LncRNA-KCNQ1OT1 Promotes the Odontoblastic Differentiation of Dental Pulp Stem Cells Via Regulating miR-153-3p/RUNX2 Axis

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Research

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Abstract

Background and Objective: Long non-coding RNAs (LncRNAs) play a key role in the odontoblastic differentiation. This study aimed to explore the role of LncRNA-KCNQ1OT1 in the odontoblastic differentiation of human dental pulp stem cells (DPSCs) and its possible mechanism.

Methods: The expression of LncRNA-KCNQ1OT1, miR-153-3p, RUNX2 in the odontoblastic differentiation was detected by qRT-PCR. Interaction between LncRNA-KCNQ1OT1 and miR-153-3p and interaction between miR-153-3p and RUNX2 were detected by dual-luciferase assay. The cell viability of DPSCs was detected by cell counting kit-8 (CCK-8), and the effect of LncRNA-KCNQ1OT1 and miR-153-3p on the odontoblastic differentiation of DPSCs was observed by alizarin red staining, alkaline phosphatase (ALP) activity assay and Western blot for RUNX2, DSPP, DMP-1.

Results: During odontoblastic differentiation of DPSCs, the expression of LncRNA-KCNQ1OT1 increased, miR-153-3p expression decreased, and RUNX2 expression increased. Dual-luciferase assay showed that LncRNA-KCNQ1OT1 sponges miR-153-3p and miR-153-3p targets on RUNX2. After LncRNA-KCNQ1OT1 and miR-153-3p expressions of DPSCs were changed, the cell viability was not notably changed, but the odontoblastic differentiation was notably changed which was confirmed with alizarin red staining, ALP activity and Western blot for RUNX2, DSPP, DMP-1.

Conclusion: LncRNA-KCNQ1OT1 promotes the odontoblastic differentiation of DPSCs via regulating miR-153-3p/RUNX2 axis, which may provide a therapeutic clue for odontogenesis.

Background

Human dental pulp stem cells (DPSCs) are a kind of adult stem cells with high proliferation, self-renewal ability and multi-differentiation potential, which are mainly derived from dental pulp tissue. DPSCs can not only differentiate into dentin, but also into bone, cartilage, fat, myogenic and other mesodermal-derived cells(1, 2). Human DPSCs are usually taken from extracted third molars or healthy premolars that need to be extracted due to orthodontics. They are easier to obtain and have less ethical pressure. Therefore, DPSCs are ideal seed cells for tissue engineering(3–5). The ability of DPSCs to differentiate into odontoblasts plays an important role in maintaining the dynamic balance of dental pulp tissue and tooth regeneration, and is the basis for future use of tissue engineering to achieve dentin regeneration(6–8). Odontoblastic differentiation of DPSCs involves many factors such as biological scaffolds, regulatory genes and signal pathways(8–12). MicroRNAs (miRNAs, miRs) as post-transcriptional inhibitors, recognize and bind to the 3'untranslated region (3'UTR) of the target gene to inhibit the translation of the target gene protein, and have complex regulatory effects on the body's physiological/pathological activities, including the process of odontoblastic differentiation(13–15). Long non-coding RNAs (LncRNAs) as competing endogenous RNAs (ceRNAs) play key role in cell cycle, migration, proliferation, differentiation and apoptosis through sponging miRNAs to regulate miRNA targets. More and more

studies have shown that LncRNAs participate in the differentiation process of odontoblasts through sponging miRNAs(10, 16, 17).

Studies have shown that runt-related transcription factor 2 (RUNX2) plays a key role in the odontoblastic differentiation. Its expression promotes the odontoblastic differentiation(18–20). In this study, the software targetscan (http://www.targetscan.org) was used to analyze which miRNAs maybe target the RUNX2, and the result showed RUNX2 may be targeted by miR-153-3p. We used LncBase Predicted v.2 database (http://carolina.imis.athena-innovation.gr) to analyze the potential LncRNAs which interact with miR-153-3p, and the results showed LncRNA-KCNQ1OT1 contains potential binding site of miR-153-3p. Jiang et al reported that miR-153-3p inhibited osteogenic differentiation of periodontal ligament stem cells via targeting KDM6A and regulating the ALP, RUNX2 and OPN transcription(21). Studies showed LncRNA-KCNQ1OT1 regulated osteogenic differentiation by sponging miR-214(22), miR-320a(23). Yu et al reported that knockdown of lncRNA-KCNQ1OT1 in tendon stem cell inhibited the osteogenic differentiation by regulating miR-138/PPARγ or RUNX2 axis(24). However, the role of lncRNA-KCNQ1OT1 and miR-153-3p in the differentiation of DPSCs into odontoblasts is still unclear. The study aimed to explore the effect of lncRNA-KCNQ1OT1 and miR-153-3p on the odontoblastic differentiation of DPSCs and confirm its regulation axis.

