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FOXA1 knockdown alleviates inflammation and enhances osteogenic differentiation of periodontal ligament stem cells via STAT3 pathway

Jin Wang[1] and Yanru Zhu[1*]

Abstract

Background Evidence has confirmed that forkhead box protein A1 (FOXA1) inhibits the osteogenic differentiation of bone marrow mesenchymal stem cells. However, whether FOXA1 regulates the osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs) to participate in periodontitis process is unclear.

Methods Lipopolysaccharide (LPS) was used to treat hPDLSCs to mimic inflammation environments. FOXA1 expression was examined by quantitative real-time PCR and western blot. The levels of IL-6 and TNF-α were evaluated by quantitative real-time PCR, ELISA and immunohistochemistry staining. hPDLSCs osteogenic differentiation was assessed by measuring alkaline phosphatase activity, alizarin red S intensity and the levels of osteogenic differentiation-related markers. Besides, the expression of signal transducer and activator of transcription 3 (STAT3) pathway-related markers were examined by western blot and immunofluorescence staining.

Results FOXA1 was upregulated in the periodontal ligament tissues of periodontitis patients, and its knockdown enhanced osteogenic differentiation of hPDLSCs. Besides, downregulation of FOXA1 suppressed inflammation levels in LPS-induced hPDLSCs. Also, FOXA1 silencing promoted the osteogenic differentiation of LPS-induced hPDLSCs by the inactivation of STAT3 pathway.

Conclusion Our data confirmed that knockdown of FOXA1 attenuated inflammation and enhanced osteogenic differentiation of LPS-induced hPDLSCs by regulating STAT3 pathway, indicating that FOXA1 might be a target for periodontitis treatment.

Keywords Periodontitis, hPDLSCs, Osteogenic differentiation, FOXA1, STAT3

Introduction

Periodontitis is mainly a chronic inflammation of the periodontal supporting tissues caused by local factors, which is also a highly prevalent chronic non-communicable disease globally [1, 2]. A large number of studies have confirmed the independent association between periodontitis and diabetes, cardiovascular disease or chronic kidney disease, which may be a risk factor [3, 4]. Currently, periodontal diagnosis is based on clinical rather than etiologic criteria and therefore provides limited treatment guidance for periodontal [5]. The treatment of periodontitis requires periodontal bone regeneration [6]. Human periodontal ligament stem cells (hPDLSCs), a newly discovered population of mesenchymal stem cells, have great potential for periodontal tissue regeneration

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[7–9]. Besides, many researches indicate that the osteogenic differentiation of hPDLSCs plays a vital function in periodontitis process [10, 11]. Therefore, it is necessary to understand the mechanism of hPDLSCs osteogenic differentiation for developing the therapeutic options of periodontitis.

In the past decades, forkhead box protein A1 (FOXA1) has been found to be associated with many cancers through acting as an anti-cancer and tumor suppressor [12–14]. Previous studies have revealed that FOXA1 plays an active role for intervertebral disc regeneration [15, 16]. Importantly, Li et al. revealed that FOXA1 downregulation facilitated the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by ERK1/2 pathway, and its downregulation effectively promoted bone healing in vivo [17]. Therefore, FOXA1 may be a vital regulator for osteogenic differentiation. However, whether FOXA1 regulates hPDLSCs osteogenic differentiation has not been reported.

Signal transducer and activator of transcription 3 (STAT3) is a member of the cytoplasmic transcription factor family and is involved in regulating multiple cell biological functions [18, 19]. Past studies have identified that the STAT3 pathway is activated in most cancers and is considered a molecular target for cancer treatment [20, 21]. Also, STAT3 plays a key role in the control of inflammation in vertebrates [22]. Meanwhile, STAT3 pathway has been confirmed to participate in the osteogenic differentiation of multiple stem cells [23, 24]. Zhang et al. suggested that PRMT5 knockdown facilitated osteogenic differentiation of hPDLSCs through STAT3/NF-κB pathway [25]. However, whether FOXA1 affects hPDLSCs osteogenic differentiation through STAT3 pathway is unknown.

Here, we found that FOXA1 silencing alleviated lipopolysaccharide (LPS)-induced inflammation and promoted hPDLSCs osteogenic differentiation through the STAT3 pathway. The discovery of FOXA1/STAT3 axis provides new directions for the clinical management of periodontitis.

