Hsa_circ_0099630 knockdown induces the proliferation and osteogenic differentiation and attenuates the apoptosis of porphyromonas gingivalis lipopolysaccharide–induced human periodontal ligament fibroblasts

Yaru Wei, Zhengjun Peng

Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, China

Contributions: (I) Conception and design: Z Peng; (II) Administrative support: Z Peng; (III) Provision of study materials or patients: Y Wei; (IV) Collection and assembly of data: Y Wei; (V) Data analysis and interpretation: Both authors; (VI) Manuscript writing: Both authors; (VII) Final approval of manuscript: Both authors.

Correspondence to: Zhengjun Peng. Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Stomatology, 56 Lingyuan Xi Road, Guangzhou 510055, China. Email: pengzhj3@mail.sysu.edu.cn.

Background: Periodontitis is an inflammatory destructive bone disease and is the most critical cause of tooth loss in adults. Recent studies have reported that circular RNAs (circRNAs) are essential in periodontitis. However, the influence and mechanism of hsa_circ_0099630 on periodontitis are not clear.

Methods: Normal periodontal tissues and inflammatory periodontal tissues were obtained from healthy patients and patients with periodontitis, respectively. Hsa_circ_0099630 was 1st identified by polymerase chain reaction (PCR) and sanger sequencing, and hsa_circ_0099630 expression was determined by real-time (RT)-quantitative PCR in periodontitis. Porphyromonas gingivalis-lipopolysaccharide (Pg-LPS) was used to construct an inflammation model in vitro. Next, cell proliferation, apoptosis, and osteogenic differentiation were monitored using Cell Counting Kit-8, flow cytometry, and western blot in the Pg-LPS-induced human periodontal ligament fibroblasts (HPLFs). The microRNA (miRNA)/messenger RNA (mRNA) axis of hsa_circ_0099630 was predicted and screened, and the function of the target genes was analyzed by a Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses.

Results: The identified hsa_circ_0099630 was upregulated in the gingival tissue of patients with periodontitis. Next, an inflammation model was constructed using Pg-LPS in the HPLFs. We discovered that Pg-LPS or hsa_circ_0099630 overexpression suppressed cell proliferation and osteogenic differentiation, and induced apoptosis in HPLFs. Additionally, hsa_circ_0099630 knockdown induced proliferation and osteogenic differentiation and prevented apoptosis in the Pg-LPS-induced HPLFs. We also screened the vast miRNA/mRNA axis associated with hsa_circ_0099630.

Conclusions: The current study uncovered the crucial role of hsa_circ_0099630 in periodontitis.

Keywords: Periodontitis; hsa_circ_0099630; osteogenic differentiation; porphyromonas gingivalis-lipopolysaccharide (Pg-LPS); human periodontal ligament fibroblasts (HPLFs)

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Introduction

Periodontitis is a chronic inflammatory disease in which host-mediated inflammation destroys the periodontal support tissue, leading to periodontal and alveolar bone resorption, and even tooth loss (1). Dental calculus, dental plaque, and bacterial infection are the main causative factors in the development of periodontitis (2). The development of periodontitis can cause an inflammatory response around the gums, with localized swelling, bleeding and other pathological changes (3). Thus, the ultimate goal of periodontal therapy is to control the inflammation and achieve the complete regeneration of the periodontal tissue (4). Human periodontal ligament fibroblasts (HPLFs) are a class of periodontal cells with multidirectional differentiation capacity (5). The osteogenic differentiation of HPLFs is the focus of research on periodontal tissue regeneration and is of great importance in periodontal therapy (6). Thus, the identification of molecular targets that promote osteogenic differentiation could provide novel ideas for the treatment of periodontitis.

