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LncRNA XIST facilitates the odontogenic differentiation of dental pulp stem cells via the FUS/ZBTB16

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Abstract

Objective: This study aims to explore the regulatory mechanism of long noncoding RNA X inactive specific transcript (lncRNA XIST) in the odontogenic differentiation of human dental pulp stem cells (hDPSCs). hDPSCs were obtained from freshly extracted third molars and identified by flow cytometry. Methodology: Odontogenic differentiation was induced in mineralized culture medium, and hDPSCs were infected with shRNA lentivirus targeting XIST or fused in sarcoma (FUS), followed by detection of alkaline phoshpatase (ALP) activity, alizarin red staining of mineralized nodules, Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) quantification of XIST expression, and Western blot analysis of FUS, ZBTB16, and odontogenic differentiation markers (DSPP and DMP1). IF-FISH was performed to detect the cellular localization of XIST and FUS. RIP assay validated the XIST and FUS binding. ZBTB16 mRNA stability was tested after actinomycin D treatment. hDPSCs were infected with oe-ZBTB16 lentivirus and further treated with sh-XIST for a combined experiment. Results: LncRNA XIST was highly expressed in hDPSCs with odontogenic differentiation. Downregulation of XIST or FUS weakened the ALP activity of hDPSCs, reduced mineralized nodules, diminished DSPP and DMP1 expressions. XIST binds to FUS to stabilize ZBTB16 mRNA and promote ZBTB16 expression. ZBTB16 overexpression partially reversed the inhibitory effect of XIST silencing on odontogenic differentiation of hDPSCs. Conclusion: In conclusion, XIST stabilizes ZBTB16 mRNA and promotes ZBTB16 expression by binding to FUS, thereby facilitating the odontogenic differentiation of hDPSCs.

Keywords: Dental pulp stem cells. Odontogenic differentiation. LncRNA XIST. FUS. ZBTB16.

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Introduction

Human dental pulp stem cells (hDPSCs) are developmentally originated from the embryonic neural crest and share some properties resembling mesenchymal stem cells (MSCs).[1] hDPSCs express MSC surface markers such as CD29, CD44, and CD105, and hold the capacity for self-renewal and multipotent differentiation.[2] Compared with other MSCs, hDPSCs exhibit greater clonality, proliferation, and mineralization potential, and can be obtained via simple minimally invasive procedures.[3] Under the stimulation of external trauma or bacterial infection, resident hDPSCs migrate to the injury site to promote the secretion of pro-regenerative cytokines in response to the inflammatory microenvironment, and then differentiate into odontoblasts, which are responsible for dental-pulp complex formation and mineralization.[4] Due to their promising potential of odontogenic differentiation, hDPSCs play a vital role in dentin-pulp repair and regeneration, representing potent cell resources for numerous regenerative medicine applications.[5] The odontogenic differentiation of hDPSCs is a complicated process that comprises various molecules.[6] Whilst acknowledging the clinical significance of odontogenic differentiation of hDPSCs, its exact mechanism remains elusive. Therefore, it is imperative to clarify the molecular mechanism of hDPSCs differentiating into odontoblasts, thereby improving their clinical applications in dentin-pulp regeneration.

Long noncoding RNAs (lncRNAs), a large class of RNA molecules with transcript length exceeding 200 nt, have been recognized as pivotal regulators in the process of odontogenic differentiation.[7] LncRNA microarray profiling has unveiled a plenty of differentially expressed lncRNAs during the process of odontogenic differentiation, implying the involvement of lncRNAs in the odontoblast-like differentiation of hDPSCs.[8] LncRNA X inactive specific transcript (XIST) results in inheritable inactivation of one of X chromosomes during embryonic development. Dysregulation of XIST has been pathologically linked with a whole spectrum of human diseases, particularly cancers.[9] On a separate note, emerging evidence has unraveled that lncRNA XIST is required for efficient osteogenic differentiation of hDPSCs, possibly due to a regulatory role in a group of mRNAs associated with this process[10]. XIST expression is elevated in odontogenic dental mesenchymal cells, and XIST dysregulation leads to the deficiency of odontogenic potential of hDPSCs.[8] Nevertheless, the exact mechanism of XIST underlying the odontogenic differentiation of hDPSCs is still unknown.

Fused in sarcoma (FUS) is a multifunctional DNA/RNA-binding protein that participates in the regulation of gene transcription, DNA repair, as well as RNA splicing, transport, and translation.[11] Existing studies have demonstrated through pathological and genetic analyses that FUS contributes significant functions in neurodegenerative diseases, typically amyotrophic lateral sclerosis and frontotemporal lobar degeneration.[12] A study detected FUS expression in the dental epithelium and dental mesenchyme of mice during early tooth development.[13] However, the role of FUS in odontogenic differentiation of hDPSCs has not been discussed yet.

The zinc finger and BTB domain-containing 16 (ZBTB16) protein-also known as the promyelocytic leukemia zinc finger-is expressed by several tissues/cell types, including neuronal, muscle, hematopoietic, respiratory, and reproductive cells,[14] and regulates many biological processes, including stem cell renewal, proliferation, cell cycle regulation, differentiation, and apoptosis.[15] ZBTB16 induces osteogenic differentiation marker genes in dental follicle cells.[16] The adeno-associated virus-shZBTB16 injection has been proven to inhibit osteogenic differentiation and reduce orthodontic tooth movement distance *in vivo*.[17] Notably, lncRNA XIST can facilitate S1P-mediated osteoclast differentiation by interacting with FUS.[18] Accordingly, we speculate the existence of an interaction between XIST and FUS/ZBTB16 in odontogenic differentiation. This work aims to establish the molecular mechanism of XIST influencing the odontogenic differentiation of hDPSCs, to confer a novel rationale for dental pulp/dentin regeneration.

Methodology

Ethics statement

This study strictly followed the *Helsinki Declaration* and was approved by the Ethics Committee of our hospital (Approval number: 2019KY018). All participants provided signed informed consent forms.

