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Original Article

Histone Demethylase Jmjd3 Regulates the Osteogenic Differentiation and Cytokine Expressions of Periodontal Ligament Cells

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Periodontal ligament (PDL) cells are critical for the bone remodeling process in periapical lesions since they can differentiate into osteoblasts and secrete osteoclastogenesis-promoting cytokines. Post-translational histone modifications including alterations of the methylation status of H3K27 are involved in cell differentiation and inflammatory reaction. The histone demethylase Jumonji domain-containing 3 (Jmjd3) specifically removes methylation of H3K27. We investigated whether Jmjd3 is involved in the osteogenic differentiation and secretion of PDL cells' inflammatory factors. Jmjd3 expression in periapical lesions was examined by immunostaining. Using siRNA specific for Jmjd3 or the specific Jmjd3 inhibitor GSK-J4, we determined Jmjd3's roles in osteogenic differentiation and cytokine production by real-time RT-PCR. The locations of Jmjd3 and NF-κB were analyzed by immunocytochemistry. Compared to healthy PDLs, the periapical lesion samples showed higher Jmjd3 expression. Treatment with GSK-J4 or Jmjd3 siRNA suppressed PDL cells' osteogenic differentiation by suppressing the expressions of bone-related genes (Runx2, Osterix, and osteocalcin) and mineralization. Jmjd3 knockdown decreased the expressions of cytokines (TNF-α, IL-1β, and IL-6) induced by lipopolysaccharide extracted from Porphyromonas endodontalis (Pe-LPS). Pe-LPS induced the nuclear translocations of Jmjd3 and NF-κB; the latter was inhibited by GSK-J4 treatment. Jmjd3 appears to regulate PDL cells' osteogenic differentiation and proinflammatory cytokine expressions.

Key words: periapical lesions, histone demethylase Jmjd3, periodontal ligament cell, osteogenic differentiation, proinflammatory cytokines

The periodontal ligament (PDL), a special tissue that connects the tooth root and alveolar bone, is related to tooth support, nutrition, sensory, and tissue repair. Fibroblasts are the major component of PDL cells and have the potential to differentiate into osteoblastic cells, cementoblast-like cells, and adipocytes [1-3]. In physiological conditions, PDL cells maintain PDL tissues in a fibrous state. In pathological conditions such as periodontitis and periapical lesions, cementoblastic/osteogenic differentiation from PDL cells is important for supporting the regeneration of the periodontium and bone tissue. PDL cells also participate in the inflammatory reaction by producing immunomodulatory cytokines. Under treatment with lipopolysaccharide (LPS), PDL cells secreted the inflammatory mediators prostaglandin E2 and interleukin-6 (IL)-6, which induced osteoclastogenesis in the

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surrounding region [4]. PDL cells were also reported to affect the migratory ability, phagocytic activity, and phenotypic maturation of the dendritic cells and macrophages. These observations indicate that PDL cells are not only structural elements of the periodontium but also influence bone remodeling and immune responses by interacting with innate immune cells [5].

Post-translational histone modifications including methylation, acetylation, phosphorylation, and ubiquitination are closely associated with eukaryotic gene expression. Histone methylation occurs on lysine and arginine residues on histone proteins. These residues exhibit in different methylated forms with lysines (K), being monomethylated (me1), dimethylated (me2), or trimethylated (me3), and arginines (R) being monomethylated (me1) or dimethylated (me2) [6]. Trimethylation on histone H3 lysine 27 (H3K27me3) leads to transcriptional repression and is reversed by the histone demethylase Jumonji domain-containing 3 (Jmjd3) (also known as kdm6b) and tetratricopeptide repeat X chromosome (Utx). Jmjd3 can regulate cell differentiation, apoptosis, tumorigenesis, and an inflammatory reaction [7-9].

Our research has demonstrated that the crucial roles of Jmjd3 in both osteoblast differentiation and apoptosis, in which BMP-2/smads and ERK signaling pathways were involved [10, 11]. It has not been established, whether Jmjd3 can regulate the differentiation and function of periodontal ligament cells. In present study, we detected the expression of Jmjd3 in PDL cells with/without periapical lesions. We also observed the effects of Jmjd3 on the expressions of osteoblast-related genes and inflammatory cytokines in PDL cells.

Materials and Methods

Materials. Alpha-modified Eagle's minimal essential medium (α-MEM) was from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was from CLARK (Richmond, VA, USA). Anti-Jmjd3 and anti-β-actin antibodies were obtained from Abcam (Cambridge, MA, USA). Antibody against NF-κB p65 (C-20) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). GSK-J4 was from Tocris Bioscience (Bristol, UK). L-ascorbic acid and β-glycerophosphate were from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection. Tissues were obtained from 22 adult patients undergoing extraction of a tooth for orthodontic or therapeutic reasons at China Medical University (Shenyang, China). The exclusion criteria of this study were: patients with systemic diseases, osteoporosis, or diabetes and patients who had been treated with an antibiotics or hormonal drugs within 3 months before this study. The study was approved by the ethics committee of China Medical University (No.20150302, Shenyang, China), and each patient signed informed consent. Specimens of apical lesions and healthy PDLs were immobilized by 10% buffered formalin for immunohistochemical analysis.

