



ORIGINAL ARTICLE

Dysregulation of lncRNA TFAP2A-AS1 is involved in the pathogenesis of pulpitis by the regulation of microRNA-32-5p

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Abstract

Objective: This study was designed to evaluate TFAP2A-AS1 expression in the dental pulp of teeth with or without pulpitis and to determine the function of TFAP2A-AS1 in pulp cells.

Methods: GSE92681 was analyzed to filter out differentially expressed lncRNAs. Pulp samples from teeth with pulpitis and healthy teeth (control) were examined using real-time (RT) quantitative polymerase chain reaction (qPCR). Human dental pulp stem cells (hDPSCs) were cultured in a specific medium for osteogenic induction, or treated with lipopolysaccharide (LPS) to simulate inflammation. The viability and apoptosis of human DPSCs (hDPSCs) were determined by XTT assay and apoptosis detection kit. Inflammation was induced by LPS and assessed by measuring the expression and release of inflammatory cytokines after TFAP2A-AS1 knockdown. Osteogenic differentiation of hDPSCs was investigated by determining expression levels of osteogenic markers and alkaline phosphatase (ALP) activity after TFAP2A-AS1 overexpression. The downstream microRNA (miRNA) was predicted. Dual-luciferase reporter was used to confirm the binding between miR-32-5p and TFAP2A-AS1.

Results: The expression of TFAP2A-AS1 was evaluated in inflamed pulp using RT-qPCR. TFAP2A-AS1 had a discriminatory ability for healthy individuals and patients with pulpitis. The expression of TFAP2A-AS1 decreased upon the osteogenic differentiation of hDPSCs, and increased upon the LPS induction. TFAP2A-AS1 can reverse the osteogenic differentiation of hDPSCs, as evidenced by decreased levels of dentine sialophosphoprotein, dentin matrix protein-1, and ALP activity. TFAP2A-AS1 knockdown can promote cell proliferation of hDPSCs and relieve LPS-induced inflammation, as evidenced by decreased levels of TNF- α , IL-1 β , and IL-6. miR-32-5p was identified as a downstream miRNA of TFAP2A-AS1.

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Conclusion: This study demonstrated the expression and potential function of TFAP2A-AS1 in the human dental pulp. TFAP2A-AS1 can inhibit odontogenic differentiation but promote inflammation in pulp cells.

KEYWORDS

inflammation, microRNA-32-5p, pulpitis, TFAP2A-AS1, osteogenic differentiation

1 | INTRODUCTION

Teeth, although small, have a complex structure comprised of components like dental pulp, each with unique features and functions.¹ Dental pulp is the soft tissue at the core of teeth, encased by the crown and root and is lined with a layer of odontoblasts.² Teeth are prone to damage from intense mechanical and chemical stress, as well as dense microbiologic colonization. This damage usually occurs in the form of caries, periodontal disease and trauma.³ If the mineralized tissues of enamel and dentine are damaged, the dental pulp would become susceptible to microbial invasion.⁴ The toxins from bacteria can penetrate the dentinal tubules. The bacterial antigens and lipopolysaccharides (LPS) can cause immunological reactions and increase the levels of immunoglobulins, prostaglandins, and other pro-inflammatory mediators in the infected pulp. In response to this insult, the dental pulp initiates a complex inflammatory response, leading to infection and inflammation. As bacteria in contact with the pulp, additional cell types of the pulp, such as stem cells, contribute greatly with a series of inflammatory-defense mechanisms, which are critical for tissue homeostasis.^{5,6} Stem cells exhibit both mesenchymal and neural characteristics.⁷ The pulpal stem cells may play a central role in the immunological responses of the dental pulp to oral microorganisms.⁸ A thorough understanding of molecular mechanism in pulpitis may lead to the identification of new therapeutic targets for this disease and enhance diagnostics strategies.

Epigenetics plays important roles in pulpitis, influencing aspects such as the inflammatory process and endodontic regeneration.^{9,10} Epigenetic processes refer to mitotically and meiotically heritable changes, independent of the DNA sequence. These processes have a complex molecular basis that involves noncoding RNAs (ncRNAs).¹¹ Since epigenetic modifications can be reversed, understanding their mechanisms could help identify new therapeutic targets for inflammatory diseases.¹² Over recent decades, modulation of ncRNA, despite lacking protein-coding function, has emerged as a new layer of gene regulation.¹¹ ncRNAs can be classified as into two main types on length (shorter or longer than

200 nt)-long noncoding RNA (lncRNA) and microRNA (miRNA).¹³ They play potential roles in dental pulp tissue, including odontogenic differentiation, immune response, and bone resorption.^{14,15} For example, MEG3, an upregulated lncRNA in inflamed pulp and LPS-treated human dental pulp cells, has been found to negatively affect inflammation and regeneration of the dentin-pulp complex in pulpitis.¹⁶ It's worth noting that lncRNA can function as natural miRNA sponges, binding to miRNAs and competitively sequestering them from their target genes, a process referred to as competing endogenous RNAs (ceRNAs). For instance, upregulated lncRNA DUXAP8 is found in pulpitis and associated with miR-18b-5p sponging function, which exacerbates the pathogenetic condition.¹⁷

In this study, GSE92681 data set from GEO database was analyzed to screen the differentially expressed lncRNAs. Then, TFAP2A-AS1 expression was evaluated in the healthy or inflamed dental pulp. Additionally, the function of TFAP2A-AS1 in osteogenic differentiation and inflammation was examined.

