

# Differential expression of long noncoding RNAs in SDF-1 $\alpha$ -induced dental pulp stem cells

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## Research

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# Abstract

## Background

SDF-1 $\alpha$  cotreatment was shown to have synergistic effects on BMP-2-induced odontogenic differentiation of human apical dental papillary stem cells (SCAP) both in vitro and in vivo. Long noncoding RNAs (lncRNAs) have an important role in the odontogenic differentiation of dental pulp stem cells (DPSCs).

## Methods

We examined the altered expression of lncRNAs in SDF-1 $\alpha$ -induced odontogenic differentiation of DPSCs by lncRNA microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. Alterations in lncRNA expression during odontogenic differentiation of DPSCs were identified. Moreover, bioinformatic analysis [Gene Ontology (GO) analysis and coding-noncoding gene coexpression (CNC) analysis] was conducted to predict the interactions of lncRNAs and identify core regulatory factors in SDF-1 $\alpha$ -induced odontogenic differentiation of DPSCs.

## Results

The microarray analysis identified 206 differentially expressed lncRNAs (134 lncRNAs with upregulated expression and 72 with downregulated expression) at 7 days post-treatment. The data demonstrated that one lncRNA, AC080037.1, regulates SDF-1 $\alpha$ -induced odontogenic differentiation of DPSCs. Our data showed that lncRNA AC080037.1 siRNA suppresses DPSCs migration and the expression of Rho GTPase induced by SDF-1 $\alpha$ . Moreover, AC080037.1 knockdown significantly affected SDF-1 $\alpha$ - and BMP-2-induced mineralized nodule formation and strongly suppressed Runt-related factor-2 (RUNX-2), DMP-1 and DSPP expression in DPSCs.

## Conclusions

Our

## Background

At present, the main treatment for pulpitis and periapical periodontitis is still root canal treatment. However, after root canal treatment, the tooth loses the nourishment of the pulp tissue and can become devitalized, brittle, and susceptible to fracture, leading to an increased incidence of extraction<sup>[1, 2]</sup>. Advanced discoveries in tissue engineering technology have provided novel insights into tooth regeneration. Human dental pulp stem cells (DPSCs), a type of mesenchymal stem cell (MSC), are isolated from dental pulp and are highly proliferative, multipotent, clonogenic cells capable of

multilineage differentiation and self-renewal<sup>[3, 4]</sup>. DPSCs are ideal seeding cells for dental pulp tissue engineering<sup>[5, 6]</sup>. To date, the regulation of DPSCs differentiation has not been fully elucidated.

During pulp injury repair, expression of the chemokine SDF-1 $\alpha$  in the pulp tissue is significantly upregulated. SDF-1 $\alpha$  can induce stem cells with repair potential to move to the damaged region and play a role in regeneration and repair<sup>[7, 8]</sup>. Our previous research found that SDF-1 $\alpha$  not only promotes the migration of apical dental papillary stem cells (SCAP) but also promotes the differentiation of SCAP into odontoblasts based on the BMP-Smad and Erk1/2 pathways<sup>[9, 10]</sup>. Nevertheless, little is known about whether other types of mechanisms, including epigenetic regulation, are involved in the SDF-1 $\alpha$ -induced osteo/odontogenic differentiation of DPSCs.

Long noncoding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides that are not translated into proteins<sup>[11]</sup>. Their functional disorders and specific roles in various diseases, especially cancers, have attracted increasing attention<sup>[12, 13]</sup>. Due to their diversity and flexibility, lncRNAs are good candidates to regulate gene expression in a temporospatial manner in response to complex situations, including during cellular differentiation<sup>[14–16]</sup>. Accumulating evidence has shown that noncoding RNA transcripts, including microRNAs and lncRNAs, are essential in stem cell proliferation and differentiation<sup>[17]</sup>. Alterations or mutations in lncRNAs have been reported to modulate multiple physiological processes. Multiple lncRNAs have been demonstrated to play regulatory roles in osteo/odontogenic differentiation from mesenchymal stem cells<sup>[18–20]</sup>. For instance, Zhong et al. reported that lncRNA CCAT1 promoted human DPSCs proliferation and differentiation via negative regulation of miRNA-218<sup>[18–20]</sup>. Nevertheless, whether lncRNAs are involved in the osteo/odontogenic differentiation of DPSCs remains unclear.

In this study, we triggered the involvement of lncRNAs in odontogenic differentiation of DPSCs by incubation with SDF-1 $\alpha$ . By lncRNA microarray analysis, alterations in lncRNA expression during SDF-1 $\alpha$ -induced odontogenic differentiation were identified. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to validate several random lncRNAs with upregulated and downregulated expression in the odontogenic differentiation of DPSCs. In addition, Gene Ontology (GO) analysis and coding-noncoding gene coexpression (CNC) analysis were conducted to predict the interactions of coding and noncoding RNAs and identify core regulatory factors in the odontogenic differentiation of DPSCs. Further analysis showed that one lncRNA, lncAC080037.1, may be involved in the regulation of SDF-1 $\alpha$ -induced odontogenic differentiation of DPSCs. These findings are important for elucidating the molecular mechanism of the differentiation of DPSCs during the restoration of dental pulp injury and will provide new ideas for pulp-dentin regeneration and clinical pulp preservation.

## Methods

### 2.1 DPSCs isolation and culture

Disease-free impacted third molars at the stage of root development (6 patients aged 12 to 18 years, males and females) were extracted from patients at the Stomatological Hospital of the Fourth Military Medical University according to a protocol approved by the Institutional Review Board of the Fourth Military Medical University. We confirmed that all methods were performed in accordance with the relevant guidelines, and informed consent was obtained from all patients. DPSCs were isolated and cultured using the method described by our previous research<sup>[9, 22]</sup>. Cells were seeded in 12-well plates at a density of  $2 \times 10^4$  cells/mL, grown to 70% confluence, and incubated in odonto/osteogenic differentiation medium containing 10% FBS, 1% penicillin-streptomycin, 10 nmol/L dexamethasone, 50 mg/L ascorbic acid and 10 nmol/L  $\beta$ -glycerophosphate (all purchased from Sigma-Aldrich) for 2 weeks. Cultures were fixed in 4% paraformaldehyde for 30 min, washed, and stained with 1% Alizarin Red S (pH = 4.2, Sigma-Aldrich) for 15 min. Then, flow cytometric examination was applied to determine the cell phenotype for CD29/PE, CD34/PE, CD45/PE, CD90/PE, CD105/PE and CD146/PE (BioLegend, San Diego, CA, USA). Cells from these clones were obtained, and passage 0 (P0) cells were cultured and expanded for subsequent experiments.

In order to verify whether the effect of SDF-1 $\alpha$  on the odontogenic differentiation of DPSCs is consistent with our previous studies. DPSCs were cultured in mineralization-inducing medium with 100ng/mL SDF-1 $\alpha$  for 7 days. Then Real-time RT-PCR and Westernblot analysis were used To detect the expression levels of osteo/odontogenic marker genes.

## **2.2 Expression microarray analysis**

### **2.2.1 RNA isolation and quality control**

Total RNA was isolated with TRIzol reagent (Invitrogen) and purified with a TaKaRa Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. RNA quantity and quality were measured by a NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis or an Agilent 2100 Bioanalyzer.

### **2.2.2 Microarray analysis**

RNA quantity and quality were measured by a NanoDrop ND-10000 (Thermo Scientific, Waltham, MA, USA), and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. An Arraystar Human LncRNA Microarray V5.0 was designed for the global profiling of human lncRNAs and protein-coding transcripts. Approximately 39,317 lncRNAs and 21,174 coding transcripts were detected by the third-generation lncRNA microarray. After quantile normalization of the raw data, lncRNAs showing significant differences in expression between the two groups were identified through *P*-value/FDR filtering.

