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The effect of DNA methylation on the miRNA expression pattern in lipopolysaccharide-induced inflammatory responses in human dental pulp cells

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ABSTRACT

Endodontic infection is a widespread oral problem. DNA methylation is a key epigenetic modification that plays important roles in various inflammatory responses, but its role in dental pulp inflammation is poorly understood. In this study, we assessed the expression of DNA methyltransferases (DNMTs) in human dental pulp cells (hDPCs) during lipopolysaccharide (LPS)-induced inflammation and found that *DNMT3B* mRNA expression was reduced and DNMT1 mRNA and protein levels decreased significantly. Pretreatment with the DNMT inhibitor 5-Aza-2'-deoxycytidine (5-Aza-CdR) significantly enhanced the expression of the inflammatory cytokines IL-6 and IL-8 in LPS-stimulated hDPCs, indicating that DNA methylation may play a role in hDPC inflammation. Studies have reported that some microRNAs (miRNAs) are involved in dental pulp infection. DNA methylation can modulate the inflammatory response by regulating miRNA expression, but this phenomenon has not yet been reported in pulp inflammation. The present study used next-generation sequencing to examine the effect of 5-Aza-CdR on the miRNA expression profile of LPS-treated hDPCs, and the results showed that 5-Aza-CdR pretreatment changed the miRNA expression pattern in hDPCs during inflammation. Among the changed miRNAs, miR-146a-5p, which is a pulp inflammation-related miRNA, demonstrated the most noticeably altered expression. miR-146a-5p could be induced by LPS in hDPCs, and 5-Aza-CdR preincubation or DNMT1 knockdown markedly increased its expression level. However, no significant difference was found in the methylation pattern of the *MIR146A* promoter with 5-Aza-CdR pretreatment or DNMT1 knockdown in LPS-stimulated hDPCs. These results indicate that DNA methylation may regulate the LPS-induced inflammatory response by changing the miRNA expression in hDPCs.

1. Introduction

Dental pulp inflammation is a common oral disease caused by bacteria and bacterial virulence factors, and it often develops into pulp necrosis or periapical disease and elicits a dental emergency (Hirsch et al., 2017). The gram-negative anaerobic bacteria *Porphyromonas gingivalis (Pg)* is an important pathogen closely associated with pulpitis and periapical periodontitis (Cao et al., 2012; Gomes et al., 2012; Robertson and Smith, 2009). As a component and the main virulence factor of the bacteria, *Pg* lipopolysaccharide (LPS) can enhance the production of inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-8 (CXCL8/IL-8), in human dental pulp cells (hDPCs), thus provoking dental pulp infection (Liu et al., 2013; Renard et al., 2016).

Epigenetic modification represents a variety of mechanisms that can result in the alteration of gene expression without a change in the nucleotide sequence (Hui et al., 2017). DNA methylation is a vital epigenetic modification that involves the addition of a methyl group to the cytosine residue in CpG dinucleotides through catalysis by DNA methyltransferases (DNMTs), such as DNMT1, DNMT3 A and DNMT3B (Seo et al., 2015). Generally, the hypermethylation of a CpG region in a

Abbreviations: LPS, lipopolysaccharide; Pg, porphyromonas gingivalis; DNMTs, DNA methyltransferases; hDPCs, human dental pulp cells; 5-Aza-CdR, 5-Aza-2'-deoxycytidine; miRNA, microRNA; IL-6, interleukin-6; IL-8, interleukin-8; IRAK1, interleukin 1 receptor associated kinase 1; TRAF6, TNF receptor associated factor 6; miR-146a, microRNA-146a-5p; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes

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gene promoter inhibits gene transcription, and the hypomethylation of a promoter drives gene expression (Hamidi et al., 2015). A growing body of evidence shows that DNA methylation plays critical roles in a variety of inflammatory conditions; in fact, aberrant DNA methylation has clinical potential as a biomarker for the prediction of diagnosis, prognosis or therapeutic response in some inflammation-related diseases (Lam et al., 2016; Mikeska and Craig, 2014; Samanta et al., 2017). However, the role of DNA methylation in dental pulp inflammation has rarely been studied, and the regulatory mechanism needs to be revealed.

MicroRNAs usually negatively modulate target gene expression at the posttranscriptional level by degrading mRNA or inhibiting mRNA translation (Sehic et al., 2017). Previous studies have shown that microRNAs play important roles in inflammatory responses and inflammatory diseases (Hao et al., 2011; Hui et al., 2017; Marques Rocha et al., 2015). MiR-181a binds directly to the 3' UTR of IL-8, leading to the downregulation of IL-8 and thus modulating hDPC inflammation (Galicia et al., 2014). The miR-133a level in endomyocardial biopsy tissues was correlated with macrophage infiltration and cardiac injury in inflammatory cardiomyopathy (Besler et al., 2016). DNA methylation could modulate inflammation and inflammatory disease by regulating miRNA expression (Kim et al., 2014; Miao et al., 2013). MiR-200c and miR-141 levels were reduced in a human prostate cancer cell line and increased by treatment with the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) or DNMT1 knockdown (Lynch et al., 2016). The binding of Twist1 and E-box recruited DNMT3 A and subsequently increased the methylation level of specific CpG sites in the *MIR186* promoter, thereby reducing miR-186 expression and decreasing its responsiveness to inflammatory signals in prostate cancer (Zhao et al., 2017). Previous studies revealed that an aberrant miRNA expression profile was implicated in the LPS-induced inflammatory response of human pulp (Mo and Xu, 2017; Zhong et al., 2012), but whether miRNA expression could be regulated by DNA methylation remains unknown.

