C for 30 sec, 95°C for 5 sec and 60°C for 30 sec (40 cycles). The level of mRNA was detected by Real Time One Step RT-qPCR (Tiangen, China) for reverse transcription and qPCR, and the conditions were: 50°C for 30 min, 95°C for 3 min, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The reaction instrument was ABI PRISM 7500 system (Applied Biosystems, USA). β -actin level was performed as the loading control. The results of relative expression were assessed by the $2^{-\Delta\Delta Ct}$.

Western blot

The transfected cells were collected, and pre-cooled RIPA lysate (Beyotime, China) was added to extract the total protein. After 10% SDS-PAGE, the protein was transferred to PVDF membranes (Millipore, USA) and blocked with 5% skim milk. Primary antibodies including anti-BMP2, anti-Osterix, anti-osteocalcin (OCN), anti-P65, anti-p-P65, anti-I κ B α , and anti-p-I κ B α were added and incubated with the membranes at 4°C overnight. After washing the membranes, the secondary antibody was added to incubate at room

temperature for 1 h. The protein bands were developed by ECL Western Blotting Substrate (Pierce, USA) and then photographed. The gray analysis was performed by Image J software 1.48U (Bethesda, USA).

Statistical analysis

The results in this study were analyzed by GraphPad Prism 6.0 (GraphPad Software, USA) and presented as mean \pm standard deviation (SD). Student's t-test was used for multiple comparisons between two groups, and one-way ANOVA was used between three or more groups. P<0.05 was deemed significant differences.

Results

The level of ANRIL was downregulated in periodontitis

Firstly, the PDL tissues were obtained from periodontitis patients and healthy people, and the expression of ANRIL was measured. According to the results of qPCR, ANRIL level was reduced in PDL tissues of patients with periodontitis, compared with the healthy control group (Fig. 1).

Identification of the osteogenic differentiation of PDLCs

To investigate the osteogenic differentiation capability, PDLCs derived from PDL tissues were cultured in DMEM with β -glycerophosphate, vitamin C, and dexamethasone. Subsequently, some of the specific markers associated with osteogenesis were evaluated. As illustrated in Fig. 2A, ALP activity was elevated in osteogenic differentiatational PDLCs. The protein levels of BMP2, Osterix, and OCN were all upregulated in PDLCs after treatment (Fig. 2B-E). Moreover, the expression of ANRIL was downregulated in differentiated PDLCs, compared with undifferentiated PDLCs (Fig. 2F).

Knockdown of ANRIL inhibited osteogenic differentiation of PDLCs

To explore the biological functions of ANRIL in PDLCs, inhibition of ANRIL expression was conducted by transfection of shRNA-ANRIL-1 and shRNA-ANRIL-2. To examine the transfection efficiency, qPCR was conducted. Compared with shRNA-NC group, the level of ANRIL was downregulated in shRNA-ANRIL-1 and shRNA-ANRIL-2 groups, especially in shRNA-ANRIL-2 group (Fig. 3A). To evaluate the effects of ANRIL on the osteogenic differentiation, ALP activity was measured. As shown in Fig. 3B, shRNA-ANRIL-2 suppressed the ALP activity, compared with shRNA-NC (Fig. 3B). Additionally, the protein expression of BMP2, Osterix, OCN was tested by western blot, and the results demonstrated that knockdown of ANRIL repressed BMP2, Osterix, OCN levels (Fig. 3C-F).

MiR-7 was identified as a target of ANRIL

The binding sites between ANRIL and miR-7 were shown in Fig. 4A. To verify the targeted relationship, a dual-luciferase reporter assay was conducted. The results demonstrated that the relative luciferase activity was decreased in HEK293T cells co-transfected with ANRIL-WT and miR-7 mimic, compared with co-transfection of ANRIL-WT and mimic NC. However, in ANRIL -MUT group, there was no difference between miR-7 mimic and mimic NC (Fig. 4B).

The expression of miR-7 was evaluated in PDL tissues and PDLCs

Subsequently, the expression of miR-7 was measured. As compared to healthy control tissues, the miR-7 level was upregulated in PDL tissues (Fig. 5A). Additionally, miR-7 level was decreased in differentiated PDLCs, compared with undifferentiated PDLCs (Fig. 5B). After knockdown of ANRIL, the expression of miR-7 was elevated, compared with the shRNA-NC group (Fig. 5C).

Downregulation of miR-7 promoted osteogenic differentiation of PDLCs

For downregulation of miR-7, miR-7 inhibitor and inhibitor-NC were transfected into differentiated PDLCs. The data of transfection efficiency illustrated that miR-7 level was reduced in the miR-7 inhibitor group, compared with the inhibitor-NC group (Fig. 6A). Then, inhibition of miR-7 enhanced ALP activity and BMP2, Osterix, OCN levels, compared with inhibitor-NC (Fig. 6B-F).

Knockdown of ANRIL inhibited osteogenic differentiation through sponging miR-7

ALP activity was declined in shRNA-ANRIL-2 group, which was not affected by inhibitor-NC but further abolished by miR-7 inhibitor (Fig. 7A). Through the results of western blot, knockdown of ANRIL inhibited BMP2, Osterix, and OCN levels. However, their levels were reversed by miR-7 downregulated (Fig. 7B-E).

Knockdown of ANRIL regulated NF-κB pathway by mediating miR-7

The protein expression of p-P65, P65, p-I κ B α , and I κ B α was measured by western blot. The data demonstrated that knockdown of ANRIL enhanced the levels of p-P65 and p-I κ B α , which were rescued by inhibition of miR-7. However, both ANRIL and miR-7 did not affect P65 and I κ B α levels (Fig. 8A and B).

Discussion

In the present study, we aimed to explore the biological functions of lncRNA ANRIL in the osteogenic differentiation in PDLCs. We found the level of ANRIL was downregulated in PDL tissues and upregulated in differential PDLCs. Knockdown of ANRIL inhibited ALP activity and BMP2, Osterix, OCN levels, suggesting that downregulation of ANRIL inhibited osteogenic differentiation of PDLCs.