Culture of primary hDPSCs

Intact, healthy, and non-carious third molars for orthodontic treatment were obtained from individuals aged 18-22 (males). Before tooth extraction, diluted povidone iodine was used for oral cleaning and disinfection. After extraction, a sterilized diamond knife was used to cut the tooth at the pulp-enamel junction, and the pulp tissue was gently removed from the tooth and soaked in a 37°C digestive solution containing 3 mg/mL of *Clostridium histolyticum* collagenase type I (Sigma Aldrich, St. Louis, MO, USA) and 4 mg/mL of *Bacillus polymyxa* collagenase type II (Gibco, Life Technologies, New York, NY, USA) for 1 h. After digestion, the sample was filtered through a cell filter with a 70 μm pore size and centrifuged at 400 g for 10 min (Falcon, Corning, New York, NY, USA). The hDPSCs were isolated and cultured as previously described.[19] Simply put, hDPSCs were selected using stro-1-labeled magnetic beads and cultured in growth medium (GM) with high sugar Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin. The cells were cultured at 37°C with 5% CO_2, and after confluence, trypsin/EDTA solution (Gibco, USA) in a 1:3 ratio was used for trypsinization and passage culture. The culture medium was changed every 48 h. The cells between the second and sixth generations were used in this study.

To obtain multipotent differentiation ability, the cells were seeded into a 24-well plate in normal growth medium (GM) at a density of 2×1.0^4 cells per well. After 24 h, the culture medium was changed to osteogenic induction medium (OM) or adipogenic induction medium (AM). The culture medium was refreshed every 48 h. On the 7th day, alkaline phosphatase (ALP) activity detection was performed, and on the 21st day, alizarin red staining (ARS) and Oil Red O staining were performed to observe the mineral deposition and lipid droplet accumulation in the cells, respectively. The adipogenic medium (AM) contained 0.1 mg/ml insulin (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), 1 mM IBMX (Thermo Fisher Scientific), and 0.2 mM indomethacin (Sigma-Aldrich). The osteogenic medium (OM) consisted of adding 5 mM glycerophosphate (Sigma-Aldrich), 50 μg/ml ascorbic acid (Sigma-Aldrich), and 250 nM dexamethasone (Sigma-Aldrich).

Flow cytometry

hDPSCs were incubated with anti-CD29 (1:1000, ab218273, Abcam, Cambridge, MA, USA), CD44 (1:10, ab269300, Abcam), CD105 (1:10, ab11415, Abcam), CD34 (1:100, ab131589, Abcam), and CD45 (1:100, ab269297, Abcam) in the dark for 30 min, with cell suspension free of antibodies in phosphate-buffered saline (PBS) as a negative control. Then, the cells were washed with PBS thrice to remove unbound antibodies. After PBS resuspension, the cells were analyzed by a flow cytometer (FACS Calibur; BD Biosciences, NJ, USA), and FlowJo software (FlowJo, Ashland, OR, USA) was used for data analysis.

Cell treatment

XIST and FUS were silenced by small-hairpin RNA (shRNA). Each shRNA plasmid was generated using one pLKO vector. Zinc finger and BTB domain containing 16 (ZBTB16) overexpression pcDNA3.1-ZBTB16 (oe-ZBTB16), sh-XIST, sh-FUS, and corresponding negative controls (oe-NC, sh-NC) were obtained from GeneChem (Shanghai, China). To generate stable cell lines, we packed the aforementioned shRNA or overexpression vector with lentivirus. Shortly, ViralPowerTM Packaging Mix (Invitrogen, Carlsbad, CA, USA) and constructed vectors were co-transfected into 293 T cells using LipofectamineTM 2000 (Invitrogen). After 48 h, the lentivirus was harvested and filtered through a 0.45μm sieve. During infection, hDPSCs were cultured in lentivirus solution containing 10 μg/mL polyene (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 24 h. To obtain stable cell lines, the cells were treated with 1 μg/mL puromycin.

ALP activity detection

After cultured in MSC osteogenic differentiation medium (OM) for 7 days, the cells were rinsed with PBS twice, fixed with 4% paraformaldehyde, and incubated with ALP staining buffer at room temperature for 20-60 min. After washing with PBS to terminate the reaction, the cells were lysed in buffer containing 0.1% Triton X100 and the ALP activity was measured using an alkaline phosphatase assay kit (Beyotime, Shanghai, China). The images were captured using a microscope (Leica Microsystems, Wetzlar, Hessen, Germany).

ARS staining

After cultured in OM for 21 days, cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min, and incubated with ARS solution at room temperature

for 1 h. Images were taken using a microscope (Leica Microsystems).

Oil red O staining

After 21 days of cultivation in AM medium, the cells were stained with Oil Red O. Briefly, cells were rinsed with PBS and fixed in 10% formalin solution for 20 min. After PBS washing, the cells were washed with 60% isopropanol, dried for 1 min, and stained with 200 µL oil red O solution (Merck, Saint Louis, MO, USA) for 20 min. Then, cells were washed twice with 60% isopropanol and once with distilled water. The results were observed under an optical microscope (Primovert, Zeiss, Jena, Germany).

qRT-PCR

The total RNA was extracted from hDPSCs using RNA-quick purification kit (YISHAN Biotechnology, Shanghai, China) and reverse transcribed into cDNA using Hifair®Ⅲ1st Strand cDNA Synthesis SuperMix for qPCR (YEASEN, Shanghai, China). Hieff®qPCR SYBR Green Master Mix (YEASEN, China) was used for qRT-PCR. The relative expression of genes was calculated using the $2^{-\Delta\Delta CT}$ method,20 with GAPDH as the internal reference for mRNA and lncRNA. Primer sequences are shown in Figure 1. The detection of target genes was carried out after virus infection and 21 days of OM incubation.