2 | MATERIALS AND METHODS

2.1 | Collection of dental pulp tissue

This study was approved by the Institutional Review Boards of Shijiazhuang Fourth Hospital (approval no. 20230019). Each patient signed a written informed consent for use of the samples. Healthy dental pulp ($n = 18$) was obtained from wisdom teeth or premolars for orthodontic reasons. Simultaneously, inflamed dental pulp samples were extirpated from carious teeth with nerve broach in 37 patients with irreversible pulpitis (the American Association of Endodontists guidelines). The two groups were matched in age and gender (Table 1). The removed tissue was immediately placed in RNeasy Lysis Buffer (Qiagen) and immediately placed in RNeasy Lysis Buffer (Qiagen) RNA Stabilization Reagent for Animal Tissue (Beyotime) and incubated at 4°C for 24 h. Then, the reagent was discarded, and tissues were transferred to a prefrozen liquid nitrogen tube with a rotating cap. After rapid freezing with liquid nitrogen, the tissues were stored at -80°C .

TABLE 1 Clinical data comparison for subjects.

Items	Normal pulp (<i>n</i> = 18)	Inflamed pulp (<i>n</i> = 37)	<i>p</i> value
Age (year)	30.1 ± 10.8	33.2 ± 8.0	.29
Gender (female/male)	7/11	16/21	.76
Pain history (days)	—	7.8 ± 4.0	—

2.2 | Cell culture, osteogenic induction, and LPS treatment

Human dental pulp stem cells (hDPSCs) from human sound third molars (Lonza) were cultured in Dulbecco's modified Eagle's medium (DMEM Sigma-Aldrich) supplemented with 10% fetal bovine serum, 50 units/mL penicillin and 50 µg/mL streptomycin (Gibco), and maintained 37°C in a 5% CO₂ incubator. Cells from passages 3 to 5 were used for experiments. To activate odontogenic differentiation, hDPSCs were cultured in a Human Dental Pulp Stem Cell Osteogenic Differentiation Medium (YaJi Biological) for 3, 7, and 14 days. mRNA levels of dentine sialophosphoprotein (DSPP) and dentin matrix protein (DMP)–1 were analyzed by real-time (RT) quantitative polymerase chain reaction (qPCR), while alkaline phosphatase (ALP) was analyzed by ALP Diagnostics Kit (Yeasen). To simulate the inflamed microenvironment of dental pulp, hDPSCs were incubated with LPS (0.1, 1, 10 µg/mL) for 12 h. TNF-α, IL-1β, and IL-6 were analyzed by RT-qPCR and the responding ELISA kits (R&D Systems).

2.3 | Cell transfection

Two siRNAs targeting TFAP2A-AS1 (siTFAP2A-AS1 and siTFAP2A-AS1-1) and siRNA negative control (siNC) were designed and synthesized by GenePharma, along with TFAP2A-AS1-overexpressed sequence (ovTFAP2A-AS1) and negative control (ovNC). For transfection, hDPSCs were cultured in antibiotic-free growth medium at about 60 confluence for 24 h and then transfected with 20 nM siRNA using Lipofectamine RNAiMAX (Invitrogen), followed by transfection efficiency verification based on the expression level of TFAP2A-AS1 by RT-qPCR.

2.4 | RNA isolation and RT-qPCR

The frozen healthy and inflamed dental pulp tissues were thawed and homogenized. Total RNA was isolated using RNeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime). Total RNA (400 ng) was subjected to reverse transcription, using the QuantiTect Reverse Transcription kit (Qiagen). The resulting cDNA was

diluted 1:20 for RT-qPCR using the SsoAdvanced Universal SYBR-Green Supermix Kit (Bio-Rad) and primers. After RT-qPCR, quantification data were normalized using the geometric mean of the selected stable reference genes (Glyceraldehyde 3-phosphate dehydrogenase for lncRNA and mRNA, and U6 for miR-32-5p), with the $2^{-\Delta\Delta Ct}$ method.

2.5 | Cell proliferation and apoptosis assays

hDPSCs proliferation was determined by Cell Proliferation kit II (XTT) (Roche, Mannheim, Germany) abiding by the manufacturer's instructions. Cell apoptosis assays were completed by flow cytometry (CytoFLEX analyzer; Beckman Coulter), within 1 h of using FITC Annexin V Apoptosis Detection Kit (BD Biosciences) based on the recommendations from the manufacturer.