Methods

DPSCs culture

This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University. The cell culture and identification was performed as described in our previous studies(13). Briefly, the pulp from normal human impacted third molars was collected and digested with 3 mg/ml collagenase type I for 1 h at 37°C. Donor of the impacted third molar had given informed consent. Single-cell suspensions of dental pulp were cultured and passaged in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C under 5 % CO_2. The fourth passage cells were used in the following experiments.

Cell transfection

DPSCs were cultured in a 6-well culture plate at a concentration of 5×10^3/ml. After 24 h, they were transfected with KCNQ1OT1 siRNA, siRNA-NC (RuiboBio, China), overexpression vectors of KCNQ1OT1 (pcDNA-KCNQ1OT1) (Sangon Biotech, China), miR-153-3p inhibitor, inhibitor–NC, miR-153-3p mimic and mimic NC (ThemoFisher, USA) with lipofectamine 2000 (ThemoFisher, USA) according to the transfection instructions, the cells were incubated in 5% CO_2, 37°C incubator and a blank control group was set up. After 48 hours, the transfected cells were taken for the following experiment.

Cell viability assay

The cell counting Kit-8 (CCK-8; Beyotime Biotechnology, China) was used to detect the viability of the DPSCs according to the manufacturer instructions, and the OD value was read at 450 nm.

Odontoblastic differentiation culture

The odontoblastic differentiation culture was performed as described in our previous study(13). Briefly, the fourth passages DPSCs were cultured in odontogenic differentiation medium containing a minimum essential medium (Invitrogen, Carlsbad, CA), 50 mg/mL a-ascorbic acid, 15% FBS, 10 mmol/L b-glycerophosphate, 10 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO), 0.292 mg/mL glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin for 14 days.

Quantitative real-time PCR (qRT-PCR)

The total RNA in cells was extracted using Trizol (Invitrogen, USA), and quantified with agarose gel (Sigma-Aldrich, USA). For LncRNA and mRNA, cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (Transgen, China). For miRNA, cDNA was synthesized using TransScript miRNA First-Strand cDNA Synthesis SuperMix (Transgen, China). GAPDH and U6 were used as the internal reference. The gene expression level was calculated with $2^{-\triangle\triangle Ct}$ method. The primer sequences were shown in the Table-1.

Luciferase reporter gene experiment

The wild-type (WT) binding fragment of lncRNA-KCNQ1OT1 or WT core sequence of 3'UTR of RUNX2 was cloned into pMIR-Report luciferase vector (Promega Corporation, USA) to construct WT-lncRNA-KCNQ1OT1 reporter vector or WT-RUNX2 reporter vector. The fragment of lncRNA-KCNQ1OT1 or core sequence of 3'UTR of RUNX2 were mutated (MU) and cloned into pMIR-Report luciferase vector to obtain MU-lncRNA-KCNQ1OT1 reporter vector or MU-RUNX2 reporter vector. The WT-lncRNA-KCNQ1OT1, MU-lncRNA-KCNQ1OT1 and miR-153-3p mimic (ThemoFisher, USA) or NC were co-transfected into HEK293 cells to investigate the relationship between lncRNA-KCNQ1OT1 and miR-153-3p. The WT-RUNX2, MU-RUNX2 and miR-153-3p mimic (ThemoFisher, USA) or NC were co-transfected into HEK293 cells to investigate the relationship between miR-153-3p and RUNX2. After transfection 48 h, relative luciferase activity was analyzed with a dual-luciferase reporter assay system (Promega Corporation, USA) according to the manufacturer instructions.

Alkaline phosphatase (ALP) activity assay

After 14 days of odontoblastic differentiation, the cells were lysed with 1% Triton X-100 for 15 min, and centrifuged at 10,000 g for 5 min, then the supernatant was collected and detected with ALP Assay Kit (Beyotime Biotechnology, China) according to the manufacturer instructions, and the OD value was read at 405 nm.

Alizarin red staining

After 14 days of odontoblastic differentiation, the cells were incubated with 2% alizarin red staining solution (Beyotime, China) for 10 min at room temperature. Then the cells were observed under an

inverted microscope and the cell mineralization was quantified with alizarin red extracted with 100 mM cetylpyridinium chloride solution (Sigma, USA), and the OD value was read at 570 nm.

Western blot analysis

After 14 days of odontoblastic differentiation, the total protein of cells was extracted by RIPA buffer (Beyotime, China). The protein was electrophoresed and transferred to PVDF membrane. After PVDF membrane blocked with 5% BSA, it was successively incubated with the primary antibody: rabbit anti-RUNX2 primary antibody (1:800, Abcam, UK), rabbit anti-DSPP (1:1000, Abcam), rabbit anti-DMP-1 (1:1000, Abcam), or mouse anti-β-actin (1:1000, Abcam, UK) and the second antibody: IRDye 700-conjugated affinity-purified goat anti-mouse (1:4,000, Rockland Immunochemicals, USA) or IRDye 800-conjugated affinity-purified goat anti-rabbit second antibody (1:4,000, Rockland Immunochemicals, USA). The relative protein expression level was analyzed with Odyssey laser scanning system (LI-COR Inc., USA).