To investigate whether DNA methylation modulates the inflammation of hDPCs by regulating miRNA, high-throughput sequencing was used to examine the effect of 5-Aza-CdR on the miRNA expression profile of Pg-LPS-stimulated hDPCs in the present study. We also screened the pulp inflammation-related miRNAs that are possibly affected by DNA methylation.

2. Materials and methods

2.1. Isolation and culture of hDPCs

Informed consent was obtained from all participants enrolled in this study, and the study was approved by the Ethical Review Board of the Guanghua School of Stomatology of Sun Yat-sen University. Healthy permanent premolars for orthodontic reasons or impacted third molars were collected from donors (18~25 years of age) undergoing tooth extraction. hDPCs were isolated and cultivated using an enzymatic method as previously described (Lu et al., 2006). Briefly, the dental pulp tissue was isolated and minced into small pieces, which were then digested in 3 mg/ml collagenase type I (Gibco, USA) for 20 min at 37 °C. Subsequently, the minced pulp tissue was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 U/ml penicillin (Gibco, USA) at 37 °C with 5% CO_2. The medium was changed every 3 days. When the cells reached 80% confluence, they were detached using trypsin/ethylene diamine tetraacetic acid (EDTA) (Gibco, USA) and subcultured at a ratio of 1:2.

2.2. Treatment with 5-Aza-CdR and transfection with DNMT1 siRNA

After hDPCs from passages 2–4 were seeded into a six-well plate for 24 h, the cell medium was replaced with 10 μg/ml *Porphyromonas gingivalis* LPS (Pg-LPS) (InvivoGen, France) for 3, 6, 12 and 24 h with or without preincubation with 10 μM 5-Aza-CdR (Sigma-Aldrich, USA) for 48 h.

For DNMT1 knockdown, DNMT1 siRNA (RiboBio, China) or negative control siRNA (catalog no. siN05815122147) (RiboBio, China) at a concentration of 50 nM was transfected into hDPCs using Lipofectamine RNAiMax transfection reagent (Invitrogen, USA). The siRNA sequences used for hDNMT1 knockdown were 5′-GGGACUGUGUCUCUGUUA UTT-3′ (sense) and 5′-AUAACAGAGACACAGU CCCTT-3′ (antisense).

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from hDPCs was extracted using TRIzol reagent following the manufacturer's instructions (Invitrogen, USA). One microgram of RNA from each sample was reverse-transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit (TaKaRa, Japan), and another one microgram of RNA was reverse-transcribed for miRNA detection using a Bulge-Loop miRNA qRT-PCR Starter Kit (RiboBio, China). Then, the reverse-transcribed products were subjected to qRT-PCR using a LightCycler 480 system with SYBR Green I Master Mix (Roche, Switzerland) according to the manufacturer's instructions. GAPDH and U6 were used as internal controls for the normalization of mRNA expression and miR-146a-5p quantification, respectively. Stem-loop primers for miR-146a-5p and U6 detection were synthesized (RiboBio, China). The primer sequences used for mRNA detection were synthesized (Invitrogen, USA) and are listed in Table 1.

2.4. Western blot analysis

Cells were harvested with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) on ice. The protein concentration of each sample was measured using a bicinchoninic acid (BCA) protein assay (Beyotime, China). Forty micrograms of protein was separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically onto polyvinylidene fluoride membranes (Millipore, USA). After blocking with TBST containing 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies against DNMT1 (1:1000; Cell Signaling Technology, USA), DNMT3B (1:1000; Cell Signaling Technology, USA), and vinculin (1:1000; Cell Signaling Technology, USA) overnight at 4 °C. After incubation with secondary antibodies (Cell Signaling Technology, USA) at 1:2000 for 1 h at room temperature and washing with TBST buffer, the target protein bands were detected with enhanced chemiluminescence reagents (Millipore, MA, USA) and scanned using an Image-Quant LAS 4000 min. system (GE Healthcare Life Sciences, USA). Band densities were quantified and normalized to vinculin using ImageJ 1.47 software (National Institutes of Health, USA).

Table 1
Primer sequences for RT-qPCR.

Gene	Primer sequence
DNMT1	F: 5′- GGCTGAGATGAGGCAAAAAG -3′ R: 5′- ACCAACTCGGTACAGGATGC -3′
DNMT3 A	F: 5'- AGGGAAGACTCGATCCTCGTC -3' R: 5′- GTGTGTAGCTTAGCAGACTGG -3′
DNMT3B	F: 5'-GCCTCAATGTTACCCTGGAA-3' R: 5′- CAGCAGATGGTGCAGTAGGA -3′
IL-6	F: 5′-TGCAATAACCACCCCTGACC-3′ R: 5′-AGCTGCGCAGAATGAGATGA-3′
IL-8	F: 5′-GGTGCAGTTTTGCCAAGGAG-3′ R: 5′-TTCCTTGGGGTCCAGACAGA-3′
GAPDH	F: 5′-TCTCCTCTGACTTCAACAGCGACA-3′ R: 5′-CCCTGTTGCTGTAGCCAAATTCGT-3′

