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Jianpeng Han
Nantong Hospital of Shanghai University (The Sixth People's Hospital of Nantong)
Xin Zhao
Nantong Hospital of Shanghai University (The Sixth People's Hospital of Nantong)
Rongrong Jiang
Affiliated Hospital of Nantong University
Chengfeng Jiang
Affiliated Hospital of Nantong University
lei li
Affiliated Hospital of Nantong University
zhiliang xu
Affiliated Hospital of Nantong University
yihua song
Affiliated Hospital of Nantong University
xingmei feng (fengxingmeint@126.com)
Affiliated Hospital of Nantong University

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Effects of miR-629-5p on odontoblast differentiation and target gene TEAD4 expression of dental pulp stem cells

Jianpeng Han,[1※] Xin Zhao,[1※] Rongrong Jiang,[2※] Chengfeng Jiang,[2] Lei Li,[2] Zhiliang Xu,,[2] Miao Wang,[2] Yihua Song,[3*] and Xingmei Feng[4*]

1、Department of Stomatology，Affiliated Nantong Hospital of Shanghai University (The Sixth People's Hospital of Nantong), Yonghe Road,Nantong, Jiangsu province, 226001, PR China.

2、Department of Stomatology, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, 226001, Jiangsu, China.

3、Department of Stomatology, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, 226001, Jiangsu, China. songyihua1986@163.com

4、Department of Stomatology, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, 226001, Jiangsu, China. fengxingmeint@126.com

※、These three authors contributed equally to this work.

*、Correspondence: songyihua1986@163.com; fengxingmeint@126.com

Abstract

TEAD4 is a member of the TEA domain (TEAD) family of transcription factors. It plays a key regulatory role in embryonic development, tissue homeostasis and cancer progression. Its expression is related to the regulation of a variety of inflammations. MicroRNA can regulate the expression of target genes and play an important role in various physiological and pathological processes. In view of the important role of TEAD4 and microRNA-629-5p (miR-629-5p) in inflammation, and based on the findings of bioinformatics research, we selected miR-629-5p as the focus of our study. In inflammatory dental pulp, we found that the expression of miR-629-5p was increased, while the expression of TEAD4 was decreased. We used Porphyromonas gingivalis lipopolysaccharide (LPS) as a stimulator of dental pulp stem cells (DPSCs) to simulate the inflammatory environment of dental pulp. The mineralization ability of LPS-stimulated DPSCs was significantly inhibited, while the level of miR-629-5p increased and the level of TEAD4 decreased. Inhibition of miR-629-5p can reverse the odontogenic defects of DPSCs treated with LPS. In addition, the expression of miR-629-5p in DPSCs was negatively correlated with the expression of TEAD4. In conclusion, miR-629-5p can inhibit the odontogenic differentiation of human dental pulp stem cells and the mechanism may be related to its role in downregulation of TEAD4 expression.

Keywords MicroRNA-629-5P, Dental pulp stem cells (DPSCs), TEAD4, Odontoblastic differentiation, Deep caries, Reversible pulpitis

Introduction

Dental caries is mediated by cariogenic bacteria. As the caries penetrates the enamel and dentin and involves dental pulp, it can cause pulptitis whose major clinical manifestation was tooth pain (Zero, Zandona, Vail, & Spolnik, 2011).During pulpitis, the inflammatory exudate may significantly raise the tissue pressure in the pulp cavity as the pulp is completely surrounded by rigid dentin walls. The microcirculation of the dental pulp is blocked, which can result in localized

or generalized pulp necrosis and death. Bacteria and their toxins have been shown to stimulate the production of proinflammatory cytokines by lymphocytes and monocytes, which is closely related to the occurrence and development of pulpitis (Adachi et al., 2007; Carrouel et al., 2013). Gram negative bacteria, especially Porphyromonas gingivalis, are initiators of pulpitis. Lipopolysaccharide (LPS), a cell wall component of Gram negative bacteria, is the main stimulating factor of dental pulp inflammation (Chung, Lee, Duraes, & Ro, 2011; Renard et al., 2016). In 2002, Gronthos et al found a novel type of cells in dental pulp with extremely similar immunophenotype as bone marrow mesenchymal stem cells which can form mineralized nodules (Gronthos et al., 2002). The cells are spindle-shaped, and have self-renewal, multidirectional differentiation capacities and strong cloning ability. These fibroblasts are later defined as dental pulp stem cells (DPSCs). The DPSCs can differentiate into odontoblasts, osteoblasts, adipocytes and chondrocytes in vitro induced by different cytokines (Cui et al., 2019; Kim et al., 2012). Previous studies have also found that DPSCs could be implanted into tooth tissue defects and effectively promoted tissue repair (Song et al., 2019; Wang et al., 2020). Therefore, DPSCs have great potential as a source of stem cell therapy and tissue engineering.

It is worth noting that some studies have shown that the differentiation and regeneration of DPSCs were significantly inhibited in the inflammatory microenvironment, which was always associated with pulpitis or deep carious(Chen, Xu, Xia, Cheng, & Zhang, 2021; Feng et al., 2018; Wang, Zhu, Qin, & Wang, 2019). In addition, the researchers demonstrated that an ex vivo LPS-induced inflammatory environment also had a negative impact on the self-renewal and differentiation potential of DPSCs (Feng et al., 2014). However, the underlying molecular mechanism remains unclear.

MicroRNAs (miRNAs) are a class of endogenous, single stranded and noncoding small RNAs, with a length of about 18-22 base pairs. They are highly conservative in the process of biological evolution and participate in regulating the expression of target genes. They mediate complex regulatory signal networks and play an important role in a variety of physiological and pathological processes (Yao, Chen, & Zhou, 2019).Increasing evidences demonstrated that microRNAs may be involved in the development and/or maintenance of inflammation and neuropathic pain (Leinders, Uceyler, Thomann, & Sommer, 2017). According to the biological prediction on the websites of Targetscan, miRDB and miRWalk, we found that miR-629-5p was closely associated with the regulation of TEAD4.