Methods

Samples collection

Periodontal ligament tissues around the tooth's root were collected from 10 periodontitis patients and 10 normal humans (undergoing orthodontics) at the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University. The specific procedures for periodontal ligament tissue collection were the same as described by Yuan et al. [26], as follows: the extracted teeth was rinsed with physiological saline, and then the periodontal ligament tissues were scraped with a periodontal scaler. A portion of the periodontal ligament tissue was used for hPDLSCs extraction, and the other part was used to make paraffin sections. This study had been approved by the Ethics Committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University. All operation procedures complied with relevant regulations, and all participants signed written informed consent.

Isolation and culture of hPDLSCs

According to the previously described [27], isolated periodontal ligament tissues (1 mm^3) from normal humans were washed by PBS, digested with collagenase type I and dispase II in α-MEM (Gibco, Grand Island, NY, USA) containing 10% FBS and 1% penicillin–streptomycin. Then, cell suspensions were collected and transferred to culture dishes with completed α-MEM at 37 °C and 5% CO_2. After selecting, hPDLSCs were obtained for later use.

Cell transfection, osteogenic induction and treatment

When reached large fusions (80–90%), hPDLSCs were cultured in osteogenic medium (Sigma-Aldrich, St. Louis, MO, USA) for 14 d to induce osteogenic differentiation. To mimic the inflammation environments, hPDLSCs were treated with different concentrations of LPS (0, 1, 5, 10, and 20 μg/mL; MedChemExpress, Monmouth Junction, NI, USA). For transfection, hPDLSCs were transfected with FOXA1 shRNA and its control (RiboBio, Guangzhou, China) by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), followed by treated with 10 μg/mL LPS in osteogenic medium. To explore the role of STAT3 on osteogenic differentiation of hPDLSCs, hPDLSCs were pre-treated with STAT3 inhibitor stattic (10 μM, Selleck Chem, Shanghai, China) before LPS stimulation.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from periodontal ligament tissues and hPDLSCs by TRIzol reagent (Invitrogen). Extracted RNAs were reverse-transcribed by PrimeScript RT Reagent Kit (Takara, Tokyo, Japan), and PCR amplification was performed using SYBR Green (Takara), cDNA and specific primers (Table 1). Relative mRNA expression of associated genes was calculated by $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control.

Western blot (WB)

hPDLSCs were lysed with RIPA buffer (Beyotime, Shanghai, China), and isolated protein was separated by SDS-PAGE and transferred to PVDF membranes. After closed with 5% skim milk, membrane was incubated with anti-FOXA1 (ab170933, 1:1000, Abcam,

Cambridge, MA, USA), anti-STAT3 (ab68153, 1:1000, Abcam), anti-p-STAT3 (ab267373, 1:1000, Abcam), anti-GAPDH (ab9485, 1:2500, Abcam) and secondary antibody (ab205718, 1:1000, Abcam). After that, membrane was incubated with BeyoECL Plus solution (Beyotime) to detect protein signals. Gray value was analyzed by Image 5.0 software with GAPDH as loading control.

Immunohistochemistry (IHC) staining

Periodontal ligament tissues were fixed in 4% formaldehyde solution, embedded in paraffin, and prepared at 4 μm-thickness sections. Basing on the SP Kit (Solarbio, Beijing, China) instructions, sections were deparaffinized in xylene, rehydrated by ethanol, treated with 3% H_2O_2 to inactivate endogenous enzymes. Before closed by goat serum, the section was pre-treated using pressure cooker heat mediated antigen retrieval with 0.01 M sodium citrate buffer (pH = 6.0) for 20 min. Then, sections were incubated with anti-periodontal ligament associated protein 1 (PLAP-1, ab201208, Abcam), anti-IL-6 (ab6672, Abcam), anti-TNF-α (ab307164, Abcam), and anti-FOXA1 (ab170933, Abcam) overnight at 4 °C, followed by treated with goat anti-rabbit IgG (ab205718, Abcam) for 30 min. After stained with DBA solution and counterstained with hematoxylin, the slides were cleared in xylene and coverslipped in neutral balsam. The positive cells of PLAP-1, IL-6, TNF-α and FOXA1 were observed under a microscope.