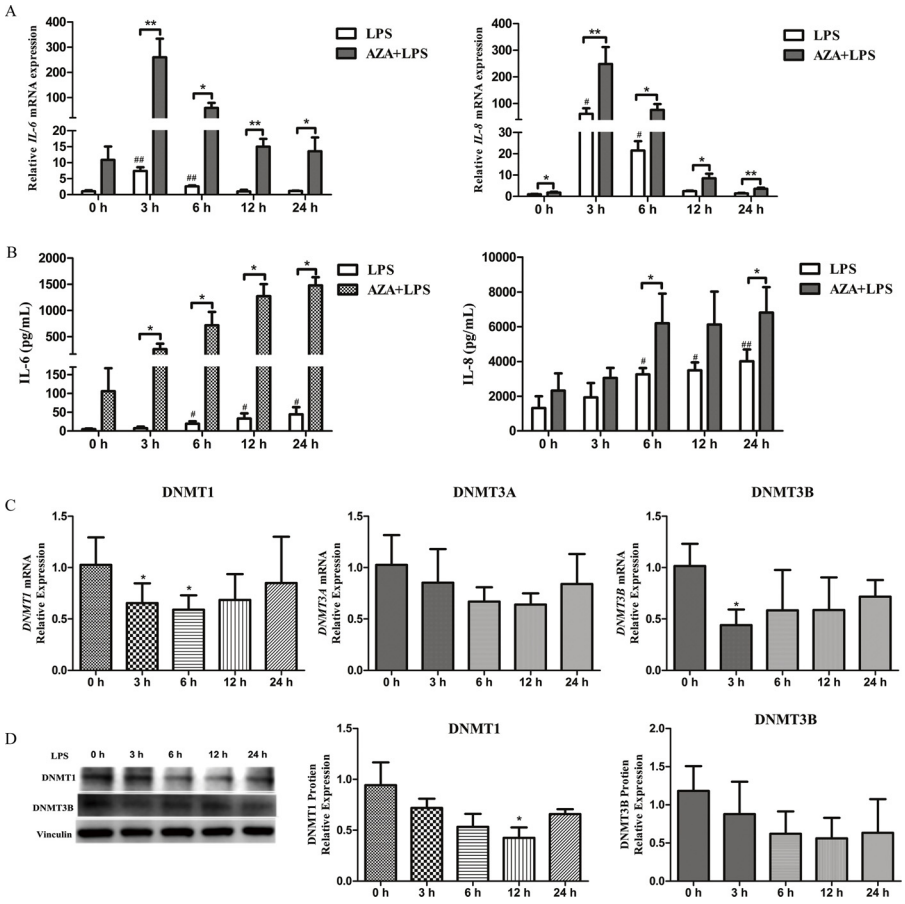

Fig. 1. The role of DNA methylation in hDPCs inflammation induced by Pg-LPS.
(A) The mRNA expression and (B) protein secretion of IL-6 and IL-8 were assessed by RT-qPCR and ELISA, respectively. GAPDH was used as an internal control for the normalization of mRNA expression (compared with the LPS group at the response time, $*P < 0.05$, $**P < 0.01$; compared with the control group at 0 h, $\#P < 0.05$, $\#\#P < 0.01$). (C) The expression of *DNMT1*, *DNMT3 A*, and *DNMT3B* mRNA in LPS-treated hDPCs was assessed by RT-qPCR. GAPDH was used as an internal control ($*P < 0.05$, $**P < 0.01$). (D) The expression of DNMT1 and DNMT3B protein in LPS-treated hDPCs was evaluated by western blotting. Vinculin was used as an internal control ($*P < 0.05$, $**P < 0.01$).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6 and IL-8 in the cell culture supernatants were analyzed using an ELISA kit (RayBiotech, USA) according to the manufacturer's protocol. The optical density (OD) value was measured at 450 nm using an automated microplate reader (Sunrise, Switzerland). The concentration of each sample was determined by the corresponding OD value and the concentration of the standard substance.

2.6. miRNA sequencing and analysis

Total RNA extracted from hDPCs using TRIzol reagent (Invitrogen, USA) was prepared for cDNA library construction and sequencing with an Illumina HiSeq 2500 system (Illumina, USA) as previously described (Xie et al., 2015). The raw small sequencing reads were filtered by eliminating low quality data, removing the 5′ adapter and discarding contaminants to obtain the clean reads. Subsequently, the clean reads were mapped to the human genome (UCSC, assembly hg19) for miRNA identification in miRBase 20.0. The expression levels of miRNA were normalized and quantified as the number of reads per million (RPM) clean tags with the following formula: RPM = (number of reads mapping to miRNA/number of reads in the clean data) $\times 10^6$.

The differentially expressed miRNAs were analyzed and had a fold change ≥ 1.2, an RPM ≥ 10, and co-upregulation or co-downregulation between the compared groups. The target genes of the miRNAs were predicted using miRanda, miRDB, TargetScan, and CLIP-seq. The target genes were then subjected to functional annotation by Gene Ontology (GO) enrichment as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses based on the DAVID tool (http://david.abcc.ncifcrf.gov/).