2.6 | Bioinformatics analysis

The bioinformatics tool, lncRNASNP v2, was used to search the possible miRNA targets of TFAP2A-AS1. Among the predicted miRNAs, miR-32-5p, known to be involved in osteogenic and adipogenic differentiation of dental pulp stem cells, was selected for further interaction studies with TFAP2A-AS1.

2.7 | Dual-luciferase reporter assay

Based on the binding sites between TFAP2A-AS1 and miR-32-5p, the wild-type and mutant binding sequence regions of miR-32-5p on the fragment of TFAP2A-AS1 were entrusted to Hanbio for chemical synthesis and inserted into pmirGLO luciferase reporter vectors (LMAI Bio). hDPSCs were transfected with reporter plasmids (wild or mutant TFAP2A-AS1) and miR-32-5p mimics or mimic negative control (NC). After 24 h of transfection, the cells processed as per the instructions of Dual-Luciferase Reporter gene assay kit (Promega). The activities of renilla luciferase and firefly luciferase were then measured using a GloMax luminometer (Promega).

2.8 | Statistics

All data were transferred to statistics software (GraphPad Prism 9) for analysis, and the Kolmogorov-Smirnov normality test was applied. Data that were not normally distributed required Mann-Whitney method for parametric analysis. Normally distributed data were compared using either one way ANOVA or *t* tests. The correlation analysis between TFAP2A-AS1 and miR-32-5p was analyzed using Pearson's correlation coefficient. The predictive performance of TFAP2A-AS1 was assessed by receiver operating characteristic (ROC) curve analysis, represented as the area under the curve (AUC). When $p < .05$, the difference was statistically significant.

3 | RESULTS

3.1 | TFAP2A-AS1 expression in healthy and inflamed dental pulp

TFAP2A-AS1 was identified as a differentially expressed lncRNA in GSE92681 (Figure 1A). Then, the expression level of TFAP2A-AS1 was assessed in the healthy and inflamed dental pulp. Results showed that pulpitis caused a sharp significant ($p < .001$) elevation in TFAP2A-AS1 expression level in patients with inflamed dental pulp when compared with the healthy ones (Figure 1B). The TFAP2A-AS1 level manifested as a well-distinguishing tool for pulpitis and health, with an AUC of 0.818 (Figure 1C). During osteogenesis of hDPSCs, the