Western blot

The total protein was extracted using radioimmunoprecipitation assay buffer plus 1 mM phenylmethylsulfonyl fluoride (ST506, Beyotime). After SDS-PAGE, the protein was transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA) and incubated with primary antibodies FUS (1:1000, ab23439, Abcam), ZBTB16 (1:1000, ab104845, Abcam), DSPP (1:1000, NBP2-92546, Novus Biological Inc., Littleton, CO, USA), DMP1 (1:1000, PA5-76874, Invitrogen), and β-actin (1:1000, ab8227, Abcam) overnight. Following incubation with the secondary antibody IgG (1:100, ab6721, Abcam), the protein bands were visualized using enhanced chemiluminescence reagent (Millipore), with β-actin as the normalized control for total protein lysis buffer. The detection of target proteins was carried out after virus infection and 21 days of OM incubation.

Bioinformatics

The binding probability between XIST and FUS, FUS and ZBTB16 mRNA was predicted with the RPISeq database (http://pridb.gdcb.iastate.edu/RPISeq/index.html).[21] RPISeq is a family of machine learning classifiers for predicting RNA-protein interactions using only sequence information. RPISeq predictions are based on Random Forest (RF) or Support Vector Machine (SVM) classifiers, trained and tested on two non-redundant benchmark datasets of RNA-protein interactions, RPI2241 and RPI369, extracted from PRIDB, a comprehensive database of RNA-protein complexes extracted from the PDB.

Immunofluorescence in situ hybridization (IF-FISH)

The cells were permeabilized with Triton-X, fixed with paraformaldehyde, and incubated with primary antibody solutions consisting of 1 µL of antibody in 100 µL of RNAse inhibited PBT for 4-6 h. After the primary antibody incubation, the coverslips were washed to remove unbound antibodies. The coverslips were then incubated for 1 h in a secondary fluorescently labelled antibody (1 µL of secondary antibody solution in 100 µL of RNAse inhibited PBT) for 1 h. After incubation, the coverslips were washed again and fixed for 10 min

Name	Sequence (5'-3)
LncRNA XIST	F: GACTAATCACCTACTTATCAGAC
	R: GGCTAGGGCTGGGGGGTTAGGG
FUS	F: ATGGCCTCAAACGATTATACCCA
	R: GTAACTCTGCTGTCCGTAGGG
ZBTB16	F: GAGATCCTCTTCCACCGCAAT
	R: CCGCATACAGCAGGTCATC
GAPDH	F: GGAGCGAGATCCCTCCAAAAT
	R: GGCTGTTGTCATACTTCTCATGG

Note: LncRNA XIST, long non-coding RNA X inactive specific transcript; FUS, FUS RNA binding protein; ZBTB16, zinc finger and BTB domain containing 16; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure 1- PCR primer sequences

with paraformaldehyde. The coverslips were then incubated at 37℃ overnight with fluorescent probes targeting XIST RNA. The FISH solution consisted of 10 μL of Cot-1 DNA, 2 uL of salmon sperm DNA and 5 μL of fluorescent probes provided by ThermoFisher. The mixture of probes and decoy DNA was suspended in 20 μL of equal hybridization solution [(20% bovine serum albumin (BSA), 20% dextran sulfate, and 4 × saline sodium citrate (SSC)] and deionized formamide. The next day, the coverslips were incubated for 20 min in a mixture of deionized formamide and SSC (Invitrogen). The coverslips were washed in 2 × then 1 × concentrations of SSC for 20 min each, then incubated for 15 min with a DAPI methanol solution, and finally observed under a confocal microscope.

RNA immunoprecipitation (RIP)

EZ-Magna RIP kit (17-701, Millipore) was used for RNA immunoprecipitation (RIP). A portion of the supernatant was taken as the input and the other portion was incubated with 1 mg magnetic beads pre-coated with IgG antibody (ab172730, Abcam) or FUS antibody (ab23439, Abcam) at 4°C overnight. The RNA complex were incubated with protease K (RIP washing buffer, 10% SDS, and 10 mg/mL protease K; Millipore) for 30 min to digest the remaining protein on the magnetic beads. Finally, the immunoprecipitated RNA was purified, followed by qRT-PCR.

RNA stability detection

hDPSCs were treated with actinomycin D (2 μg/mL; Sigma-Aldrich) for 0, 3, 6, and 12 h respectively. The total RNA was collected at designated time points. qRT-PCR was performed for ZBTB16 mRNA stability analysis.

Statistical analysis

Data analysis and map plotting were performed using the SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The measurement data are presented as mean ± standard deviation. The data were examined for normal distribution and homogeneity of variance. The *t*-test was adopted for comparisons between two groups. One-way or two-way analysis of variance (ANOVA) was employed for the comparisons between multiple groups, following Tukey's multiple comparison test. A $p < 0.05$ indicated a significant difference.

Results

LncRNA XIST is highly expressed in *hDPSCs* with odontogenic differentiation

Flow cytometry identification of our cultured primary hDPSCs showed positive results for CD29, CD44, and CD105, and negative results for CD34 and CD45 (Figure 2A). The ALP activity of cells was enhanced after 7 days of incubation in OM (P=0.0058, Figure 2B). After 21 days, ARS staining showed massive calcified nodules (P=0.0045, Figure 2C) and significantly elevated expressions of DSPP and DMP1 in hDPSCs (P<0.0001, Figure 2D). These results indicated that hDPSCs were differentiated into odontoblasts after OM incubation. Moreover, hDPSCs incubated in OM showed an obviously increased XIST expression profile (P=0.0006, Figure 2E). We speculated that XIST was related to the odontogenic differentiation of hDPSCs. Meanwhile, we incubated hDPSCs in AM, and after 21 days, we observed a large number of lipid droplets in the cells, indicating that hDPSCs had the capacity of adipogenic differentiation (Figure 2F). Briefly, these results suggest that the hDPSCs we cultured have multi-directional differentiation ability.

Silencing of *XIST* inhibits the odontogenic differentiation of *hDPSCs*

We silenced the expression of XIST in hDPSCs through lentiviral infection (P=0.0008, Figure 3A). Silencing of XIST resulted in a weakened ability of hDPSCs to differentiate into odontoblasts, a significant decrease in ALP activity on the 7th day of differentiation induction (P=0.0156), and ARS-stained nodules on the 21st day (P=0.0100) (Figure 3B-C), as well as a significant decrease in the expressions of DSPP (P=0.0003) and DMP1 (P<0.0001) (Figure 3D).