The purpose of this study was to analyze the role of microRNA in the odontoblastic differentiation of human DPSCs in the inflammatory microenvironment. We demonstrated that miR-629-5p could negatively regulate its target gene TEAD4 in DPSCs, and up-regulation of miR-629-5p by LPS stimulation could inhibit the expression TEAD4 and its downstream genes of odontogenic differentiation (RUNX2 and DSPP).

Materials and methods

Sample collection and cell cultures

Inflamed tissue samples were collected according to the guidelines of the Ethics Committee of the Affiliated Hospital of Nantong University, and the informed consent of the donor was obtained. Healthy dental pulp was derived from healthy sample teeth of 18 individuals aged 18-30 years. The tooth are the third molars extracted due to the tooth impaction. Inflammatory pulp

tissue was collected from the third molars of 18 patients (18-30 years old) diagnosed with reversible pulpitis caused by deep caries. All participants had no history of periodontal infection, root lesions and systemic diseases. Individuals having habit of smoking and drinking were excluded from the study. Then, we randomly selected three teeth from each group, and in a sterile environment, the teeth were split to expose the pulp cavity. Dental pulp tissue was obtained and digested with 3 mg/mL collagenase solution at 37 ° C for 1 h and passed through 70 μm cell filter (BD Falcon) to obtain the single cell suspension. The cell suspension was placed in a 25 cm^2 Petri dish and cultured at 37 ° C in Dulbecco's modified Eagle medium (DMEM) at 5 ° C, which was supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. 5% CO2 atmosphere. The medium was changed every three days. When the cells reached 85% to 90% fusion, they were subcultured in a ratio of 1:3. Cells from the third generation were used in all experiments. We named the cells derived from normal pulps N-DPSC, and the cells derived from Inflamed pulps I-DPSC.

Western blot analysis

Total protein lysates were extracted from harvested cells using RIPA buffer supplemented with 1 mM PMSF and loading buffer (Beyotime, Shanghai, China). Protein concentrations were determined using a BCA protein assay kit (Beyotime), according to the manufacturer's specifications. Equal amounts of protein (100 μg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred onto polyvinylidene fluoride (Invitrogen, Thermo Fisher Scientific, MA) membranes in a blotting apparatus (Bio-Rad, Richmond, CA) at 300 mA for 90 min. Membranes were blocked with 5% non-fat milk for 2 h at 37°C, and then incubated overnight at 4°C with primary antibodies: GADPH (1:1000, anti-rabbit; Santa Cruz), RUNX2(1:800, anti-mouse; Sigma), DSPP(1:200, anti-rabbit; Santa Cruz) and TEAD4 (1:500, anti-rabbit; Santa Cruz). Then the membranes were probed with HRP-conjugated anti-rabbit or anti-mouse IgG antibody (Beyotime) for 2 h at room temperature. Finally, the protein bands were visualized using Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific).

RNA isolation and real-time RT-PCR

Total RNA was collected using TRIzol Reagent (Invitrogen, CA) as per the manufacturer's instructions. To quantify miR-629-5p's expression, complementary DNA (cDNA) was synthesized using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), followed by quantitative polymerase chain reaction (PCR) with AceQ qPCR SYBR Green Master Mix (without ROX) (Vazyme,Shanghai, China). We used small nuclear RNA U6 as the normalizing control. For GADPH, RUNX2, TEAD4 and DSPP detection,cDNA was synthesized using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). AceQ qPCR SYBR Green Master Mix (without ROX) (Vazyme) was then used for quantitative PCR of these genes. GAPDH was used for normalization. We use a Light Cycler 480 Real-Time PCR System (Roche Diagnostic, Mannheim, Germany) to test these levels. The primer sequences used in the experiment were as follows:GAPDH:5¢-GAAGGTGAAGGTCGGAGTC-3¢, 5¢-GAAGATGGTGATGGGATTTC-3¢; RUNX2: 5¢-TCAACGATCTGAGATTTGTGGG-3¢, 5¢-TCAACGATCTGAGATTTGTGGG-3¢; DSPP:5¢-GGAGACAAGACCTCCAAGAGTA-3¢,5¢-TGCTGGGACCCTTGATTTCTA-3¢; and TEAD4:5¢-AGGTGGTGGAGAAAGTTGAGACA-3¢,5¢-CGGTGGATGCGGTAAGAGTAG-3¢.

The 2^-ΔΔCt method was used for relative quantification of the gene expression。

Odontoblastic differentiation

DPSCs (2×10^4 cells/dish) were cultured in 35 mm culture dishes (Costar, Cambridge, MA) in odontoblastic differentiation medium containing a minimum essential medium (Invitrogen, Carlsbad, CA), 15% FBS (Gibco-BRL; Life Technologies, Inc., Gaithersburg, MD), 10 mmol/L β-glycerophosphate, 50 mg/mL α-ascorbic acid, 10 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO), 0.292 mg/mL glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin G for 21 d, replacing the medium every 2 d.

Alizarin red staining and ALP assay

The mineralization potential of the cells was assessed by Alizarin red staining when cells were cultured with the osteogenic medium for 14 d. The cells were fixed with 4% paraformaldehyde for 1 h and then incubated with 40 mM Alizarin red S (Sigma) for 15 min in the dark. ALP staining was carried out after osteogenic induction for 14 d using the ALP assay kit (Beyotime) according to the manufacturer's instructions.

MicroRNA (miRNA) selection and transfection

In this experiment, we first explored the relationship between miRNA and TEAD4, and investigated the influence of inflammation. After screening, we selected miR-629-5p, and the binding of miR-629-5p and TEAD4 were predicted by using TargetScan, miRWalk, and microDB databases.