### **2.2.3 RNA labelling and array hybridization**

Sample labelling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Briefly, each RNA sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). The labelled cRNAs were purified by an RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labelled cRNAs (pmol Cy3/ $\mu$ g cRNA) were measured by a NanoDrop ND-1000. One microgram of each labelled cRNA was fragmented by adding 5  $\mu$ L of 10 $\times$  Blocking Agent and 1  $\mu$ L of 25 $\times$  Fragmentation Buffer and then heating the mixture at 60°C for 30 min. Finally, 25  $\mu$ L of 2 $\times$  GE Hybridization buffer was added to dilute the labelled cRNA. Fifty microlitres of hybridization solution was dispensed into the gasket slide and assembled in the lncRNA expression microarray slide. The slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned using the Agilent DNA Microarray Scanner (part number G2505C). Agilent Feature Extraction software (version 11.0.1.1) was used to analyse the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed lncRNAs with statistical significance between the two groups were identified through *P*-value/FDR filtering. Differentially expressed lncRNAs between the two samples were identified through fold change filtering.

#### **2.2.4 Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated using TRIzol reagent (Invitrogen) and then reverse transcribed using SuperScript<sup>TM</sup> III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The levels of five lncRNAs with upregulated and five with downregulated expression included in the microarray study were measured by qRT-PCR using 2X PCR master mix (Arraystar). The primer sequences are shown in Table 1.

#### **2.2.5 Bioinformatics analysis**

The GO project provides a controlled vocabulary to describe gene and gene product attributes in any organism (<http://www.geneontology.org>). The categories cover three domains: biological process, cellular component and molecular function. Fisher's exact test in Bioconductor's topGO was used to determine if there was more overlap between the DE list and the GO annotation list than would be expected by chance. The *P*-value produced by topGO denotes the significance of GO term enrichment in the DE genes. The lower the *P*-value, the more significant the GO term is (*P*-value  $\leq$  0.05 is recommended). A CNC network and mRNA target gene network were constructed to identify the interactions among genes and to locate core regulatory factors that played an important role in these networks.

### **2.3 Small interfering RNA (siRNA) knockdown of gene expression**

DPSCs were transfected with an siRNA targeting lncRNA AC080037.1 and a nontargeting control (NC) siRNA (Dharmacon, Chicago, IL, USA). lncRNA AC080037.1 siRNA was diluted to the working concentration in RNase-free water. DPSCs were transfected with 25 nM siRNA using DharmaFECT

Transfection Reagent (Dharmacon) according to the manufacturer's siRNA transfection protocol. Briefly, cells were transfected with siRNA upon reaching 70–80% confluence. The effectiveness of siRNA knockdown was determined by quantifying the level of lncRNA AC080037.1 gene expression via real-time RT-PCR after 48 h. In addition, the cells were pretreated with lncRNA AC080037.1 siRNA at 25 nM for 48 h at 37°C prior to SDF-1 $\alpha$  or BMP-2 stimulation to block lncRNA AC080037.1 expression in subsequent experiments.

## 2.4 RT-qPCR

Total RNA was extracted using TRIzol reagent (TaKaRa, Bio, Inc., Shiga, Japan), and 1  $\mu$ g of total RNA per sample was reverse transcribed into cDNA using a Prime Script RT reagent kit (TaKaRa). The expression levels of target genes were quantified through real-time RT-PCR using the SYBR Premix Ex Taq II kit (TaKaRa) and tested in an ABI 7500 Real-time RT-PCR System (Biosystems 7500 System, Foster City, CA, USA). The primers are shown in Table 2, and GAPDH was used as the housekeeping gene. All reactions were run in triplicate.

## 2.5 Cell transmigration assay

DPSCs (p3) were loaded in the upper chambers of 24-well 8- $\mu$ m pore Transwell inserts (Corning, NY, USA) at  $5 \times 10^3$  cells/well. The following 4 groups were included: a control A group, in which the lower chamber contained only normal culture medium; a control B+SDF-1 $\alpha$  group, in which the lower chamber medium was supplemented with 100 ng/mL SDF-1 $\alpha$ ; and NC siRNA and lncRNA AC080037.1 siRNA groups, in which DPSCs were preincubated with siRNA (100 ng/mL) (Sigma-Aldrich) for 24 h and then used in Transwell assays with 100 ng/mL SDF-1 $\alpha$  in the lower chamber. After incubation for 24 h at 37°C, nonmigrated cells on the upper side of the Transwell chamber were removed, and cells that had transmigrated to the lower side were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for evaluation under a microscope. Five fields were randomly selected from each chamber and subjected to cell counting.

## 2.6 Alizarin Red staining and quantification

DPSCs were seeded in 12-well plates at a density of  $1 \times 10^5$  cells/well. These samples were divided into three groups. After growing to 80% confluence, the cells were transfected with siRNA according to the manufacturer's protocol. The transfection medium was replaced with mineralizing medium after 48 h. The cells were pretreated with SDF-1 $\alpha$  at 100 ng/mL for 2 h at 37°C before BMP-2 (100 ng/mL, PeproTech, Inc.) stimulation. Each group included 5 wells, and the medium was changed every 3 days. After 2 weeks, the cells were observed under a light microscope. For mineralization analysis, cultures were washed 3 times with PBS, fixed in 4% polyoxymethylene for 1 h, washed with distilled water 3 times, and then stained with Alizarin Red S (Sigma-Aldrich) (pH = 4.2) for 15 min at room temperature. The excess dye was removed by washing 5 times with distilled water; 0.5 mL of PBS was then added to each well,

and images were acquired. To quantify Alizarin Red staining, we used 10% cetylpyridinium chloride (Sigma-Aldrich) to elute the stain and incubated it for 15 min. The stain was dissolved, and the concentration was determined by the absorbance at 562 nm as measured by a microplate reader (Power Wave 340, Bio-TEK, VT, USA).

## 2.7 Western blot analysis

DPSCs (p4) were seeded in 6-well plates and grown to 80% confluence at 37°C in a 5% CO<sub>2</sub> incubator. The cells were transfected with siRNA as described above. At 3 and 7 days, the samples were lysed with RIPA buffer supplemented with protease inhibitors and subjected to ultrasonication at a low frequency. The supernatant containing the total protein was harvested after centrifugation, and the protein concentration was measured using a BCA protein assay reagent (Beyotime). Primary antibodies against CDC42, RhoA, RAC1/2/3, Runx-2 (1:1000, Cell Signaling Technology, MA, USA), DMP-1 (1:1000, Abcam), DSPP (1:1000, Santa Cruz Biotechnology) and GAPDH (1:3000, Cell Signaling Technology) were used according to the manufacturer's instructions. Proteins were extracted, subjected to SDS-polyacrylamide gels and then transferred to 0.22 µm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in QuickBlock™ Blocking Buffer (Beyotime) for 1 h and incubated with primary antibodies at 4°C overnight. After being washed in TBST 3 times, the membranes were incubated with dilutions of the appropriate secondary antibodies for 1 h at room temperature, followed by incubation with an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA, USA) for a few seconds. The resulting signals were captured using a ChemiDoc MP system (Bio-Rad) and Image Lab software (Bio-Rad). GAPDH was used as an internal control. The quantities of the phosphorylated proteins were calculated by normalizing the p-form to the total amount.

## 2.8 Statistical analyses

The lncRNA expression differences between different groups of cells were analysed using a two-sample t-test. Differences were considered significant at  $P < 0.05$ . The data reported in this study are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using SPSS Statistics 20.0 software. The statistical significance of the differences between two groups was analysed via one-way ANOVA at a significance level of  $P < 0.05$ .