Statistical analysis

The SPSS 22.0 software was used to analyze the data of this research. The data of this study were presented as mean ± standard deviation (M ± SD). Comparison among groups was tested with independent t test or one-way analysis of variance (ANOVA) followed by Tukey's test. The Pearson analysis was used to analyze the correlation. Statistical graph was made using GraphPad Prism 7 software. $P < 0.05$ was considered statistically significant.

Results

LncRNA-KCNQ1OT1, miR-153-3p and RUNX2 expression levels during odontoblastic differentiation

During the odontoblastic differentiation of DPSCs, the expressions of LncRNA-KCNQ1OT1, miR-153-3p and RUNX2 were detected at 0, 1, 3, 7, 14 d. The relative expression levels of LncRNA-KCNQ1OT1, miR-153-3p and RUNX2 were quantified with the expression level at 0 d when the DPSCs did not begin the odontoblastic differentiation. On the 1 d of differentiation of odontoblasts, the expressions of LncRNA-KCNQ1OT1 and RUNX2 increased, and miR-153-3p expression level decreased, and they reached a peak at 3 d, then their expression levels at 7, 14 d was similar to that at 3 d (Fig. 1A-C). Spearman correlation analysis showed that LncRNA-KCNQ1OT1 and RUNX2 expression levels were positively correlated; LncRNA-KCNQ1OT1 and miR-153-3p expression levels were negatively correlated; miR-153-3p and RUNX2 expression levels were negatively correlated (Fig. 1D-F).

The effect of LncRNA-KCNQ1OT1 on the cell viability and odontoblastic differentiation of DPSCs

After transfection, the LncRNA-KCNQ1OT1 expression level was detected by qRT-PCR. LncRNA-KCNQ1OT1 expression level of DPSCs transfected with pcDNA-KCNQ1OT1 significantly increased, and

that of DPSCs transfected with KCNQ1OT1 siRNA notably decreased (Fig. 2A). The viability of DPSCs was assessed with CCK-8 kit. Compared with control, the differences of OD value among groups were not statistically significant. The transfection did not affect the viability of DPSCs (Fig. 2B).

After 14 days of culture, the odontoblastic differentiation of DPSCs was detected by ALP activity, Alizarin red staining and Western blot for RUNX2, DSPP, and DMP-1. The result of ALP activity assay showed the OD value of the pcDNA-KCNQ1OT1 group was higher than that of control and NC groups, while the OD value of the KCNQ1OT1 siRNA group was lower than that of control and NC groups (Fig. 2C). The result of Alizarin red staining displayed that mineralized bone matrix increased in the pcDNA-KCNQ1OT1 group and decreased in the KCNQ1OT1 siRNA group comparison with the control and NC groups (Fig. 2D). The result of Western blot showed the protein expression levels of RUNX2, DSPP, and DMP-1 in the pcDNA-KCNQ1OT1 group increased and in the KCNQ1OT1 siRNA group decreased compared with the control and NC groups (Fig. 2E).

Overexpression of miR-153-3p reverses the promotion of odontoblastic differentiation induced by LncRNA-KCNQ1OT1

After transfection, LncRNA-KCNQ1OT1 and miR-153-3p expression levels were detected by qRT-PCR. LncRNA-KCNQ1OT1 expression level of DPSCs transfected with pcDNA-KCNQ1OT1 or pcDNA-KCNQ1OT1 + mimic or pcDNA-KCNQ1OT1 + mimic NC significantly increased (Fig. 3A), and miR-153-3p expression level of DPSCs transfected pcDNA-KCNQ1OT1 or pcDNA-KCNQ1OT1 + mimic NC significantly decreased, and miR-153-3p expression level of DPSCs transfected with pcDNA-KCNQ1OT1 + mimic was similar to the control group (Fig. 3B). In addition, the results of CCK-8 assay showed the transfection did not affect the viability of DPSCs (Fig. 3C).

After 14 days of odontoblastic differentiation, the mineralized bone matrix, ALP activity and protein expression levels of RUNX2, DSPP and DMP-1 in the pcDNA-KCNQ1OT1 group increased comparison with the control group. While DPSCs were co-transfected with pcDNA-KCNQ1OT1 and miR-153-3p mimic, the mineralized bone matrix, ALP activity, RUNX2, DSPP, and DMP-1 protein expression levels were close to the control group. When DPSCs were co-transfected with pcDNA-KCNQ1OT1 and mimic NC, the mineralized bone matrix, ALP activity, RUNX2, DSPP, and DMP-1 protein expression levels were similar to the pcDNA-KCNQ1OT1 group (Fig. 3D-F).