The miR-629-5p control, mimic and inhibitor (Biomics Biotech, Nanjing, China) were transfected into DPSCs according to the instructions of Lipofectamine 3000 (Thermo, MA, USA). According to preliminary experiments, the final concentrations of mimic and inhibitor transfection were 20 nM and 100 nM, respectively, and the efficiency of transfection was verified by qRT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

After stimulating cells with 1 μg/mL concentrations of LPS, the supernatant was collected, and interleukin-6 (IL-6) and interleukin-8 (IL-8) expression levels were measured according to the manufacturer's protocol of Human IL-6 ELISA Assay kit (BD Bio-sciences, San Jose, CA, USA) and Human IL-8 ELISA Assay kit (R&D systems, Minneapolis, MN, USA).

Immunofluorescent staining

The experiment of cell fluorescence was carried out according to the procedure previously described by our research group (Jiang et al., 2021).DPSCs were planted with a density of 1×10^5 cells/mL. After 24 h of cell attachment, DPSCs were fixed by 4% paraformaldehyde for 1 h. The primary antibody was diluted in proportion with Immunostaining Blocking/Primary Antibody Dilution Buffer (Sangon Biotech) and incubated overnight at 4°C. DPSCs were incubated with the corresponding secondary antibody for 2 h at room temperature. Nuclei were stained with DAPI (1:1000; Santa Cruz) and the cells were mounted on an inverted fluorescent microscope and photographed.

Statistical analysis

All the experiments were repeated at least three times independently and the data were presented as mean ± standard deviation. Two group comparisons were tested with two-tailed paired Student's t-test. All statistical evaluation was performed with GraphPad Prism 6.01. One-way analysis of variance was applied to analyze the difference among multiple groups. Value of $p < 0.05$ was considered statistically significant.

Result

The expression of TEAD4 in dental pulp decreased under inflammatory microenvironment

We collected pulp tissues from normal and inflamed teeth. The RT-PCR assay proved that the expression of TEAD4 was significantly reduced in pulp tissues with reversible pulpitis (Fig. 1a). We further studied the expression of TEAD4 in the cells derived from normal pulps（N-DPSC）and inflamed pulps (I-DPSC), both under the odontogenic induction environment. We found that the expression of TEAD4 was significantly decreased in I-DPSC (Figs. 1b. 1c). Alizarin red staining and ALP staining showed that the odontogenic capacity of I-DPSC group was decreased.

LPS stimulation inhibited the expression of TEAD4 in DPSCs

According to our previous report (Bao et al., 2019), we used 1 μg/mL LPS from P. gingivalis for stimulation of DPSCs to simulate the inflammatory microenvironment. After treatment with LPS, DPSCs were incubated with normal medium for 7 d, and the cell supernatant was harvested. IL-6 and IL-8 were detected by ELISA assay. We found that under the stimulation of LPS, the levels of IL-6 and IL-8 significantly increased (Fig. 2a). Western blotting and RT-PCR assay showed that LPS treatment decreased the expression of TEAD4 in DPSCs (Figs. 2b, 2c).

Overexpression of TEAD4 reversed LPS-induced damage of odontogenic capacity of DPSCs

To confirm the relationship between TEAD4 and the odontogenic ability of LPS-stimulated DPSCs, we transfected TEAD4 overexpression plasmid into DPSCs. The specificity and effectiveness of TEAD4 overexpression were verified by RT-PCR (Fig. 3a). After 14 d of mineralization induction, alizarin red staining and ALP staining showed that overexpression of TEAD4 significantly enhanced the mineralized bone matrix formation and ALP activity in DPSCs stimulated by LPS, and the level was similar as the normal DPSCs (Figs. 3b, 3c). Subsequently, we used RT-qPCR and Western blot analysis to determine the mRNA and protein levels of odontoblast marker genes, Runx2 and DSPP (Fig. 3d). We clearly found that overexpression of TEAD4 significantly increased the mRNA and protein expression of odontoblast-related genes in LPS-stimulated DPSCs (Fig. 3e). The above results imply that TEAD4 is involved in rescue of the LPS-induced damage of odontogenic capacity of DPSCs.

miR-629-5p regulates the expression of TEAD4

To further investigate the upstream factors of TEAD4, Targetscan, miRDB and miRWalk databases were used to predict which miRNA may target TEAD4. Among the expected candidates, we focused on miR-629-5p (Fig. 4a). To verify its role in inflammatory pulp, we detected miR-629-5p in the normal pulp from the healthy teeth, inflamed pulp from the carious teeth and LPS-treated DPSCs by qRT-PCR. It was found that under the stimulation of inflammation, the

expression of miR-629-5p in dental pulp tissues and DPSCs increased significantly (Fig. 4b). To verify whether miR-629-5p acts on TEAD4, we overexpressed or knocked down miR-629-5p in DPSCs (Fig. 4c). We found that the expression of TEAD4 decreased after overexpression of mir-629-5p; whereas increased after miR-629-5p knockdown (Figs. 4d, e). Immunofluorescence assay further confirmed that the expression of TEAD4 decreased significantly after miR-629-5p overexpression (Fig. 4f). Collectively, these findings demonstrated that miR-629-5p negatively regulated TEAD4.

Effect of miR-629-5p on odontoblast differentiation

Finally, we examined the effect of miR-629-5p on odontoblastic differentiation ability of DPSCs. We found that overexpression of miR-629-5p resulted in decreased expression level of DSPP and RUNX2, while knocking down of miR-629-5p could significantly upregulated the expression of the two odontoblastic markers (Figs.5a,b). The result of qRT-PCR is the same (Fig.5c). Alizarin red staining and ALP staining further verified that the odontoblastic differentiation ability of DPSCs was decreased after overexpression of miR-629-5p and increased after knockdown of miR-629-5p (Fig.5d). These results indicate that miR-629-5p can target TEAD4 and regulate the odontoblastic differentiation of DPSCs.