Immunohistochemical analysis. PDLs of diseased apical tissues and normal apical tissues were immobilized by 10% buffered formalin and embedded in paraffin. We then sliced the tissues into 6-μm sections, dehydrated and deparaffinized the sections, and blocked them with horse serum. The sections were then incubated with anti-Jmjd3 (1 : 200) antibody overnight at 4°C. After being washed with phosphate-buffered saline (PBS), the sections were incubated with a biotinylated secondary antibody (Maixin Bio, Fuzhou, China) and avidin-biotin-peroxidase complex (Maixin Bio). The reaction was then carried out with a 3,3'-diaminobenzidine kit (Maixin Bio).

Cell culture. SH-9 immortalized human PDL cells were maintained in α-MEM supplemented with 10% FBS (growth medium) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air [12]. To induce osteogenic differentiation, 6 mM β-glycerophosphate and 50 μg/mL ascorbic acid were added to the medium as the differentiation medium. To induce the expression of cytokines, we supplemented the growth medium with 10μg/mL lipopolysaccharide from Porphyromonas endodontalis (Pe-LPS) as follows. The specific Jmjd3 inhibitor GSK-J4 diluted in PBS was used to inhibit the demethylase activity of Jmjd3.

Porphyromonas endodontalis LPS (Pe-LPS) Preparation. Porphyromonas endodontalis (P. endodontalis, ATCC® 35406™) was purchased from the Central Laboratory of Capital Medical University (Beijing, China) and cultured anaerobically at 37°C. We extracted P. endodontalis LPS by the hot phenol water method [13]. Based on our previous findings, we used 10 μg/mL Pe-LPS for the follow-up experiments in the present study [14].

Gene expression analysis. The RNA was extracted with the Trizol purification system in accordance with the manufacturer's protocol (Invitrogen, Carlsbad, CA,

USA). After purification, cDNA was synthesized using a PrimeScript reverse transcription kit (Takara, Shiga, Japan). A quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out with a 7500 real-time RT-PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR *Premix Ex Taq*TM (Takara, Shiga, Japan).

The Primer sequences in this study were as follows: Gapdh: Forward, 5'-GCACCGTCAAGGCTGAG AAC-3' Reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. Jmjd3: Forward, 5'-CACGCGGCTCGTGTATGTA-3' Reverse, 5'-GGGTCACAGCTAGCATTGGAA-3'. Runx2: Forward, 5'-ACAACCACAGAACCAC AAG-3' Reverse, 5'-TCTCGGTGGCTGGTAGTGA-3'. Osterix: Forward, 5'-GCCAGAAGCTGTGAAAC CTC-3' Reverse, 5'-GCTGCAAGCTCTCCATA ACC-3'. Osteocalcin (OCN): Forward, 5'-AGGGCA GCGAGGTAGTGAAG-3' Reverse, 5'-AAGGGCAAG GGGAAGAGGAAAG-3'. Tumor necrosis factor-alpha (TNF-α): Forward, 5'-GACAAGCCTGTAGCCCATG TTGTA-3' Reverse, 5'-CAGCCTTGGCCCTTGAAG A-3'. IL-1β: Forward, 5'-CTTCTTCGACACATGGG ATAAC-3' Reverse, 5'-TTTGGGATCTACACTCTCT CCAGC-3'. IL-6: Forward, 5'-AAGCCAGAGCTGTG CAGATGAGTA-3' Reverse, 5'-TGTCCTGCAGCCAC TGGTTC-3'.

SDS-PAGE and Western blot analysis. Total protein of cells were extracted with a protein extraction kit (Bio-Rad, Hercules, CA, USA). Extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The PVDF membranes were placed in TBST (Tris-buffered saline, 0.1% Tween 20) containing 5% non-fat milk to seal off the non-specific binding site, and then incubated overnight at 4°C with either an primary anti-Jmjd3 antibody (diluted at 1 : 1000 v : v) or anti-β-actin antibody (diluted at 1 : 2000 v : v). After incubation for 2 h at room temperature with the secondary antibody conjugated with Dylight fluorescence 800 (LI-COR, Lincoln, NE, USA) (diluted at 1 : 20000 v : v), the protein bands were analyzed by the Odyssey CLX imaging system (Li-Cor).

Small interfering RNA (siRNA) transfection. The duplexes of each small interference RNA (siRNA) targeting Jmjd3 and negative controls (non-silencing siRNA or scramble siRNA) were obtained from Invitrogen. With the help of Lipofectamine2000 (Invitrogen), the siRNAs were transfected into the cells. The target sites were as follows: siJmjd3, 5'-GGGAA GUUUCGAGAGUCCUACCUUU-3', 5'-AAAGGUA GGACUCUCGAAACUUCCC-3'; and siCont, 5'-UU CUCCGAACGUGUCACGUTT-3', 5'-ACGUGACAC GUUCGGAGAATT-3'.