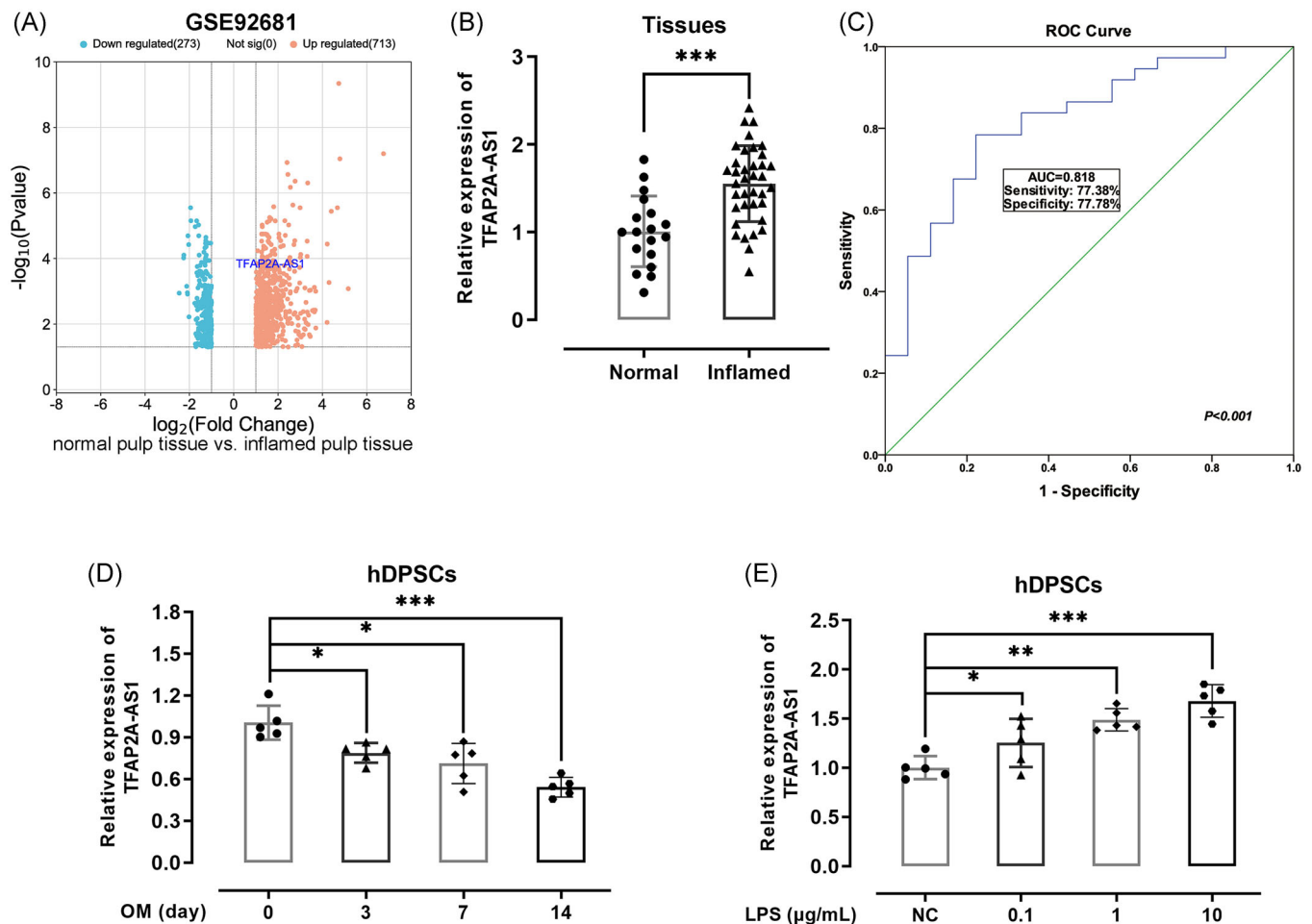


FIGURE 1 The expression levels of TFAP2A-AS1 in dental pulp. (A) TFAP2A-AS1 was one of the differentially lncRNA in GSE92681 data set. (B) TFAP2A-AS1 expression level in healthy and inflamed pulp tissue was determined by real-time quantitative polymerase chain reaction (RT-qPCR). (C) The area under the curve (AUC) was analyzed by receiver operating characteristics (ROC) in predicting pulpitis. (D) TFAP2A-AS1 expression in osteogenic-differentiating hDPSCs was assayed by RT-qPCR assay. (E) The expression of TFAP2A-AS1 in LPS-induced hDPSCs was determined by RT-qPCR. * $p < .05$, *** $p < .001$. hDPSCs, human dental pulp stem cells. LPS, lipopolysaccharide.

TFAP2A-AS1 level decreased ($p < 0.05$) upon the culture time (Figure 1D). LPS exposure dose-dependently increased the expression of TFAP2A-AS1 in hDPSCs (Figure 1E).

3.2 | TFAP2A-AS1 overexpression inhibited osteogenesis in hDPSCs

The influence of TFAP2A-AS1 overexpression on the osteogenesis of hDPSCs was evaluated by the expression of osteogenic markers, including DSPP mRNA, DMP-1 mRNA, and ALP activity. TFAP2A-AS1 was overexpressed successfully (Figure 2A). According to the results, the seeded cells without TFAP2A-AS1 overexpression had higher DSPP mRNA ($p < .05$) and DMP-1 mRNA ($p < .05$) expression levels (Figure 2B,C). Analysis of ALP activity also showed a significant decrease ($p < .05$) in ALP activity due to TFAP2A-AS1 overexpression (Figure 2D). These results demonstrated that TFAP2A-AS1 overexpression of in hDPSCs can inhibit hDPSCs differentiation into osteoblasts.

3.3 | TFAP2A-AS1 knockdown reversed LPS-induced effects in hDPSCs

The impact of TFAP2A-AS1 knockdown on LPS-induced effects in hDPSCs was evaluated through the levels of inflammatory factors (TNF- α , IL-1 β , and IL-6), cell proliferation, and cell apoptosis. In comparison with siNC group, the expression level of TFAP2A-AS1 was sharply decreased in siTFAP2A-AS1 group ($p < .001$) and siTFAP2A-AS1-1 group ($p < .01$) (Figure 3A). Compared to siNC group, downregulation of TFAP2A-AS1 in hDPSCs greatly reduced the mRNA expression levels of TNF- α (Figure 3B), IL-1 β (Figure 3C), and IL-6 (Figure 3D) ($p < .05$), as well as their protein levels ($p < .05$) (Figure 3E,F,G). TFAP2A-AS1 knockdown significantly decreased ($P < 0.05$) hDPSCs cell apoptosis (Figure 3H), while hDPSCs proliferation increased upon TFAP2A-AS1 downregulation (Figure 3I). Consequently, downregulation of TFAP2A-AS1 can protect hDPSCs from LPS-induced inflammation and injury.