Discussion

In this study, we first found a decrease of TEAD4 expression in the inflammatory pulp of reversible pulpitis caused by deep caries. Then 1 μg/ml LPS from primomonas gingivalis was used to stimulate the DPSCs to simulate an inflammatory microenvironment. We interestingly found the expression of miR-629-5p was downregulated in the inflamed pulps, and a negative correlation with TEAD4 was verified by overexpression and knockdown of miR-629-5p.

It is well known that pulpitis may developed from deep dental caries. Chronic inflammation may persist in the pulp, inducing permanent loss of normal tissue and reducing innate repair capacities ((Farges et al., 2015). Previous studies demonstrated that a variety of transcription factors played an important role in the development of pulpitis from dental caries (Xu et al., 2018; Song et al., 2017). As a multifunctional protein, TEAD4 plays an indispensable role in early embryonic development, inflammation control, angiogenesis and mesenchymal stem cell differentiation (Joshi et al., 2017; Ma et al., 2014; Teng et al., 2016; Zhu et al., 2020)

MiRNA can play an important role in the pulp of dental caries. Moreover, studies have shown that miR-629-5p can be a potential diagnostic marker of asthma (Maes et al., 2016). Downregulation of miR-629-5p is involved in inflammatory regulation (Gougelet & Colnot, 2013; Lin et al., 2013). miR-629-5p has long been widely considered as a biomarker of many cancers (Li et al., 2020; Lu et al., 2018). Recent studies have shown that miR-629-5p is associated with cellular stress, inflammatory response and pain, including rheumatoid arthritis, virus or bacterial induced neuroinflammation (Maes et al., 2016; Munoz-Culla et al., 2014; Zhang et al., 2015). Detection of miR-629-5p in plasma can be a new diagnostic basis for chronic obstructive pulmonary disease. (Soeda et al., 2013).

TEAD4, as a member of transcriptional enhancer factor (TEF) family of transcription factors, participates in a variety of cell biology regulation ((Kim & Gumbiner, 2019; Shuai et al., 2020). For example, TEAD4 plays an important role in regulating embryonic development and muscle regeneration (Joshi et al., 2017; Wang et al., 2018). The current study explored its effects

on odontoblast differentiation of DPSCs. According to the prediction website, we found that miR-629-5p was closely related to the regulation of TEAD4.

As shown in our datas, the level of TEAD4 in LPS-stimulated DPSCs is reduced. Moreover, we surprisingly found that the weakened odontogenic capacity was significantly reversed when TEAD4 was overexpressed in LPS-stimulated DPSCs. Therefore, the reduction of TEAD4 is closely associated with a decrease in DPSCs odontogenic capacity under the inflammatory condition. In addition, we discovered that the inhibition of miR-629-5p in LPS-stimulated DPSCs can lead to upregulation of TEAD4 and opposing changes were induced by the mimic of miR-629-5p. These findings validate our hypothesis that under LPS stimulation, miR-629-5p is elevated in DPSCs, which can lead to the inhibition of TEAD4, ultimately resulting in decreased odontogenic potential. Our findings also indicate that miR-629-5p may be a potential avenue for a new therapeutic approach for pulpitis associated with deep caries, though further studies are essential to disclose how TEAD4 further regulates the downstream odontogenic differentiation related factors.

In conclusion, miR-629-5p may be upregulated in inflammatory pulp of dental caries and LPS-treated DPSCs. miR-629-5p negatively regulated its target gene TEAD4, and inhibited the expression of its downstream genes of odontogenic differentiation. As a result, the odontoblastic differentiation ability of DPSCs is weakened (Fig. 6).

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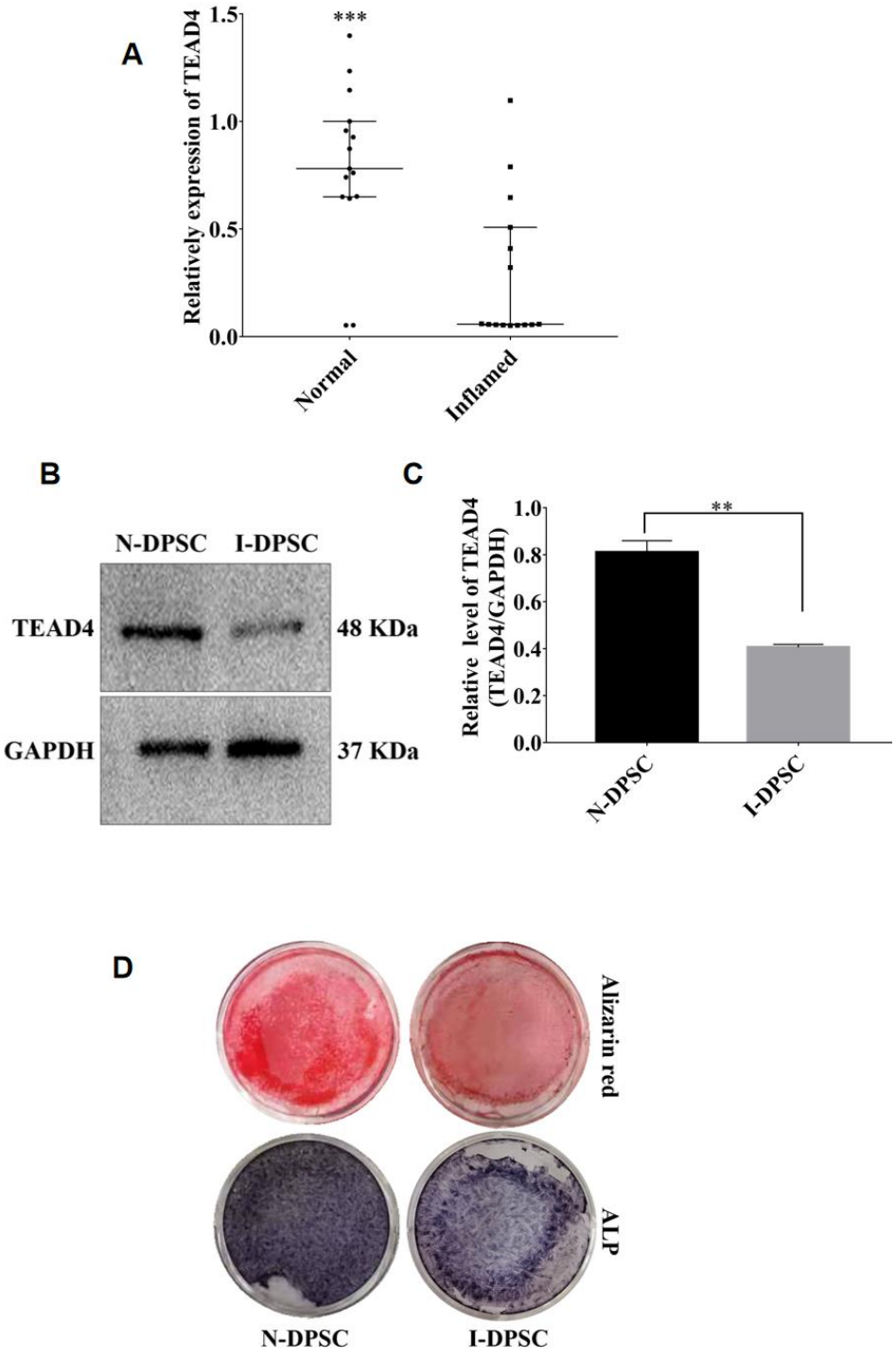