# Results

## 3.1 Isolation and characterization of DPSCs

In this study, human DPSCs derived from enzymatic digestion of dental pulp explants

were cultured, and the primary cells presented clone-like growth after they were incubated for 3 to 4 days (Fig. 1a). Putative stem cells obtained from the cell clones that were grown for 2 weeks were

characterized by odonto/osteogenic differentiation tests and flow cytometry. Calcified nodules were observed at Day 14, and the Alizarin Red staining intensity was significantly increased (Fig. 1b). Flow cytometry was used to characterize surface DPSC molecules. These cells were negative for CD34 and CD45 (Fig. 1c-d) and positive for the markers of mesenchymal stem cells CD29, CD90, CD105 and CD146 (Fig. 1e-h). These results correspond with previous studies reporting that DPSCs were successfully isolated from dental pulp.

## 3.2 Effect of SDF-1 $\alpha$ on the odontogenic differentiation of DPSCs

When DPSCs were treated for 7 days, the gene expression level of RUNX-2, DMP-1, and DSPP in DPSCs were significantly up-regulated in the treated group than that in their control group (Fig. 2a-b) (\* $P < 0.05$ ). Meanwhile, the protein expression level of RUNX-2, DMP-1, and DSPP in SDF-1 $\alpha$  group were significantly higher than that in Control group (Fig. 2c-d) (\* $P < 0.05$ ). Total RNA of the successfully differentiated cells was subsequently used for lncRNA microarray analysis.

## 3.3 RNA quality control

RNA quantification and quality assurance were performed by using a NanoDrop ND-1000. The O.D. A260/A280 ratio for all samples was in the range of 1.8–2.2, while the O.D. A260/A230 ratios were greater than 1.8 (Table 2). Then, RNA integrity and gDNA contamination were assessed by denaturing agarose gel electrophoresis, and the results are presented in Fig. 3.

## 3.4 Changes in the expression of lncRNAs after DPSC induction by SDF-1 $\alpha$

To determine the differential expression of lncRNAs during SDF-1 $\alpha$  induction in DPSCs, we used scatterplots, volcano plots and box plots of the lncRNA expression profiles because of their universality and perceptual intuition (Fig. 4a-d). In the present study, RNA was collected after 7 days of treatment with 100 ng/mL SDF-1 $\alpha$ . Significant upregulation of expression was found for 134 lncRNAs, but 72 lncRNAs showed the opposite trend (fold change  $> 1.5$  or  $< 1.5$ ,  $P < 0.05$ ) (Fig. 4c). In addition, cluster analysis showed significant differences in lncRNA expression between the two groups (Fig. 4a). Tables 3 and 4 reveal that the expression of ENST00000521002 (fold change, 2.3698723) and T183545 (fold change, 9.0026448) showed the most notable upregulation and downregulation, respectively. In this part, we found that lncRNA AC080037.1 expression was upregulated, and some studies confirmed that it was related to the transmission and osteo/odontogenic differentiation of DPSCs. These results demonstrated that lncRNAs underwent transitional alterations during SDF-1 $\alpha$  induction in DPSCs.

Then, qRT-PCR was utilized to verify the results of the microarray analysis, and six differentially expressed lncRNAs (ENST00000584975, T183545, BIG-lncRNA-547, ENST00000521002, ENST00000542427 and ENST00000538349) were selected for qRT-PCR analysis. The qRT-PCR results of the randomly selected lncRNAs were consistent with the microarray analysis results (Fig. 4e-j).



## 3.5 GO and CNC analysis

GO analysis was performed to determine the significantly changed functions obtained from the differentially expressed lncRNAs of DPSCs during SDF-1 $\alpha$  induction. A total of 142 GO functions ( $P < 0.05$ ) were obtained; 92 were enhanced and 50 were decreased in the treatment group. The negative logarithm of the  $P$  value ( $- \text{LgP}$ ) was applied to show the correlation of gene expression levels and the relevant biological processes (Fig. 5a-f). The GO functions enriched from the genes with upregulated expression were related to receptor internalization, organelle fission, wound healing, G1 to G0 transition, drinking behaviour, mitochondrial respiratory chain complex III assembly, respiratory chain complex III assembly, regulation of attachment of spindle microtubules to the kinetochore, synaptic vesicle budding, and several others. The GO functions enriched from the genes with downregulated expression included detection of stimulus, sodium-independent organic anion transport, positive regulation of double-strand break repair via nonhomologous end joining, negative regulation of double-strand break repair via homologous recombination, regulation of rhodopsin mediated signalling pathway, positive regulation of isotype switching, and several others.

Next, a CNC network was constructed to identify the interactions and degree of significance among the differentially expressed mRNAs and lncRNAs based on clustering coefficients and degrees. 8 core regulatory genes were identified including SPAG5, MX2, APLN, CDC42, DMP-1, RUNX-2, RhoA and Rac1 (Additional file 1: Fig. S1).

## 3.6 LncRNA AC080037.1 siRNA suppresses SDF-1 $\alpha$ -induced DPSCs migration

We selected one candidate lncRNA, AC080037.1, which was identified as a possible core regulatory factor of DPSCs according to the CNC network, to investigate its function in DPSCs. First, we confirmed the knockdown effect of siRNA transfection on the gene expression of lncRNA AC080037.1 in DPSCs through real-time RT-PCR at 3 days and 7 days (Fig. 6c). Compared with that of the control and NC groups, the expression of lncRNA AC080037.1 was substantially reduced by transfection of siRNA AC080037.1 ( $*P < 0.05$ ). Then, we detected the effect of lncRNA AC080037.1 on the migration of DPSCs induced by SDF-1 $\alpha$ . The Transwell migration assay (Fig. 6a,b) showed that more DPSCs were localized on the lower side of the membrane in the control + SDF-1 $\alpha$  group than in the control group after 24 h of treatment ( $P < 0.05$ ). When cells were pretreated with lncRNA siRNA and then treated with 100 ng/mL SDF-1 $\alpha$ , the number of transmigrated cells was significantly decreased compared with that of the NC group and the control + SDF-1 $\alpha$  group ( $P < 0.05$ ) (Fig. 6b). There was no significant difference between the control + SDF-1 $\alpha$  and NC groups ( $*P > 0.05$ ).

## 3.7 LncRNA AC080037.1 knockdown affects the expression of Rho GTPase in DPSCs

Then, we detected the effect of lncRNA AC080037.1 knockdown on the expression of Rho GTPase in DPSCs. As shown in Fig. 5d-f, qRT-PCR demonstrated that the CDC42, RhoA and Rac1/2/3 gene

expression levels were decreased in the siRNA-treated group on Day 3 compared to those in the NC groups ( $*P < 0.05$ ). In addition, after 3 days of stimulation, no significant difference in their expression levels was observed between the control and NC groups. The protein expression levels of CDC42, RhoA and Rac1/2/3 were similar to the gene expression levels. CDC42, RhoA and Rac1/2/3 expression was strongly reduced by lncRNA AC080037.1 siRNA treatment at 3 days. No differences in RhoA and Rac1/2/3 protein levels were observed between the control and NC groups (Fig. 5g-h).

### **3.8 LncRNA AC080037.1 (siRNA) silencing inhibits SDF-1 $\alpha$ and BMP2-induced odontogenic differentiation of DPSCs**

To confirm whether lncRNA AC080037.1 could stimulate odontogenic differentiation of DPSCs induced by SDF-1 $\alpha$  and BMP-2, we performed lncRNA AC080037.1 knockdown experiments. After 2 weeks of induction, Alizarin Red S staining showed that the mineralized bone matrix was notably weakened in the BMP2-induced DPSCs after lncRNA AC080037.1 knockdown compared to that of the control and NC groups (Fig. 7a,b). As demonstrated by quantitative real-time PCR, the expression of lncRNA AC080037.1 was significantly decreased in DPSCs after transfection with siRNA at Day 7 (Fig. 6c). RT-PCR showed significantly downregulated mRNA expression of RUNX2, DMP-1 and DSPP in the BMP2-induced DPSCs transfected with lncRNA AC080037.1 siRNA (Fig. 7c-e). Furthermore, Western blot results showed that the expression of RUNX2, DMP-1 and DSPP was downregulated at 7 days post-induction in the siRNA-depleted DPSCs compared to the control and NC cells (Fig. 7f-g). The above results suggested a key contributory role for lncRNA AC080037.1 in osteogenic and odontogenic differentiation induced by SDF-1 $\alpha$  and BMP-2 in DPSCs.