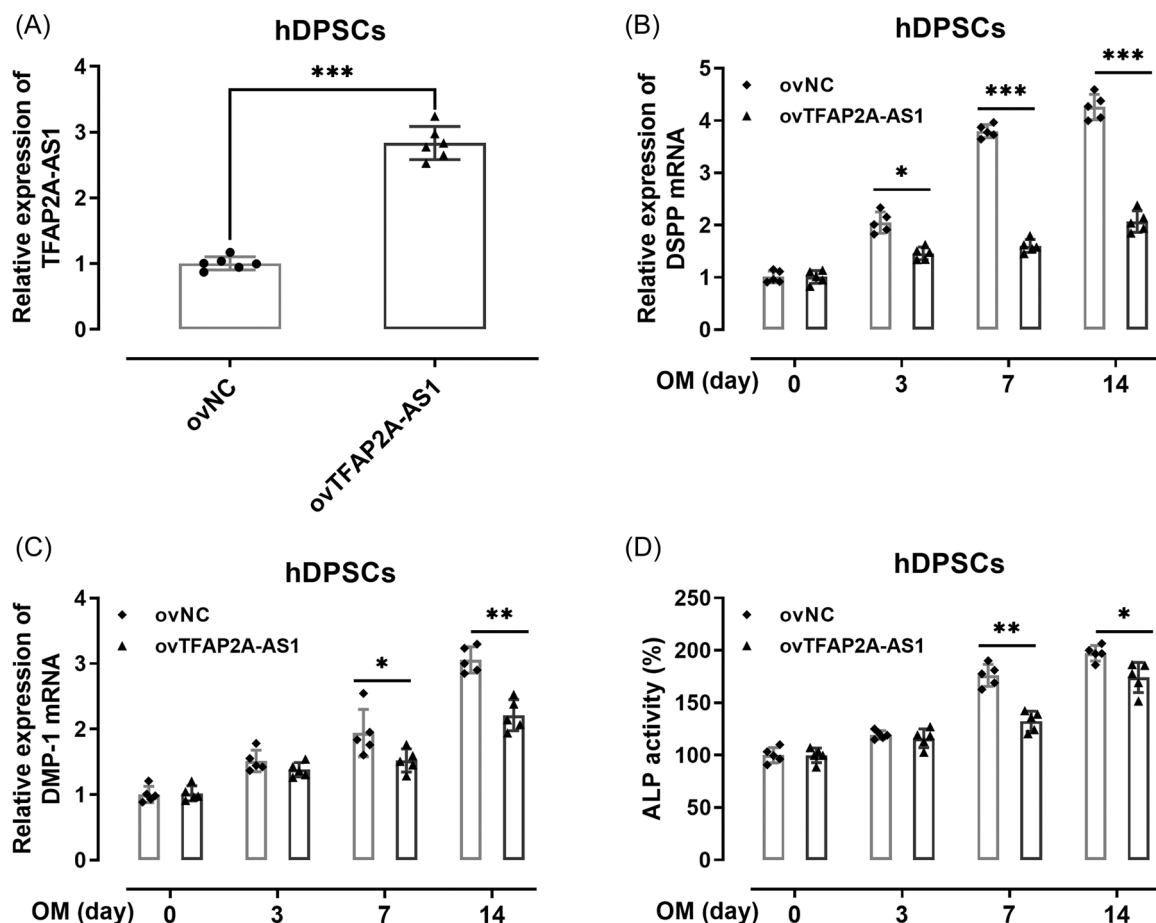


FIGURE 2 Overexpression of TFAP2A-AS1 impeded odontogenic differentiation of hDPSCs. (A) TFAP2A-AS1 expression level increased upon the transfection by RT-qPCR. The mRNA expression of DSPP (B) and DMP-1 (C) was analyzed by real-time quantitative polymerase chain reaction (RT-qPCR). (D) Alkaline phosphatase (ALP) activity assay was determined by ALP Diagnostics Kit. * $p < .05$, *** $p < .001$. hDPSCs, human dental pulp stem cells.