Fig1 The expression of TEAD4 in dental pulp decreased under inflammatory microenvironment . a TEAD4 expression was analyzed in normal (n = 15) and pulpitis dental pulp tissue (n = 15) using qRT-PCR. b The protein expression of TEAD4 in DPSCs cultured in odontogenic differentiation medium and complete medium (as control) was analyzed by Western blot (WB) . c Quantitative data of the WB assay. d Alizarin red staining and ALP staining indicated that the odontogenic capacity of DPSCs derived from carious teeth is weakened as compared with those derived from normal pulps.

**P < 0.01, ***P < 0.001.

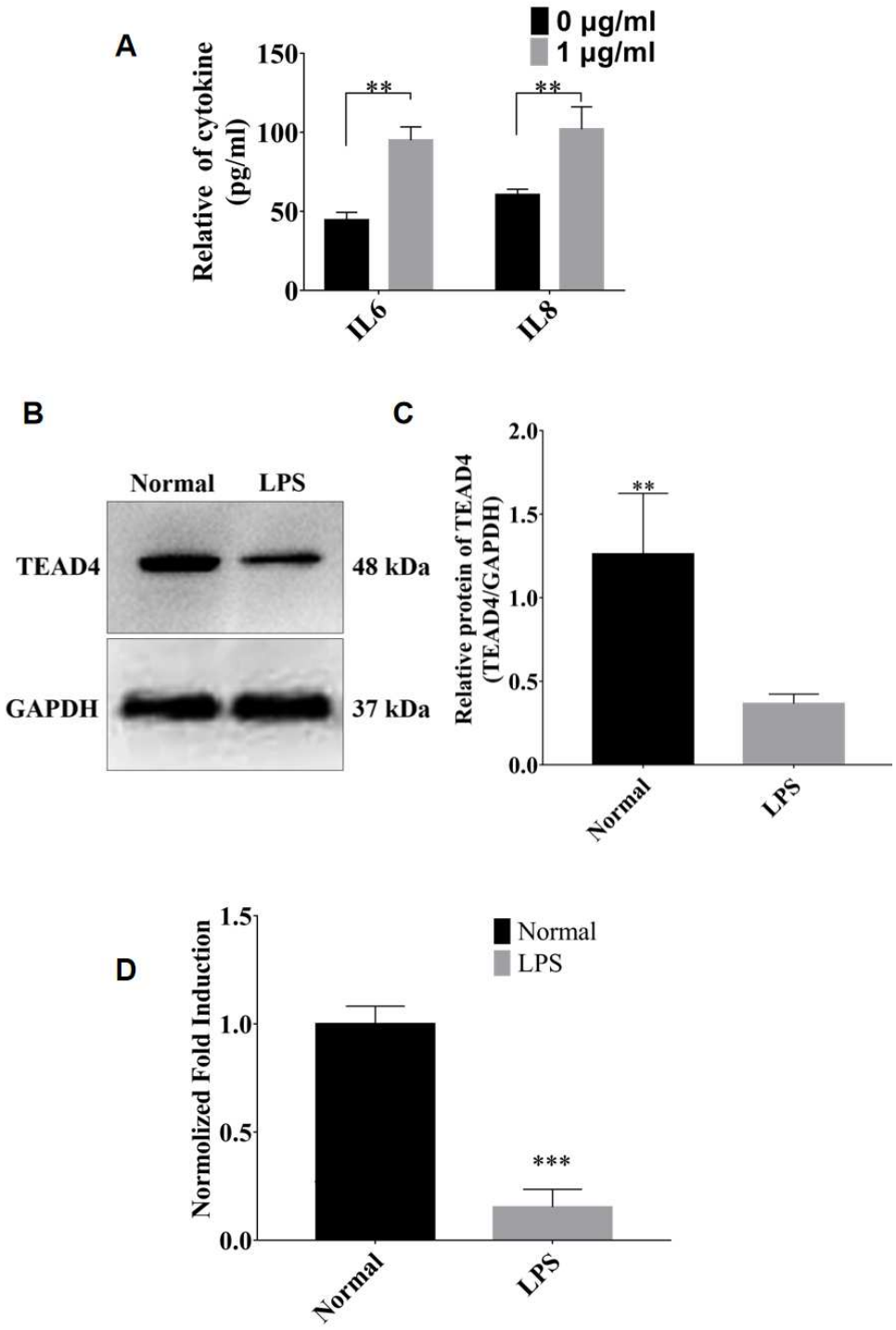

Fig2 The pulpitis model was induced by LPS stimulation.