XIST binds to FUS to stabilize *ZBTB16 mRNA* and promote its protein expression

We speculate that XIST may affect the mRNA stability of ZBTB16 by binding to FUS, thereby regulating its protein expression. Based on the IF-FISH assay, we observed that XIST and FUS were co-localized in the cytoplasm of cells (Figure 4A). RPISeq database predicted the binding probability between XIST, FUS, and ZBTB16 mRNA (Figure 4B). RIP assay validated the binding relation between XIST and FUS, as well as FUS and ZBTB16 mRNA (P<0.05, Figure 4C). We silenced FUS expression in hDPSCs

(P<0.0100, Figure 4D-E), and found that ZBTB16 mRNA stability was significantly abated (P<0.0100, Figure 4F). After OM incubation, the expression of ZBTB16 in hDPSCs increased (P<0.0100, Figure 4G-H), and downregulation of XIST or FUS significantly repressed ZBTB16 expression (P<0.0100, Figure 4I-J). Briefly, the binding of XIST and FUS facilitates ZBTB16 protein expression by stabilizing its mRNA stability.

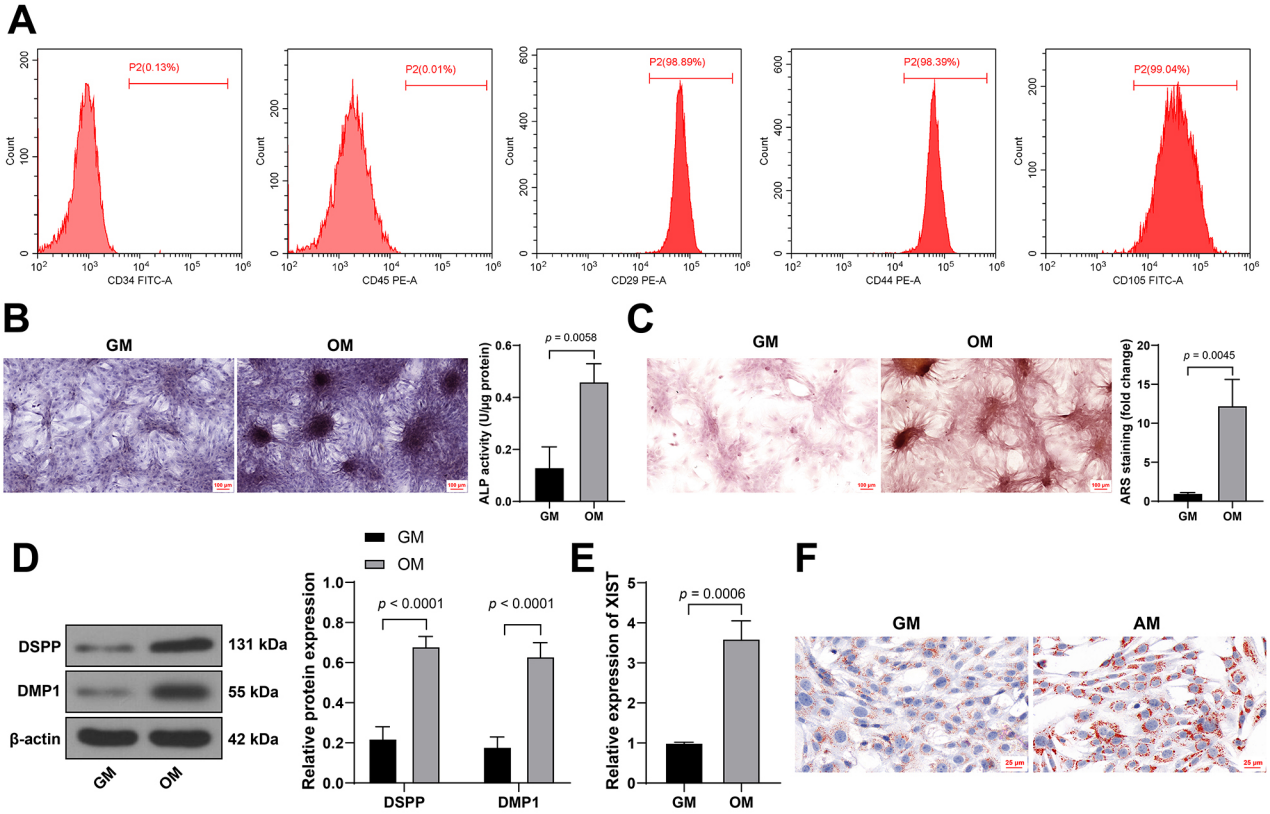

Figure 2- LncRNA XIST is highly expressed in hDPSCs with odontogenic differentiation. Intact, healthy, and non-carious third molars for orthodontic treatment were obtained from individuals aged 18-22 years. A: Detection of cell surface markers (CD29, CD44, CD105, CD34, CD45) by flow cytometry; hDPSCs were incubated in either GM (growth medium) or OM (osteogenic differentiation medium). After 7 days, B: detection of ALP activity. After 21 days, C: ARS observation of mineralized nodules; D: Western blot analysis of DSPP and DMP1 expressions in hDPSCs; E: qRT-PCR quantification of XIST relative expression in hDPSCs. hDPSCs were cultured in AM (adipogenic differentiation medium), and after 21 days, F: Oil Red O staining was performed to observe lipid droplet formation. Cell experiments were conducted in independent triplicate. Data in panels BCE were analyzed by the t-test, and data in panel D were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test.