Recently, lots of researches revealed the role of ANRIL. Polymorphisms at the ANRIL gene are associated with the risks of many human diseases, including malignancy, cardiovascular disease, bone mass, obesity, and type 2 diabetes [15]. Generally, ANRIL is a prognostic biomarker and an oncomiR in human cancers, such as lung cancer, gastric cancer, esophageal squamous cell carcinoma [17]. In addition, dysregulation of ANRIL promotes the development of atherosclerosis and leads to coronary heart disease through mediating single nucleotide polymorphisms and injuring endothelial cell [18, 19]. Furthermore, ANRIL mediates VEGF to effects on diabetic retinopathy [20]. Several lncRNAs have been reported to be involved in osteogenic differentiation, such as PCAT1 [21], MEG3 [22, 23], TWIST1 [24], MSC-AS1 [25]. However, the roles of ANRIL in osteogenic differentiation, especially in PDLCs, are still unknown. The results of this study indicated that ANRIL downregulation had inhibited effects on the osteogenic differentiation of PDLCs.

Besides, the molecular mechanism of the differentiation induced by ANRIL was further investigated. miR-7 was regarded as a sponge of ANRIL. Zhao et al. have suggested that miR-7 abolishes the attenuation of oxidative injury of human trabecular meshwork cells induced by ANRIL [26]. Shu et al. have reported that silence of ANRIL exacerbates H9c2 cell injury induced by hypoxia by miR-7-5p/SIRT1 axis [27]. Li et al. have revealed that ANRIL mediates the migration and invasion of T-cell acute lymphoblastic leukemia via miR-7-5p/TCF4 axis [28]. In our study, we also verified that ANRIL sponge to miR-7 through dual-luciferase reporter assay.

Accumulating evidence shows that miR-7 is involved in human diseases. For example, miR-7 may function as a tumor suppressor and regulates cellular processes, including growth, metastasis, differentiation, and apoptosis [29]. Besides cancers, abnormal expression of miR-7 is a potential biomarker for type 2 diabetes, acute pancreatitis, and Alzheimer's Disease [30–32]. Moreover, miR-7 plays functional roles in osteoarthritis through regulating proliferation, apoptosis, and inflammation [33]. In periodontitis, miR-7 level was reduced during osteogenic differentiation, mediated by circular RNA ADR1as to repress osteoblastic differentiation of PDLCs [34]. In the present study, the results demonstrated that the expression of miR-7 was increased in PDL tissues and reduced in differential PDLCs. Downregulation of miR-7 promoted osteogenic differentiation of PDLCs. Moreover, knockdown of ANRIL inhibited osteogenic differentiation via sponging miR-7.

Non-canonical NF-κB pathway related to immune deficiencies and abnormal activation of the pathway leads to the pathogenesis of a variety of autoimmune and inflammatory diseases [35]. Moreover, NF-κB pathway is also involved in osteogenic differentiation [36, 37]. There are several studies have revealed that ANRIL and miR-7 mediate NF-κB signaling. Overexpression of ANRIL facilitates angiogenesis of

diabetes mellitus via activation of NF-κB pathway [38]. MiR-7 suppresses pancreatic cancer progression through inactivating NF-κB pathway [39]. In the present study, we found knockdown of ANRIL enhanced the levels of p-P65 and p-IκBa, which were rescued by inhibition of miR-7. These results suggested that downregulation of ANRIL activating inhibited osteogenic differentiation of PDLCs through NF-κB pathway by sponging miR-7.

Conclusions

The expression of ANRIL was decrease in PDL tissues, with miR-7 level was increased. MiR-7 was identified as a sponge of ANRIL. The levels of ANRIL and miR-7 were elevated and reduced in differential PDLCs, respectively. Importantly, knockdown of ANRIL inhibited osteogenic differentiation by sponging miR-7 through activating NF-κB signaling pathway, suggesting that ANRIL will be contributed to periodontitis.

Abbreviations

ALP: alkaline phosphatase; DMEM: dulbecco's modified eagle medium; lncRNA: long non-coding RNA; miRNA: microRNA; PDL: periodontal ligament; mut: mutant; NC: negative control; PDLCs: periodontal ligament cells; qPCR: quantitative polymerase chain reaction; WT: wild type.

Declarations

Ethics approval and consent to participate

This study protocol was approved by the Ethics Committee of Beihua University Affiliated Hospital. Written informed consent was provided prior to the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and analysed during the current study are not available due to hospital policy but are available from the corresponding author on reasonable request.

Competing interest

The authors declare no conflict of interest.

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Authors' contribution

XL and YZ designed the work and collected the data. XL did the experiments and conducted statistical analysis. XL contributed to writing the manuscript. Review and editing the manuscript was done by YZ. The authors have read the approved the final manuscript.

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Not applicable.

References

1. Fischer RG, Lira Junior R, Retamal-Valdes B, et al. **Periodontal disease and its impact on general health in Latin America. Section V: Treatment of periodontitis.** Braz Oral Res. 2020;34(supp1 1):e026.
2. Genco RJ, Borgnakke WS. **Diabetes as a potential risk for periodontitis: association studies.** Periodontol 2000. 2020;83(1):40-5.
3. Khan S, Barrington G, Bettoli S, Barnett T, Crocombe L. **Is overweight/obesity a risk factor for periodontitis in young adults and adolescents?: a systematic review.** Obes Rev. 2018;19(6):852-83.
4. Ebersole JL, Graves CL, Gonzalez OA, et al. **Aging, inflammation, immunity and periodontal disease.** Periodontol 2000. 2016;72(1):54-75.
5. Cerajewska TL, Davies M, West NX. **Periodontitis: a potential risk factor for Alzheimer's disease.** Br Dent J. 2015;218(1):29-34.
6. Fagundes NCF, Almeida APCPSC, Vilhena KFB, Magno MB, Maia LC, Lima RR. **Periodontitis As A Risk Factor For Stroke: A Systematic Review And Meta-Analysis.** Vasc Health Risk Manag. 2019;15:519-32.
7. Hirashima S, Kanazawa T, Ohta K, Nakamura KI. **Three-dimensional ultrastructural imaging and quantitative analysis of the periodontal ligament.** Anat Sci Int. 2020;95(1):1-11.
8. Zhu Y, Ai R, Ding Z, et al. **LncRNA-01126 inhibits the migration of human periodontal ligament cells through MEK/ERK signaling pathway.** J Periodontal Res. 2020;55(5):631-41.
9. Paraskevopoulou MD, Hatzigeorgiou AG. **Analyzing MiRNA-LncRNA Interactions.** Methods Mol Biol. 2016;1402:271-86.
10. Huang Y. **The novel regulatory role of lncRNA-miRNA-mRNA axis in cardiovascular diseases.** J Cell Mol Med. 2018;22(12):5768-75.