**a** The concentrations of interleukin-6 (IL-6) and interleukin-8 (IL-8) in cell supernatant were detected by ELISA. **b** WB analysis of TEAD4 protein expression in normal and 1μg/ML LPS-stimulated DPSCs (LPS). **c** Quantitative analysis of the protein levels detected by WB . **d** mRNA expression of TEAD4 was detected by qRT-PCR. *P < 0.05, **P < 0.01.

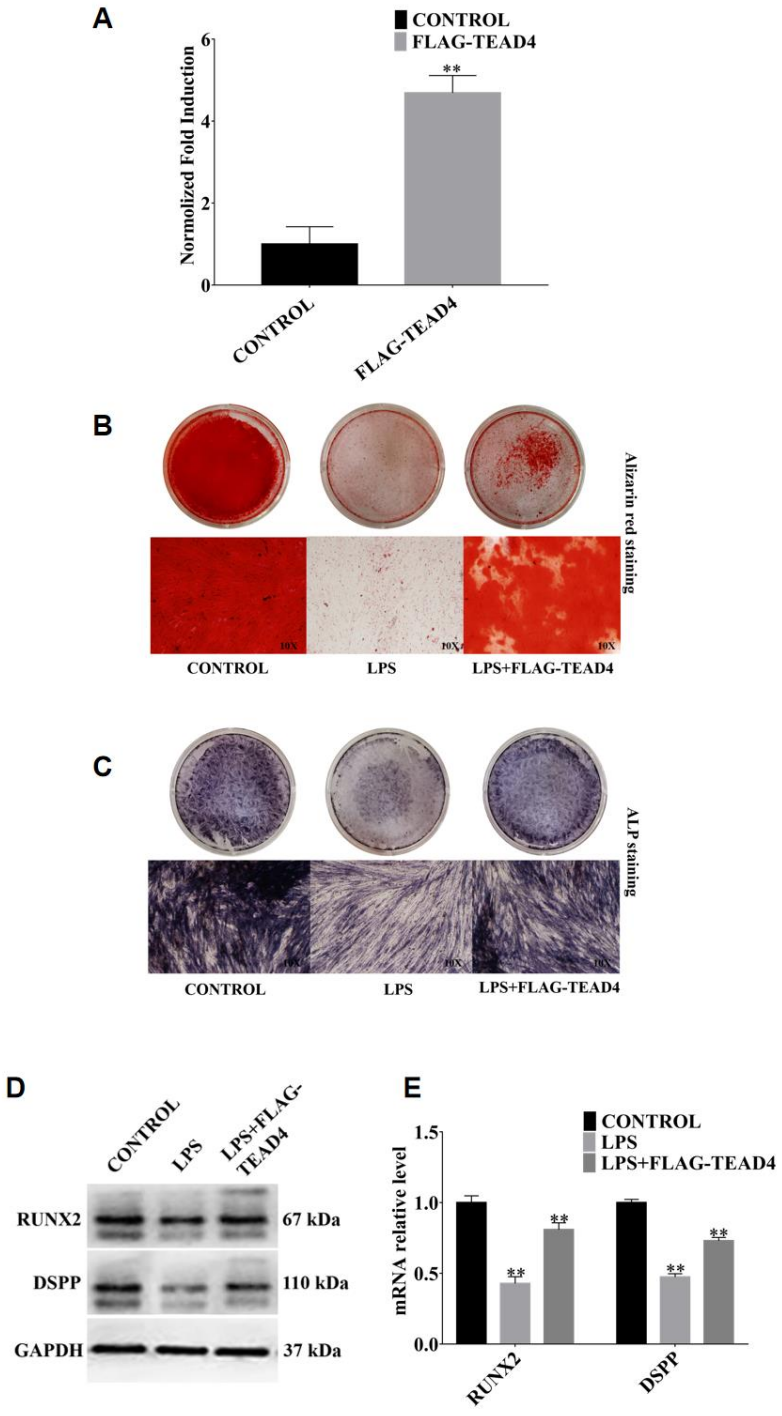

FIG3 Overexpression of TEAD4 restores LPS-induced damage of odontogenic differentiation capacity of DPSCs. **a** The efficiency of TEAD4 overexpression was detected by qRT-PCR. **b**, Osteogenic differentiation was determined by alizarin red staining. **c** Osteogenic differentiation was determined by ALP staining. **d** Western blot analysis of the expression of odontoblast differentiation-related proteins RUNX2 and DSPP. **e** The mRNA expression of TEAD4, RUNX2 and DSPP was detected by qRT-PCR. *P < 0.05, ***P < 0.001