Circular RNAs (circRNAs) are a special class of non-coding RNAs with a circular structure (7). Approximately 15% of circRNAs are derived from transcripts and most contain exonic sequences (8). Compared to linear RNAs, the circular structure of circRNAs is resistant to digestion by ribonuclease R (9). As a result, circRNAs are less susceptible to degradation and have a more stable structure. CircRNAs are also characterized by a wide distribution, high conservation, and tissue expression specificity (10). CircRNAs have been reported to act as microRNA (miRNA) "sponges" and regulate the expression of the parental genes involved in disease processes, including skin damage, osteoarthritis, tumors, cardiovascular diseases, neurological diseases, and endocrine diseases (11,12). Thus, circRNA has become an ideal diagnostic marker and therapeutic target for diseases with critical clinical applications. Recent studies have also shown that circRNAs participate heavily in multiple processes, such as tissue regeneration, stem cell proliferation, and osteogenic differentiation (13-15). Further, the knockdown of circ_0138959 was shown to attenuate the pyroptosis of HPLFs through the miRNA-527/caspase-5 axis. However, the role of circRNAs in regulating the osteogenic differentiation of HPLFs is currently unclear.

In our preliminary experiment, we found that hsa_circ_0099630 was highly expressed in the gingival tissue of patients with periodontitis by detecting the expression of multiple circRNAs. Based on the circbase database, we discovered that hsa_circ_0099630 is located in chr12:97885421-97924637, its best transcript is NR_024037, and its gene symbol is rhabdomyosarcoma 2 associated transcript (RMST). Additionally, a recent microarray screening study showed that hsa_circ_0099630 was upregulated in chronic sinusitis with nasal polyps (16). Thus, we speculated that hsa_circ_0099630 may be relevant to inflammation and may play a crucial role in periodontitis. Illumina sequencing results had showed that the expression levels of hsa_circ_0099630 were increased in periodontitis tissues compared with control periodontium (17). Recently, the expression of hsa_circ_0099630 was found decreased in inflamed periodontal ligament cells (iPDLCs) compared with healthy PDLCs (hPDLCs), and overexpression hsa_circ_0099630 suppressed iPDLCs proliferation and osteogenic differentiation (18). Thus, the role of hsa_circ_0099630 in periodontitis is confusing.

In our study, we explored the possibility that hsa_circ_0099630 might influence the development progression of periodontitis. We first cultured HPLFs and constructed inflammatory models using porphyromonas gingivalis–lipopolysaccharide (Pg-LPS) based on research (19). We then verified the effects of hsa_circ_0099630 overexpression on the proliferation, apoptosis. and osteogenic differentiation of HPLFs, and the effects of hsa_circ_0099630 silencing on these functions in Pg-LPS-induced HPLFs. We also bioinformatically predicted the miRNA/messenger RNA (mRNA) axis associated with hsa_circ_0099630 and analyzed the possible functions and regulatory pathways of the target genes. Thus, our study provides potential research ideas for periodontal therapy. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4209/rc).

Methods

Tissue samples

Normal periodontal tissues were obtained from the extracted interrupted 3rd molars of 20 healthy patients. Equal amounts of inflammatory periodontal tissues were obtained from 60 patients with periodontitis. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of the Hospital of Stomatology, Sun Yat-sen University (No. 2020-07-162). All the participants

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signed a written informed consent form. The tissues were stored at –80 ℃.

Cell culture

The HPLFs were supplied by Procell (Wuhan, China), and were grown in Dulbecco's Modified Eagle Medium (Sigma, USA) containing 10% fetal bovine serum (Invitrogen, USA) at 37 ℃ with 5% carbon dioxide.

Cell treatment

The HPLFs were 1st processed with Pg-LPS to build the inflammation model (20). The hsa_circ_0099630 overexpression plasmid (OE-circ), empty vector (OE-CTRL), hsa_circ_0099630 shRNAs (shcirc), and control (shCTRL) were all provided by Integrated Biotech Solutions (Shanghai, China). The HPLFs were inoculated in 6-well plates and cultured for 24 h. Lipofectamine 3000 reagent (Invitrogen, USA) was used to transfect the HPLFs with OE-circ and OE-CTRL, respectively. After 48 h, the transfected cells were harvested, and the transfection efficiency was assessed by real-time (RT)-quantitative polymerase chain reaction (qPCR). Similarly, the Pg-LPS-induced HPLFs were transfected with shcirc and shCTRL, respectively.

RT-qPCR

Total RNAs were separated from the ground periodontal tissues or the treated HPLFs using a TRIzol kit (Invitrogen, USA). After the purity assessment, complementary DNA (cDNA) was generated using the BestarTM RT-qPCR kit (DBI Bioscience, China) with 2 μg of RNA. Next, the RT-PCR was conducted with the SYBR Green qPCR Mix kit (Sparkjade, China). Relative gene expression was counted using the $2^{-\Delta\Delta Ct}$ method.