2.7. Evaluation of MIR146 A promoter methylation with MassARRAY spectrometry

Genomic DNA samples were isolated and purified with a DNA extraction kit (BioTeke, China). The sequence of the *MIR146 A* gene from NCBI databases was analyzed and used to design the primers for the detection of methylation of the *MIR146A* promoter with Agena EpiDesigner. The sodium bisulfite-converted DNA samples were amplified with custom-designed primers and then transcribed, followed by cleavage with RNase A into small fragments covering the CpG sites. Individual CpG sites were then quantitatively analyzed for their methylation level by the MassARRAY platform (Sequenom, USA) at Beijing Genomic Institute (BGI), as previously described (Xu et al., 2016). The primers used are presented below:

5': aggaagagagGGGGTAGAGGAAGGTAGTTAAGGTT, and 3': cagtaatacgactcacta tagggagaaggctTCTACCTAATCTTCTCCCCAAAAAC.

2.8. Statistical analyses

At least three independent experiments were performed for each assay, and the data are shown as the means ± standard deviation (SD). Comparisons between the experimental groups were analyzed using Student's *t*-test or nonparametric Mann-Whitney U test with SPSS 20.0 software (SPSS, Chicago, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The role of DNA methylation in hDPC inflammation induced by Pg-LPS

To investigate whether DNA methylation is involved in the inflammatory response of hDPCs, the effect of the DNA methyltransferase

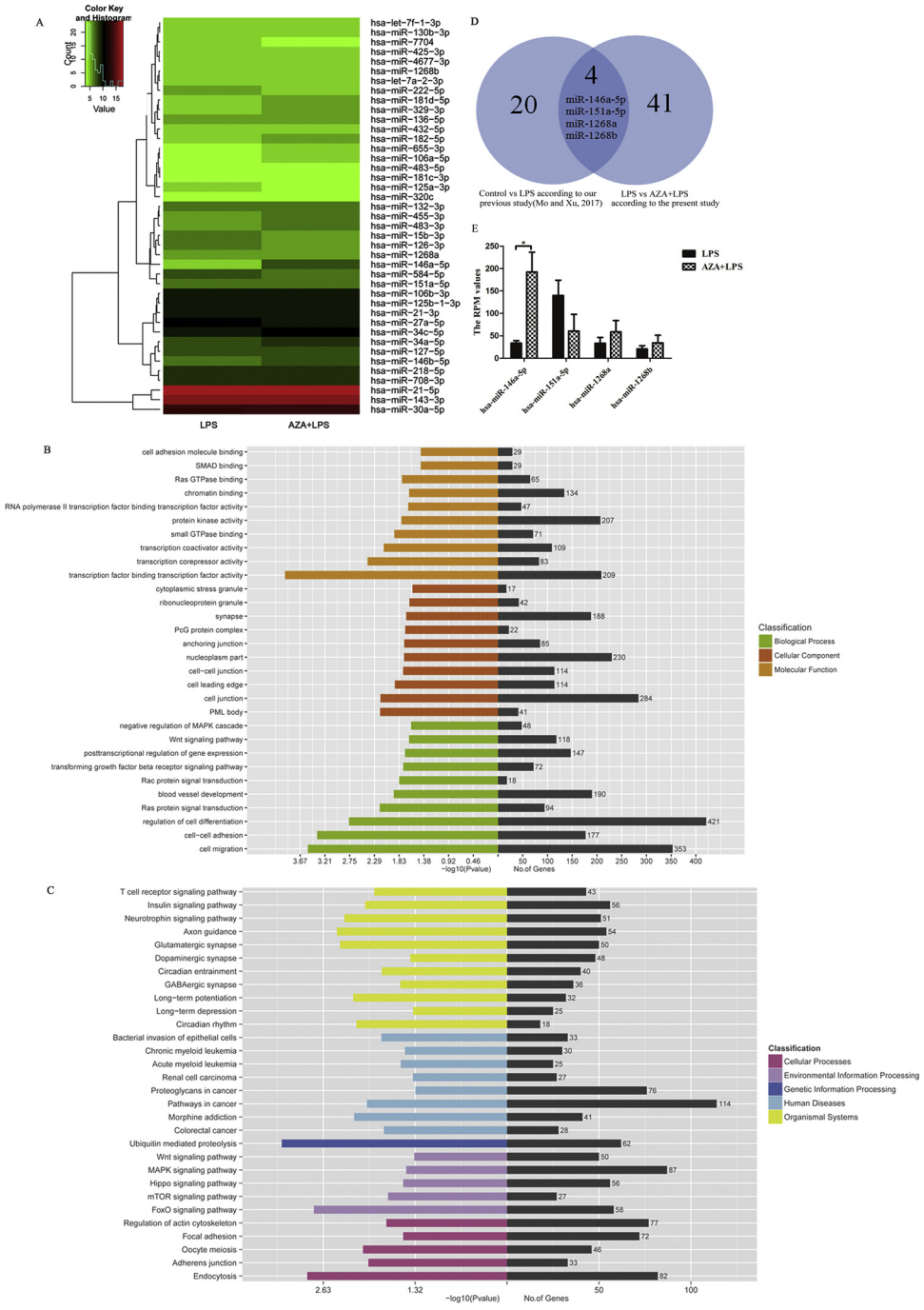

Fig. 2. Differentially expressed miRNAs in LPS-stimulated hDPCs pretreated with 5-Aza-CdR and their functional enrichment analyses.
(A) Differentially expressed microRNAs in LPS-stimulated hDPCs pretreated with 5-Aza-CdR. Each row represents a microRNA, and each column represents the corresponding sample. The colors of the rectangle from green to red indicate mean RPM values from low to high, respectively. (B) GO enrichment analysis and (C) KEGG enrichment analysis of the predicted target genes of differentially expressed miRNAs. (D, E) A subset of 4 miRNAs among 20 differentially expressed miRNAs in hDPCs inflammation as we previously reported (Mo and Xu, 2017), were differentially expressed in response to pretreatment with 5-Aza-CdR in LPS-treated hDPCs in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