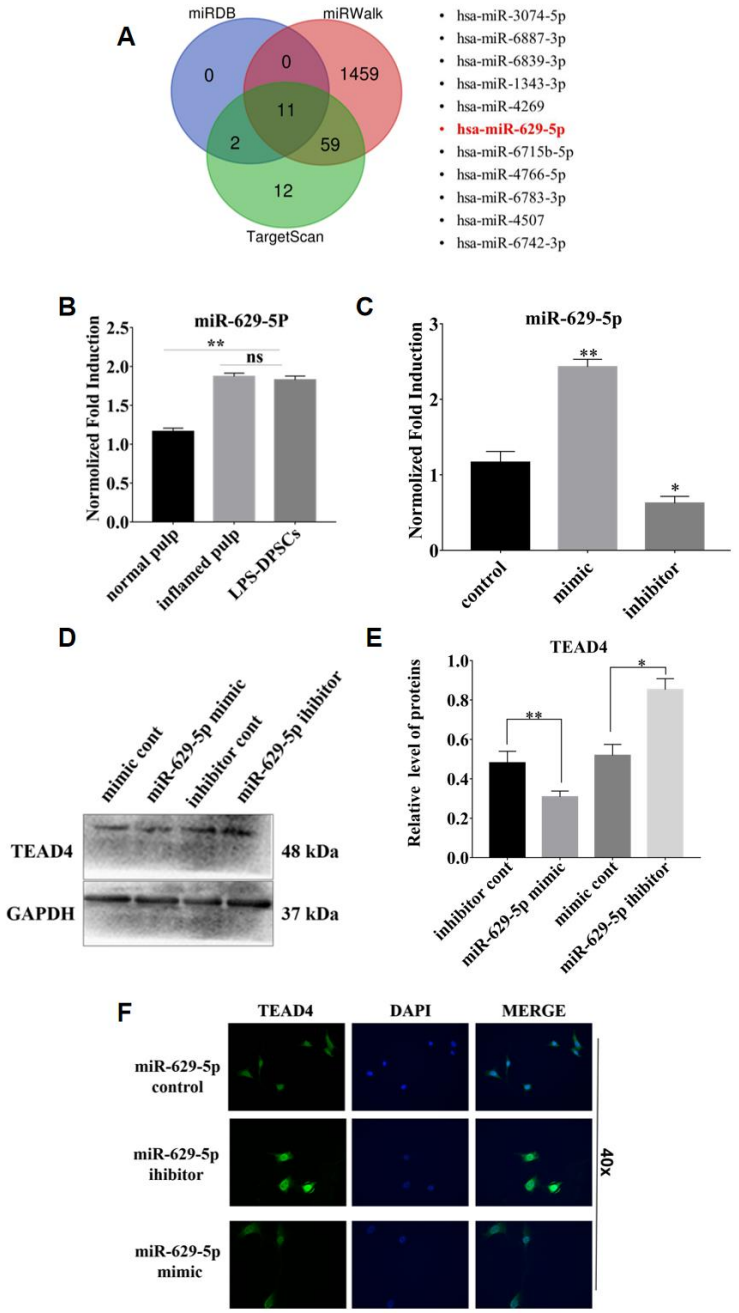

Fig4 miR-629-5p regulates TEAD4 expression. **a** Bioinformatics analysis was applied to predict the target of miR-629-5p and TEAD4 by Targetscan, Mirdb and Mirwalk databases. **b** qRT-PCR was used to detect the level of miR-629-5p in the normal pulp ,inflamed pulp and LPS-treated DPSCs **c** DPSCs were over-expressed and inhibited by miR-629-5p mimic and inhibitor respectively (mimic,inhibitor group), and treated with the same amount of PBS as a control group. Transfection efficiency was detected by qRT-PCR.; **d,** The protein level of TEAD4 in control, mimic and inhibitor group was detected by Western blot. **e** The mRNA level of TEAD4 of thse three groups were detected by qRT-PCR. **f** The expression of TEAD4 (green) in control, mimic and inhibitor group were examined by cytofuorescence. Nuclei are stained with DAPI (blue). Scale bar is 20 μm. *P < 0.05, **P < 0.01,***P < 0.001.