## **Discussion**

Exogenous signal receptors such as cytokine receptors, chemokine receptors, and other types of G protein-coupled receptors can sense external signals, thereby causing internal cell responses and establishing polarity. Studies have shown that when pulp tissue is damaged, the level of SDF-1 $\alpha$  in the pulp tissue is significantly upregulated<sup>[23]</sup>. SDF-1 $\alpha$  can activate DPSCs with repair potential and further induce DPSCs to move to the damaged site and play an important role in the regeneration of odontoblasts and the formation of restorative dentin.

As in our previous research, SDF-1 $\alpha$  was shown to have a synergistic effect on BMP-2-induced odontogenic differentiation of human SCAP<sup>[10]</sup>. In the present study, RNA was collected after 7 days of treatment with SDF-1 $\alpha$ , and the altered expression of lncRNAs in the osteo/odontogenic differentiation of DPSCs was investigated. The present results identified transitional, global alterations of lncRNAs, which were involved in key signalling pathways for odontoblast differentiation. For example, 134 lncRNAs showed significantly upregulated expression, and 72 lncRNAs showed significantly downregulated expression. The qRT-PCR results were consistent with those of the microarray. Notably, lncRNA AC080037.1 expression was upregulated in our study; this change may be related to the recombination of

microtubules and actin. The present data further suggested that one lncRNA, lncRNA AC080037.1, is essential for efficient odontogenic differentiation of DPSCs.

In the present study, GO and CNC analyses were conducted to predict the biological functions and underlying mechanisms of the differentially expressed lncRNAs during migration and differentiation. GO analyses mainly include the following three aspects: biological process, cellular component and molecular function. The top 10 of the results pathways of GO enrichment analysis were selected as the principal nodes of the DAG, and the results of GO analysis are presented as DAG, in which the branch represents inclusion, and the defined functions ranging from top to bottom decreased. In addition, the related GO terms are displayed together through their inclusive relationships, and the depth of the colour represents the degree of enrichment. We found that several factors related to cytoskeletal changes and vesicle synapses, including synaptic vesicle budding, protein localization to the cytoskeleton, positive regulation of blood vessel diameter, vascular endothelial cell proliferation, and regulation of attachment of spindle microtubules to the kinetochore, were enriched in the GO analysis.

lncRNAs exhibit a variety of different cellular localizations and functional regulatory modes. These molecules play an important role in regulating the differentiation and signal transmission of mesenchymal stem cells. Different lncRNAs can affect cell adhesion, migration and differentiation by reorganizing the cytoskeletal structure and regulating the expression of Rho GTPase pathway molecules<sup>[24]</sup>.

After the chemokine SDF-1 $\alpha$  binds to the G protein-coupled receptor CXCR4, it mediates the activation of Rho family GTPases (CDC2, RhoA and Rac1) and participates in the regulation of the cytoskeleton, the formation of cell membrane protrusions and cell adhesion<sup>[25]</sup>. The Rho GTPase and Rho signalling pathways play an important role in the polarization and even the complete differentiation process of odontoblasts<sup>[26]</sup>. CDC42 is an important member of the Rho family of proteins. This molecule can bind to guanine trinucleotides (GTPs) and play an important role as a "molecular switch" in cell signal transduction, regulating the signal pathways formed by multiple polarities and cell connections. The formation of polar protein complexes and the localization of polar protein complexes are essential steps in the establishment of cell top-bottom polarity<sup>[27]</sup>. Studies have reported that GTPases RhoA and Rac1 are important for amelogenin<sup>[28]</sup>. In this study, we found that knockdown of lncRNA AC080037.1 decreased the expression of CDC42, RhoA and Rac1 in DPSCs.

Studies have shown that lncRNAs can directly interact with cytoskeletal proteins, change the three-dimensional structure of cells, and regulate the cytoskeleton through the Rho GTPase signalling pathway<sup>[24]</sup>. Therefore, the interaction among lncRNAs, the Rho GTPase signalling pathway and the cytoskeleton is the basis for cell movement, which ultimately leads to cell polarization and migration. Studies have reported that lncRNA-H19 activates the CDC42/PAK1 pathway by targeting miR-15b to promote the proliferation, migration and invasion of liver cancer cells. Shi S et al. found that lncRNA XLOC010623 can activate the TIAM1/Rac1 and RhoA-ROCK2 signalling pathways, thereby increasing the expression of focal adhesion proteins and inducing the migration of adipose tissue-derived stem cells<sup>[29]</sup>.

Van Grembergen O et al. found that in the breast cancer cell line MDA-MB-231, LINC00152 regulates target genes involved in cytoskeletal remodelling, including tubulin tyrosine ligase and Rho GTPase Rhobtb3<sup>[30]</sup>.

Moreover, in this study, we used RUNX-2, DSPP and DMP-1 as markers of odontoblastic differentiation in DPSCs. RUNX-2, a transcription factor, is crucial and necessary in the early stage of osteogenic differentiation<sup>[31, 32]</sup>. The expression of DSPP, which encodes dentin phosphoprotein (DPP) and dentin sialoprotein (DSP), was observed during odontoblastic differentiation<sup>[31, 32]</sup>. Furthermore, other studies have used DSPP as a marker for odontoblastic differentiation<sup>[34, 35]</sup>. In addition, the DMP-1 gene has been reported to be present during development of the tooth, and thus, it is important in the identification of odontoblastic differentiation<sup>[36–38]</sup>. Our study showed that knockdown of lncRNA AC080037.1 before the addition of SDF-1 $\alpha$  and BMP-2 resulted in decreased expression of RUNX-2, DMP-1 and DSPP. Therefore, we believe that lncRNA AC080037.1 is involved in the regulation of DPSCs after SDF-1 $\alpha$  induction in this experiment, but the site of action requires further exploration.

## Conclusions

In conclusion, differential expression patterns of lncRNAs were shown in DPSCs before and after SDF-1 $\alpha$  induction in the microenvironment. The results showed that 134 lncRNAs had significantly upregulated expression, while 72 lncRNAs had downregulated expression. This result indicates that multiple different regulators are involved in inducing osteo/odontogenic differentiation of DPSCs, which provides a reference for subsequent experiments. In addition, GO analyses and CNC analysis were utilized to preliminarily investigate the potential mechanism of odontogenic differentiation of DPSCs. Furthermore, our results highlighted the significant involvement of one lncRNA, AC080037.1, in the positive regulation of the osteo/odontogenic differentiation of DPSCs and indicated that lncRNA AC080037.1 could be a potential target in regenerative endodontics. We will further assess this molecule in future experiments, examine its role in osteo/odontogenic differentiation of DPSCs and explore its regulatory mechanism and target genes during this process.

## Declarations

### Ethics approval

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards and was approved by the Ethical Committee of Hospital of Stomatology, Fourth Military Medical University, Xi'an, China (Protocol Number: IRB-REV-2021019). Written informed consent was obtained from each patient, under the approved guidelines set by the Fourth Military Medical University, Stomatological Hospital.

### Data Availability

The microarray datasets supporting the conclusions of this article are available (GEO accession:GSE183162). The other relevant datasets have been uploaded as part of additional files.

### **Competing interests**

The authors have no conflicts of interest regarding the publication of this article to declare.