Alizarin red staining. After incubation with osteogenic differentiation medium for 21 days, cells were washed twice with PBS and fixed with 3.7% formaldehyde for 10 min. Next, 60 mM Alizarin red (Sigma-Aldrich) solution was added to each well for staining for 10 min.

Immunocytochemical analysis. The cells seeded on the cover slips were pretreated with 3.7% formaldehyde at room temperature for 10 min and then permeabilized with methanol for 20 min at −20°C. Samples were equilibrated with 4% bovine serum albumin (BSA) in PBS for 40 min in a humidified atmosphere and then incubated at 4 °C overnight with anti- NF-κB or Jmjd3 antibody (diluted at 1 : 500 v : v). The cover slides were rinsed three times with PBS over a 20-min period and then incubated with anti-rabbit Alexa Fluor 488 IgG (Invitrogen/Thermo Fisher Scientific) for 40 min and 10 μg/mL Hoechst 33342 for 20 min, both diluted 1 : 500 in 4% BSA. Washing steps were performed with PBS. The cover slips were then mounted with fluorescent mounting medium (Dako Cytomation, Carpinteria, CA, USA). The cells were visualized with an Olympus BX50 microscope equipped with epi-fluorescence illumination (using U-MNIBA and WU filters for green and blue fluorescence, respectively).

Statistical analysis. Each experiment was repeated at least three times, and the data are reported as the mean±SEM. Differences between two or more groups were analyzed by a one-way analysis of variance (ANOVA) and the Newman-Keuls post-hoc test. Probability p-values < 0.01 were considered significant.

Results

Jmjd3 expression was increased in periapical lesions. We evaluated the expression of Jmjd3 in the PDL tissue samples obtained from patients with and without periapical lesions by conducting an immunohistochemical analysis. The PDL tissue from patients with periapical lesions exhibited higher level of Jmjd3 staining compared to the healthy PDL tissue (Fig. 1).

The expression of Jmjd3 was increased during the

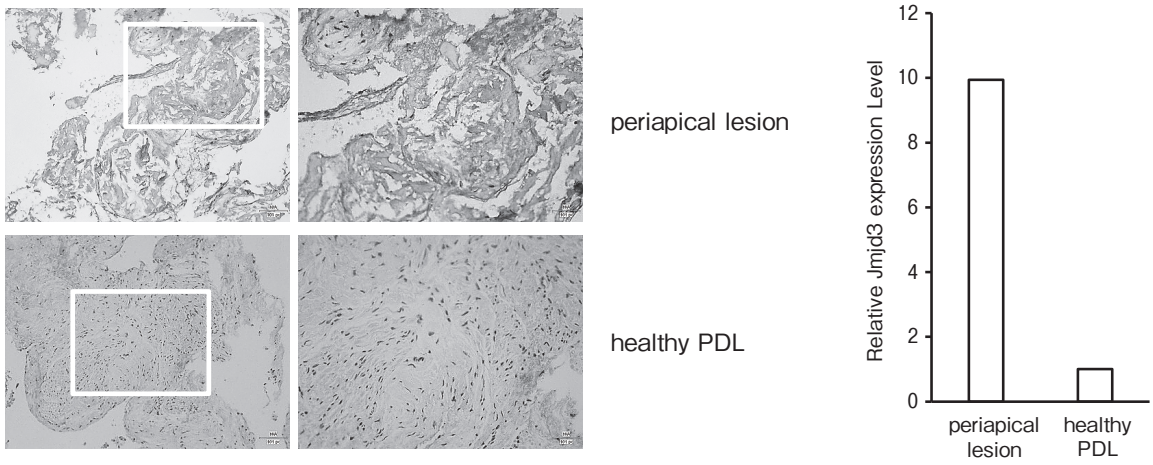

Fig. 1 Expression of Jmjd3 increased in periapical lesions. Representative images of Jmjd3 expression in healthy periodontal ligament and periapical lesions tissue, as determined by immunohistochemistry staining. Increased staining was observed in periapical lesions (Left). The immunohistochemical staining data was quantified by ImageJ software (v0.5.1, National Institutes of Health, Bethesda, MD, USA) (Right).

osteogenic differentiation of SH-9 cells. SH-9 cells were cultured in osteogenic differentiation medium for the indicated periods (Fig. 2). As determined by the real-time RT-PCR, the expressions of Jmjd3 (Fig. 2A) was increased, as were those of bone-related genes including Runx2 (Fig. 2C), Osterix (Fig. 2D), and OCN (Fig. 2E). The results of the Western blot analysis revealed that the intensity of a band corresponding to Jmjd3 increased from day 0 to day 7 (Fig. 2B). The levels of β-actin (used as the internal control) did not change during the culture period (Fig. 2B). SH-9 cells were cultured in osteogenic differentiation medium for 21 days. The mineralization of the cells were assessed by Alizarin red staining (Fig. 2F).

