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Jumonji domain-containing protein 3 regulates the early inflammatory response epigenetically in human periodontal ligament cells

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ABSTRACT

Objective: To investigate the role of the histone 3 lysine 27 trimethylation (H3K27me3) demethylase Jumonji domain-containing protein 3 (Jmjd3) in the epigenetic regulation of the inflammatory response in human periodontal ligament cells (HPDLs).

Design: HPDLs were stimulated with lipopolysaccharide from *E. coli*. The expression of Jmjd3 in HPDLs was examined by quantitative real-time polymerase chain reaction (Q-PCR), Western Blot and immunofluorescent staining. Potential target genes were selected by silencing Jmjd3 and were confirmed by Chromatin Immunoprecipitation (ChIP).

Results: Q-PCR, Western Blot and immunofluorescent staining revealed that the expression of Jmjd3 was increased in inflamed HPDLs. Knockdown of Jmjd3 led to the suppression of inflammation-induced up-regulation of interleukin-6 and interleukin-12. Moreover, ChIP assays demonstrated that Jmjd3 was recruited to the promoters of interleukin-6 and interleukin-12b and this recruitment was associated with decreased levels of trimethylated histone 3 lysine 27 (H3K27).

Conclusions: It was concluded that Jmjd3 regulated the activation of interleukin-6 and interleukin-12b in the early inflammatory response of HPDLs via demethylation of H3K27me3 at promoters. This molecular event may play an important role in the regulation of the inflammatory response in HPDLs.

1. Introduction

The periodontal ligament is a specialized connective tissue located between the alveolar bone and cementum (Beertsen, McCulloch, & Sodek, 1997). It is composed of heterogeneous cell populations and is predominated by fibroblasts. Microbial stimuli, including gram-negative bacterial pathogens (specifically their membranous lipopolysaccharides) from infected pulp, can cause periodontal ligament infection, leading to apical periodontitis (Graves, Oates, & Garlet, 2011; Lu et al., 2017; Nair, 2006; Yue et al., 2016). Apical periodontitis is an inflammatory disorder of periradicular tissue, mostly resulted from the infection of endodontic origin, characterized by the destruction of the periodontal ligament and alveolar bone in the apical area (Yue et al., 2016). In this scenario, pro- and anti-inflammatory cytokines, secreted by immune cells, periodontal ligament cells (HPDLs) and osteoblasts, play a major role in the regulation of inflammatory immune responses within the periodontal ligament microenvironment (Araujo-Pires et al., 2014; Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). As a consequence, HPDLs plays a vital role in apical periodontitis, and the inflammatory cytokines are critical determinants throughout the entire process of lesion progression.

It is commonly known that epigenetic regulation (including the modification of histones) plays a central role in physiological and pathological processes. Histone methylation status takes part in maintaining or suppressing gene activity. Specifically, methylation of histone 3 lysine 27 (H3K27) keeps the chromatin in a closed state so that the promoters of specific genes are not accessible to the transcriptional machinery, leading to gene silencing (Gallagher et al., 2015). Jumonji domain-containing protein 3 (Jmjd3), as a H3K27 demethylase, can open up the chromatin and activate target gene expression by

Abbreviations: Jmjd3, Jumonji domain-containing protein 3; H3K27me3, histone 3 lysine 27 trimethylation; HPDLs, human periodontal ligament cells; PBS, phosphate-buffered saline; Q-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin Immunoprecipitation; H3K27, histone 3 lysine 27; DAPI, 4′,6-diamidino-2-phenylindole, siCTR, control small interfering RNA transfection group; siJMJD3, Jmjd3 small interfering RNA transfection group

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specifically catalyzing the removal of a methyl group from trimethylated H3K27 (H3K27me3) (Xiang et al., 2007). Jmjd3 was previously shown to regulate inflammation in macrophages, monocytic cells and bone marrow cells (Das et al., 2012; De Santa et al., 2007, 2009) by controlling cytokine gene expression. Additionally, Jmjd3 has been found to be acutely up-regulated during dental pulp inflammation (Hui et al., 2014). However, there is little research on the role of Jmjd3 in periodontal ligament cell inflammation. In this study, we investigated the biological function of Jmjd3 in periodontal ligament inflammation and explored its molecular mechanism of action in HPDLs in vitro.

2. Materials and methods

2.1. Cell culture

Periodontal ligament tissues were collected from healthy human premolars, which were extracted from children aged between 12 and 14 years old for orthodontic reasons. The HPDL on the middle of the root was acquired and cultured in Dulbecco's modified Eagle medium (DMEM; HyClone, Beijing, China) supplemented with 20% fetal bovine serum (HyClone) and an antibiotic solution (100 U/mL penicillin and 100 U/mL streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. Cells that grew out from the collected tissues were passaged in DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). The cells were used between the third and fifth passages. Lipopolysaccharides from *Escherichia coli* 055:B5 (Sigma-Aldrich) were used to stimulate inflammation in HPDLs.