### **Authors' Contributions**

Min Xiao and Bo Yao contributed equally to this work. QY contributed to the conception and design of the study. MX and BY performing experimental procedures and drafting the manuscript. YB and XM contributed to data acquisition, analysis and interpretation. JW analyzed the data. All authors read and approved the final manuscript.

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### **Consent for publication**

Not applicable.

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## Tables

**Table 1.** qRT-PCR Primers

Genes	forward primer	reverse primer
CDC42	CCATCGGAATATGTACCGACTG	CTCAGCGGTCGTAATCTGTCA
RhoA	AGCCTGTGGAAAGACATGCTT	TCAAACACTGTGGGCACATAC
RAC1/2/3	ATGTCCGTGCAAAGTGGTATC	CTCGGATCGCTTCGTCAAACA
RUNX-2	CCCGTGGCCTTCAAGGT	CGTTACCCGCCATGACAGTA
DSPP	GCATTTGGGCAGTAGCATGG	CTGACACATTTGATCTTGCTAGGAG
DMP-1	ACTGTGGAGTGACACCAGAACACA	AGCTGCAAAGTTATCATGCAGATCC
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
lncRNA AC080037.1	ACGGTGCAACTTCCATCTTACT	TGGAACCATCACTCATACAAACAC

**Table 2.** RNA Quantification and Quality Assurance by NanoDrop ND-1000

Sample	OD260/280 Ratio	OD260/230 Ratio	Conc. (ng/μl)	Quantity (ng)
Control 1	1.98	2.28	1431.16	85869.60
Control 2	1.98	2.33	1222.86	73371.60
Control 3	1.99	2.27	1007.95	60477.00
Induction group 1	1.99	2.28	1165.06	46602.40
Induction group 2	1.99	2.07	897.12	35884.80
Induction group 3	1.96	2.16	887.15	35486.00

**Table 3.** The top 10 up-regulated expressed lncRNAs determined by microarray.

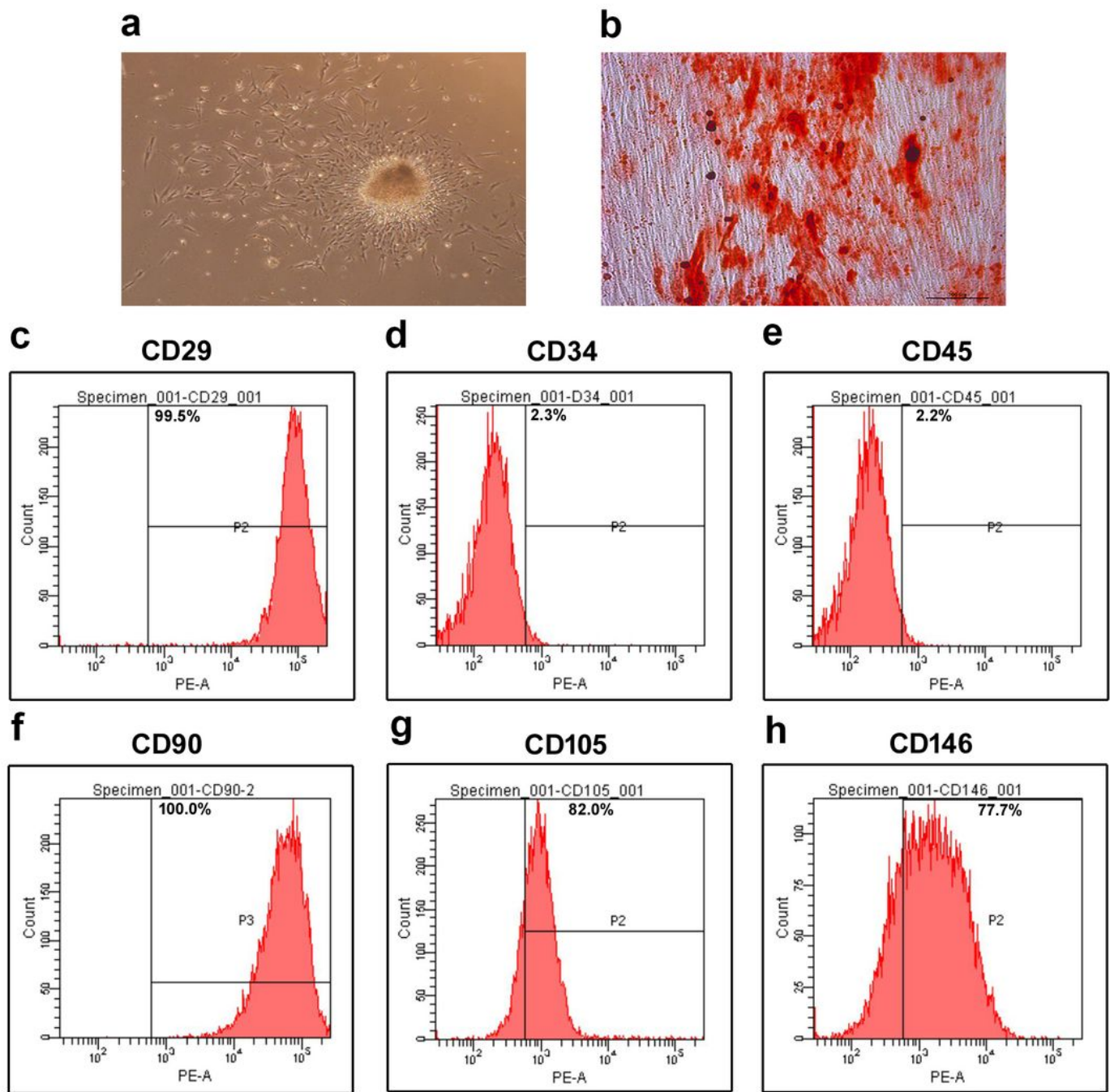


Transcript_ID	P-value	Fold Change
ENST00000521002	0.005568589	2.3698723
ENST00000591400	0.033484458	2.3198148
ENST00000443776	0.033595669	2.2168101
TCONS_00028615	0.039949741	2.0929568
ENST00000600222	0.048408125	1.9943659
ENST00000485153	0.002524464	1.9094213
ENST00000609338	0.018694563	1.8875282
ENST00000563759	0.026047232	1.8305166
T132737	0.016610563	1.8240722
ENST00000542427	0.038243253	1.8077866