The inhibition of Jmjd3 demethylase activity impaired the osteogenic differentiation of SH-9 cells. To explore whether the demethylase activity of Jmjd3 regulate osteogenic differentiation, we cultured SH-9 cells in osteogenic differentiation medium for 7 days with GSK-J4, a potent inhibitor of Jmjd3. The treatment with GSK-J4 suppressed the expressions of Runx2 (Fig. 3A), Osterix (Fig. 3B), and OCN (Fig. 3C), but not that of Jmjd3 (Fig. 3D), as shown by a real-time RT-PCR.

The silencing of Jmjd3 expression impaired osteogenic differentiation and mineralization. To further assess the roles of Jmjd3 in the osteogenic differentiation of SH-9 cells, we performed gene silencing experiments. The transient silencing of Jmjd3 by siRNA transfection into SH-9 cells markedly suppressed the expressions of Jmjd3, Runx2, Osterix, and OCN compared to those in the siCont cells, as revealed by a real-time RT-PCR (Fig. 3E). We also cultured siCont and siJmjd3 cells in the osteogenic differentiation medium for 21 days, and the mineralization were then assessed by Alizarin red staining. The intensity of staining was decreased in the siJmjd3 cells compared to that in the siCont cells (Fig. 3F).

The silencing of Jmjd3 inhibited the expressions of cytokines and NF-κB translocation. As described in Figure 4, SH-9 cells were cultured in the growth medium with 10 μg/mL Pe-LPS for the indicated periods, and the expressions of Jmjd3, TNF-α, IL-1β, and IL-6 were detected by real-time RT-PCR. Jmjd3 expression was induced by Pe-LPS, as were the expressions of TNF-α, IL-1β, and IL-6 (Fig. 4A). We also cultured siCont and siJmjd3 cells in the growth medium with 10 μg/mL Pe-LPS for 6 h. The silencing of Jmjd3 inhibited the expressions of TNF-α (Fig. 4C), IL-1β (Fig. 4D), and IL-6 (Fig. 4E) as well as that of Jmjd3 (Fig. 4B) as shown by real-time RT-PCR.

To investigate whether Jmjd3 regulates the expression of proinflammatory cytokines by affecting the NF-κB pathway, we cultured SH-9 cells in growth medium with 10 μg/mL Pe-LPS for 15, 30, and 60 min. The cells were then subjected to indirect immunostain-