inhibitor 5-Aza-CdR on LPS-induced inflammation in hDPCs was studied. As shown in Fig. 1A and B, the mRNA expression and protein secretion of the inflammatory indicators IL-6 and IL-8 were increased after Pg-LPS stimulation. Compared to LPS treatment alone, 5-Aza-CdR preincubation significantly enhanced the mRNA expression of *IL-6* and *IL-8*; the increase in amplitude peaked at 3 h and then slowed at 12 h and 24 h. Similarly, 5-Aza-CdR significantly increased the protein levels of IL-6 and IL-8 in a time-dependent manner.

Then, the mRNA and protein levels of DNMTs in LPS-stimulated hDPCs were measured. The results demonstrated that the *DNMT1* mRNA level was significantly reduced after LPS treatment, with the minimum level occurring at 6 h after stimulation (Fig. 1C). A similar pattern of DNMT1 protein expression was observed (Fig. 1D). *DNMT3 A* mRNA expression was decreased, but the decrease was not significant. *DNMT3B* mRNA expression was reduced, and the difference was statistically significant at 3 h (Fig. 1C), whereas the reduction in its protein expression was not statistically significant (Fig. 1D). These data suggest that DNA methyltransferases, especially DNMT1, play a role in hDPC inflammation.

3.2. The effect of 5-Aza-CdR on the microRNA expression profile of LPS-treated hDPCs

To investigate whether DNA methylation modulates the inflammatory response of hDPCs by regulating miRNA expression, the effect of 5-Aza-CdR on the miRNA expression profile in LPS-treated hDPCs was detected with an Illumina HiSeq 2500 sequencing platform. The results showed that 5-Aza-CdR pretreatment changed the miRNA expression pattern compared with LPS stimulation alone. We screened a total of 41 differentially expressed miRNAs; 22 were upregulated, and

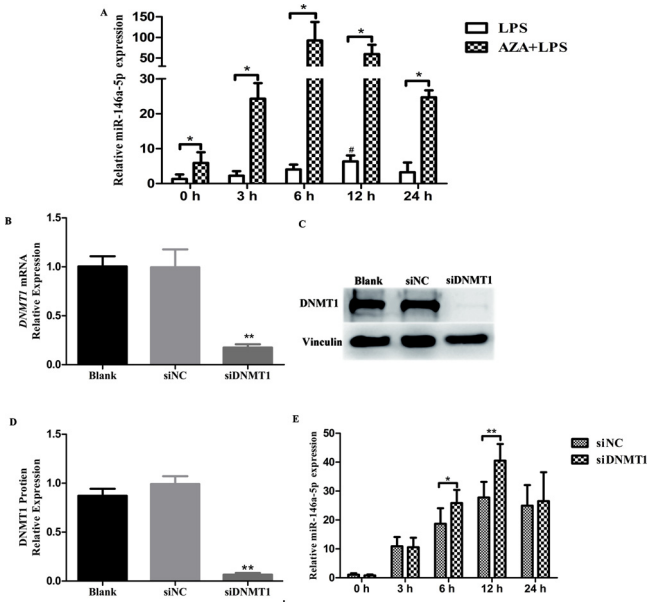

Fig. 3. The effect of 5-Aza-CdR treatment or DNMT1 knockdown on the expression of *MIR146A* induced by LPS in hDPCs.
(A) The relative *MIR146A* expression after pretreatment with 5-Aza-CdR in LPS-stimulated cells was assessed by stem-loop RT-qPCR (compared with the LPS group at the response time, $*P < 0.05$; compared with the control group at 0 h, $\#P < 0.05$). U6 was used as an internal control. (B–D) The mRNA and protein expression of DNMT1 were evaluated by RT-qPCR or western blotting after DNMT1 knockdown (compared with the blank control and siNC group, $**P < 0.01$). GAPDH and vinculin were used as internal controls for the normalization of DNMT1 mRNA and protein expression, respectively. (E) The effect of DNMT1 knockdown on *MIR146A* expression was determined by stem-loop RT-qPCR in LPS-stimulated hDPCs (compared with the siNC group,$*P < 0.05$,$**P < 0.01$).

19 were downregulated (Fig. 2A).

Then, the target genes of 41 differentially expressed miRNAs were predicted through miRanda, TargetScan, CLIP-seq, and miRDB algorithms and subjected to GO and KEGG pathway enrichment analyses. The results showed that the enriched GO terms included transcription regulation, MAPK cascade regulation, transforming growth factor β receptor signaling pathway, and cell migration and differentiation (Fig. 2B). The KEGG pathway enrichment analysis showed that various signaling pathways were involved, such as the MAPK signaling pathway, mTOR signaling pathway, Wnt signaling pathway, and T cell receptor signaling pathway (Fig. 2C).