**Table 4.** The top 10 down-regulated expressed lncRNAs determined by microarray.

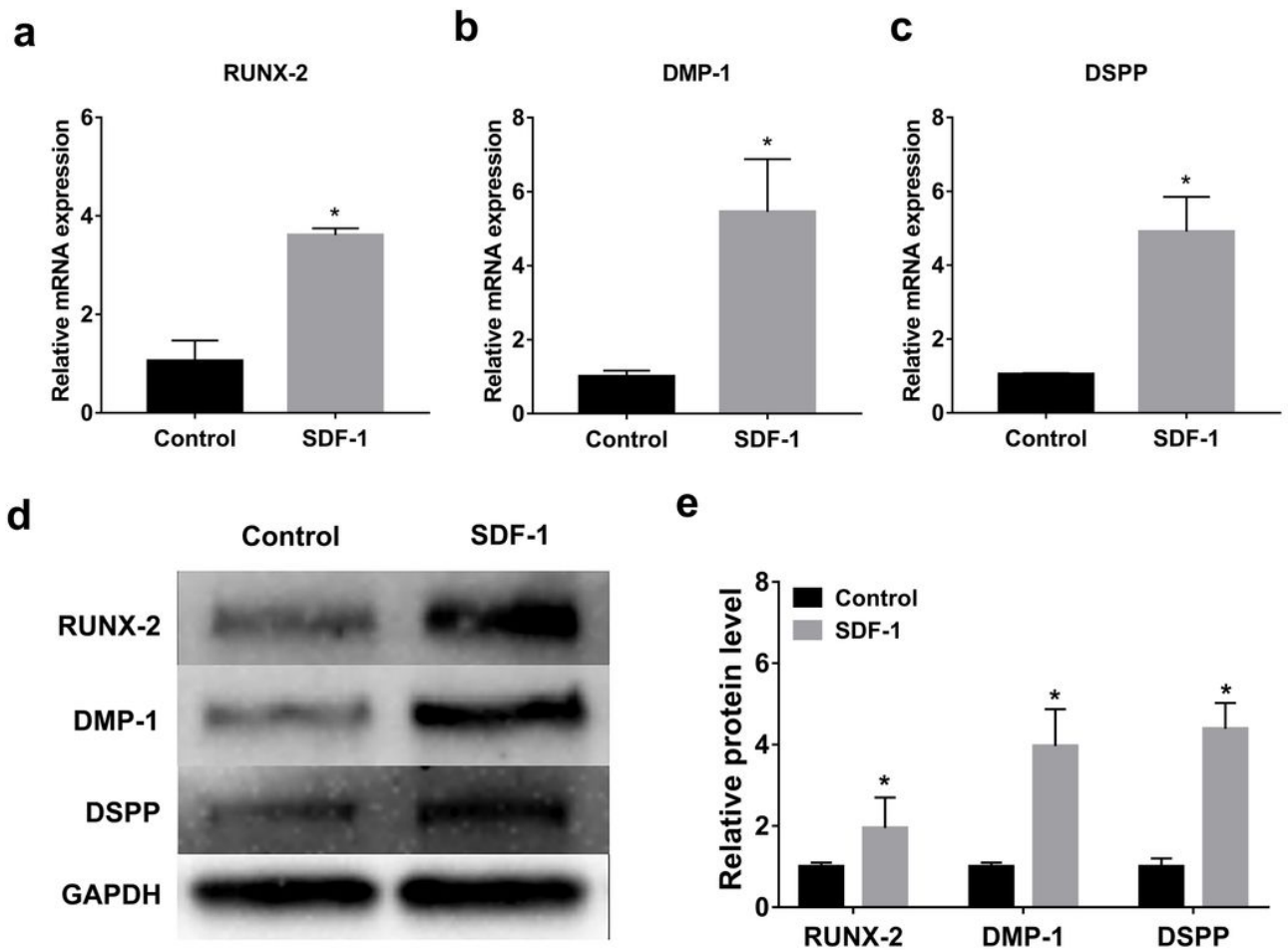
Transcript_ID	P-value	Fold Change
T183545	0.017243588	9.0026448
BIG-lncRNA-547.1	0.048121993	4.5696956
T356015	0.049988556	2.5679189
TCONS_00003744	0.008115508	2.0335586
FTMT22400010498	0.04619673	2.0223942
ENST00000584898	0.049271263	1.9672648
T104971	0.009285972	1.9279512
ENST00000418985	0.036751388	1.9069384
NR_036608	0.020398345	1.8754223
ENST00000650190	0.028465501	1.8236636

## Figures



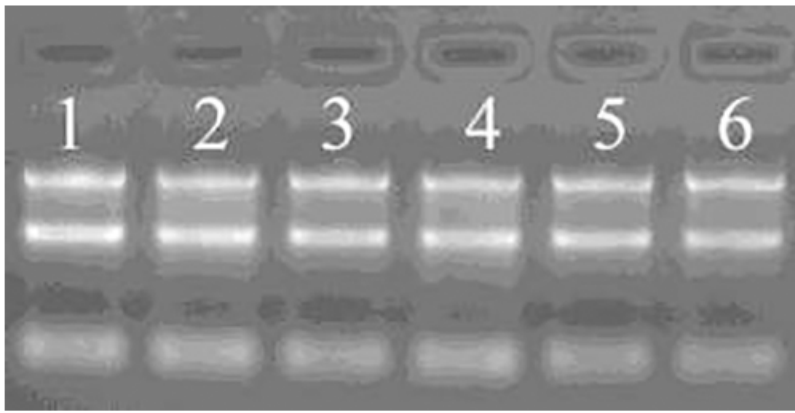
**Figure 1**

Osteo/odontogenic differentiation and surface molecule characterization of DPSCs. (a) Morphological observation of primary culture DPSCs on day 4. (b) Alizarin red S staining for mineralized nodules after odontogenic induction for 14 days. Surface markers and the osteo/odontogenic potential of DPSCs: (c) CD29, (d) CD34, (e) CD45, (f) CD90, (g) CD105 and (h) CD146. (scale bar = 100  $\mu\text{m}$ )



**Figure 2**

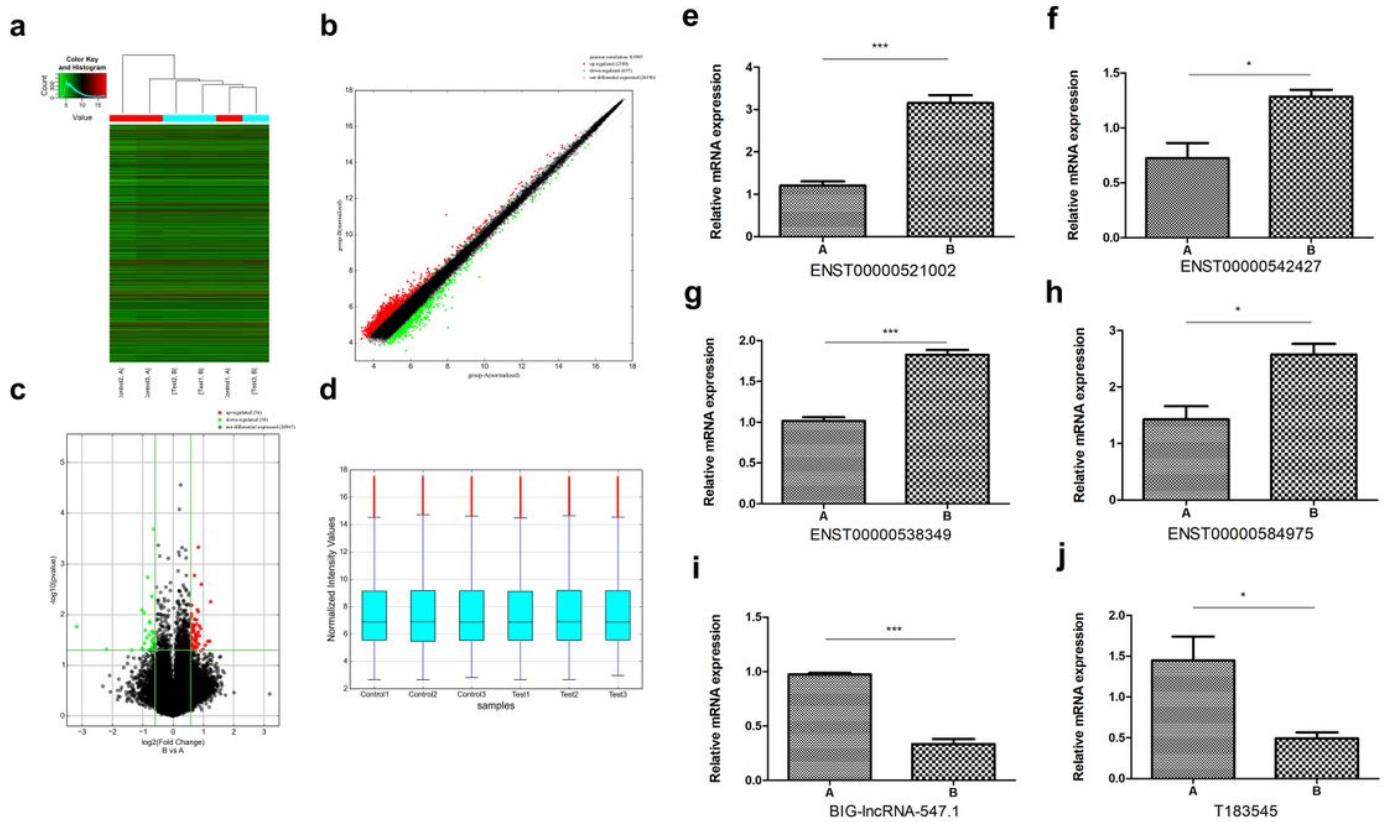
SDF-1 affects RUNX-2, DMP-1 and DSPP expression. Total RNA and protein were extracted from cells, and real-time RT-PCR and Western blot analyses were performed to quantify RUNX-2, DMP-1 and DSPP expression. (a,b) Relative gene expression levels of RUNX-2, DMP-1 and DSPP on day 7. The expression of all target genes was normalized to that of GAPDH. (c,d) Protein expression of RUNX-2, DMP-1, and DSPP on day 7. Data are shown as the mean  $\pm$  SD; treated group versus control group: \*  $P < 0.05$ .