ELISA

Supernatant of hPDLSCs was incubated with the corresponding ELISA Kits (ab9324, ab285312; Abcam) to measure the IL-6 and TNF-α levels according to kit instructions.

Alkaline phosphatase (ALP) activity assay

ALP Assay Kit (Beyotime) was used to measure ALP activity. Briefly, hPDLSCs were lysed using RIPA buffer, and supernatant was extracted from cell lysates. Supernatant was seeded in 96-well plate, incubated with chromogenic substrate (Beyotime) for 10 min, and terminated with termination solution. After that, absorbance was assessed at 405 nm to calculate ALP activity.

Table 1 Primer sequences used for qRT-PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
FOXA1	GCAATACTCGCCTTACGGCT	TACACACCTTGGTAGTACGCC
TNF-α	GACAAGCCTGTAGCCCATGT	GGAGGTTGACCTTGGTCTGG
IL-6	ACTCACCTCTTCAGAACG AATTG	CCATCTTTGGAAGGTTCA GGTTG
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC

Alizarin red S (ARS) staining

The mineralization nodules of hPDLSCs were determined by ARS staining kit (Beyotime). Briefly, hPDLSCs were washed with PBS, fixed by paraformaldehyde and stained with ARS solution for 30 min. Then, the mineralization nodules were observed under microscope, and ARS intensity was detected using microplate reader.

Immunofluorescence (IF) staining

hPDLSCs were treated with 4% paraformaldehyde, 0.2% Triton X-100 and goat serum. After that, cells were incubated with anti-FOXA1 (ab170933, Abcam), anti-p-STAT3 (ab267373, Abcam), secondary antibodies, and stained with DAPI solution (Beyotime). The signals of FOXA1 and p-STAT3 were observed under a microscope.

Statistical analysis

The original photos of all staining and protein figures are shown in Supplementary file 1. Data are presented as mean ± SD. All analyses were performed using GraphPad Prism 7.0 software. Student's *t*-test was used for comparison between the 2 groups, and one-way ANOVA followed by Tukey's post-hoc tests were used for comparison among multiple groups. $P < 0.05$ was considered as significant.

Results

FOXA1 was overexpressed in periodontitis patients and LPS-induced hPDLSCs

To determine whether the collected periodontal ligament tissue was successful, we evaluated the expression of the periodontal ligament marker PLAP-1 using IHC staining. The results showed that PLAP-1 was significantly expressed in the periodontal ligament tissues (Supplementary Fig. 1). Through qRT-PCR and WB, we found that FOXA1 was higher in the periodontal ligament tissues of periodontitis patients than that in normal humans (Fig. 1A-B). Meanwhile, IHC staining suggested that the levels of IL-6 and TNF-α were elevated in the periodontal ligament tissues of periodontitis patients (Fig. 1C). Furthermore, we detected FOXA1 expression in hPDLSCs treated with different concentrations of LPS. The results revealed that FOXA1 mRNA and protein levels were markedly increased in LPS-induced hPDLSCs in a concentration-dependent manner (Fig. 1D-E). Besides, qRT-PCR showed the enhanced IL-6 and TNF-α mRNA levels in LPS-induced hPDLSCs, which was confirmed by ELISA (Fig. 1F-G). Above results indicated that high FOXA1 expression might be related to periodontitis process.

Silencing of FOXA1 promoted osteogenic differentiation of hPDLSCs

To study the role of FOXA1 in the osteogenic differentiation of hPDLSCs, hPDLSCs were transfected with sh-FOXA1. FOXA1 mRNA and protein levels were significantly decreased in hPDLSCs transfected with sh-FOXA1 (Fig. 2A-B). Functional experiments revealed that silencing of FOXA1 facilitated ALP activity and ARS intensity (Fig. 2C-D). Besides, FOXA1 knockdown enhanced the protein levels of osteogenic differentiation-associated markers (Runx2, COL1A1 and SP7) (Fig. 2E). The above data indicated that FOXA1 might inhibit osteogenic differentiation of hPDLSCs.

FOXA1 knockdown restrained inflammation in LPS-induced hPDLSCs.