The study was approved by the Ethics Committee of the State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, Sichuan, China. Additionally, we obtained the approval of each patient or his or her parent.

2.2. Immunofluorescent staining

HPDLs were seeded on glass slides. After reaching 30% confluence, cells were treated with lipopolysaccharide for indicated times, and fixed in 4% (v/v) paraformaldehyde. Next, the cells were washed, permeabilized with 0.25% Triton 100, and then washed again with phosphate-buffered solution (PBS). Finally, the cells were incubated with an anti-Jmjd3 (1:50; omnimabs, Alhambra, CA) antibody or PBS (as negative control group) overnight at 4 °C and subjected to immunofluorescent staining. Coverslips were mounted on slides, and slides were viewed under a fluorescence microscope.

2.3. Rat

Fifteen 6-week-old male Wistar rats(weighting 170–200 g) were used in this study. The rats were obtained from a commercial farm (Dashuo, Chengdu, China) and housed in isolated cages throughout the experimental period. They were fed standardized diet of hard briquettes and water, and maintained under a 12-h light/dark cycle (lights on from 08:00 to 20:00) at a temperature of 22 °C. The experimental apical periodontitis were established by opening the pulp chamber of the left maxillary first molar, and the right maxillary first molar was used as control. Exposed teeth were kept open to the oral environment. Rats were killed at 5, 7 and 10 days after pulp exposure respectively (n = 5 in each group). The protocol was approved by the Animal Ethics Committee of Sichuan University.

2.4. Preparation of tissues

The maxillae of each rat was removed immediately after death and was fixed in 4% paraformaldehyde in PBS at 4 °C for 24 h and were decalcified with 10% EDTA for 8 weeks. Finally, the maxillae were embedded in paraffin and cut into 5 μm-thick sections parallel to the long axis of the tooth. The sections included at least one root canal and periapical tissue of maxillary first molar was used for analysis. Haematoxylin-eosin staining was carried out to analyze the situation of inflammation. Immunohistological staining was performed to evaluate the expression of Jmjd3.

2.5. Immunohistochemistry

Immunohistochemistry was performed according to the instructions of SP-9001 kit (Zhongshanjinqiao, Beijing, China). Sections were subjected to epitope recovering in pancreatic enzymes by 0.1% (V/V) at 37 ℃ for 20 min. And then nonspecific immunoglobulin binding was blocked by 5% (V/V) bovine serum albumin for 30 min at 37 ℃. Sections were incubated with rabbit polyclonal antibodies against Jmjd3 (1:100; omnimabs, Alhambra, CA), overnight at 4℃. Then, sections were stained. Specimens were developed with a 3,3′-Diaminobenzidine tetrahydrochloride chromogen kit (Zhongshanjinqiao, Beijing, China) and counterstained with hematoxylin.

2.6. Transfection with small interfering RNA

HPDLs were transfected with a small interfering RNA targeting Jmjd3 or a control small interfering RNA (Santa Cruz Biotechnology, Santa Cruz, CA) using the Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were collected after transfection with small interfering RNA for analysis of messenger RNA and protein levels.

2.7. Real-time quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from cells using the TRIZOL reagent (Life Technologies, Carlsbad, CA, USA). First strand complementary DNA synthesis from the total RNA template was performed with the PrimeScript RT reagent kit with genomic DNA Eraser (Perfect Real Time; Takara, Dalian, Liaoning, China). The resulting complementary DNAs were subjected to Q-PCR with SYBR Premix Ex TaqTM II (Perfect Real Time; Takara) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) or in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Expression levels of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primer sequences used for Q-PCR reactions are listed in Table 1.

Table 1
All primer sequences used for PCR reactions.

Primer	Forward (5'-3')	Reverse (5'-3')
GAPDH	GTCTTCACCACCATGGAGAAG	GTTGTCATGGATGACCTTGGC
Jmjd3	CACTGGAGCAAGTGTGGAAC	GTCTGTTCAGAGTTGCAGCC
IL-1β	CAGCCAATCTTCATTGCTCA	TCGGAGATTCGTAGCTGGAT
IL-6	GTGCCTCTTTGCTGCTTTCAC	GGTACATCCTCGACGGCATCT
IL-8	TTCAGAGACAGCAGAGCACA	AGCACTCCTTGGCAAAACTG
IL-12b	TTCTTCATCAGGGACATCATCAAAC	GTCAGGGAGAAGTAGGAATGTGGA