PCR

The divergent primer and convergent primer were designed based on the sequences of hsa_circ_0099630. The genomic DNA (gDNA) of the HPLFs was extracted in accordance with the instructions and stored at –20 ℃. Next, total RNA was extracted from the HPLFs, reverse transcribed into cDNA, and stored at –20 ℃. Glyceraldehyde 3-phosphate dehydrogenase primers and 2 primers for hsa_circ_0099630 were added to the extracted gDNA and cDNA for PCR amplification. After the products were obtained, agarose gel electrophoresis was performed. The PCR amplification product of hsa_circ_0099630 in the above cDNA was subjected to Sanger sequencing.

Cell Counting Kit-8 (CCK-8)

The HPLFs were treated based on the experiment purpose after being inoculated in 96-well plates for 12 h of incubation. After 48 h of treatment, CCK-8 reagent (Dojindo, Japan) was added to the cells (10 μL per well). After continuing the incubation for 2 h, the absorbance of each well at 450 nm was tested by a microplate reader (Bio-Rad, USA).

Flow cytometry

Groups of HPLFs were collected and washed with pre-cooled phosphate buffered solution (PBS). After centrifugation, the HPLFs were suspended in binding buffer (1×) and the concentration was adjusted to 1×10^6 cells/mL. The cell suspensions were incubated with 5 μL of AnnexinV-fluorescein isothiocyanate (FITC) for 10 min and 5 μL of propidium iodide (PI) for 5 min. PBS (500 μL) was then added to the HPLFs. FITC (515 nm) and PI (560 nm) were evaluated using flow cytometer (BD Biosciences), and cell apoptosis was analyzed using FlowJo (Version 8.8.6, TreeStar, San Carlos, CA, USA).

Alizarin red staining

The groups of HPLFs were cultured in osteogenic induction medium (conventional medium supplemented with 10 mmol/L of sodium β-glycerophosphate, 0.1 μmol/L of dexamethasone, and 0.05 of mmol/L ascorbyl-2-phosphate) for 2 weeks. After PBS washing, the HPLFs were fixed in 4% paraformaldehyde and stained with 0.1% alizarin red solution at 37 ℃ for 30 min. After washing with doble distilled water (ddH_2O), red or purplish red stained calcified nodules were observed microscopically to assess the stromal mineralization capacity of the HPLFs.

Alkaline phosphatase (ALP) activity

The groups of HPLFs were inoculated in 24-well plates at 2×10^4 cells/well. Based on the reagent instructions, 200 μL of cell lysis solution was added to the HPLFs, and the plates were stored at 4 ℃ overnight. The plates were shaken for

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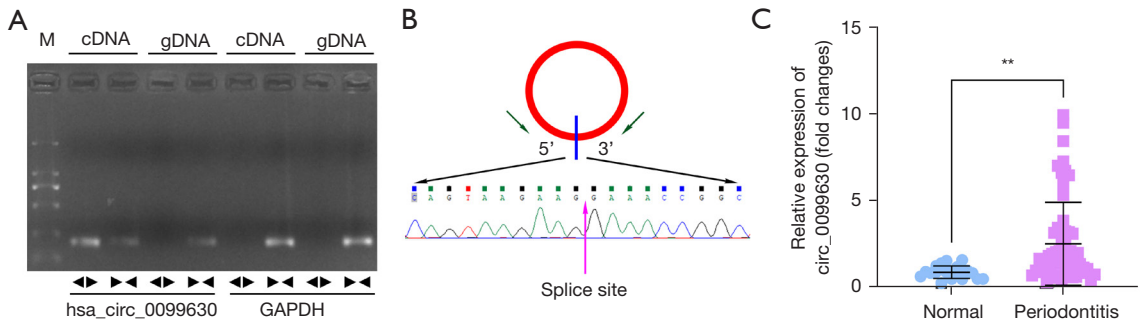

Figure 1 Identification and expression of hsa_circ_0099630 in the gingival tissue of patients with periodontitis. (A) The level of hsa_circ_0099630 was tested by PCR using the RNA generated by cDNA and gDNA from the HPLFs. (B) The splice site of hsa_circ_0099630 was verified using Sanger sequencing. (C) The level of hsa_circ_0099630 was determined with RT-qPCR in the gingival tissues of the periodontitis patients (n=60) and healthy controls (n=20). **, P<0.01. PCR, polymerase chain reaction; cDNA, complementary DNA; gDNA, genomic DNA; HPLFs, human periodontal ligament fibroblasts; RT-qPCR, real-time quantitative PCR.