To further explore the effect of FOXA1 on inflammation process in periodontitis, hPDLSCs were transfected with sh-FOXA1 and treated with LPS. FOXA1 mRNA and protein levels were increased in LPS-induced hPDLSCs, while this effect could be reversed by sh-FOXA1 (Fig. 3A-B). Through qRT-PCR and ELISA, we found that FOXA1 silencing abolished LPS-mediated the promotion effects on IL-6 and TNF-α levels in hPDLSCs (Fig. 3C-D). These data showed that FOXA1 might promote inflammation in LPS-induced hPDLSCs.

FOXA1 silencing promoted osteogenic differentiation of LPS-induced hPDLSCs via STAT3 pathway

To further explore FOXA1 roles and mechanisms in periodontitis process, we examined the effect of sh-FOXA1 on the osteogenic differentiation of LPS-induced hPDLSCs. As shown in Fig. 4A-B, ALP activity and ARS intensity were suppressed in LPS-induced hPDLSCs, while FOXA1 silencing eliminated these effects. In addition, the inhibitory effect of LPS treatment on the Runx2, COL1A1 and SP7 levels in hPDLSCs could be abolished by FOXA1 silencing (Fig. 4C). Given the role of STAT3 pathway in the osteogenic differentiation of hPDLSCs [25], we also detected the expression of STAT3-related markers. The results of WB and IF staining showed that FOXA1 silencing reverted LPS-mediated the enhancing effect on p-STAT3/STAT3 levels in hPDLSCs (Fig. 4C-D). To further confirm the role of STAT3 on osteogenic differentiation of hPDLSCs, hPDLSCs were treated with STAT3 inhibitor stattic and LPS. The results showed that stattic treatment indeed reduced p-STAT3/STAT3 protein level and promoted the protein levels of osteogenic markers (RUNX2, COL1A1 and SP7) in LPS-induced inflammatory models of hPDLSCs (Supplementary Fig. 2A-B), indicating that STAT3 suppressed osteogenic differentiation in hPDLSCs. Taken together, FOXA1 inhibited osteogenic differentiation in LPS-induced hPDLSCs through the activation of the STAT3 pathway.