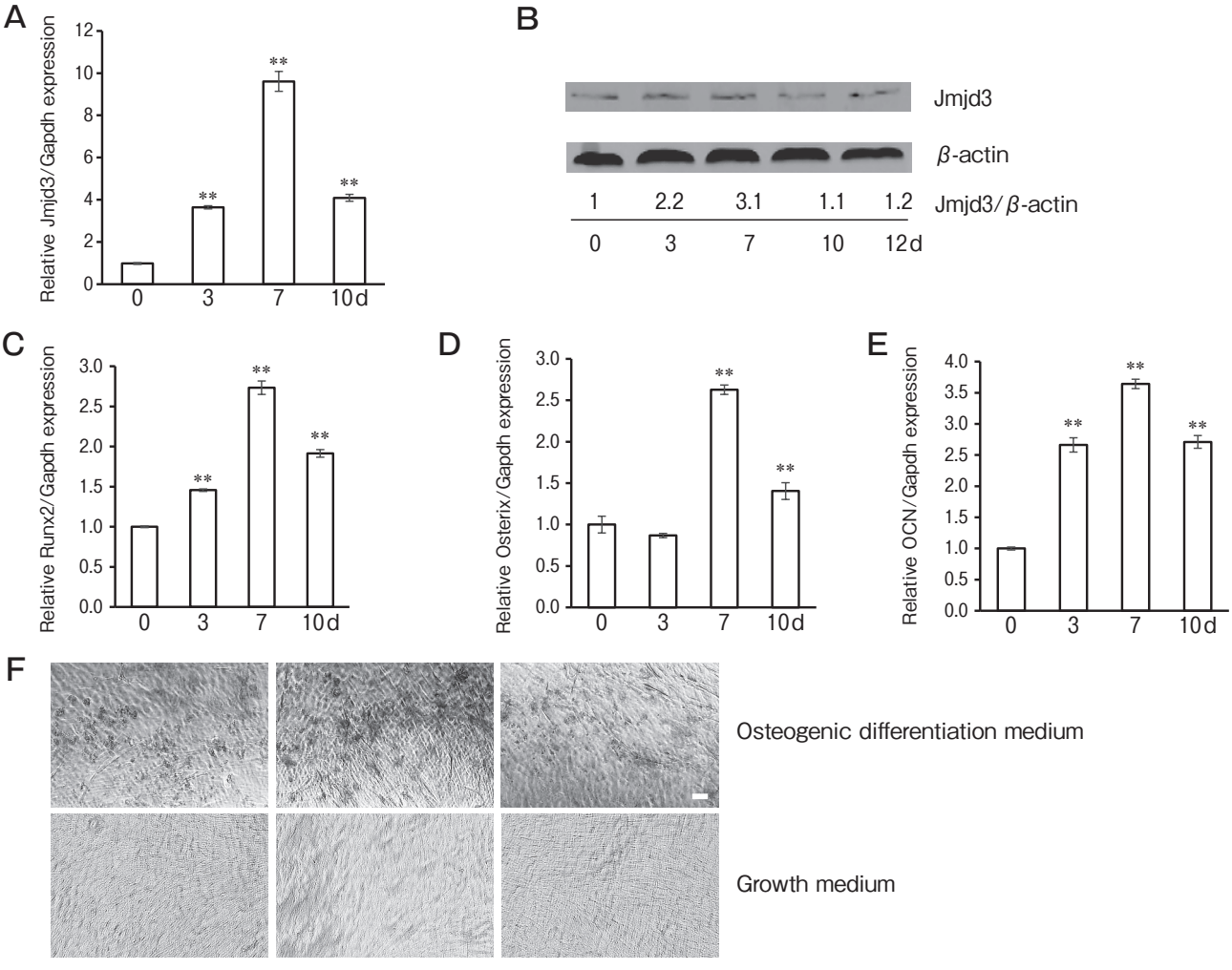

Fig. 2 Expression of Jmjd3 increased in PDL cells treated with osteogenic differentiation medium. SH-9 cells were cultured in osteogenic differentiation medium for the indicated periods. The expression of Jmjd3 was examined by Real-time RT-PCR (**A**) and Western blot (**B**). The expressions of osteoblast-related markers including Runx2 (**C**), Osterix (**D**), and OCN (**E**) were examined by Real-time RT-PCR. Mineralization of SH-9 cells cultured for 21 days with osteogenic differentiation medium (**F**, upper) and growth medium (**F**, lower) was determined by Alizarin red staining. Values from real-time RT-PCR data represent the mean ± S.E.M. of representative analysis from 3 separate experiments. $^{**}p<0.01$. The Western blot data were quantified by Image J software. Bar indicates 20 μm.

ing using an anti-NF-κB antibody or Jmjd3 antibody. After treatment with Pe-LPS for 30 min, the Jmjd3 and NF-κB entered into the cell nuclei simultaneously (Fig. 5A). SH-9 cells were pretreated with 10 μM GSK-J4 for 2 h prior to Pe-LPS treatment. The nuclear translocation of NF-κB was analyzed by immunostaining. Nuclear translocation of NF-κB was observed in cells treated with Pe- LPS (Fig. 5B, upper). However, in GSK-J4-pretreated cells, NF-κB was localized mainly in the cytoplasm (Fig. 5B, lower).

Discussion

Epigenetic regulation, including post-translational histone modification, is an important mechanism related to various bone diseases such as osteoporosis and osteoarthritis [15]. Jmjd3 is an H3K27me3 demethylase that has emerged as an important player in osteoblastic differentiation and immune response of bone destruction [16, 17]. Periapical lesions are thought to be the result of a local inflammatory response, *i.e.*, a microbial infection in the root canal and inflammatory