11. Bhan A, Soleimani M, Mandal SS. **Long Noncoding RNA and Cancer: A New Paradigm.** *Cancer Res.* 2017;77(15):3965-81.
12. Liao K, Xu J, Yang W, You X, Zhong Q, Wang X. **The research progress of LncRNA involved in the regulation of inflammatory diseases.** *Mol Immunol.* 2018;101:182-8.
13. Sánchez-Muñoz F, Martínez-Coronilla G, Leija-Montoya AG, et al. **Periodontitis may modulate long-non coding RNA expression.** *Arch Oral Biol.* 2018;95:95-9.
14. Ju C, Liu R, Zhang YW, et al. **Mesenchymal stem cell-associated lncRNA in osteogenic differentiation.** *Biomed Pharmacother.* 2019;115:108912.
15. Kong Y, Hsieh CH, Alonso LC. **ANRIL: A lncRNA at the CDKN2A/B Locus With Roles in Cancer and Metabolic Disease.** *Front Endocrinol (Lausanne).* 2018;9:405.
16. Gholami L, Ghafouri-Fard S, Mirzajani S, et al. **The lncRNA ANRIL is down-regulated in peripheral blood of patients with periodontitis.** *Noncoding RNA Res.* 2020;5(2):60-6.
17. Li Z, Yu X, Shen J. **ANRIL: a pivotal tumor suppressor long non-coding RNA in human cancers.** *Tumour Biol.* 2016;37(5):5657-61.
18. Chen L, Qu H, Guo M, et al. **ANRIL and atherosclerosis.** *J Clin Pharm Ther.* 2020;45(2):240-8.
19. Chi JS, Li JZ, Jia JJ, Zhang T, Liu XM, Yi L. **Long non-coding RNA ANRIL in gene regulation and its duality in atherosclerosis.** *J Huazhong Univ Sci Technolog Med Sci.* 2017;37(6):816-22.
20. Thomas AA, Feng B, Chakrabarti S. **ANRIL: A Regulator of VEGF in Diabetic Retinopathy.** *Invest Ophthalmol Vis Sci.* 2017;58(1):470-80.
21. Yu L, Qu H, Yu Y, Li W, Zhao Y, Qiu G. **LncRNA-PCAT1 targeting miR-145-5p promotes TLR4-associated osteogenic differentiation of adipose-derived stem cells.** *J Cell Mol Med.* 2018;22(12):6134-47.
22. Wang Q, Li Y, Zhang Y, et al. **LncRNA MEG3 inhibited osteogenic differentiation of bone marrow mesenchymal stem cells from postmenopausal osteoporosis by targeting miR-133a-3p.** *Biomed Pharmacother.* 2017;89:1178-86.
23. Liu Y, Zeng X, Miao J, et al. **Upregulation of long noncoding RNA MEG3 inhibits the osteogenic differentiation of periodontal ligament cells.** *J Cell Physiol.* 2019;234(4):4617-26.
24. Xu Y, Qin W, Guo D, et al. **LncRNA-TWIST1 Promoted Osteogenic Differentiation Both in PPDLSCs and in HPDLSCs by Inhibiting TWIST1 Expression.** *Biomed Res Int.* 2019 Jun 23;2019:8735952.
25. Zhang N, Hu X, He S, Liu J, Zhang M, Jin Z. **LncRNA MSC-AS1 promotes osteogenic differentiation and alleviates osteoporosis through sponging microRNA-140-5p to upregulate BMP2.** *Biochem Biophys Res Commun.* 2019;519(4):790-6.
26. Zhao J, Sun H, Zhang JM, et al. **Long non-coding RNA ANRIL down-regulates microRNA-7 to protect human trabecular meshwork cells in an experimental model for glaucoma.** *Eur Rev Med Pharmacol Sci.* 2019;23(8):3173-82.
27. Shu L, Zhang W, Huang C, Huang G, Su G, Xu J. **lncRNA ANRIL protects H9c2 cells against hypoxia-induced injury through targeting the miR-7-5p/SIRT1 axis.** *J Cell Physiol.* 2020;235(2):1175-83.

28. Li G, Gao L, Zhao J, Liu D, Li H, Hu M. **LncRNA ANRIL/miR-7-5p/TCF4 axis contributes to the progression of T cell acute lymphoblastic leukemia.** Cancer Cell Int. 2020;20:335.
29. Li M, Pan M, You C, Dou J. The Therapeutic Potential of miR-7 in Cancers. Mini Rev Med Chem. 2019;19(20):1707-16.
30. Wan S, Wang J, Wang J, et al. **Increased serum miR-7 is a promising biomarker for type 2 diabetes mellitus and its microvascular complications.** Diabetes Res Clin Pract. 2017;130:171-9.
31. Lu P, Wang F, Wu J, et al. **Elevated Serum miR-7, miR-9, miR-122, and miR-141 Are Noninvasive Biomarkers of Acute Pancreatitis.** Dis Markers. 2017;2017:7293459.
32. Akhter R. Circular RNA and Alzheimer's Disease. Adv Exp Med Biol. 2018;1087:239-243.
33. Zhou X, Jiang L, Fan G, et al. Role of the ciRS-7/miR-7 axis in the regulation of proliferation, apoptosis and inflammation of chondrocytes induced by IL-1 β . Int Immunopharmacol. 2019;71:233-40.
34. Li X, Zheng Y, Zheng Y, et al. Circular RNA CDR1as regulates osteoblastic differentiation of periodontal ligament stem cells via the miR-7/GDF5/SMAD and p38 MAPK signaling pathway. Stem Cell Res Ther. 2018;9(1):232.
35. Sun SC. The non-canonical NF-kappaB pathway in immunity and inflammation. Nat Rev Immunol. 2017;17(9):545-58.
36. Xue N, Qi L, Zhang G, Zhang Y. **miRNA-125b Regulates Osteogenic Differentiation of Periodontal Ligament Cells Through NKIRAS2/NF-kappaB Pathway.** Cell Physiol Biochem. 2018;48(4):1771-81.
37. Wang YJ, Zhang HQ, Han HL, Zou YY, Gao QL, Yang GT. **Taxifolin enhances osteogenic differentiation of human bone marrow mesenchymal stem cells partially via NF-kappaB pathway.** Biochem Biophys Res Commun. 2017;490(1):36-43.
38. Zhang B, Wang D, Ji TF, Shi L, Yu JL. Overexpression of lncRNA ANRIL up-regulates VEGF expression and promotes angiogenesis of diabetes mellitus combined with cerebral infarction by activating NF-kappaB signaling pathway in a rat model. Oncotarget. 2017;8(10):17347-59.
39. Xia J, Cao T, Ma C, et al. **miR-7 Suppresses Tumor Progression by Directly Targeting MAP3K9 in Pancreatic Cancer.** Mol Ther Nucleic Acids. 2018;13:121-32.