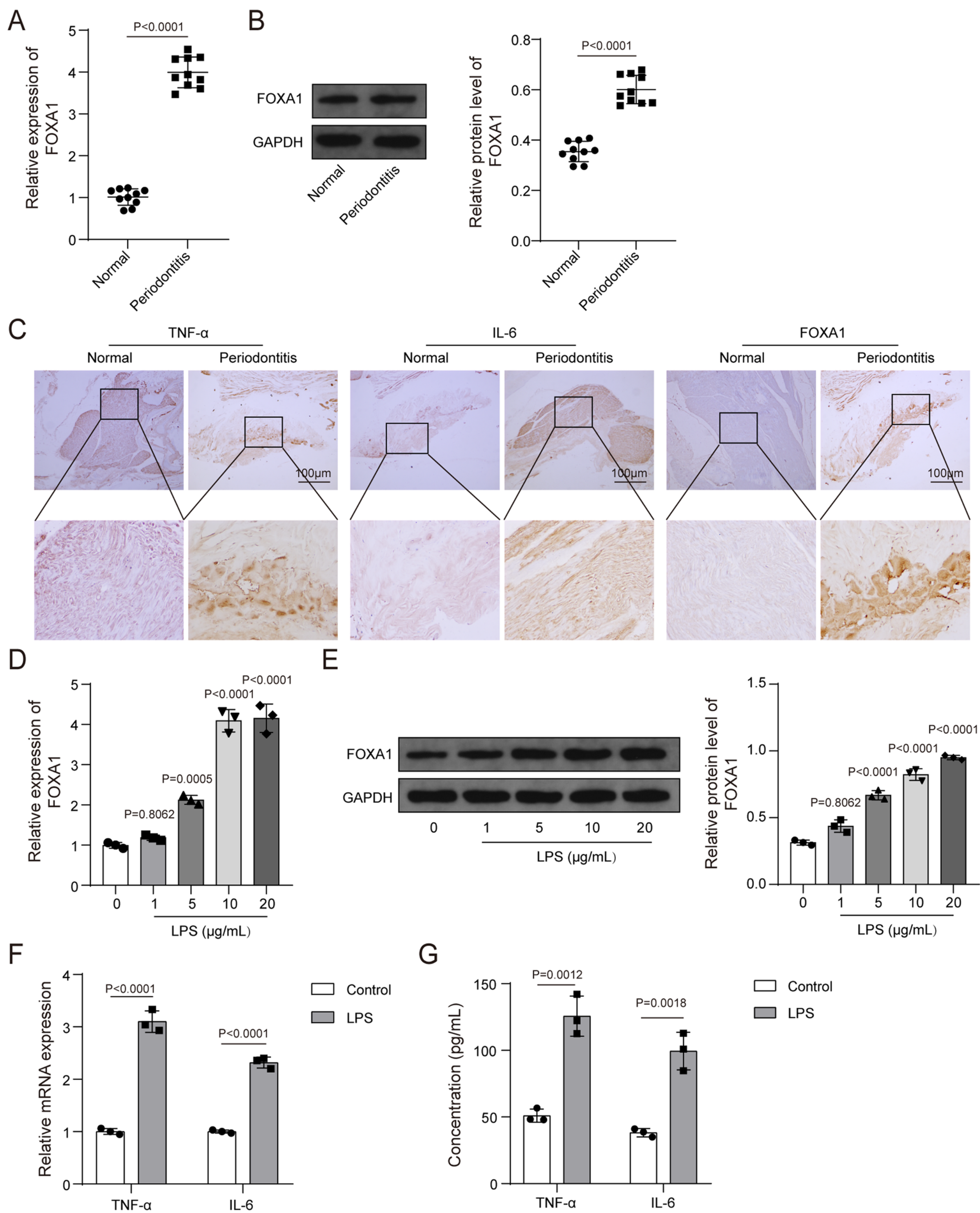
Discussion

Early symptoms of periodontitis are not obvious, but the teeth begin to loosen or even fall off when the periodontitis develops to the middle and late stages, which seriously affect people's quality of life [2]. Importantly, periodontal disease may play a role in the initiation or progression of systemic disease [28]. For patients with periodontitis, surgery, dental implants or laser treatment have their limitations [29, 30]. Therefore, the development of new treatment options for periodontitis is urgent. In this study, we found that FOXA1 knockdown could promote the osteogenic differentiation of LPS-induced hPDLSCs by inactivating STAT3 pathway, suggesting that FOXA1 might be a molecular target for treating periodontitis.

In the past, research on FOXA1 has focused on cancer. FOXA had been reported to be upregulated in gastric cancer tissues, and its downregulation inhibited cell proliferation and invasion [31]. Qiu et al. found that FOXA1 was overexpressed in endometrial cancer, and its silencing inhibited cell proliferation and tumor growth [32]. Currently, FOXA1 has been less studied in regulating stem cell osteogenic differentiation and cellular inflammation. Previous study confirmed that FOXA1 downregulation promoted BMSCs osteogenic differentiation [17], and inhibited the inflammation of alveolar macrophages under LPS conditions [33]. These data verify that FOXA1 may have a role in inhibiting osteogenic differentiation and promoting inflammation. In this, our results revealed that FOXA1 was highly expressed in periodontitis patients and LPS-induced hPDLSCs. Consistent with previous studies [17, 33], we further suggested that FOXA1 silencing

(See figure on next page.)

Fig. 1 FOXA1 expression in periodontitis patients and LPS-induced hPDLSCs. **A**-**B** qRT-PCR and WB analysis were used to test the mRNA and protein levels of FOXA1 in the periodontal ligament tissues of periodontitis patients ($n = 10$) and normal humans ($n = 10$). **C** The levels of IL-6, TNF-α and FOXA1 in the periodontal ligament tissues of periodontitis patients and normal humans were evaluated by IHC staining. **D**-**E** The mRNA and protein levels of FOXA1 were measured by qRT-PCR and WB analysis in hPDLSCs treated with different concentrations of LPS (1, 5, 10, and 20 μg/mL) ($n = 3$). **F**-**G** IL-6 and TNF-α levels in hPDLSCs treated with or without LPS were detected by qRT-PCR and ELISA ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$