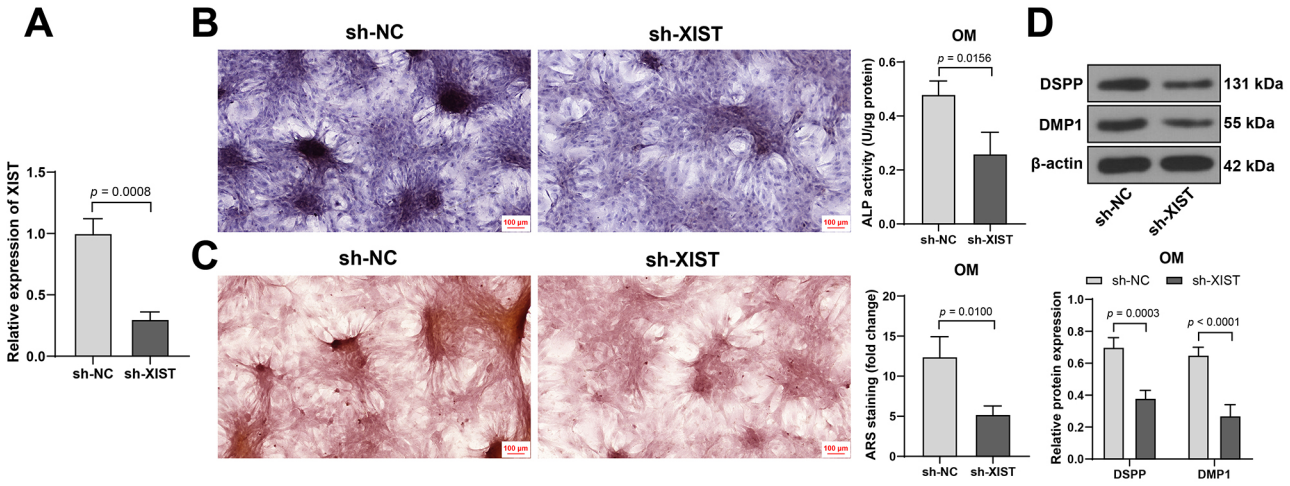

Figure 3- Silencing of XIST inhibits the odontogenic differentiation of hDPSCs. hDPSCs were infected with small hairpin RNA XIST (sh-XIST) lentivirus, with sh-NC as a control, and stably expressed cells were screened using puromycin. A: qRT-PCR quantification of XIST expression in hDPSCs. hDPSCs were cultured in OM (osteogenic differentiation medium). After 7 days, B: detection of ALP activity. After 21 days, C: ARS observation of mineralized nodules; D: Western blot analysis of DSPP and DMP1 expressions in hDPSCs. Cell experiments were conducted in independent triplicate. Data in panels ABC were analyzed by the t-test, and data in panel D were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test.