Figures

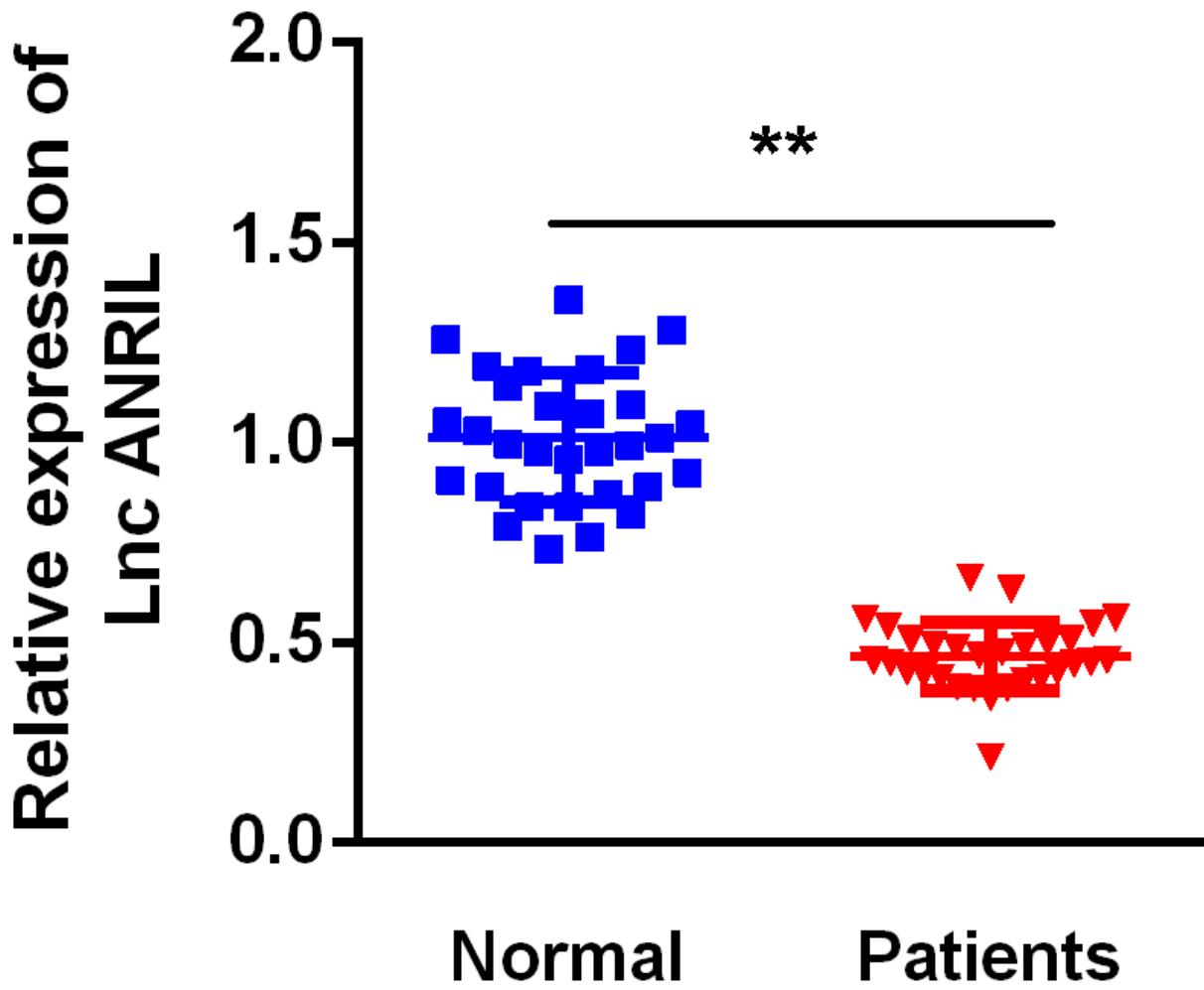


Figure 1

The expression of ANRIL was decreased in PDL tissues of periodontitis patients. PDL tissues from 30 patients with periodontitis and 30 healthy people, and then ANRIL level was detected by qPCR. **P<0.01.