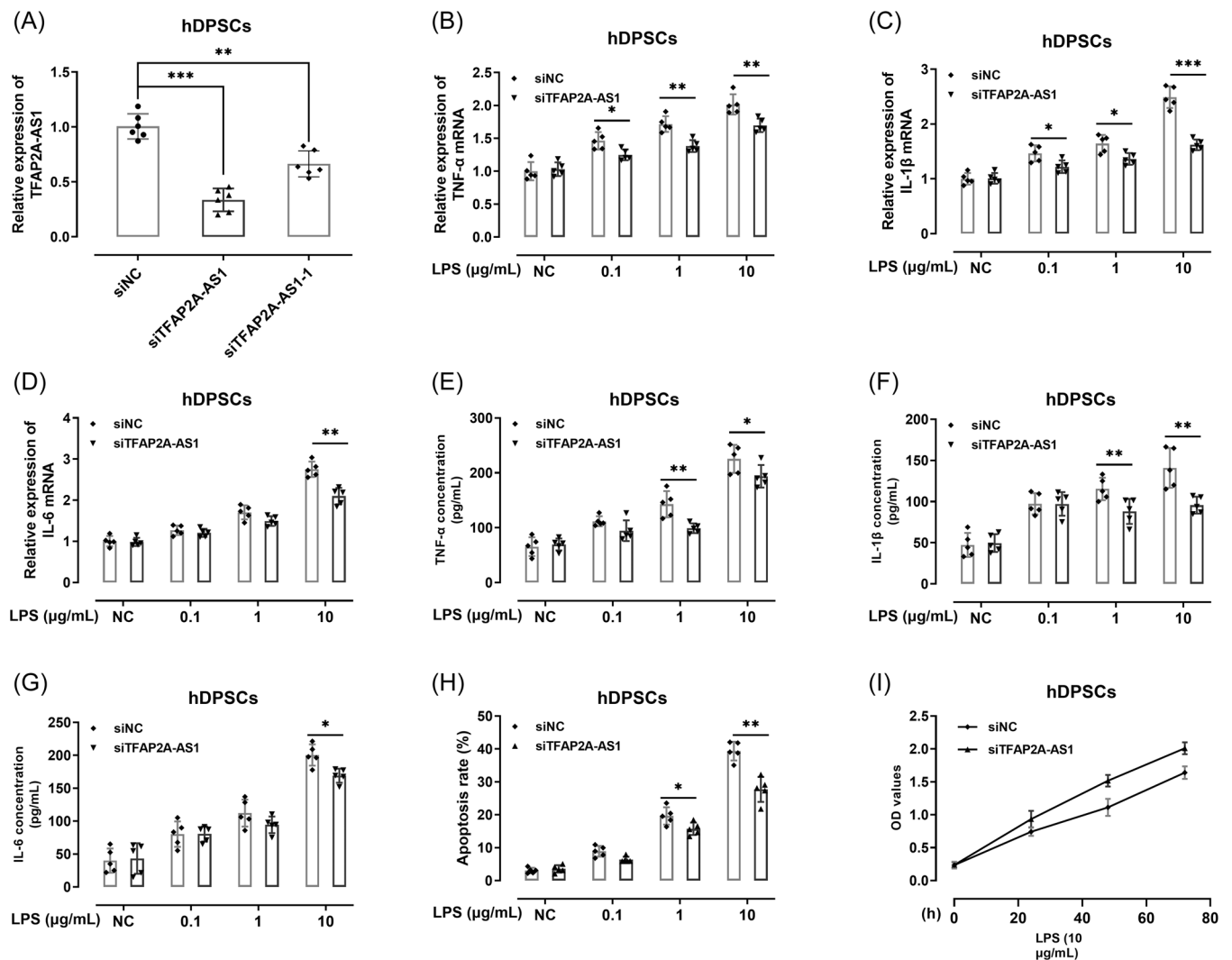


FIGURE 3 Knockdown of TFAP2A-AS1 prohibited the inflammasome and cell apoptosis, but promote cell proliferation of hDPSCs. (A) TFAP2A-AS1 expression level decreased upon the transfection of siRNA by RT-qPCR. Expression of TNF- α (B), IL-1 β (C), and IL-6 mRNA (D) in hDPSCs was analyzed by real-time quantitative polymerase chain reaction (RT-qPCR). TNF- α (E), IL-1 β (F), and IL-6 (G) levels in culture supernatants of hDPSCs were determined by responding ELISA kits. (H) Cell death assay of transfected or non-transfected hDPSCs. (I) Cell proliferation of hDPSCs, transfected or non-transfected, was assayed by Cell Proliferation kit II (XTT). * p < .05, ** p < .01, *** p < .001. hDPSCs, human dental pulp stem cells.

3.4 | miR-32-5p was a direct target of TFAP2A-AS1

Based on the search results from lncRNASNP, we focused on miR-32-5p (Figure 4A), which is related to inflammation¹⁸ and osteogenic differentiation of dental pulp stem cells.¹⁹ TFAP2A-AS1 downregulation can raise the expression level of miR-32-5p, while its upregulation can reduce the expression level of miR-32-5p (p < .01) (Figure 4B). The expression level of miR-32-5p was inversely correlated with that of TFAP2A-AS1 in inflamed pulp (Figure 4C). Under wild-type TFAP2A-AS1 condition, luciferase activity was significantly attenuated by miR-32-5p overexpression (Figure 4D). In

contrast, no major differences of mutant TFAP2A-AS1 were noticed regarding miR-32-5p-mediated luciferase reporter gene activity.