Lane 1: Total RNA of Control 1  
 Lane 2: Total RNA of Induction group 1  
 Lane 3: Total RNA of Control 2  
 Lane 4: Total RNA of Induction group 2  
 Lane 5: Total RNA of Control 3  
 Lane 6: Total RNA of Induction group 3

**Figure 3**

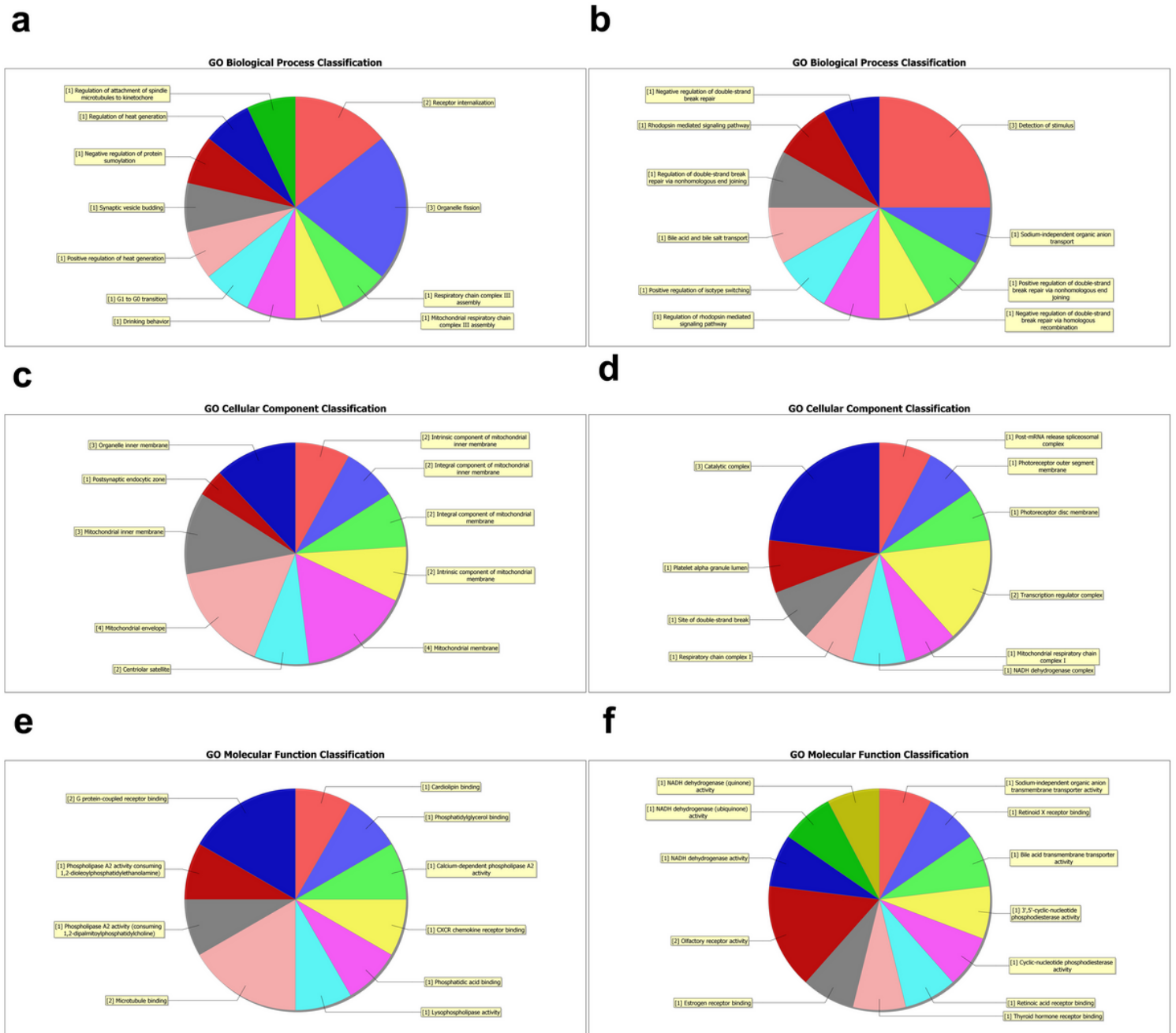
RNA Integrity and gDNA contamination test by Denaturing Agarose Gel Electrophoresis. The 28S and 18S ribosomal RNA bands were fairly sharp, intense bands. The intensity of the upper band was about twice that of the lower band.



**Figure 4**

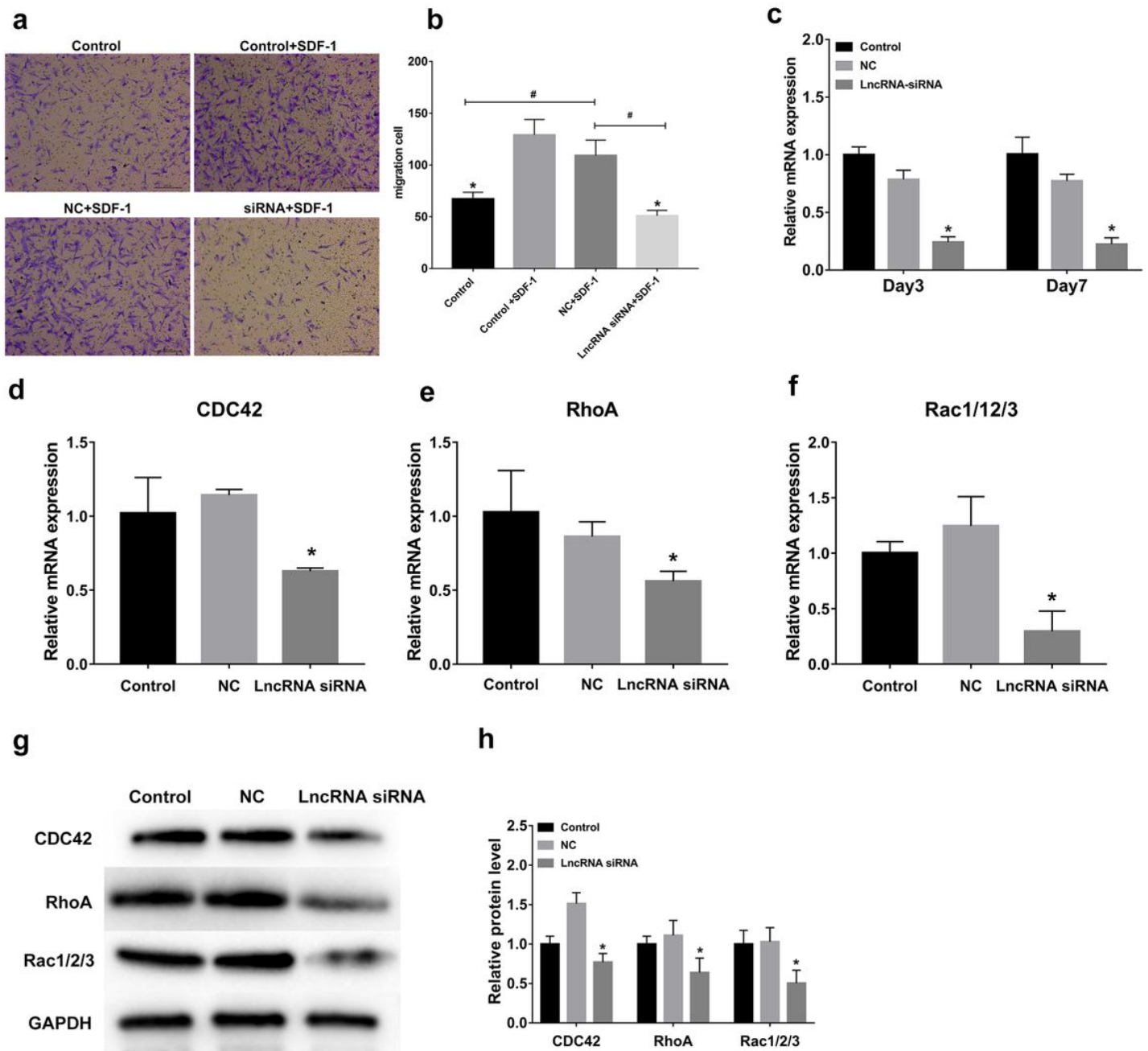
The different expression of lncRNA amongst SDF-1 $\alpha$ -induced DPSCs and uninduced DPSCs. Scatter plot (b), Volcano plot (c), Box plot (d), of lncRNA. Box plots was a convenient way to visualize the distributions of a dataset. We could find there were 134 lncRNAs were significantly up-regulated and 72 lncRNAs were