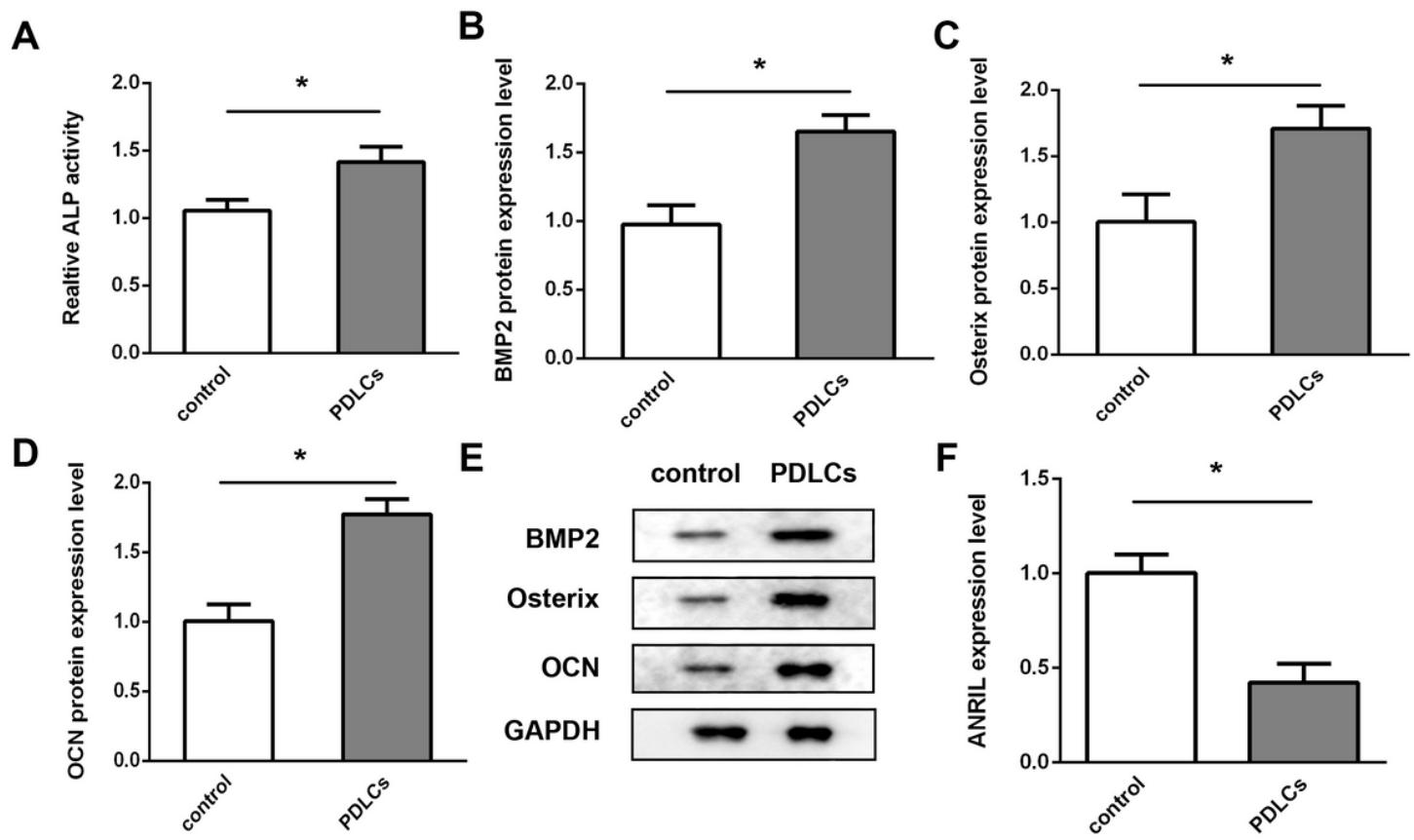


Figure 2

Identification of successfully establishing of the osteogenic differentiation model. (A) ALP activity was detected in undifferentiated and differentiated PDLCs. The relative protein expression normalized to GAPDH was calculated, including (B) BMP2, (C) Osterix, and (D) OCN. (E) The protein levels of osteogenic-related markers as BMP2, Osterix, and OCN were tested by western blot. GAPDH was performed as the housekeeping control. (F) The expression of ANRIL was measured in undifferentiated and differentiated PDLCs by qPCR. * $P<0.05$.