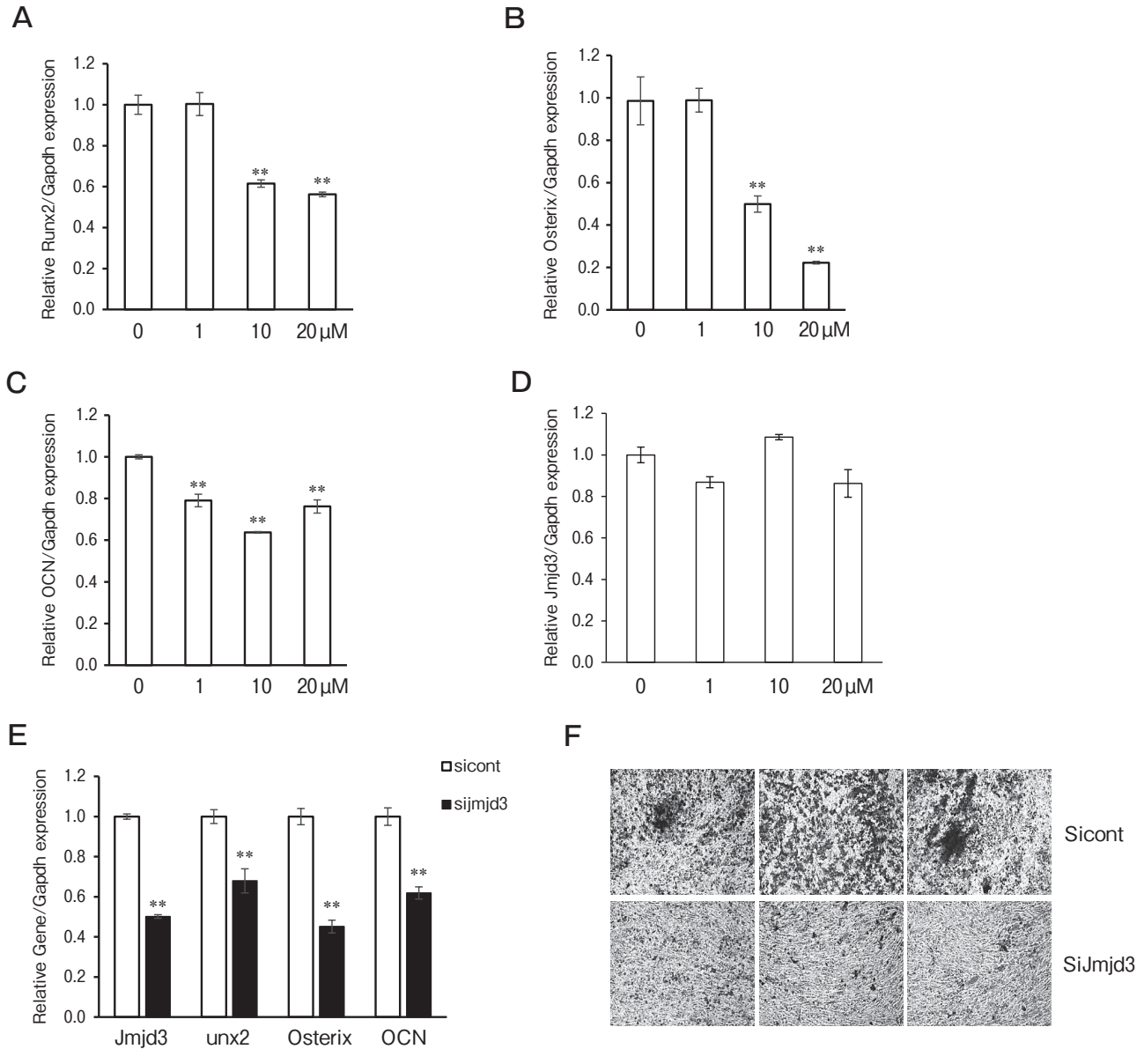

Fig. 3 Inhibition or knockdown of Jmjd3 impaired osteogenic differentiation of PDL cells. SH-9 cells were cultured in osteogenic differentiation medium for 7 days with the indicated dose of GSK-J4. The expressions of Runx2 (**A**), Osterix (**B**), OCN (**C**) and Jmjd3 (**D**) were examined by real-time RT-PCR. SH-9 cells were transfected with siRNA targeting for Jmjd3 (siJmjd3) or non-specific siRNA (siCont). siCont and siJmjd3 cells were cultured in osteogenic differentiation medium for 7 days. The expressions of Jmjd3, Runx2, Osterix, and OCN were examined by Real-time RT-PCR (**E**). siCont and siJmjd3 cells were cultured in osteogenic differentiation medium for 21 days. Mineral deposition was determined by Alizarin red staining (**F**). Values represent the mean ± S.E.M. of representative analysis from 3 separate experiments. $^{**}p<0.01$.

cytokines that are mediated in the periodontal tissue. They are caused by the destruction of the integrity of the periodontal ligament and progress with the destruction of the alveolar bone. PDL cells are thought to contribute significantly to the maintenance of adjacent tissues, due to their ability to differentiate into osteoblastic cells and secrete inflammatory cytokines [18]. In the present study, higher levels of Jmjd3 expression were detected in the samples from patients with periapical lesions compared to the samples with healthy PDLs. These