10 min to break up the cells into suspension. The suspensions (30 µL per well) were transferred to a 96-well plate. Next, the cells were treated with 50 µL of substrate solution at 37 ℃ for 15 min, and 150 µL of color developer. The absorbance value was assessed at 520 nm by a microplate reader.

Western blot

The proteins were 1st extracted using radioimmunoprecipitation assay (RIPA) lysis buffer from the treated HPLFs. After quantifying the protein concentration, proteins (5 µg) were mixed with loading buffer, denatured, separated on 12% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) gels, and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck, Billerca, MA, USA). After blocking, the bands were incubated with primary antibodies (Abcam) overnight at 4 ℃, and a secondary antibody (1:5,000, Abcam) for 1 h. The bands were the processed with electrochemiluminescence (ECL) reagents (Thermo, Waltham, MA, USA), and the results were observed under Tanon-5200CE (Biotanon, Shanghai, China).

Bioinformatics analysis

The hsa_circ_0099630-miRNA interaction was predicted using the RNAhybrid and miRanda databases. The miRNA-mRNA interaction was predicted using miRDB, TargetScan, and miRTarBase databases.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Functional annotations of the target genes were identified using the GO (21,22) and KEGG databases (22,23).

Statistical analysis

The data are shown as the mean ± standard deviation. The experiments were performed at least 3 times. The statistical analysis was conducted with SPSS 20.0 (SPSS, Chicago, IL) with the Student's *t*-test (for 2 group comparisons) or a 1-way analysis of variance (for multiple group comparisons). A P value <0.05 was considered significant.

Results

Identification and expression of hsa_circ_0099630 in the gingival tissue of patients with periodontitis

To identify hsa_circ_0099630, we performed PCR and Sanger sequencing to confirm the back-splicing junction of hsa_circ_0099630. As *Figure 1A* shows, we discovered that the divergent primers amplified hsa_circ_009963 in the cDNA but not the gDNA, suggesting the stable existence of hsa_circ_0099630. Through sequencing, we found that the splice site of hsa_circ_0099630 was CAGTAAGAAGGAAACCGGC, which was consistent with the database (see *Figure 1B*). We also found that hsa_circ_0099630 was highly expressed in the gingival tissues of the periodontitis patients relative to that in the normal

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tissues of the healthy controls (see *Figure 1C*). Further, we discovered that hsa_circ_0099630 expression was associated with the order of severity and grade of periodontitis in 60 cases of periodontitis patients (see *Table 1*). Thus, we revealed that hsa_circ_0099630 was upregulated in patients with periodontitis.

Pg-LPS prevents proliferation and osteogenic differentiation, induces apoptosis, and upregulates hsa_circ_0099630 in HPLFs

To further assess the effect of hsa_circ_0099630 on periodontitis, we 1st constructed an inflammation model using Pg-LPS. As *Figure 2A* shows, the cell viability in the HPLFs was significantly decreased in the Pg-LPS group relative to the control group. Further, the apoptosis of the HPLFs was dramatically enhanced in the Pg-LPS group compared to that of the control group (see *Figure 2B*). We also discovered that the osteogenic differentiation ability of the HPLFs was markedly diminished after Pg-LPS treatment (see *Figure 2C*). Additionally, relative to the control group, ALP activity was also notably weakened in the Pg-LPS group (see *Figure 2D*). The RT-qPCR data showed that Pg-LPS stimulation caused a prominent increase in hsa_circ_0099630 expression in the HPLFs (see *Figure 2E*). The western blot results showed that RUNX2, Collagen I, osteocalcin, and Osterix expressions were significantly lower in the Pg-LPS group than the control group (see *Figure 2F*). In general, cell proliferation and osteogenic differentiation were prominently weakened, while apoptosis was enhanced in the Pg-LPS-induced inflammatory model.