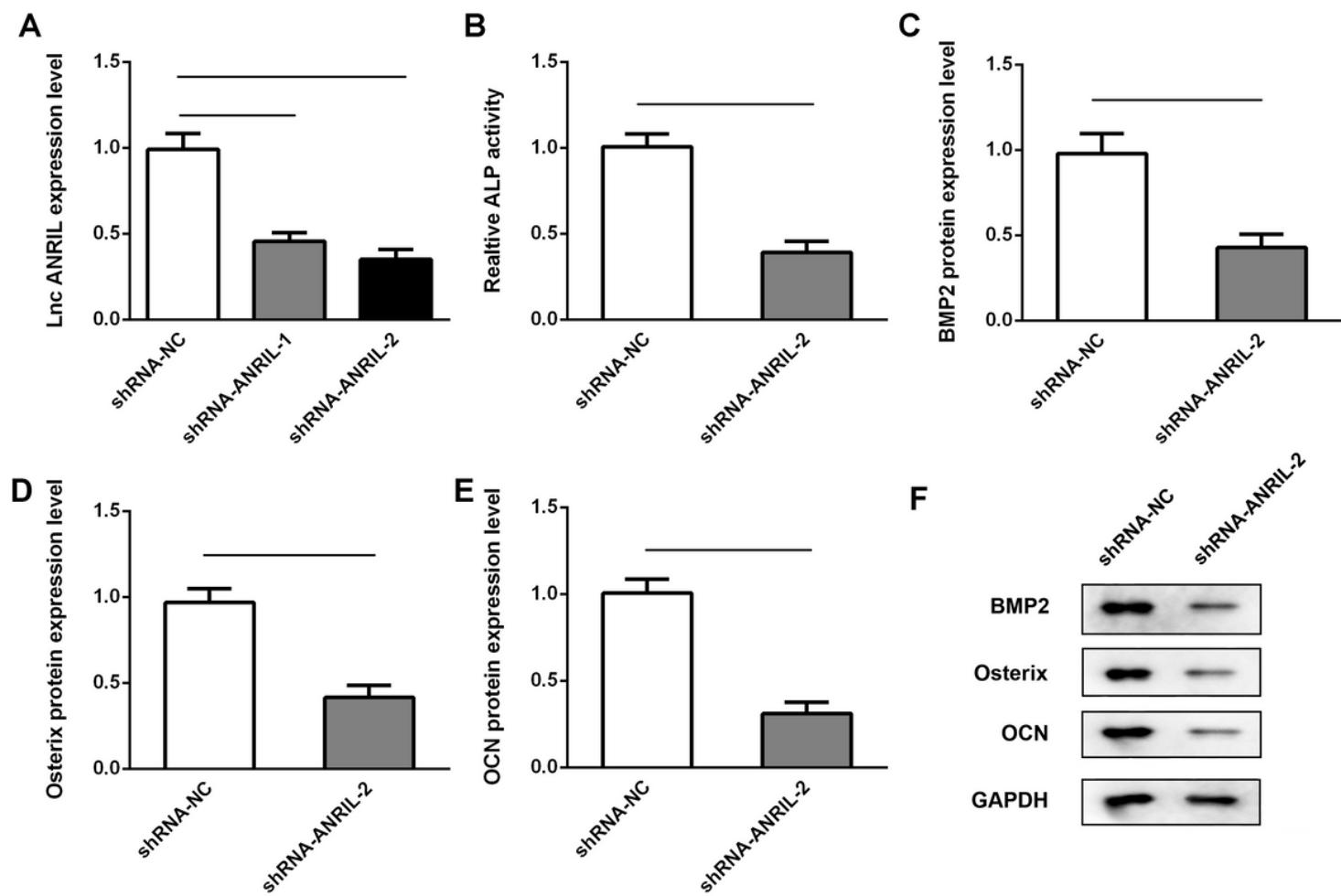


Figure 3

Inhibition of ANRIL suppressed osteogenic differentiation of PDLCs. (A) The transfection efficiency was measured by qPCR when PDLCs transfection of shRNA-ANRIL-1, shRNA-ANRIL-2, and shRNA-NC. (B) ALP activity was detected post-transfection. The protein levels of (C) BMP2, (D) Osterix, (E) OCN quantified, (F) which were detected by western blot post-transfection. GAPDH was used as an internal control. *P<0.05.

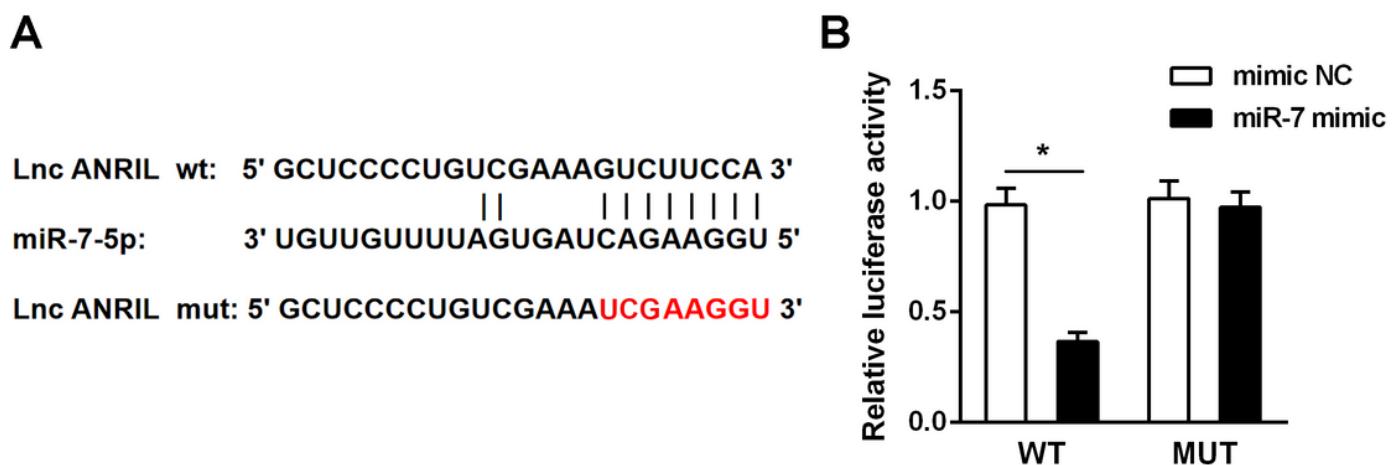
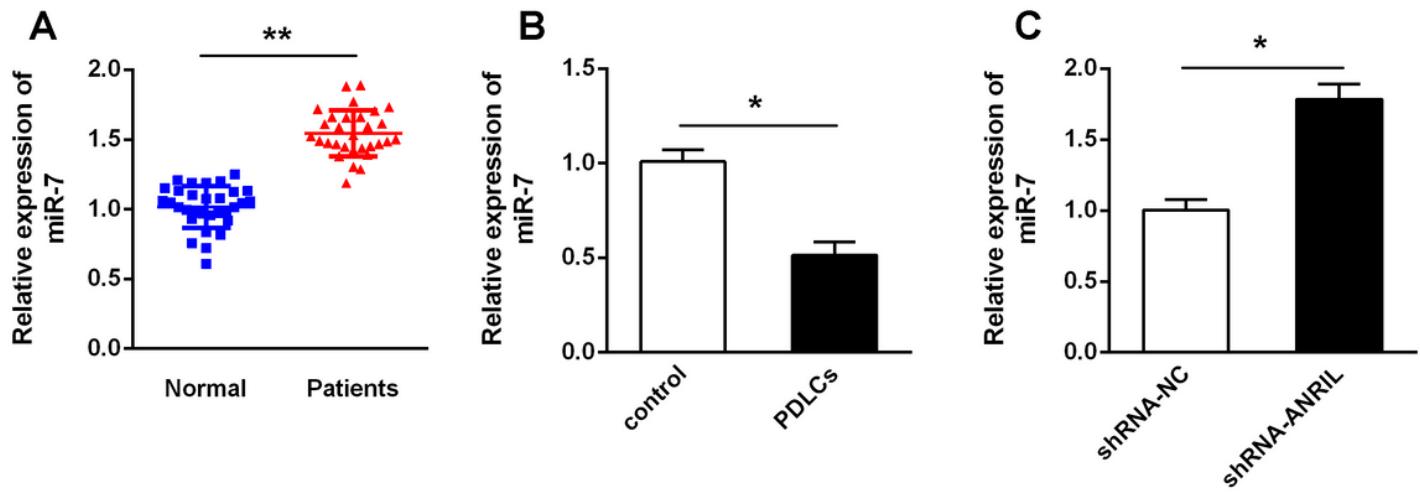


Figure 4

ANRIL sponged to miR-7. (A) The binding sites between ANRIL and miR-7 were shown. The mutant sequences of ANRIL were also shown. (B) HEK 293T cells were co-transfected with ANRIL-WT or ANRIL-MUT together with miR-7 mimic or mimic NC, and the relative luciferase activity was detected. *P<0.05.