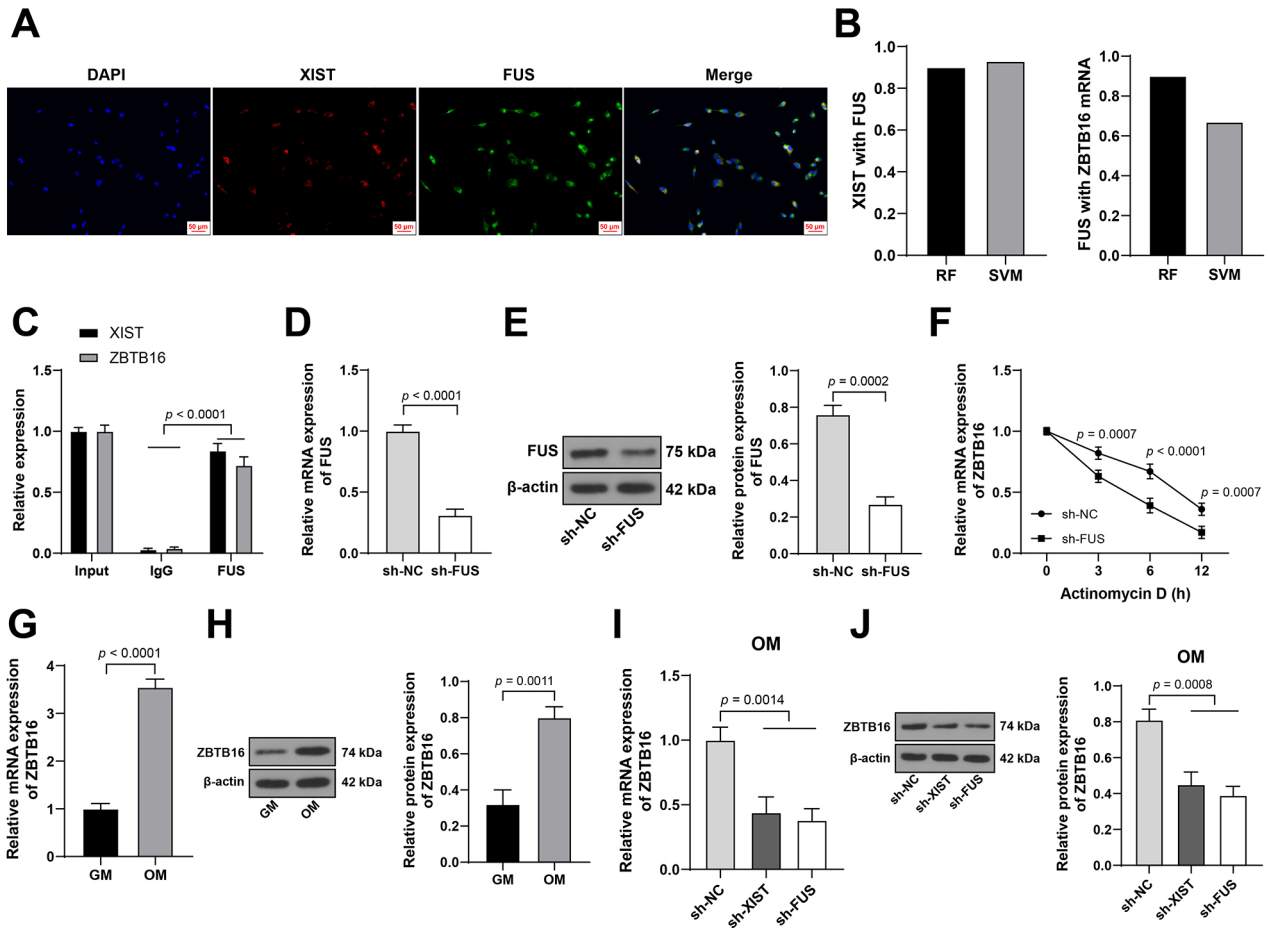

Figure 4- XIST binds to FUS to stabilize ZBTB16 mRNA and promote its protein expression. A: Observation of the localization of XIST and FUS in cells using IF-FISH assay. B: Prediction of the binding probability between XIST, FUS, and ZBTB16 mRNA using the RPISeq database (http://pridb.gdcb.iastate.edu/RPISeq/index.html), RF, Random Forest; SVM, Support Vector Machine. C: RIP validation of the binding between XIST and FUS, and between FUS and ZBTB16 mRNA. hDPSCs were infected with sh-FUS packaged lentivirus, with sh-NC as a control, and stably expressed cells were screened using puromycin. D-E: qRT-PCR and Western blot detection of FUS expression in cells. F: After actinomycin D treatment (2 μg/mL), detection of ZBTB16 mRNA expression. G-J: qRT-PCR and Western blot detection of ZBTB16 expression in cells under different treatments. Cell experiments were conducted in independent triplicate. Data in panels ACDFG were analyzed by the t-test. Data in panels BE were analyzed by two-way ANOVA, and data in panels HI were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

Downregulation of *FUS* inhibits the odontogenic differentiation of *hDPSCs*

We induced odontogenic differentiation in cells with low FUS expression and found a significant decrease in ALP activity (P=0.0162) and the number of ARS-stained nodules (P=0.0097) (Figure 5A-B), as well as a significant decrease in DSPP (P=0.0001) and DMP1 (P=0.0001) expressions (Figure 5C). Such outcome indicates that downregulation of FUS inhibits the odontogenic differentiation of hDPSCs.

Overexpression of *ZBTB16* partially reverses the inhibitory effect of *XIST* silencing on the odontogenic differentiation of *hDPSCs*

Finally, we overexpressed ZBTB16 in cells (P=0.0001, Figure 6A-B) and then treated these cells in combination with sh-XIST. Compared with sh-XIST treatment alone, the expressions of DSPP and DMP1 were significantly elevated after sh-XIST + oe-ZBTB16 treatment (P<0.0001; Figure 6B), and the ALP activity (P=0.0400) and ARS-stained nodules (P=0.0149) of cells were increased (Figure 6C-E), indicating that overexpression of ZBTB16 partially reverses the inhibitory effect of XIST silencing on the odontogenic differentiation of hDPSCs.

Discussion

hDPSCs have gained increasing popularity in relation to dental tissue engineering and regenerative medicine because of their properties of easy accessibility, multi-lineage differentiation, and self-renewal potential.[5] LncRNAs are implicated in a wide array of cellular processes including cell proliferation and differentiation, and aberrant lncRNA profiling