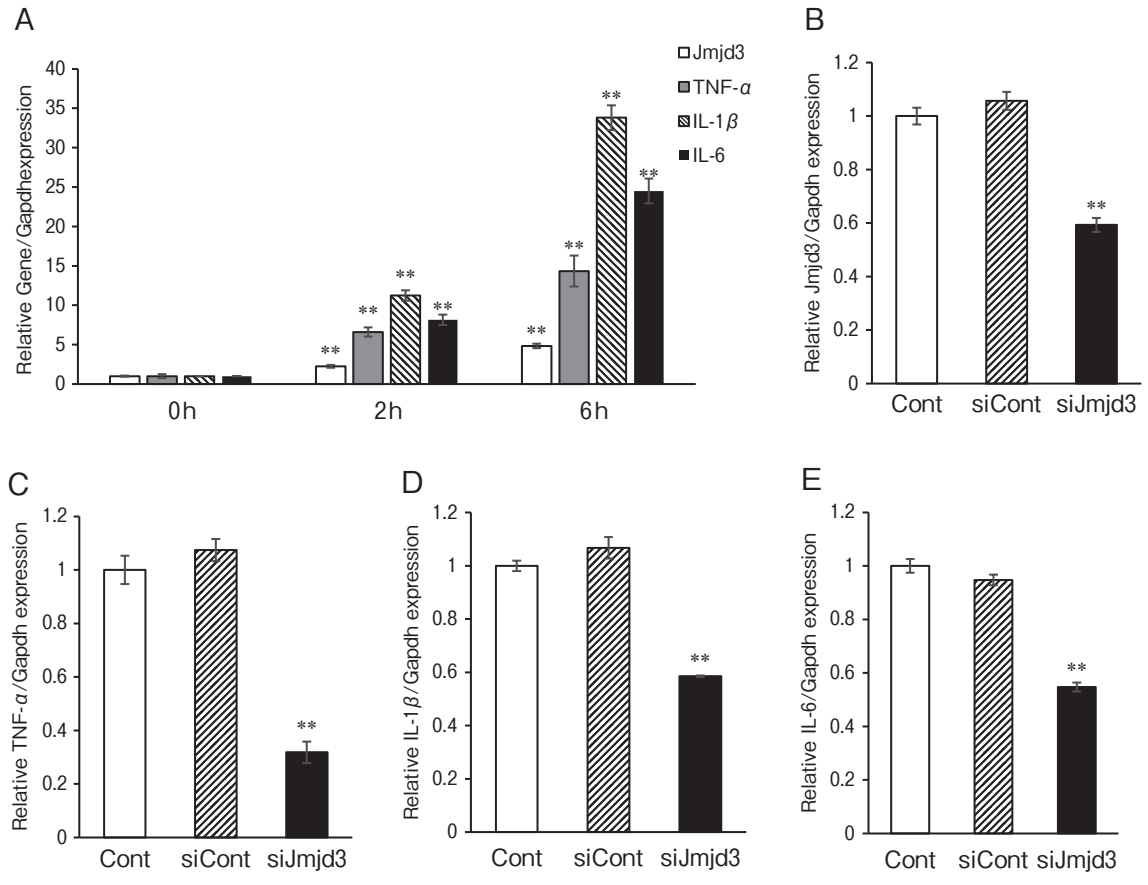

Fig. 4 Knockdown of Jmjd3 decreased the expressions of cytokines of PDL cells through NF-κB signaling pathway. SH-9 cells were cultured in growth medium treated with 10 μg/mL Pe-LPS for 0, 2, 6 h, respectively. The expressions of Jmjd3, TNF-α, IL-1β, and IL-6 were examined by real-time RT-PCR (**A**). No-treatment (Cont), siCont and siJmjd3 cells were cultured in growth medium with 10 μg/mL Pe-LPS for 6 h. The expressions of Jmjd3 (**B**), TNF-α (**C**), IL-1β (**D**), and IL-6 (**E**) were examined by Real-time RT-PCR. Values represent the mean ± S.E.M. of representative analysis from 3 separate experiments. $^{**}p<0.01$.

findings imply that Jmjd3 is involved in the functional reaction of PDL cells in response to a periapical infection and inflammation. In addition to the PDL cells, the apical lesions were full of immune cells. Future studies should address the role of Jmjd3 in immune cells in apical lesions.

Since PDL cells have the ability to differentiate into osteoblastic cells, we next examined the involvement of Jmjd3 in the expressions of osteoblast-related genes in PDL cells. The Jmjd3 expression gradually increased in PDL cells cultured with osteoblast differentiation medium, with the peak at day 7, and this was accompanied by increases in the expressions of osteoblast-related genes. In the process of osteoblast differentiation, the transcription factors Runx2 and Osterix play regulatory roles to control the expressions of bone-related genes such as osteopontin, bone sialoprotein, and OCN [1,19-24]. These bone-related genes ultimately participate in the differentiation and mineralization of osteoblasts [25,26]. Our previous study revealed that Jmjd3 regulates Runx2 and Osterix expressions via the modification of the H3K27me3 levels on their promoter regions [10]. In the present study, the inhibition or silencing of Jmjd3 suppressed the expressions of Runx2, Osterix, and OCN; this was also shown by the results of the Alizarin red staining. These observations suggest that Jmjd3 is responsible for osteoblastic properties in PDL cells.

We further investigated the role of Jmjd3 in PDL cells' expression of inflammatory cytokines. Porphyromonas endodontalis is a gram-negative anaerobic microorganism that is most commonly isolated from