Hsa_circ_0099630 silencing accelerates proliferation and attenuates apoptosis of Pg-LPS-induced HPLFs

Based on the above results, hsa_circ_0099630 was highly expressed in the Pg-LPS-induced HPLFs. We further overexpressed hsa_circ_0099630 in the HPLFs and silenced hsa_circ_0099630 in the Pg-LPS-induced HPLFs. The RT-qPCR data showed that hsa_circ_0099630 overexpression significantly increased hsa_circ_0099630 expression, and hsa_circ_0099630 silencing significantly decreased hsa_circ_0099630 expression mediated by the. Pg-LPS in HPLFs (see *Figure 3A*). The CCK-8 results showed that hsa_circ_0099630 overexpression significantly suppressed cell viability in the HPLFs, and hsa_circ_0099630 silencing signally increased cell viability, which were inhibited by the Pg-LPS in the HPLFs (see *Figure 3B*). We also found that hsa_circ_0099630 overexpression significantly accelerated the apoptosis of HPLFs, and hsa_circ_0099630 silencing notably restrained cell apoptosis, which were induced by Pg-LPS in the HPLFs (see *Figure 3C*). Overall, we showed that the knockdown of hsa_circ_0099630 induced proliferation and prevented apoptosis in the Pg-LPS-induced HPLFs.

Hsa_circ_0099630 knockdown induces osteogenic differentiation of Pg-LPS-induced HPLFs

The Alizarin red staining results showed that hsa_circ_0099630 overexpression or Pg-LPS treatment led to a notable decrease in the osteogenic differentiation capacity of HPLFs, and hsa_circ_0099630 silencing caused a notable increase in the osteogenic differentiation capacity, which was attenuated by Pg-LPS (see *Figure 4A*). Similarly, hsa_circ_0099630 overexpression or Pg-LPS observably reduced ALP activity in the HPLFs, and hsa_circ_0099630 silencing markedly elevated ALP activity in the Pg-LPS-induced HPLFs (see *Figure 4B*). Additionally, the western blot data showed that hsa_circ_0099630 overexpression or Pg-LPS significantly downregulated RUNX2, Collagen I, osteocalcin, and Osterix in the HPLFs, and hsa_circ_0099630 silencing notably upregulated RUNX2, Collagen I, osteocalcin, and Osterix in the Pg-LPS-induced HPLFs (see *Figure 4C*). Thus, we further verified that the knockdown of hsa_circ_0099630 also promoted osteogenic differentiation in the Pg-LPS-induced HPLFs.

MiRNA/mRNA axis of hsa_circ_0099630

More importantly, we also investigated the possible miRNA/mRNA axis of hsa_circ_0099630 by a bioinformatics analysis. First, we determined the location of hsa_circ_0099630 in the HPLFs by examining the levels of hsa_circ_0099630 in the cytoplasm and nucleus. Our data showed that hsa_circ_0099630 was mainly expressed in the cytoplasm (see *Figure 5A*). Second, we explored the potential miRNAs regulated by hsa_circ_0099630. Based on a condition of >2 binding sites to hsa_circ_0099630, we screened 5 eligible miRNAs; that is, miR-182, miR-1200, miR-338, miR-576, and miR-623 (see *Figure 5B*). Next, we further analyzed the target genes of the 5 miRNAs and obtained the intersection target genes by a Venn diagram. We identified 153 target genes associated with 5 miRNAs (see *Figure 5C*).