Among the changed miRNAs, a subset of miRNAs (miR-146a-5p, miR-151-5p, miR-1268a and miR-1268b) exhibited aberrant expression in LPS-treated hDPCs in our previous sequencing data (Mo and Xu, 2017) as well as differential expression in response to 5-Aza-CdR preincubation in this study (Fig. 2D and E). Of these four miRNAs, only miR-146a-5p has been revealed to play a role in hDPC inflammation (Liu et al., 2016; Shu et al., 2014; Sipert et al., 2014; Wang et al., 2012). This miRNA was also the only one that was noticeably induced by 5-Aza-CdR treatment in the present sequencing analysis. Thus, miR-146a-5p was chosen for further verification.

3.3. The expression level of MIR146 A is regulated by DNA methylation in LPS-treated hDPCs

According to the above results, miR-146a-5p was screened for further verification. Stem-loop RT-qPCR showed that LPS significantly induced miR-146a-5p expression in hDPCs. Compared to LPS stimulation alone, 5-Aza-CdR markedly upregulated *MIR146A* expression, with the peak expression occurring at 6 h (Fig. 3A); these results were concordant with the sequencing results.

To survey the impact of DNMT1 on *MIR146A* expression, DNMT1 was knocked down in hDPCs. The results showed that the DNMT1 mRNA and protein expression levels were significantly reduced after DNMT1 siRNA transfection (Fig. 3B–D). Compared to LPS-treated cells transfected with negative control siRNA, DNMT1 knockdown cells had significantly increased *MIR146A* expression at 6 h and 12 h (Fig. 3E). These data demonstrated that the expression level of *MIR146 A* was regulated by DNA methylation in LPS-treated hDPCs.

3.4. The effect of DNA methylation on MIR146 A promoter methylation in LPS-treated hDPCs

To further explore whether DNA methylation regulates the methylation level of the *MIR146A* promoter directly, the Sequenom MassARRAY platform was used to measure the methylation of the *MIR146A* promoter covering 24 CpG sites (Fig. 4A). The DNA methylation analysis showed that the methylation levels in the *MIR146A* promoter were not significantly different before and after LPS exposure (Fig. 4B); under both conditions, the promoter had a low methylation level. There were no significant differences in the methylation pattern of the *MIR146A* promoter in LPS-treated hDPCs with 5-Aza-CdR pretreatment or DNMT1 knockdown (Fig. 4C and D).

4. Discussion

LPS from the cell walls of gram-negative bacteria can penetrate into the dental pulp and trigger inflammatory responses, which play a vital role in endodontic infection (Parolia et al., 2014; Renard et al., 2016). IL-6 and IL-8, which are usually detected in the inflamed pulp, are regarded as important diagnostic biomarkers and mediators of pulp inflammation (Elsalhy et al., 2013; Zanini et al., 2017). DNA methylation is a key epigenetic modification that plays a fundamental role in gene transcription, and its role in inflammatory responses has recently attracted increasing interest (Diomede et al., 2017; Iwaya et al., 2018). Nevertheless, whether DNA methylation is involved in LPS-induced dental pulp inflammation is still unclear. In the present study, hDPCs were pretreated with 5-Aza-CdR to determine the role of DNA methylation in LPS-induced inflammation. The results showed that 5-Aza-CdR pretreatment enhanced the expression of IL-6 and IL-8 induced by LPS. Meanwhile, the expression of DNMT1 was downregulated in LPS-stimulated cells. These results indicated that DNA methylation played a role in the inflammatory response of hDPCs and that DNMT1 was possibly involved.

Previous studies indicated that DNA methylation could modulate inflammation and inflammatory disease by regulating miRNA expression (Kim et al., 2014; Lynch et al., 2016; Miao et al., 2013). Our previous study revealed a dysregulated miRNA expression pattern in LPS-stimulated hDPCs (Mo and Xu, 2017). To explore whether the aberrantly expressed miRNAs are regulated by DNA methylation, the present study determined the effect of 5-Aza-CdR on the miRNA expression pattern using high-throughput sequencing. The results indicated that the miRNA expression profile was altered in response to 5-Aza-CdR pretreatment in LPS-stimulated hDPCs. Then, 41 differentially expressed miRNAs were screened for the prediction of target genes and subjected to a functional enrichment analysis. GO analysis showed that the targets of these miRNAs were involved in MAPK cascade regulation, the transforming growth factor β receptor signaling pathway, and cell migration and differentiation, which are related to the inflammatory immune process (Cooper et al., 2010; Garate et al., 2013; Zhao et al., 2014). KEGG pathway analysis implicated inflammatory immune pathways, such as the MAPK signaling pathway, the Wnt signaling pathway, and the T cell receptor signaling pathway. These data indicated that there may exist a complex interplay of the signaling pathways in DNA methylation-regulated inflammation of hDPCs.