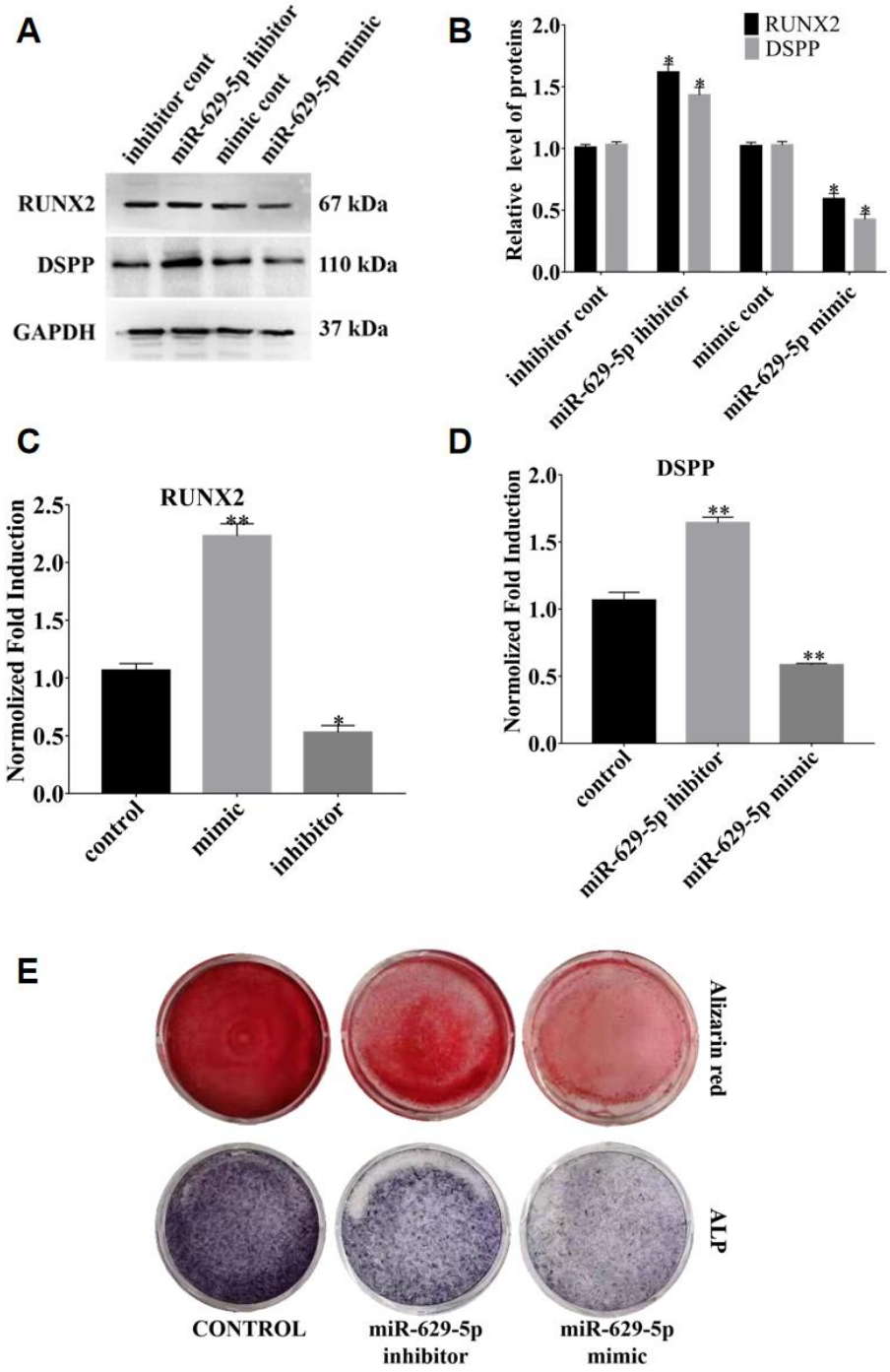

Fig5 Effect of miR-629-5p on odontoblast differentiation. **a and b** The expression of odontoblast differentiation-related proteins RUNX2, DSPP in the control, mimic, inhibitor group was detected by Western blot analysis and quantitative analysis of expression levels of protein were shown. **c and d** The expression of RUNX2 and DSPP in the control, mimic, and inhibitor group were evaluated by RT-PCR.**e** The effect of miR-629-5p overexpression and knockdown on mineralization capacity of DPSC was evaluated by alizarin red and ALP staining. *P < 0.05 **P < 0.01 ***P < 0.001

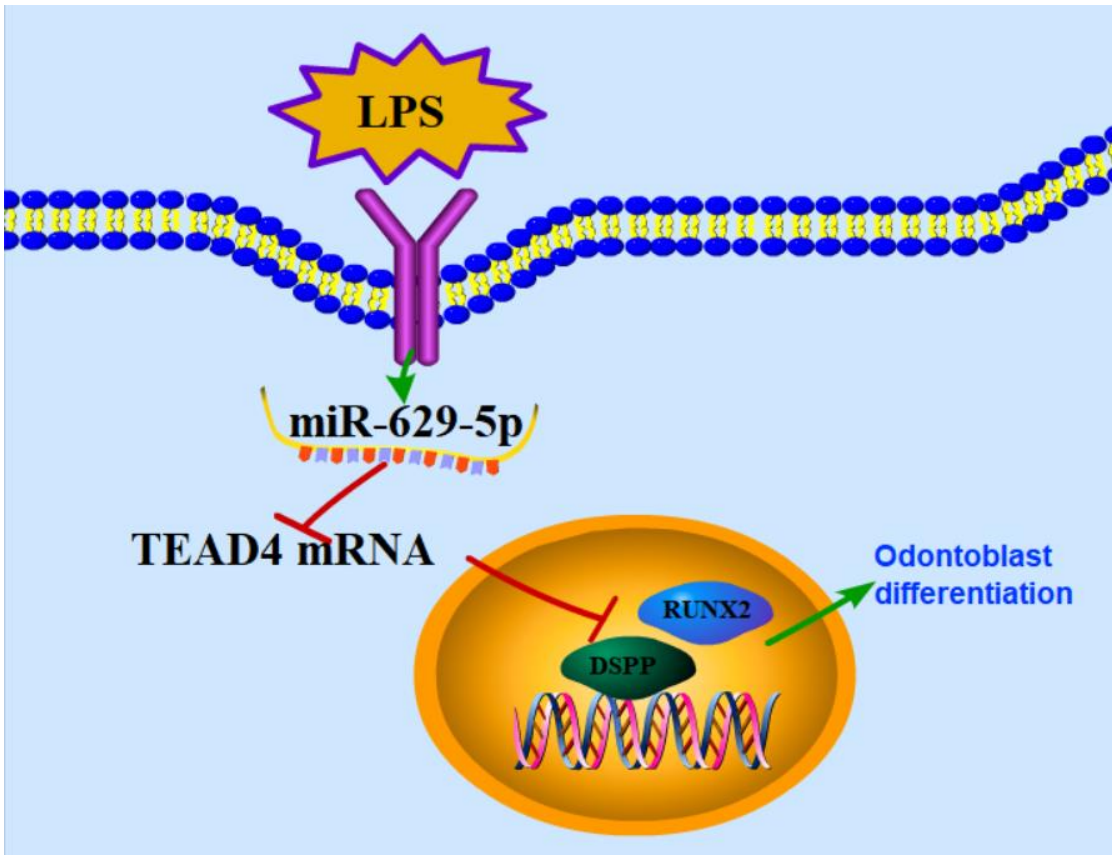

Fig6 schematic diagram showing the interaction between miR-629-5p and TEAD4 in odontogenic differentiation of dental pulp stem cells. In this study, LPS was used to stimulate dental pulp stem cells to simulate the microenvironment of pulp associated with inflammatory. We find that the expression of mir-629-5p improved, and miR-629-5p inhibited the expression of TEAD4，resulting in downregulation of downstream genes of odontogenic differentiation. Then affected odontoblast differentiation of DPSCs,and further affected the progress of reversible pulpitis.