We also performed GO and pathway analyses of these

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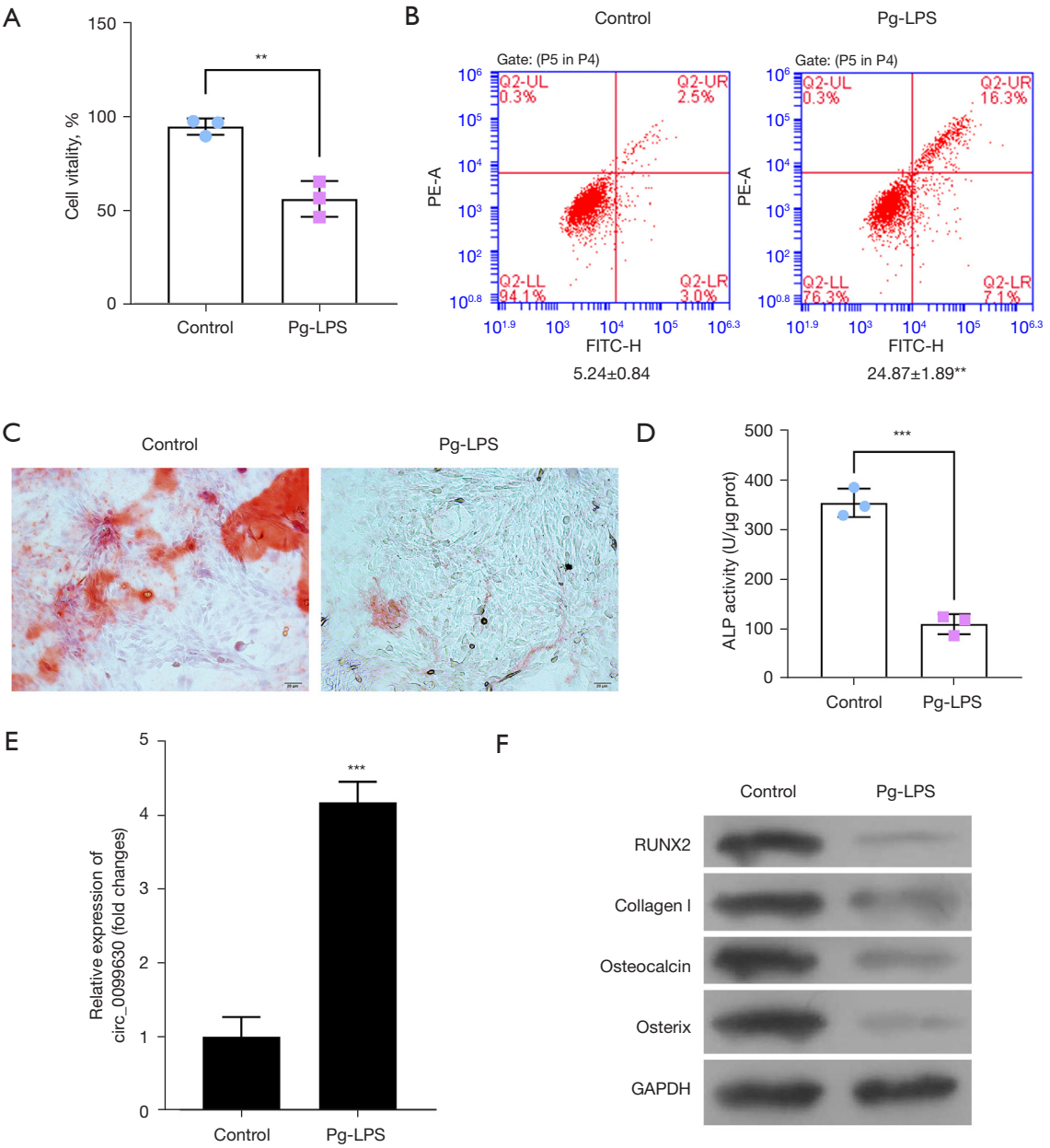

Figure 2 Pg-LPS prevents proliferation and osteogenic differentiation, induces apoptosis, and upregulates hsa_circ_0099630 in HPLFs. Pg-LPS was 1st applied to stimulate HPLFs to construct a model of inflammation. (A) Cell proliferation was monitored by CCK-8. (B) Cell apoptosis was assessed by flow cytometry. (C) Bone formation was confirmed using Alizarin red staining. Magnification, 200×. (D) ALP activity was tested by a kit. (E) The level of hsa_circ_0099630 was examined by RT-qPCR. (F) The levels of osteogenic differentiation-related proteins were identified by western blot. **, P<0.01; ***, P<0.001. Pg-LPS, porphyromonas gingivalis–lipopolysaccharide; HPLFs, human periodontal ligament fibroblasts; CCK-8, cell Counting Kit-8; ALP, alkaline phosphatase; RT-qPCR, real-time quantitative polymerase chain reaction; FITC, fluorescein isothiocyanate.