To further investigate the miRNAs regulated by methylation during

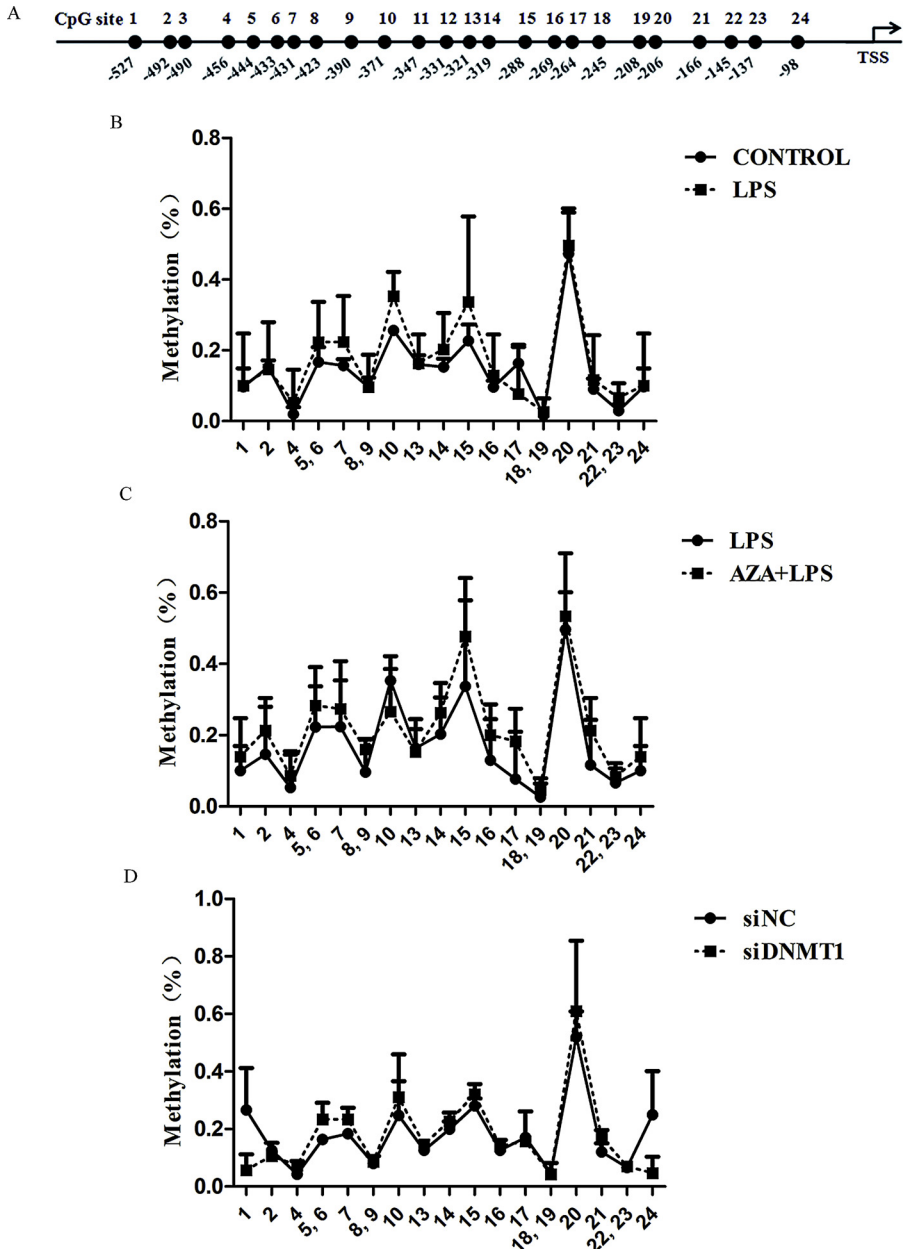

Fig. 4. Quantitative methylation analysis of CpG sites in the *MIR146A* promoter in hDPCs.
(A) The schematic diagram of CpG sites in the human *MIR146A* promoter. (B) The methylation analysis of the *MIR146A* promoter in the LPS group and the control group. (C) The methylation level of the *MIR146A* promoter in the LPS group and the 5-AZA + LPS group. (D) The methylation analysis of the *MIR146A* promoter in the DNMT1 siRNA and the siNC group in LPS-stimulated hDPCs (the abscissa in panels A, B, and C refers to CpG sites. $^{*}P < 0.05$).

the inflammation, we analyzed the miRNAs aberrantly expressed in the present sequencing that were also abnormally expressed in LPS-stimulated hDPCs in our previous report (Mo and Xu, 2017). Four miRNAs, namely, miR-146a-5p, miR-151-5p, miR-1268a and miR-1268b, were thus screened. Among these miRNAs, miR-146a-5p, the only pulp inflammation-related miRNA, was most noticeably induced. Previous studies showed that miR-146a-5p (also named miR-146a) is upregulated in LPS-treated hDPCs (Mo and Xu, 2017; Shu et al., 2014; Sipert et al., 2014; Wang et al., 2012). The gain- and loss-of-function analyses indicate that miR-146a reduces the protein levels of IRAK1 and TRAF6 and suppresses the expression of IL-6 and IL-8, thus playing a negative regulatory role in hDPC inflammation (Shu et al., 2014). 5-Aza-CdR was shown to increase *MIR146A* expression in prostate cancer cell lines (Wang et al., 2014). Consistent with these reports, miR-146a-5p was increased after LPS stimulation, and 5-Aza-CdR preincubation markedly increased its expression level in LPS-induced cells in the present study. Furthermore, DNMT1 knockdown significantly upregulated *MIR146A* expression in LPS-treated hDPCs. These data indicated that the *MIR146 A* transcription level was modulated by DNA methylation in hDPC inflammation.