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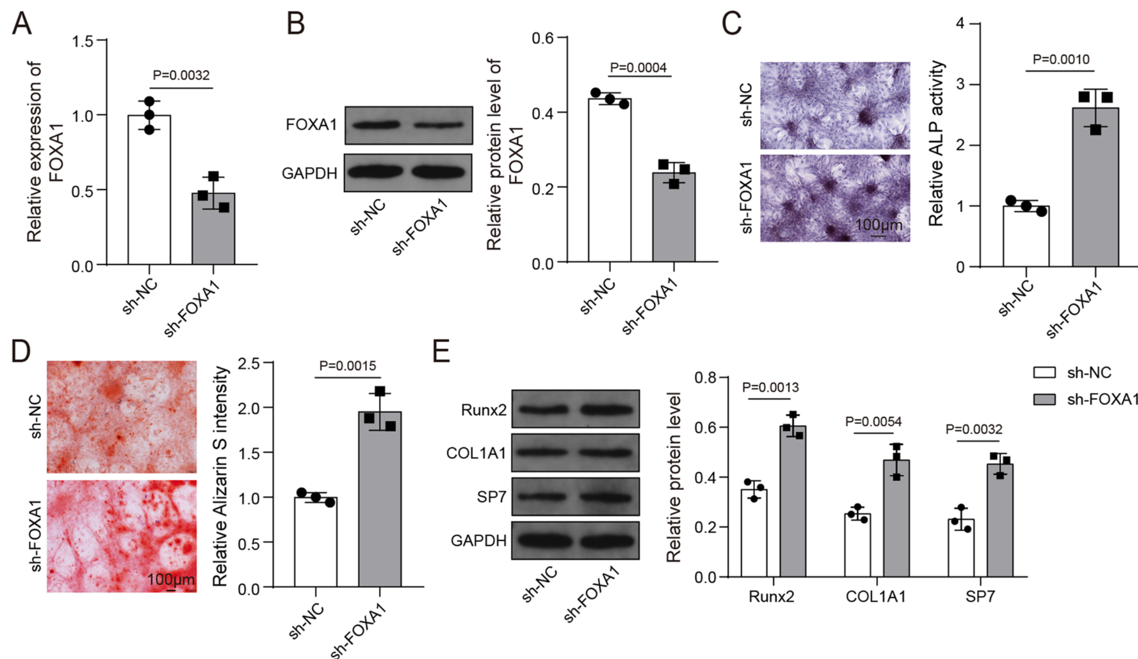

Fig. 2 Effect of sh-FOXA1 on osteogenic differentiation of hPDLSCs. hPDLSCs were transfected with sh-NC/sh-FOXA1 and induced osteogenic differentiation with osteogenic medium ($n=3$). **A**-**B** The mRNA and protein levels of FOXA1 were assessed by qRT-PCR and WB analysis. **C** ALP Assay Kit was performed to evaluate ALP activity. **D** ARS staining was used to measure mineralized areas. **E** The protein levels of Runx2, COL1A1, and SP7 were tested by WB analysis. $^{**}P<0.01$, $^{***}P<0.001$