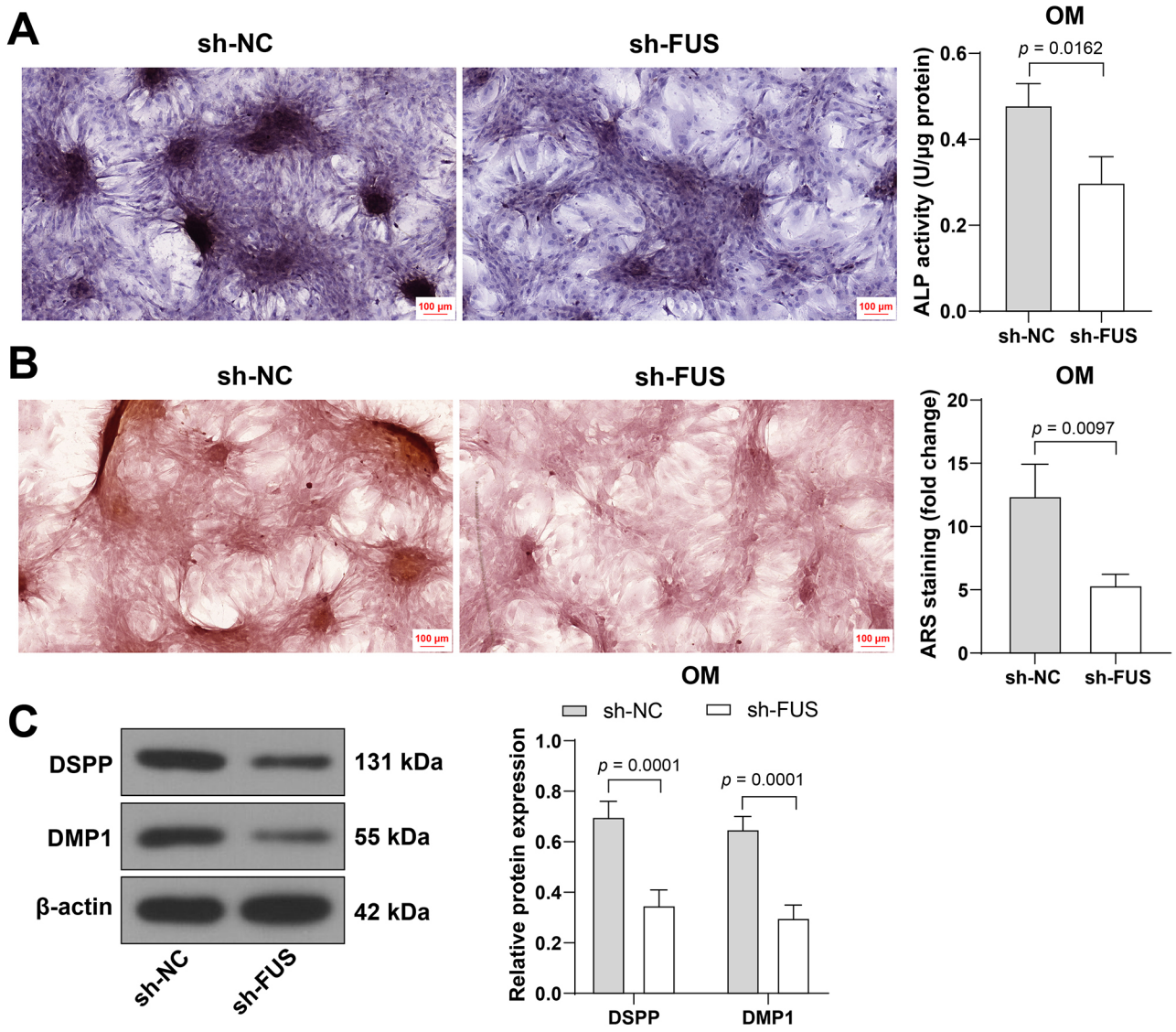

Figure 5- Downregulation of FUS expression inhibits the odontogenic differentiation of hDPSCs. hDPSCs were infected with sh-FUS packaged lentivirus, with sh-NC as a control, and stably expressed cells were screened using puromycin. hDPSCs were cultured in OM (osteogenic differentiation medium). After 7 days, A: detection of ALP activity. After 21 days, B: ARS observation of mineralized nodules; C: Western blot analysis of DSPP and DMP1 expressions in hDPSCs. Cell experiments were conducted in independent triplicate. Data in panels AB were analyzed by the t-test, and data in panel C were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test.

has been linked to the loss of odontogenic potential.[8] For example, lncRNA H19/SAHH axis epigenetically regulates odontogenic differentiation of hDPSCs by inhibiting the DNMT3B-mediated methylation of DLX3.[22] Microarray has also revealed numerous lncRNA-associated ceRNA networks during odontogenic differentiation of hDPSCs.[23] Our results elucidate that lncRNA XIST is highly expressed in hDPSCs with odontogenic differentiation, and XIST binds to FUS to stabilize the mRNA of ZBTB16 and promote its protein expression. Specifically, XIST facilitates odontogenic differentiation of hDPSC via the FUS/ZBTB16.

In this study, we isolated and cultured primary hDPSCs. Flow cytometry results demonstrated that hDPSCs were positive for CD29, CD44, and CD105, and negative for CD34 and CD45. Apart from these MSC markers, hDPSCs also express bone markers in the osteogenic medium including DSPP, DMP-1, and ALP. DSPP is a precursor protein encoding two major non-collagenous proteins necessary for dentin formation: dentin sialoprotein and phosphoprotein.[24] DSPP is involved in the formation of the periodontium as well as tooth structures. DSPP-deficient mice present furcation involvement, cementum, and alveolar bone defect.[25] DMP1, an acidic protein that is essential to the mineralization of bone and dentin, can be found as proteolytically processed fragments in the mineralized tissues.[26] DMP1 is essential for osteogenesis and odontogenesis during both embryonic and postnatal development.[27] ALP, an