LncRNA-KCNQ1OT1 act as a sponge of miR-153-3p and miR-153-3p target on RUNX2

Luciferase reporter gene experiment was used to verify that LncRNA-KCNQ1OT1 act as a sponge of miR-153-3p and miR-153-3p target on RUNX2. The result showed that when the binding fragment of LncRNA-KCNQ1OT1 or core sequence of 3'UTR of RUNX2 was mutated, the luciferase relative activity of mimic group was similar to the NC group. However, in the WT-LncRNA-KCNQ1OT1 or WT-RUNX2 reporter gene system, the relative activity of luciferase in mimic group was significantly lower than that in NC group.

Discussion

Odontoblasts are terminally differentiated cells from DPSCs and are one of the main cells that form dental tissues. Odontoblastic differentiation is the prerequisite for dentin formation. Studying the influence of various signaling pathways on the differentiation of odontoblasts, regulating the expression of various signaling pathways, and promoting the differentiation of odontoblasts will be of great significance for the treatment of various dentin-related diseases. More and more researches prove that LncRNAs and miRNAs play key roles in odontoblastic differentiation(10, 16, 17). In this study, we found that LncRNA-KCNQ1OT1 promoted the odontoblastic differentiation of DPSCs via regulating miR-153-3p/RUNX2 axis.

RUNX2 is one of the members of the Runt family, which is an specific transcription factor of odontoblastic differentiation of DPSCs(18). In this study, during the odontoblastic differentiation of DPSCs, the expression of RUNX2 increased, which is consistent with the research results of other scholars(25). Studies showed many miRNAs regulated the odontoblastic differentiation via targeting on RUNX2(15, 26, 27). In this study, we used targetscan software to found miR-153-3p have the binding site of RUNX2 3'UTR. Jiang et al reported that miR-153-3p inhibited osteogenic differentiation of periodontal ligament stem cells via targeting KDM6A and regulating the ALP, RUNX2 and OPN transcription(21). The odontoblastic differentiation process is similar to the osteogenic differentiation. Therefore, in this study, the expression level of miR-153-3p during odontoblastic differentiation of DPSCs was detected and the result showed it decreased in the process and miR-153-3p expression level was negatively correlated with RUNX2 expression level. The luciferase reporter gene experiment confirmed that RUNX2 is a target of miR-153-3p. The results indicated that miR-153-3p was indeed involved in the odontoblastic differentiation of DPSCs, and it was a negative regulatory factor.

The mechanism by which the expression of miR-153-3p decreases during the differentiation of odontoblasts is still unclear. LncRNAs as ceRNAs can sponge miRNAs. We used LncBase Predicted v.2 database to found LncRNA-KCNQ1OT1 contained the potential binding site of miR-153-3p. Study showed that knockdown of LncRNA-KCNQ1OT1 in tendon stem cell inhibited the osteogenic differentiation(24). The result of qRT-PCR in this study showed LncRNA-KCNQ1OT1 increased during odontoblastic differentiation of DPSCs and its expression level was negatively correlated with miR-153-3p. The luciferase reporter gene experiment confirmed that LncRNA-KCNQ1OT1 sponges miR-153-3p. The results indicated that LncRNA-KCNQ1OT1 positively regulated the odontoblastic differentiation of DPSCs. Therefore, we constructed pcDNA-KCNQ1OT1 or KCNQ1OT1 siRNA to transfect DPSCs to enhance or downregulate LncRNA-KCNQ1OT1 expression level. The result of CCK-8 showed LncRNA-KCNQ1OT1 expression changes had no effect on the cell viability. The odontoblastic differentiation of DPSCs was detected by alizarin red staining, ALP activity and Western blot for RUNX2, DSPP and DMP-1, and the findings showed down-regulated LncRNA-KCNQ1OT1 expression inhibited the odontoblastic differentiation of DPSCs, while LncRNA-KCNQ1OT1 overexpression promoted the odontoblastic differentiation of DPSCs. When DPSCs were co-transfected with pcDNA-KCNQ1OT1 and miR-153-3p

mimic, DPSCs overexpressed LncRNA-KCNQ1OT1 and miR-153-3p at the same time, LncRNA-KCNQ1OT1's promotion of odontoblast differentiation was reversed.

In summary, our data demonstrated that LncRNA-KCNQ1OT1 promotes the odontoblastic differentiation of DPSCs via regulating miR-153-3p/RUNX2 axis, which may provides a therapeutic clue for odontogenesis.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

GF and XF conceived and designed the experiments. XL, XC, JX, ML, DH and YL performed the experiments. XL and XF analyzed the data. XL and XC wrote the paper. All authors read and approved the final manuscript.