significantly down-regulated. The results of cluster analysis on the microarray analysis showed significant differences between the two groups. Quantitative Real-time Polymerase Chain Reaction. We chose 4 up-regulated lncRNAs (e-h) and 2 down-regulated lncRNAs (i-j) randomly to test the accuracy of microarray analysis by qRT-PCR.



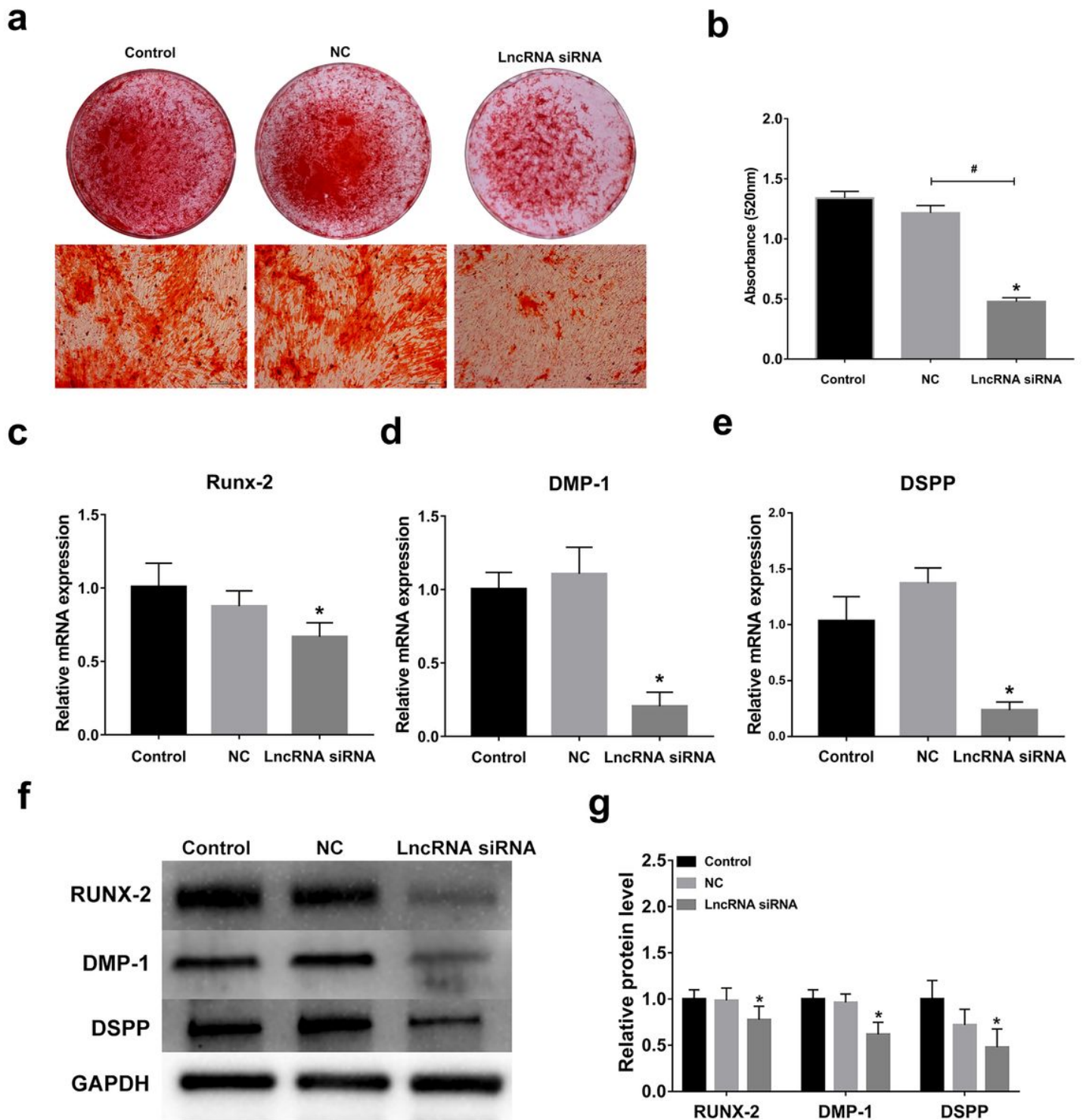
**Figure 5**

Gene Ontology (GO) analysis which covers three domains: biological process (a and d), cellular component (b and e) and molecular function (c and f).



**Figure 6**

(a) Transmembrane migration of DPSCs as determined by microscopy and 0.1% crystal violet staining. (scale bar = 200  $\mu$ m) (b) Numbers of migrated cells in each group. (c) After transfection with LncRNA AC080037.1 siRNA for 3 and 7 days, DPSCs were subjected to mRNA analysis. Total RNA and protein were extracted from cells, and real-time RT-PCR (d-f) and Western blot analyses (h-i) were performed to quantify CDC42, RhoA and Rac1/2/3 expression at day 3. The expression of all target genes was normalized to that of GAPDH. The quantitative results are presented the means  $\pm$  SD of three independent experiments. Compared with the NC group: \* $P$ <0.05.



**Figure 7**

Effect of LncRNA AC080037.1 on the odontogenic differentiation of DPSCs mediated by SDF-1 $\alpha$  and BMP-2. (a) Alizarin red S staining of DPSCs: After transfection with LncRNA AC080037.1 siRNA, cells were incubated with 100 ng/mL SDF-1 $\alpha$  and BMP-2 for 14 days. (b) Quantitative assessment of alizarin red S staining of DPSCs. (c-e) Relative gene expression levels of LncRNA AC080037.1, RUNX-2, DMP-1 and DSPP at day 7; The expression of all target genes was normalized to that of GAPDH. (f-g) The protein

expression of RUNX-2, DMP-1, and DSPP on day 7. Data are shown as the mean  $\pm$  SD; treated group versus NC group: \*  $P < 0.05$ .

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [figS1.png](#)