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Table 1 Correlation between hsa_circ_0099630 expression and clinicopathological features in 60 patients with periodontitis

Clinicopathologic characteristics	N	Low expression	High expression	P value
Age (years)				0.1762
<60	28	18	10	
≥60	32	15	17	
Sex				0.1896
Male	28	17	11	
Female	32	14	18	
BMI (kg/m^2)				0.4951
Normal, 18.5–24.99	15	7	8	
Overweight, 25–29.99	23	11	12	
Obesity I, 30–34.99	22	8	14	
Order of severity				0.0022
Slight	22	14	8	
Medium	21	5	16	
Severe	17	3	14	
Grade of periodontitis				0.0004
Level 0	21	15	6	
Level 1	25	7	18	
Level 2	14	2	12	

Level 0: a healthy periodontium and up to 1 proximal site with loss of attachment ≥3 mm; Level 1: presence of proximal attachment and loss ≥3 mm in ≥2 nonadjacent teeth; and Level 2: presence of proximal attachment loss ≥5 mm in ≥30% of teeth.

target genes. As the GO analysis showed, the enriched GO-biological process terms mainly included the response to hydroxyurea, and fibroblast proliferation; the enriched GO-cellular component terms mainly included the cell leading edge, and postsynaptic density; the enriched GO-molecular function terms mainly included dynamin binding, protein kinase activity, and protein phosphatase I binding (see *Figure 5D*). The KEGG analysis results showed that the enriched pathways mainly included human cytomegalovirus infection, the cGMP-PKG signaling pathway, and the estrogen signaling pathway (see *Figure 5E*). Thus, we examined the vast miRNA/mRNA axis of hsa_circ_0099630, and preliminarily identified the possible function of the target genes.

Discussion

Chronic inflammation can affect the repair and regeneration of bone tissue, probably because inflammation can alter the cellular microenvironment (24). Periodontitis, as an infectious disease, is a major cause of loose and missing teeth in adults (25). It has been reported that periodontitis is mainly caused by G-bacterial infections (26). Among them, Pg, a melanin-producing anaerobic bacillus, is recognized as the main causative agent of chronic periodontitis (27). LPS, as the primary component of the G-bacterial cell wall, is a key factor for Pg (28). Research has shown that LPS participates in the destruction of periodontal tissues (29). LPS not only acts directly on periodontal tissue causing tissue destruction, but also acts as a potential cell activator to stimulate the activation and differentiation of osteoclasts (30). Pg-LPS, as a key inflammatory mediator, can activate the host immune system (31). Pg-LPS can also enter the blood circulation and produce a certain inflammatory response to the blood vessel wall (32). Studies have shown that Pg-LPS can act on mononuclear macrophages, induce the production of inflammatory cytokines, and participate in the development