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Tables

Table-1 The primer sequences for qRT-PCR

Gene	Primer sequences (5'-3')
lncRNA-KCNQ1OT1	Forward: ACTCACTCACTCACTCACT
	Reverse: CTGGCTCCTTCTATCACATT
miR-153-3p	Forward: ACACTCCAGCTGGGTTGCATAGTCACAAA
	Reverse: CAGTGCGTGTCGTGGAGT
RUNX2	Forward: TGCCACCTCTGACTTCTGC
	Reverse: GATGAAATGCCTGGGAACTG
U6	Forward: GTGCTCGCTTCGGCAGCACAT
	Reverse: TACCTTGCGAAGTGCTTAAAC
GAPDH	Forward: AGGTGAAGGTCGGAGTCAAC
	Reverse: CGCTCCTGGAAGATGGTGAT

Figures

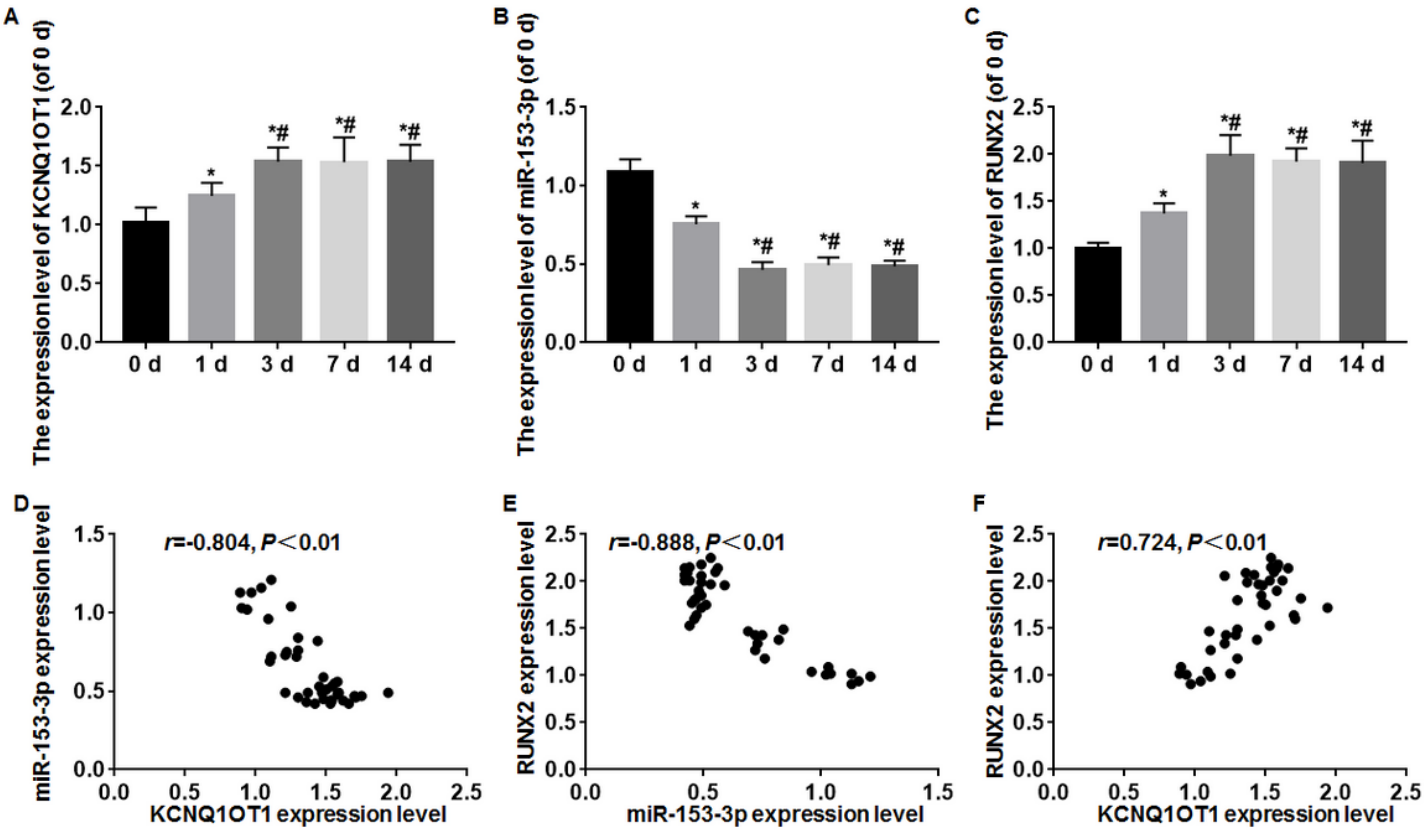

Figure 1

LncRNA-KCNQ1OT1, miR-153-3p and RUNX2 expression levels during odontoblastic differentiation were detected by qRT-PCR. (A) On the 1 d of differentiation of odontoblasts, the expression of LncRNA-KCNQ1OT1 increased and reached a peak at 3 d. (B) On the 1 d of differentiation of odontoblasts, miR-153-3p expression level decreased and reached a peak at 3 d. (C) On the 1 d of differentiation of odontoblasts, the expression of RUNX2 increased and reached a peak at 3 d. (D) LncRNA-KCNQ1OT1 expression level was negatively correlated with miR-153-3p expression level. (E) miR-153-3p expression level were negatively correlated with RUNX2 expression level. (F) LncRNA-KCNQ1OT1 expression level was positively correlated with RUNX2 expression level. * vs. 0 d, $P < 0.05$; # vs. 1 d, $P < 0.05$.