In addition to miR-146a-5p, the members of the miR-146 and miR-181 families, namely, miR-146b-5p, miR-181c-3p and miR-181d-5p, were increased in the 5-Aza-CdR pretreatment group according to the present sequencing. MiR-146b-5p is considered to be endotoxin-responsive, acting in a similar but not exactly the same way as miR-146a-5p (Paterson and Kriegel, 2017). MiR-146b-5p is overexpressed in macrophages of atherosclerosis (AS) patients and modulates inflammation by targeting TRAF6 (Lin and An, 2017). The miR-181 family contains mainly miR-181a-3p/5p, miR-181b-3p/5p, miR-181c-3p/5p and miR-181d-3p/5p. MiR-181a-5p and miR-181b-5p are reported

to regulate inflammation by targeting IL-6 and IL-8 (Chan et al., 2013; Galicia et al., 2014). MiR-181c-3p is significantly elevated in esophageal squamous cell carcinoma and its high expression is related with the poor prognosis (Chen et al., 2015). MiR-181d-5p plays an anti-oncogenic role in osteosarcoma via targeting FOXP1 (Chen et al., 2018). Our previous report indicated that the expression levels of miR-146b-5p, miR-181c-3p and miR-181d-5p did not significantly change after LPS stimulation in hDPCs; this finding suggests that these miRNAs might not be affected in hDPC inflammation (Mo and Xu, 2017).

5-Aza-CdR has a broad function in the production of pro- and anti-inflammatory molecules (Seelan et al., 2018). Exposure to 5-Aza-CdR in chondrocytes enhances the expression of IL-1, TGF-β and nitric oxide synthase (NOS) (Zhao et al., 2015). In contrast, another study revealed that 5-Aza-CdR treatment enhances the expression of the anti-inflammatory molecules liver X receptor α (LXRα) and PPARγ and suppresses the release of TNF-α, IL-6 and CCL2 in macrophages (Cao et al., 2014). We found that 5-Aza-CdR markedly increased not only the expression of the inflammatory cytokines IL-6 and IL-8 but also the level of the anti-inflammatory molecule *MIR146A* in LPS-induced hDPCs; the greatest increases in IL-6 and IL-8 mRNA levels occurred at 3 h and subsequently slowed at 12 h and 24 h, and the most noticeable increase in miR-146a occurred at 6 h. We speculated that in the initial phase of inflammation, IL-6, IL-8 and miR-146a were induced by LPS stimulation. Then, the continuous augmentation of *MIR146A* induced by 5-Aza-CdR may inhibit the production of IL-6 and IL-8, which exhibited a smaller increase in the later phase of inflammation, thus blunting the excessive pulpal inflammatory response. According to Figs. 1 and 3, IL-6 and IL-8 mRNA and protein levels did not correlate at the later time points after LPS stimulation, and a similar tendency was observed when 5-Aza-CdR induced robust *MIR146A* expression upon inflammation. Generally, the half-life of mRNA is shorter than that of protein; thus, the expression of mRNA generally occurs earlier than protein in the process of gene expression (Chan et al., 2018; Yang et al., 2016). Additionally, a single gene could be targeted by multiple miRNAs via mRNA degradation or mRNA translation inhibition (Na and Kim, 2013). The dysregulated miRNAs in our study might function in a cooperative or antagonistic way and result in the expression patterns of IL-6 and IL-8.

5-Aza-CdR was reported to increase *MIR146A* expression by decreasing the DNA methylation level of the *MIR146 A* promoter, thereby promoting apoptosis in prostate cancer cell lines and inhibiting prostate tumor growth (Wang et al., 2014). Although we found that 5-Aza-CdR pretreatment or DNMT1 knockdown enhanced *MIR146A* expression in LPS-stimulated hDPCs, the methylation analysis showed no significant difference in the methylation pattern of *MIR146A* promoter, contrary to the above report. Cardoso et al. found no differences in the methylation patterns of the *TLR2* and *CD14* gene promoters in normal and inflamed dental pulp. Thus, it was supposed that hypomethylation of these two genes was a common feature in dental pulp (Cardoso et al., 2014). In the present study, the methylation status of CpG sites in the *MIR146A* promoter were both low before and after LPS exposure, suggesting that its hypomethylation might also be a common feature in hDPCs. Promoters with low methylation levels seem to be less responsive to demethylation treatment (Rubinstein et al., 2010). As such, the hypomethylation status of the *MIR146A* promoter may make it less susceptible to 5-Aza-CdR pretreatment or DNMT1 knockdown in hDPCs. Moreover, the effect of 5-Aza-CdR on gene expression arises not only from promoter demethylation but also from the demethylation of upstream genes (e.g., genes encoding transcription factors), regulatory elements (e.g., enhancers), or the gene body (Jjingo et al., 2012; Seelan et al., 2018). Thus, alterations in *MIR146A* expression may be due to demethylation of the gene body or other CpG sites not detected in this study, or, more likely, DNA methylation indirectly regulates *MIR146A* expression upon the LPS-induced inflammatory response in hDPCs. More studies are necessary to further elucidate the role of DNA methylation-mediated miRNA expression in regulating the inflammatory response in hDPCs.

5. Conclusions

In summary, this study demonstrated that DNA methylation plays an important role in the inflammation of hDPCs. Pretreatment with the DNA methyltransferase inhibitor 5-Aza-CdR could change the miRNA expression profile of LPS-stimulated hDPCs. These findings contribute to the understanding of the epigenetic mechanisms underlying dental pulp inflammation and provide new insight into the development of endodontic infection.

Conflict of interest

The authors declare that they have no conflict of interest.

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