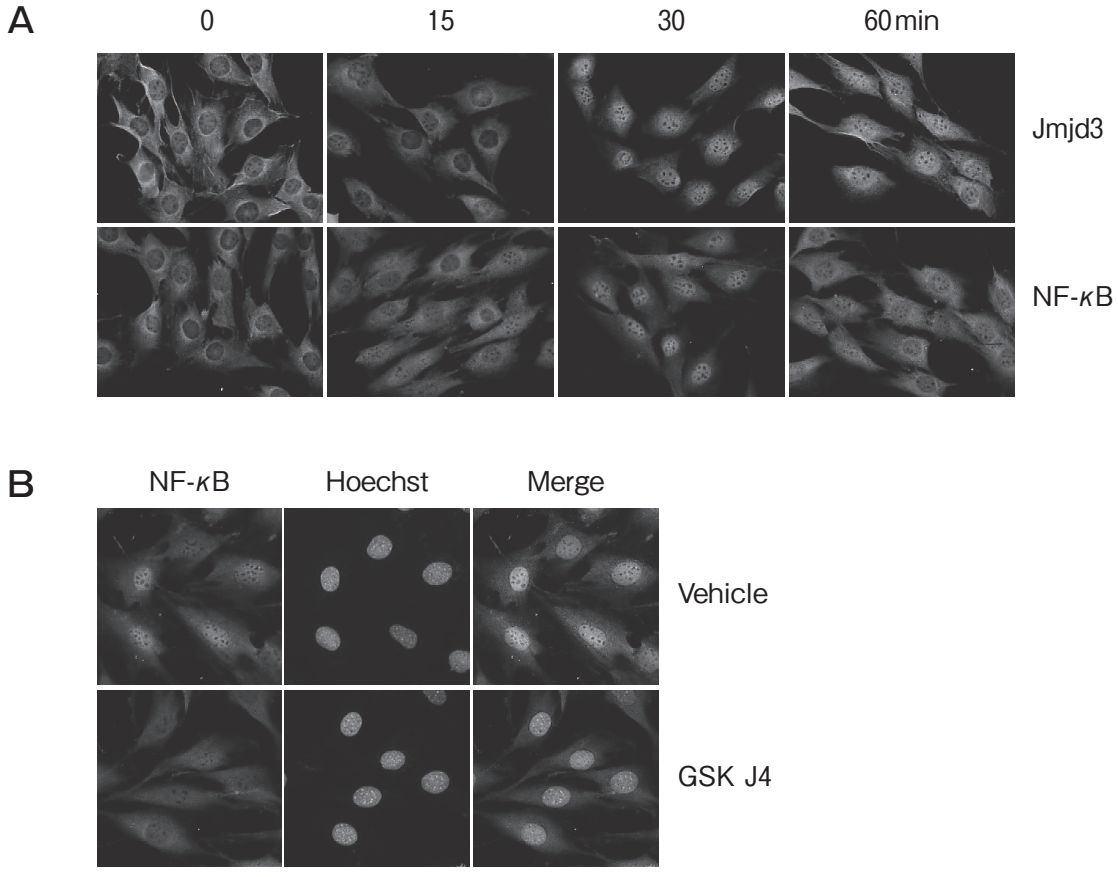

Fig. 5 SH-9 cells were cultured in growth medium with 10 μg/mL Pe-LPS for 15, 30 and 60 min, respectively. The cells were subjected to indirect immunostaining using an anti-NF-κB antibody or Jmjd3 antibody. When cultured for 30 min, the Jmjd3 and NF-κB entered the nucleus simultaneously (**A**). SH-9 cells were cultured in growth medium with 10 μg/mL Pe-LPS for 15 min pretreated with or without 10 μM GSK-J4 for 2 h. The cells were subjected to indirect immunostaining using an anti-NF-κB antibody and Hoechst 33342. NF-κB localized to the nuclei of vehicle cells (**B**, upper) and the cytoplasm of GSK-J4 treated cells (**B**, lower). Bar indicates 20 μm.

infected root canals and periapical lesions [27]. LPS is the primary virulence factor of P. endodontalis, and it induces expressions of cytokines including TNF-α, IL-1β, and IL-6, which are potent immunologic mediators associated with periapical lesions and related to bone destruction [13, 28, 29]. In the present investigation, Pe-LPS induced the expressions of Jmjd3 as well as TNF-α, IL-1β, and IL-6 in PDL cells. Knockdown of Jmjd3 decreased the expression of these cytokine in PDL cells. The silencing of Jmjd3 did not affect the cell vitality cultured in the osteogenic differentiation medium [10]. NF-κB is an important transcription factor for cytokine expression. NF-κB is activated and translocated to the nucleus in response to LPS in numerous cell types. Our earlier research demonstrated that P. endodontalis LPS affects the viability and cytokine production of osteoblasts and promotes osteoclastogenesis via the NF-κB signaling pathway [14, 30]. Pe-LPS stimulated NF-κB translocation and activation in PDL cells, accompanied by the phosphorylation and degradation of I-κB [14]. GSK-J4 is a selective enzyme activity inhibitor of H3K27 histone demethylases including Jmjd3 and UTX. In this study, pretreatment with GSK-J4 significantly inhibited the NF-κB nuclear translocation induced by Pe-LPS, which indicates that Jmjd3 regulates Pe-LPS-induced cytokine expressions through the NF-κB signaling pathway. It was reported that Jmjd3 up-regulates the expressions of IL-6 and IL-12b in an H3K27 demethylation dependent manner [31]. These results identified the pivotal role of Jmjd3 during the inflammatory response in PDL cells.

In conclusion, we demonstrated that periapical

lesions show higher levels of Jmjd3 expression compared to the levels in healthy tissue. Knockdown of Jmjd3 impaired the expressions of osteoblast-related genes. Pe-LPS induced the expressions of Jmjd3 as well as TNF-α, IL-1β, and IL-6, which were suppresed by the inhibition of Jmjd3 through a blockade of NF-κB nuclear translocation. These findings reveal a novel function of the histone demethylase Jmjd3 in both osteogenic differentiation and cytokine expressions of PDL cells. Whether these functions would lead to the bone destruction of periapical tissue during periapical inflammation remains to be further studied.

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