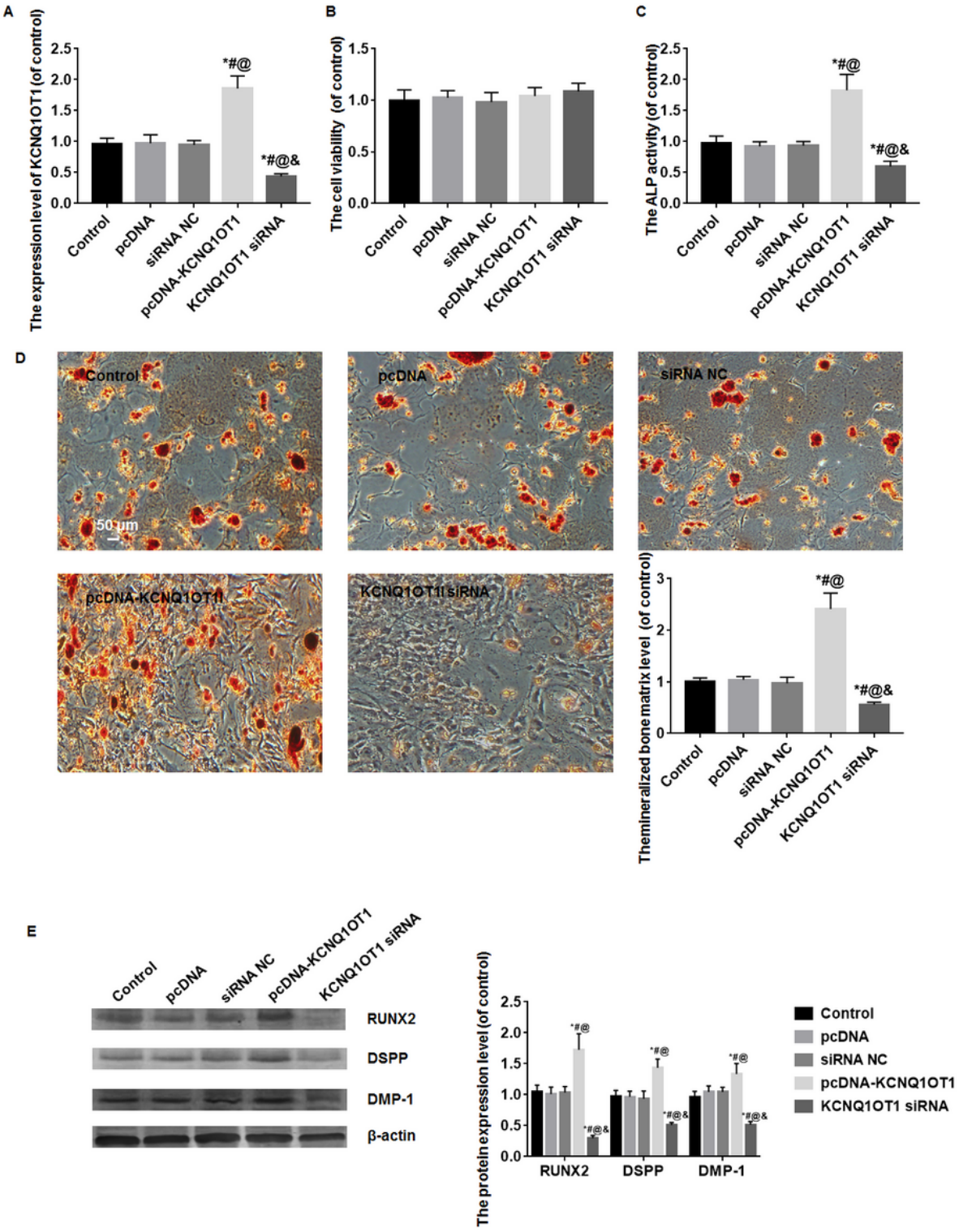

Figure 2

The effect of LncRNA-KCNQ1OT1 on the cell viability and odontoblastic differentiation of DPSCs. (A) LncRNA-KCNQ1OT1 expression level of DPSCs transfected with pcDNA-KCNQ1OT1 significantly increased, and that of DPSCs transfected with KCNQ1OT1 siRNA notably decreased. (B) The transfection did not affect the viability of DPSCs. (C) After 14 days of odontoblastic differentiation, ALP activity in the pcDNA-KCNQ1OT1 group increased and in the KCNQ1OT1 siRNA group decreased comparison with the

control and NC groups. (D) After 14 days of odontoblastic differentiation, the mineralized bone matrix in the pcDNA-KCNQ1OT1 group increased and in the KCNQ1OT1 siRNA group decreased comparison with the control and NC groups. (E) After 14 days of odontoblastic differentiation, RUNX2, DSPP, and DMP-1 protein expression levels in the pcDNA-KCNQ1OT1 group increased and in the KCNQ1OT1 siRNA group decreased comparison with the control and NC groups. Bar = 50 μm. * vs. control group, P < 0.05; # vs. pcDNA group, P < 0.05; @ vs. siRNA NC group, P < 0.05; & vs. pcDNA-KCNQ1OT1 group, P < 0.05.