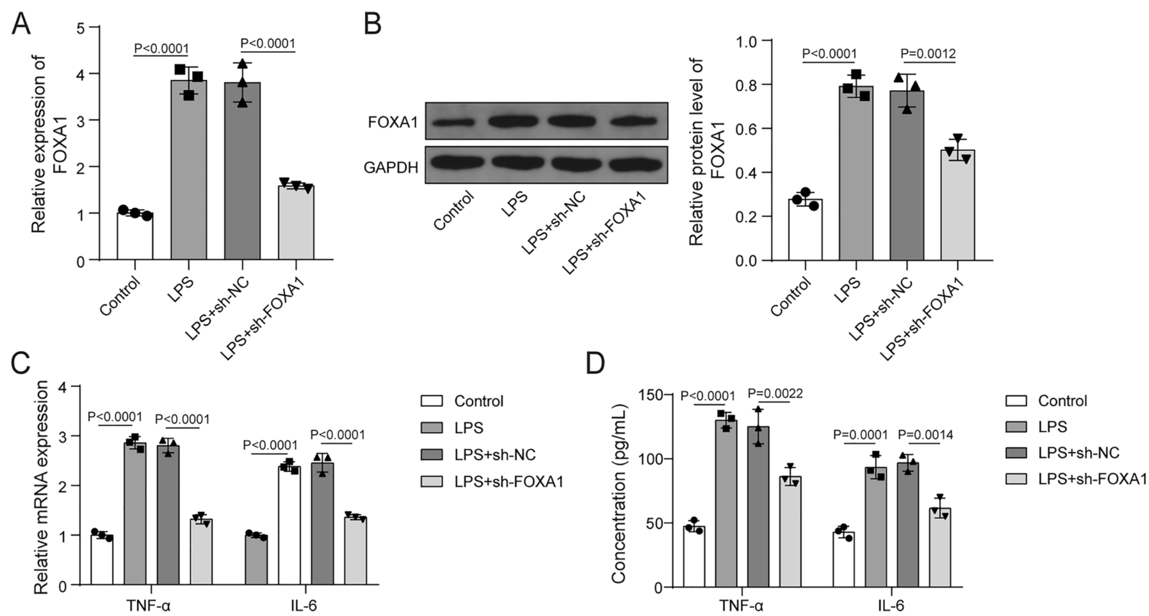

Fig. 3 Effect of sh-FOXA1 on inflammation in LPS-induced hPDLSCs. hPDLSCs were transfected with sh-NC/sh-FOXA1 and treated with LPS in osteogenic medium ($n=3$). Non-treated cells were used as control ($n=3$). **A**-**B** qRT-PCR and WB analysis were used to detect the mRNA and protein levels of FOXA1. **C**-**D** IL-6 and TNF-α levels were measured by qRT-PCR and ELISA. $^{**}P<0.01$, $^{***}P<0.001$

promoted hPDLSCs osteogenic differentiation and suppressed LPS-induced inflammation. This indicates that the FOXA1 may inhibit the osteogenic differentiation and promote inflammation to speed up the process of periodontitis.