4 | DISCUSSION

Pulpitis is one of the most common oral diseases. Its main clinical manifestations include spontaneous and paroxysmal pain, cold- and hot-stimulating pain, and nocturnal pain, which seriously affects the quality of life of patients.²⁰ Previous studies prove that lncRNA is involved in the inflammatory process of the immune system, and is related to pulpitis.²¹ In a study by HUANG

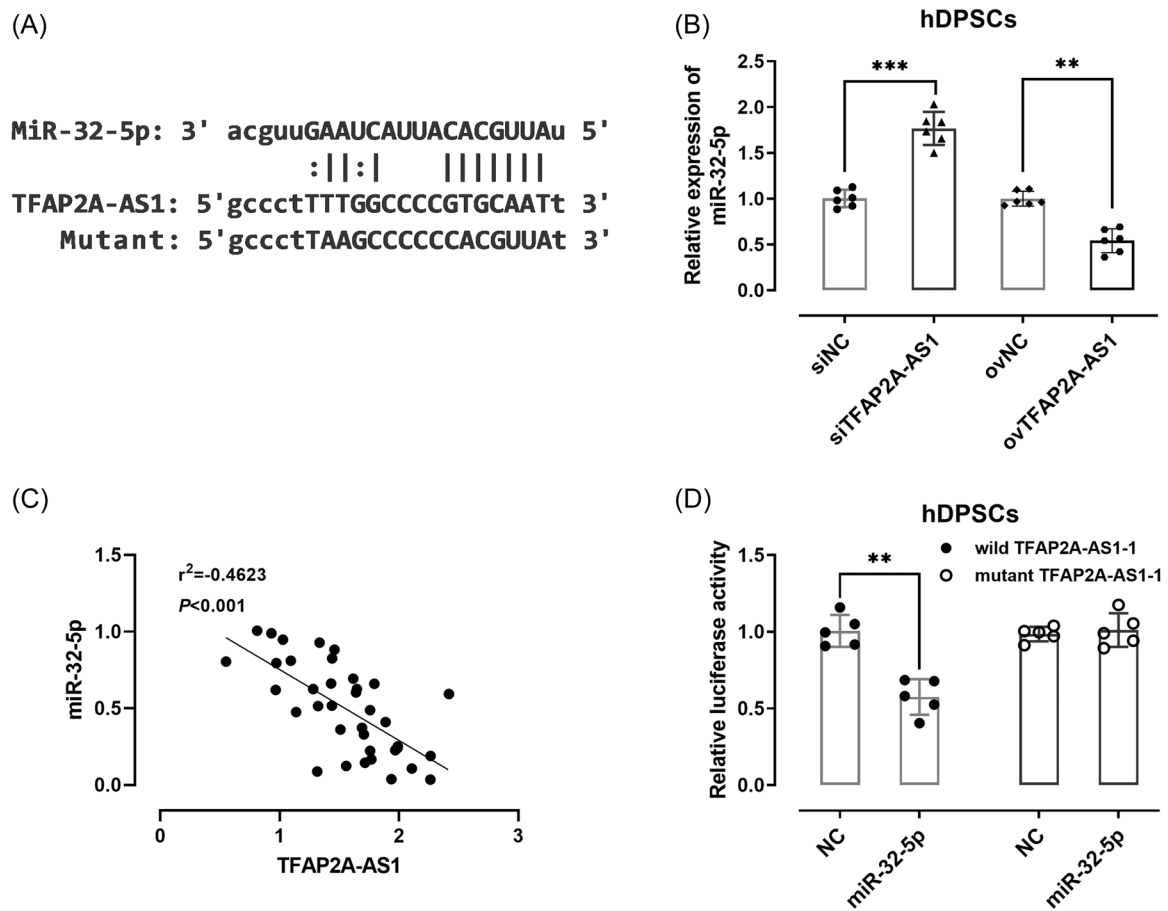


FIGURE 4 TFAP2A-AS1 acted as a sponge for miR-32-5p. (A) The binding site between TFAP2A-AS1 and miR-32-5p was predicted. (B) The expression level of miR-32-5p was analyzed using RT-qPCR after TFAP2A-AS1 overexpression or knockdown. (C) The expression level of TFAP2A-AS1 was inversely correlated with that of miR-32-5p. (D) Relative luciferase activity was determined after cotransfection of wild or mutant TFAP2A-AS1 with miR-32-5p mimics, or the corresponding control oligonucleotide in hDPSCs. $**p < .01$, $***p < .001$. hDPSCs, human dental pulp stem cells.

et al., lncRNA expression in pulpitis and normal human dental pulp tissues was assessed. They found significant differences in lncRNA expression between normal dental pulp tissue and pulpitis-affected tissue, suggesting lncRNA could play a crucial role in the pathogenesis of pulpitis.²² In our study, we analyzed GSE92681 data set, and identified TFAP2A-AS1 as a differentially expressed lncRNA in pulpitis. TFAP2A-AS1 has been reported to be related to the immune response in breast cancer treatment and oral squamous cell carcinoma progress.^{23,24} Subsequently, we identified TFAP2A-AS1 expression profiles in pulpitis tissues and found it was upregulated in pulpitis tissues compared with the healthy pulp. After induction of odontoblastic differentiation, TFAP2A-AS1 expression level was decreased in hDPSCs. In contrast, if hDPSCs were treated with LPS, TFAP2A-AS1 expression level increased. These findings imply TFAP2A-AS1 may be involved in the progress of pulpitis.