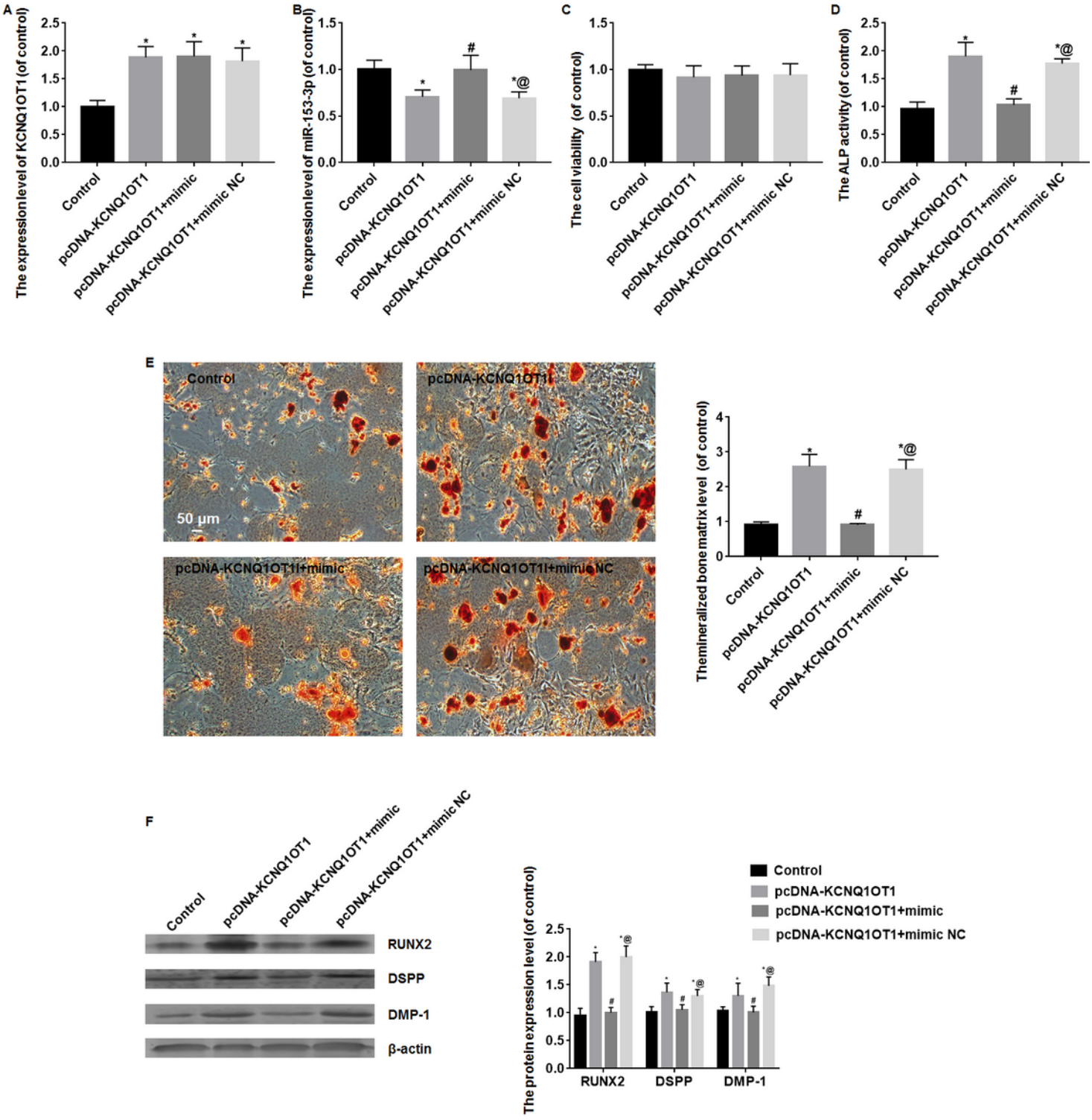

Figure 3

Overexpression of miR-153-3p reverses the promotion of odontoblastic differentiation induced by LncRNA-KCNQ1OT1. (A) LncRNA-KCNQ1OT1 expression level of DPSCs transfected with pcDNA-KCNQ1OT1 or pcDNA-KCNQ1OT1+mimic or pcDNA-KCNQ1OT1+mimic NC significantly increased. (B) miR-153-3p expression level of DPSCs transfected pcDNA-KCNQ1OT1 or pcDNA-KCNQ1OT1+mimic NC significantly decreased, and miR-153-3p expression level of DPSCs transfected with pcDNA-KCNQ1OT1+mimic was similar to the control group. (C) The results of CCK-8 assay showed the transfection did not affect the viability of DPSCs. (D-F) After 14 days of odontoblastic differentiation, the ALP activity, mineralized bone matrix, RUNX2, DSPP, and DMP-1 protein expression levels in the pcDNA-KCNQ1OT1 group increased comparison with the control group. While DPSCs were co-transfected with pcDNA-KCNQ1OT1 and miR-153-3p mimic the ALP