**Figure 5**

MiR-7 expression in PDL tissues and PDLCs. (A) The level of miR-7 was tested in PDL tissues from periodontitis patients and healthy control by qPCR. (B) MiR-7 level was detected by qPCR in undifferentiated and differentiated PDLCs. (C) The expression of miR-7 was measured by qPCR after PDLCs were transfection of shRNA-NC and shRNA-ANRIL-2. *P<0.05. **P<0.01.

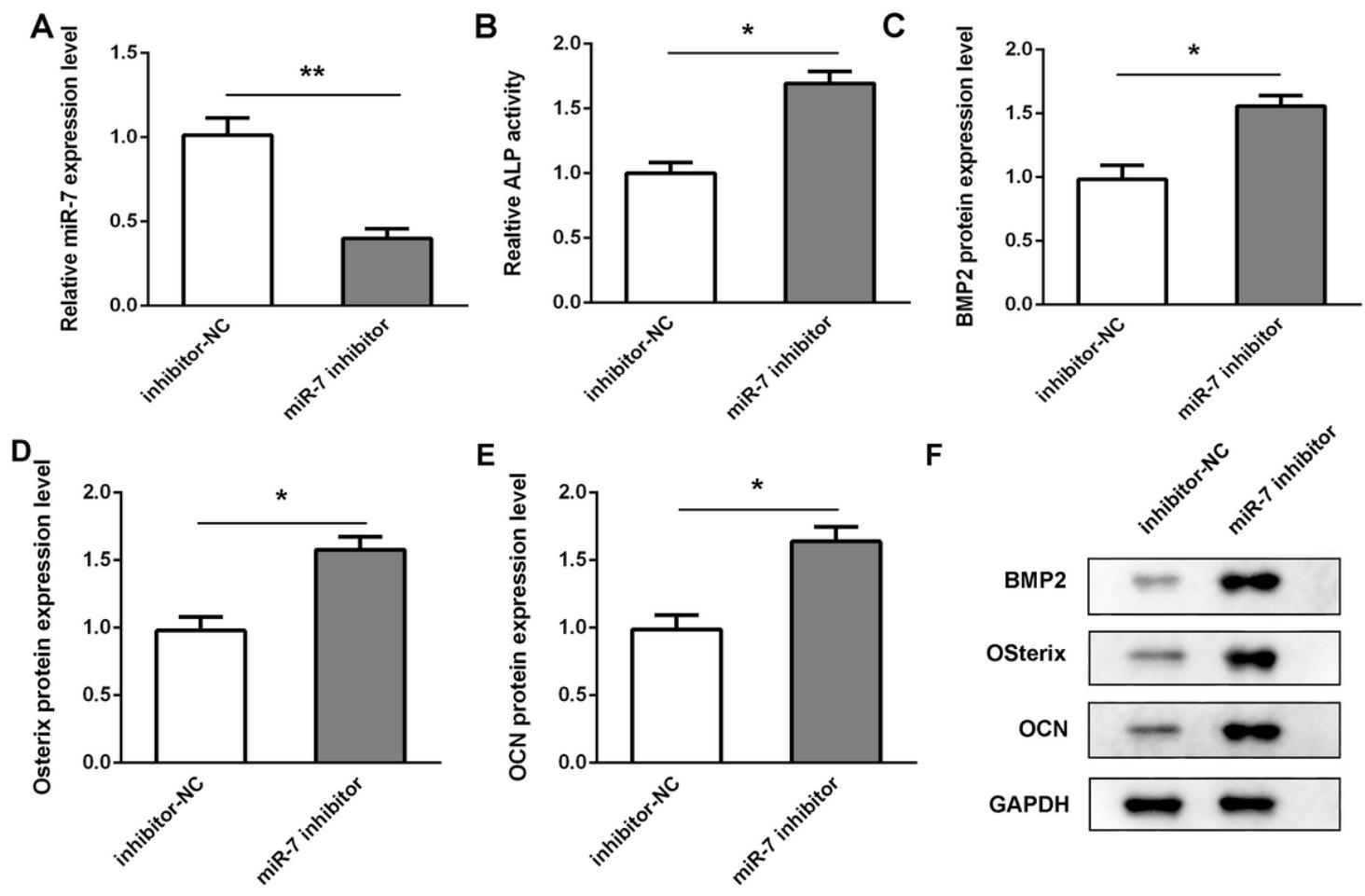


Figure 6

MiR-7 downregulated facilitated PDLCs osteogenic differentiation. (A) The transfection efficiency was measured by qPCR in PDLCs transfected of miR-7 inhibitor and inhibitor-NC. (B) After transfection, ALP activity was evaluated. The protein levels of (C) BMP2, (D) Osterix, (E) OCN were quantified, (F) which were assessed by western blot. *P<0.05. **P<0.01.