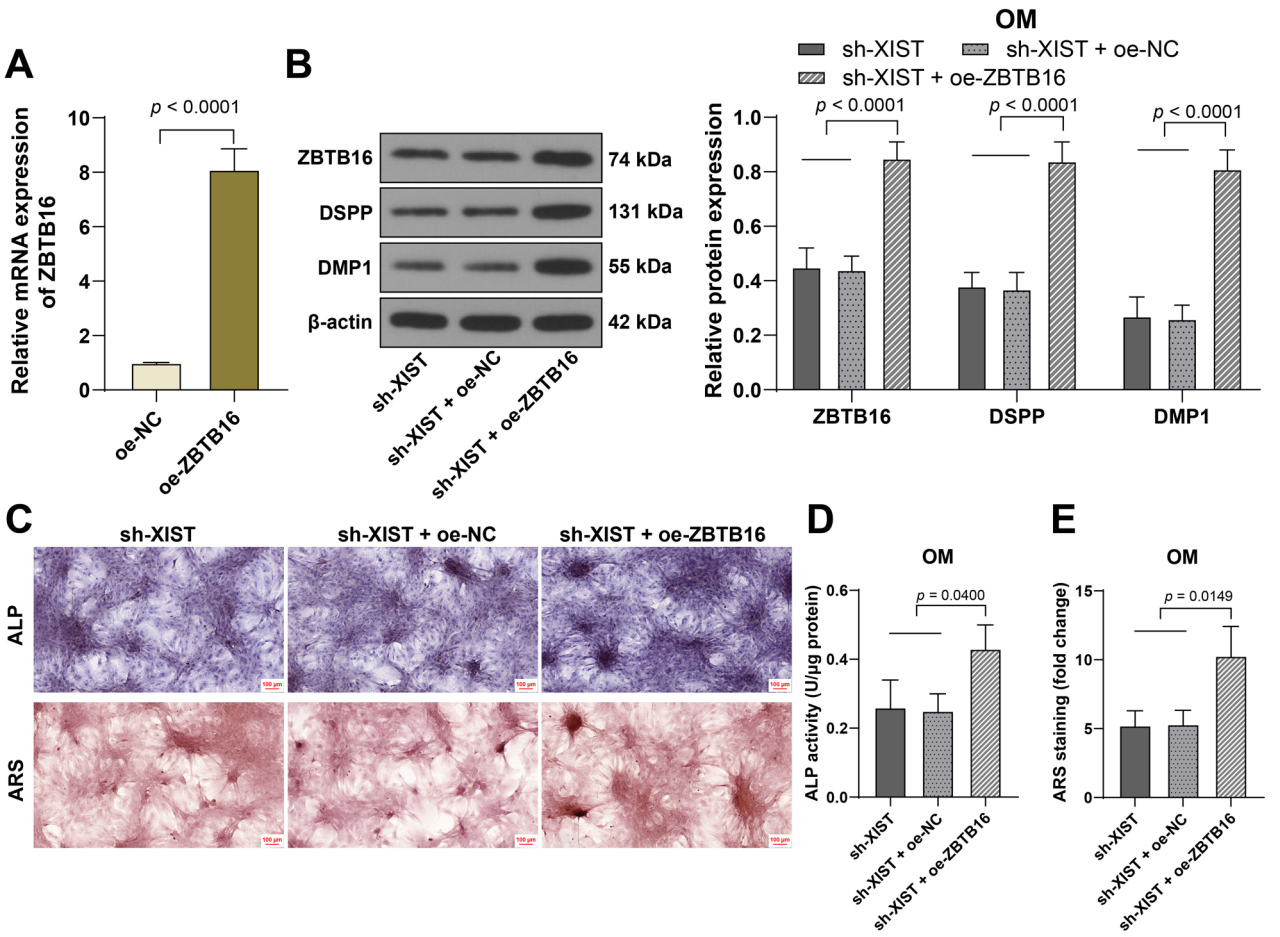

Figure 6- Overexpression of ZBTB16 partially reverses the inhibitory effect of XIST silencing on the odontogenic differentiation of hDPSCs. hDPSCs were infected with oe-ZBTB16 packaged lentivirus, with oe-NC as a control, and stably expressed cells were screened using puromycin. A: qRT-PCR quantification of ZBTB16 expression in hDPSCs. hDPSCs were cultured in OM (osteogenic differentiation medium). After 21 days, B: Western blot analysis of ZBTB16, DSPP, and DMP1 expressions in hDPSCs. C-E: Detection of ALP activity (at 7th day of OM culture) and ARS observation of mineralized nodules (at 21st day of OM culture). Cell experiments were conducted in independent triplicate. Data in panel A were analyzed by the t-test. Data in panel B were analyzed by two-way ANOVA, and data in panels DE were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

enzyme effective in mineral deposition, is upregulated in the early stage of odontoblast maturation.[28] After 7 days of OM incubation, the ALP activity of hDPSCs was augmented; and after 21 days of incubation, a large number of calcified nodules were observed, and DSPP and DMP1 expressions were dramatically increased. These results confirmed that OM incubation induced the odontogenic differentiation of hDPSCs. LncRNA XIST is a known RNA molecule indispensable for X chromosome inactivation.[9] XIST has been identified to play vital roles in irreversible pulp neural inflammation by forming ceRNA regulatory networks.[29] XIST expression is dramatically downregulated in cultured dental mesenchymal cells but upregulated in odontogenic mesenchymal cells.[8] Our results revealed a significant increase in XIST expression after OM incubation, which indicated that XIST might participate in the odontogenic differentiation process of hDPSCs. Downregulation of XIST in hDPSCs weakened the differentiation ability of hDPSCs into odontoblasts, reduced the ALP activity and mineralized nodules, and diminished the expressions of DSPP and DMP1.

Thereafter, we shifted to investigate the downstream mechanism of XIST influencing the odontogenic differentiation of hDPSCs. XIST has been reported to assist the osteoclast differentiation by interacting with FUS.[18] Aberrant accumulation of FUS in degenerating neurons characterizes the neuropathology of amyotrophic lateral sclerosis and frontotemporal lobar degeneration.[12] However, the role of FUS in odontogenic differentiation of dental pulp stem cells has not been reported before. We are the first to illustrate the particular relation of FUS with odontogenic differentiation of hDPSCs. The FUS expression pattern is dynamically regulated during early tooth development in mice. FUS is abundantly expressed in the odontoblasts and pulp proliferation zone of mice at postnatal day 14.[13] For the first time,

we demonstrated that FUS downregulation repressed the odontogenic differentiation of hDPSCs.

ZBTB16 modulates a wide range of biological processes encompassing stem cell maintenance and proliferation, cell differentiation, musculoskeletal development, hematopoiesis, metabolism, and immunity.[30,31] ZBTB16 expression is significantly elevated during the osteogenesis process of MSCs and has a positive correlation with MSC osteogenesis.[32] After the induction of osteogenic differentiation, ZBTB16 expression is notably increased in dental follicle cells, the precursor cells of cementoblast.[16] Knockdown of ZBTB16 attenuates ALP activity and mineralized nodules in hDPSCs, and also impairs the markers of odontogenic differentiation.[19] We speculated that XIST affected the ZBTB16 mRNA stability by binding to FUS, thereby regulating its protein expression. RIP assay validated the binding between XIST and FUS, as well as between FUS and ZBTB16 mRNA. ZBTB16 expression was elevated after OM incubation, and downregulation of XIST or FUS evidently restrained the ZBTB16 expression. Mechanistically, XIST promotes the expression of ZBTB16 by binding to FUS and thereby promotes the odontogenic differentiation of hDPSC. Similarly, lncRNA XIST is demonstrated to interact with FUS and increase the SPHK1 stability, indicating its ability in promoting osteoclast differentiation through SPHK1/S1P/ERK signaling pathway.[18]

Conclusion

To conclude, XIST stabilizes ZBTB16 mRNA and promotes its expression by binding to FUS, thereby facilitating the odontogenic differentiation of hDPSC. Our study is limited to the cellular level, lacking clinical data analysis or animal validation *in vivo*. In addition to binding to RNA binding proteins, XIST may also regulate odontogenic differentiation of cells via other mechanisms. Not limited to ZBTB16, there may also be other downstream target genes involved in odontogenic differentiation. In the future, we will validate the mechanism of odontogenic differentiation in animals and explore other downstream target genes of XIST.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

Cheng, Ruiqing: Conceptualization (Lead); Data curation (Lead) Formal analysis (Lead); Validation (Lead); Writing – original draft (Lead); Writing – review & editing (Lead). **Sun, Honglei:** Data curation (Equal); Investigation (Equal); Validation (Equal) **Qiao, Xiaotong:** Data curation (Equal); Methodology (Equal); Visualization (Equal). **Chen, Xuefang:** Conceptualization (Equal), Formal Analysis (Equal), Writing – review & editing (Equal).

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