activity, mineralized bone matrix, RUNX2, DSPP, and DMP-1 protein expression levels were close to the control group. Bar = 50 μm. * vs. control group, P < 0.05; # vs. pcDNA-KCNQ1OT1 group, P < 0.05; @ vs. pcDNA-KCNQ1OT1+mimic group, P < 0.05.

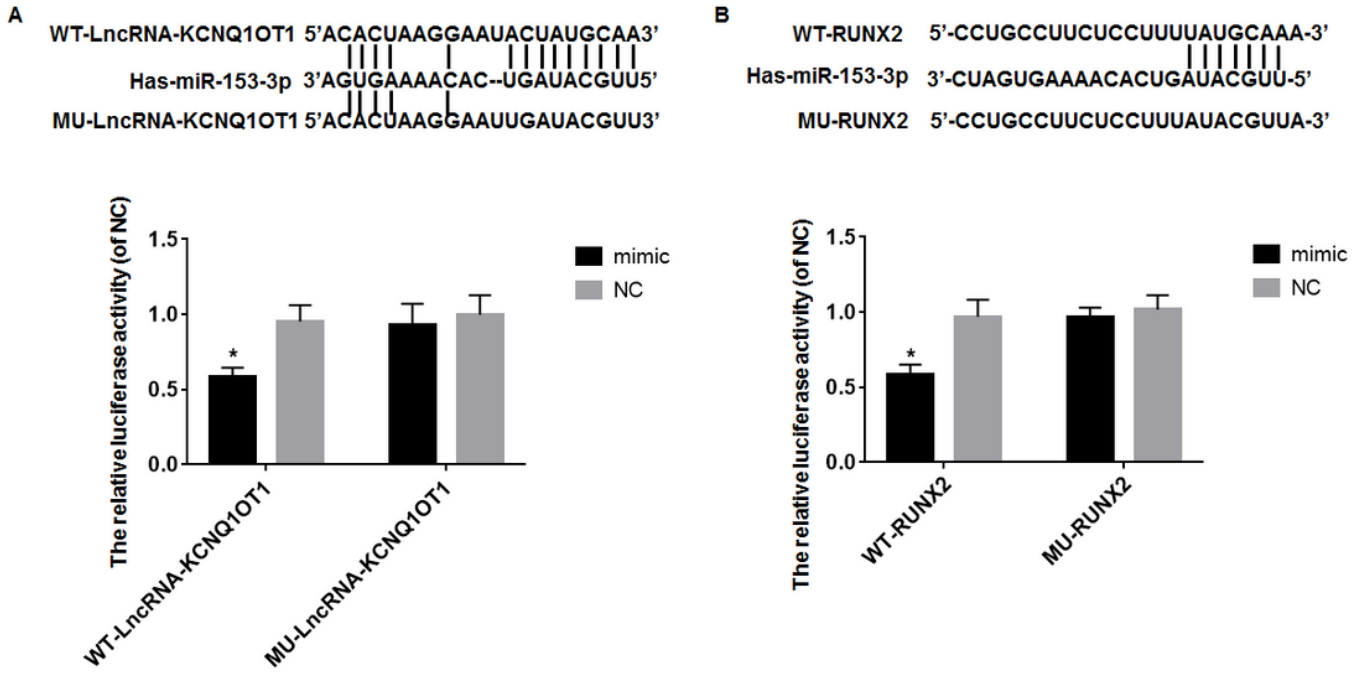

Figure 4

LncRNA-KCNQ1OT1 act as a sponge of miR-153-3p and miR-153-3p target on RUNX2. (A) In the WT-LncRNA-KCNQ1OT1 reporter gene system, the relative activity of luciferase in mimic group was significantly lower than that in NC group. When the binding fragment of LncRNA-KCNQ1OT1 was mutated, the luciferase relative activity of mimic group was similar to the NC group. (B) In the WT-RUNX2 reporter gene system, the relative activity of luciferase in mimic group was significantly lower than that in NC group. When the core sequence of 3'UTR of RUNX2 was mutated, the luciferase relative activity of mimic group was similar to the NC group. * vs. NC group, P < 0.05.