Functional pulp tissue is a prerequisite for completing root formation during tooth development and eruption. The odontoblasts lined in pulp can polarize and secrete a collagenous matrix, to physiologically and continuously form dentine.²⁵ The composition of dentine is similar to that of bone. This study explored the regulatory mechanism of TFAP2A-AS1 in the odontogenic differentiation. TFAP2A-AS1 was overexpressed in hDPSCs, followed by differentiating into odontoblasts in vitro. The expression profiles of osteogenic markers in TFAP2A-AS1-downregulated hDPSCs were then examined and compared to those in non-downregulated cells. The results showed that the upregulation of TFAP2A-AS1 inhibited the osteo/odontogenic differentiation potential of DPSCs. Similarly, lncRNA-Ankrd26 from dental pulp stem cells can promote dental pulp restoration through osteoblastic differentiation of mesenchymal stem cells.²⁶ lncRNA SNHG1, LINC00968, and linc02349 can also promote the odontogenic differentiation of hDPSCs.²⁷⁻²⁹ However,

other lncRNAs, such as DANCR and LINC01133, have inhibitory effects on bone differentiation of hDPSCs.^{30,31} This study provides a new target for the regenerative treatment of endodontic diseases and enhances understanding of hDPSCs functions.

The cellular response to pulpal infection involves several resident cells, such as odontoblasts and fibroblasts, as well as immune cells. The recruited immune cells at the site of infection release the effector molecules including cytokines, chemokines and other pro-inflammatory mediators.³² The severity of inflammation dictates the outcome of dental pulp infection, which could result in regeneration, repair, or necrosis. hDPSCs play an important role in the regenerative and inflammatory response of the pulp to trauma and injury. Increasingly studies have evaluated the role of lncRNAs in the cellular and immune mediators in diseased dental pulps.¹¹ For example, an in-vitro study revealed an upregulation of PVT1 in the pulpitis cell model, which facilitates injury to human dental pulp cells caused by LPS.³³ This in-vitro study demonstrated TFAP2A-AS1 knockdown can reverse the LPS-induced increase in inflammatory mediators. Moreover, lncRNAs may regulate the proliferation and apoptosis of hDPSCs. lncRNA H19 is known to enhance the proliferation capability of hDPSCs.³⁴ In the current study, TFAP2A-AS1 showed an inhibitory effect on proliferation capability and a promoting effect on the apoptotic capability of hDPSCs. These data provide insight into the regulatory effects of TFAP2A-AS1 on inflammatory response and growth of hDPSCs.

Considering the ceRNA function of lncRNAs, we further investigated the potential downstream miRNA for TFAP2A-AS1. Ultimately, miR-32-5p was verified to be a miRNA of TFAP2A-AS1 in hDPSCs. In oxidized low-density lipoprotein-induced human umbilical vein endothelial cells, miR-32-5p can suppress the expression of inflammatory factors including IL-1 β , IL-6, TNF- α , ICAM-1, and VCAM-1.¹⁸ This indicates the anti-inflammatory function of miR-32-5p in cell inflammation. Notably, miR-32 can be activated by enamel matrix proteins and regulate osteogenic and adipogenic differentiation of human exfoliated deciduous teeth.¹⁹ This miRNA potentially plays an important role in hDPSCs differentiation. Therefore, it was speculated that TFAP2A-AS1 may function in diseased dental pulps via sponging miR-32-5p.

The use of TFAP2A-AS1 could serve as a promising epigenetic biomarker for diagnosing pulpitis. Moreover, its role in various biological processes of pulpitis may highlight its potential as an epigenetic therapy. However, this study, conducted in our single center, has some limitations, including a small sample size and in vitro

experiments. Therefore, future research is needed to explore the roles of TFAP2A-AS1 in pulpitis in vivo and the feasibility of a TFAP2A-AS1-inhibiting therapy.

5 | CONCLUSION

This study demonstrates TFAP2A-AS1 expression and potential function in human dental pulp. The expression pattern of TFAP2A-AS1 in human inflamed pulp was higher than that in healthy pulp. TFAP2A-AS1 appears to hinder odontogenic differentiation, but it promotes inflammation in pulp cells. These findings suggest that TFAP2A-AS1 may contribute to the pathogenesis of pulpitis. Further research is needed to elucidate mechanism of TFAP2A-AS1 in pulpal diseases.

AUTHOR CONTRIBUTIONS

Mingming Liu: Conceptualization; data curation; writing—original draft; writing—review and editing. **Weijing Jia:** Conceptualization; Data curation; investigation; writing—review and editing. **Lin Bai:** Conceptualization; data curation; investigation; writing—review and editing. **Qiaolin Lin:** Conceptualization; data curation; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest in this study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study protocol was approved by The Ethics Committee of Shijiazhuang Fourth Hospital and followed the principles outlined in the Declaration of Helsinki. In addition, informed consent has been obtained from the participants involved.

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