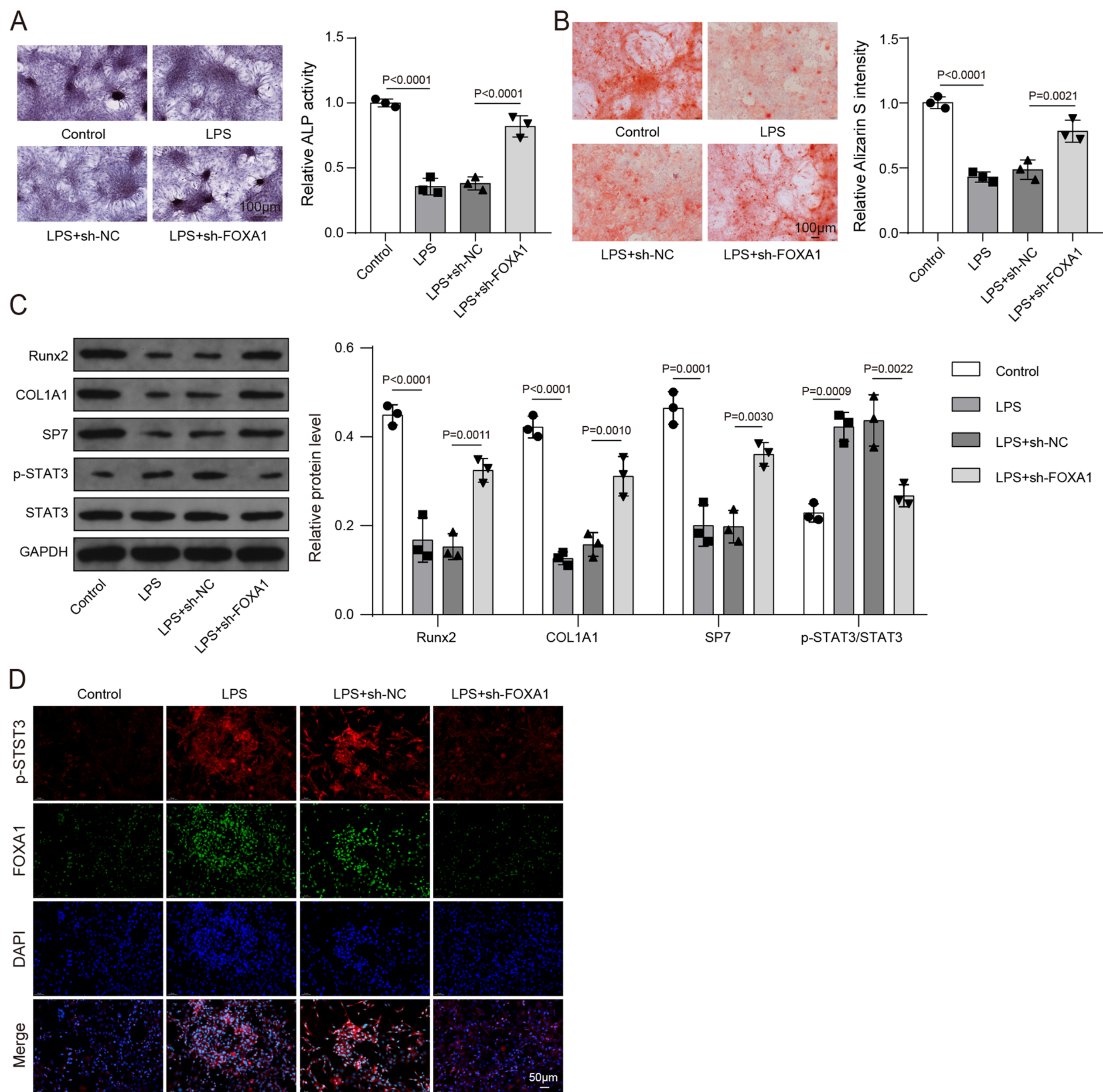

Fig. 4 Effect of sh-FOXA1 on osteogenic differentiation and STAT3 pathway of LPS-induced hPDLSCs. hPDLSCs were transfected with sh-NC/sh-FOXA1 and treated with LPS in osteogenic medium ($n=3$). Non-treated cells were used as control (n = 3). **A** ALP Assay Kit was used to detect ALP activity. **B** Mineralization area was evaluated with ARS staining. **C** WB analysis was used to test the protein levels of Runx2, COL1A1, SP7, p-STAT3 and STAT3. **D** IF staining was performed for detecting FOXA1 and p-STAT3 signals. $^{**}P<0.01$, $^{***}P<0.001$

Activation of the STAT3 pathway appears in a variety of diseases, including breast cancer [34], liver fibrosis [35] and osteosarcoma [36]. Knockdown of hnRNPA2/B1 inhibited STAT3 phosphorylation to regulate breast cancer cell apoptosis and autophagy [37]. Sun et al. demonstrated that METTL3 enhanced malignant progression of colorectal cancer through activation of the JAK1/STAT3 pathway [38]. Also, the research of STAT3 pathway in osteogenic differentiation of stem cells has attracted a lot of attention. It had been reported that PRMT5 downregulation alleviated inflammation and promoted osteogenic differentiation in LPS-induced hPDLSCs through inactivating STAT3/NF-κB pathway [25], indicating that STAT3 pathway was activated by LPS conditions in hPDLSCs. Consistent with this study, we found that LPS treatment activated STAT3 pathway

in hPDLSCs through the detection of p-STAT3/STAT3 levels, and FOXA1 silencing suppressed the activity of STAT3 pathway in LPS-induced hPDLSCs. The evidence suggests that FOXA1 may facilitate periodontitis process by promoting the activity of STAT3 pathway.

Periodontal ligament (PDL) cells have certain stem cell characteristics and can differentiate into the PDL, alveolar bone, and cementum, which are considered to be the main cells for the regeneration and repair of natural periodontal tissue [26]. In this study, we demonstrated that FOXA1/STAT3 axis can inhibit the osteogenic differentiation of PDL stem cells. Therefore, targeted inhibition of FOXA1/STAT3 axis may accelerate periodontal tissue regeneration by promoting osteogenic differentiation, thereby alleviating the progression of periodontitis.

Conclusions

Taken together, we confirmed that FOXA1 knockdown alleviated inflammation and enhanced osteogenic differentiation in LPS-induced hPDLSCs through inhibiting the STAT3 pathway. Of course, there are limitations to our study. In the future, rescue experiments are needed to determine the function of STAT3 pathway in FOXA1-mediated osteogenic differentiation, and in vivo experiments are required to detect the importance of FOXA1/STAT3 axis in periodontitis progression. Collectively, these findings provide new ideas for the clinical treatment of periodontitis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-024-05286-7.

Below is the link to the electronic supplementary material.Supplementary file1 (TIF 1418 kb)

Supplementary file2 (TIF 2652 kb)

Supplementary file3 (DOCX 11343 kb)

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Author contributions
Jin Wang and Yanru Zhu wrote the main manuscript text. Jin Wang and Yanru Zhu prepared Figs. 1, 2, 3, 4. All authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Conflicts of interest
The authors declare no competing interests.

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