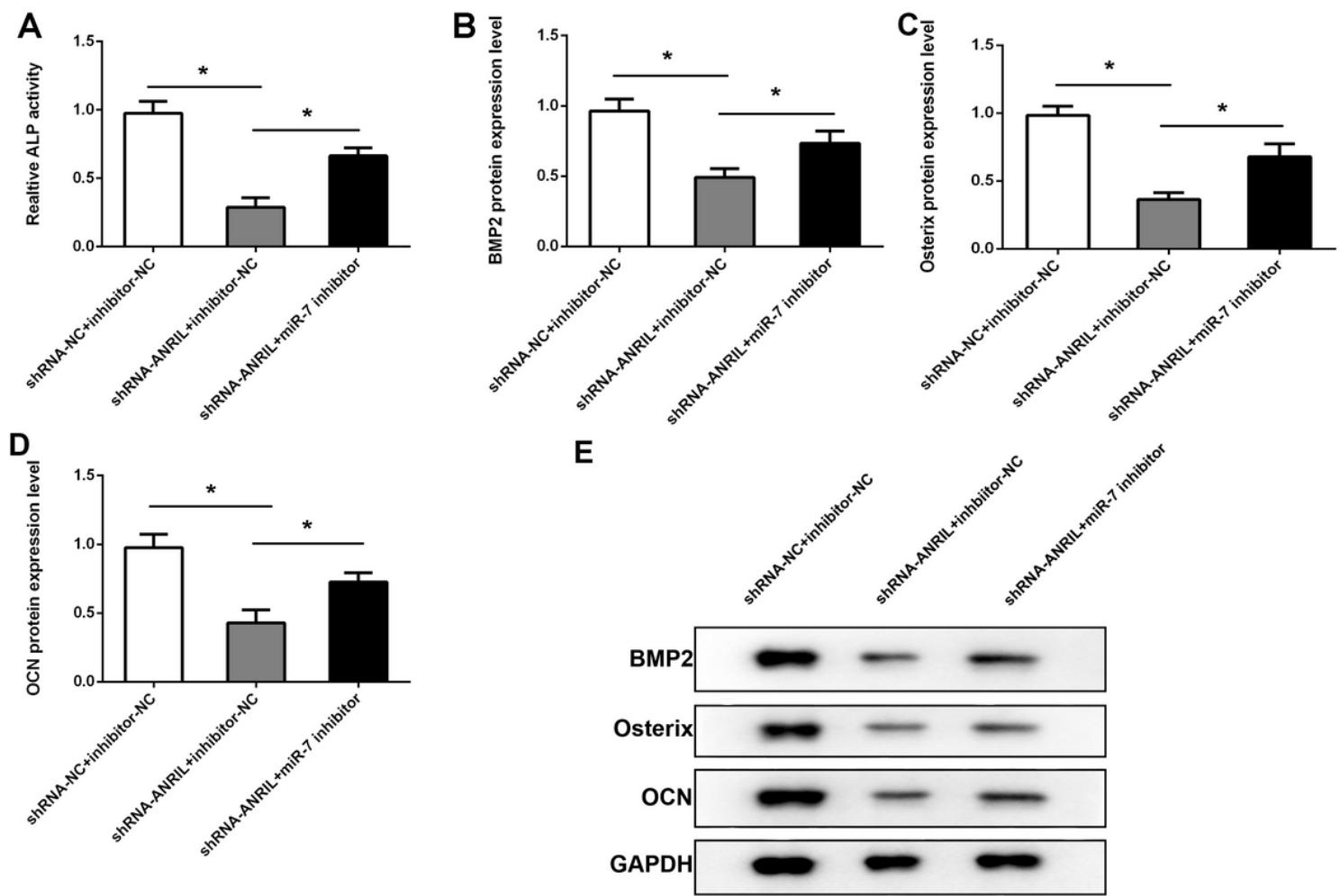


Figure 7

ANRIL knockdown suppresses osteogenic differentiation of PDLCs via regulating miR-7. (A) ALP activity was tested in PDLCs after transfection of shRNA-ANRIL-2 and miR-7 inhibitor. (B) Gray analysis for western blot. (C) BMP2, (D) Osterix, (E) OCN protein levels were assessed by western blot. *P<0.05.

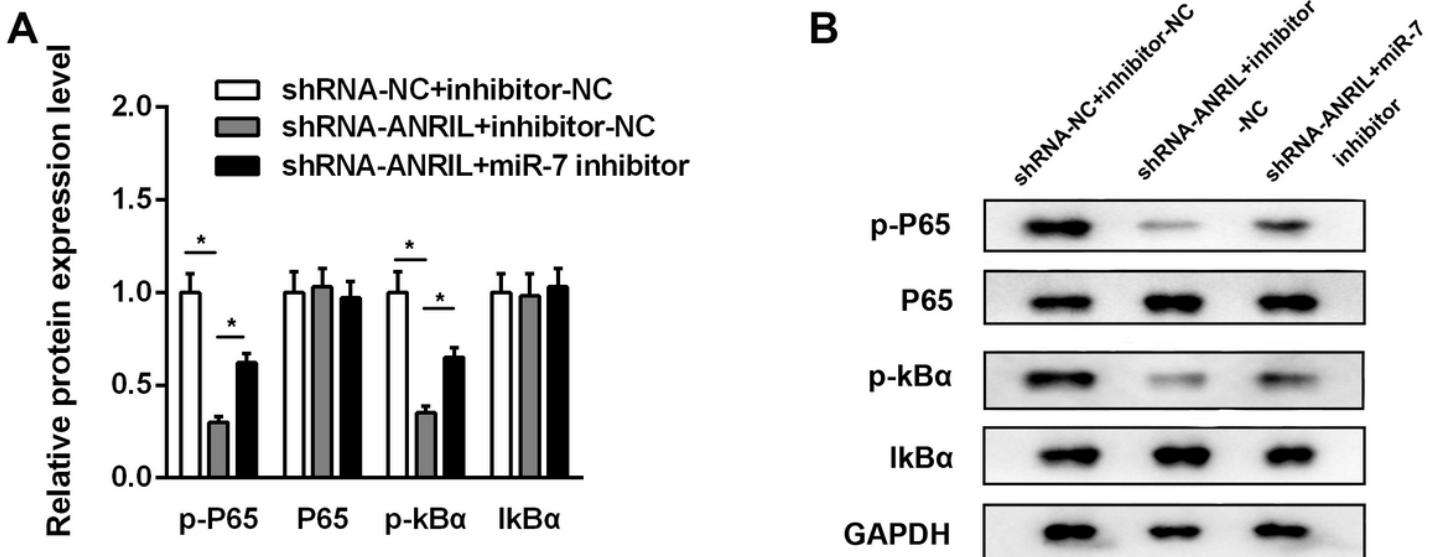


Figure 8

ANRIL activated NF- κ B pathway by inhibiting miR-7. (A) The relative protein expression of p-P65, P65, p-IkBa, and IkBa was quantified by normalizing to GAPDH level. (B) The protein of p-P65, P65, p-IkBa, and IkBa was examined by western blot.*P<0.05.