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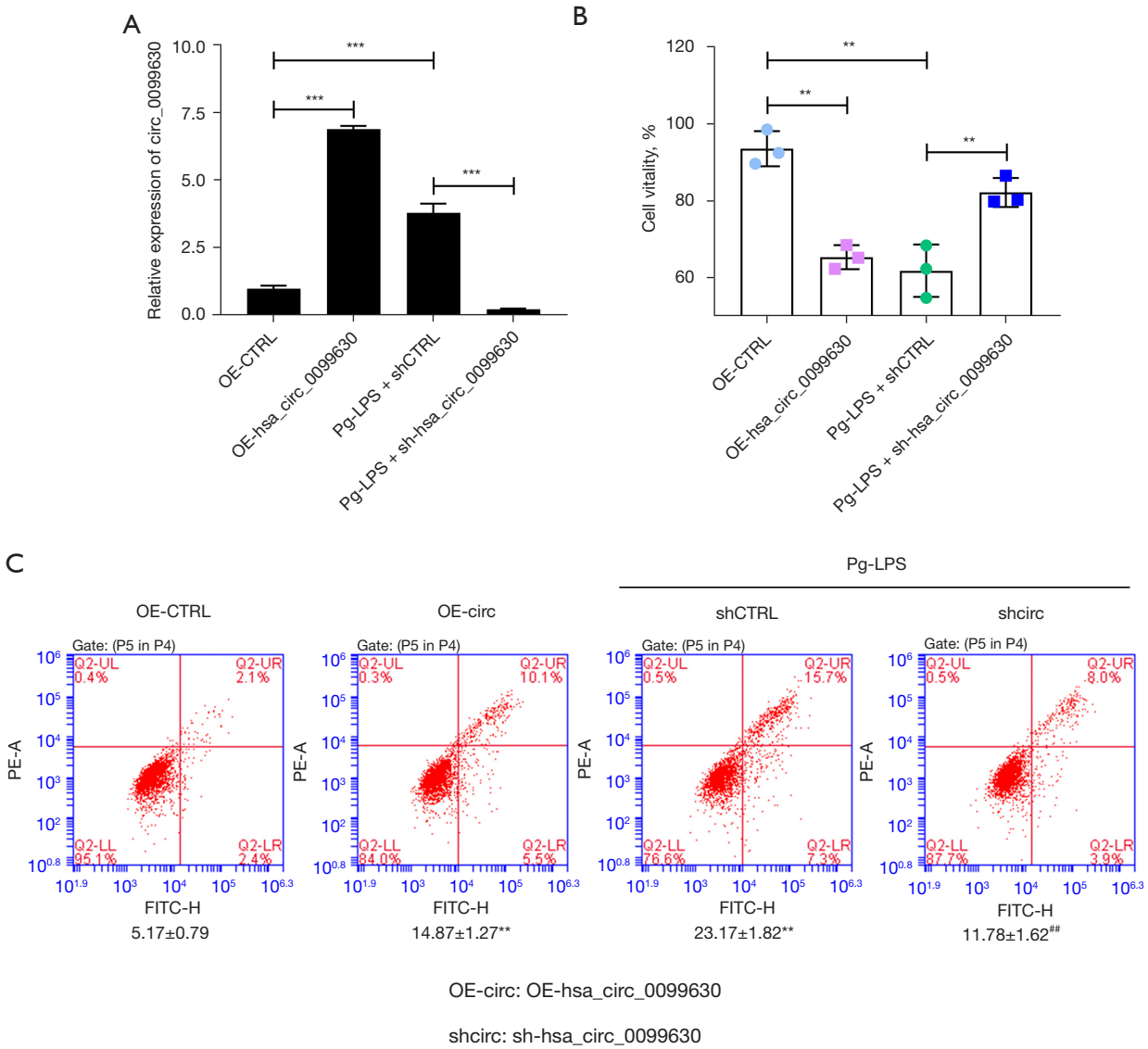

Figure 3 Hsa_circ_0099630 silencing accelerates proliferation and attenuates apoptosis of Pg-LPS-induced HPLFs. The HPLFs were transfected with hsa_circ_0099630 overexpression plasmids or hsa_circ_0099630 shRNAs after Pg-LPS stimulation. (A) RT-qPCR exhibited the expression change of hsa_circ_0099630. (B) CCK-8 displayed the change in cell proliferation. **, P<0.01; ***, P<0.001. (C) Flow cytometry was conducted to evaluate cell apoptosis in each group. **, P<0.01 *vs.* OE-CTRL group; ##, P<0.01 *vs.* Pg-LPS + shCTRL group. Pg-LPS, porphyromonas gingivalis–lipopolysaccharide; HPLFs, human periodontal ligament fibroblasts; shRNA, short hairpin RNA; RT-qPCR, real-time quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; OE, overexpression; CTRL, control; FITC, fluorescein isothiocyanate.

of periodontitis (33,34). Pg- LPSs, as cellular inflammation inducers, can be applied to construct inflammatory cell models, as reported in previous studies (35,36). In our study, we also used Pg-LPS to stimulate HPLFs to construct inflammatory model cells. Our data showed that Pg-LPS treatment suppressed the proliferation and induced the apoptosis of HPLFs.

The regeneration of periodontal tissue has been the focus of periodontal disease research (37). HPLFs have been reported to have a multidirectional differentiation ability and to participate in tissue repair and regeneration (38). Thus, the study of molecular regulatory mechanisms

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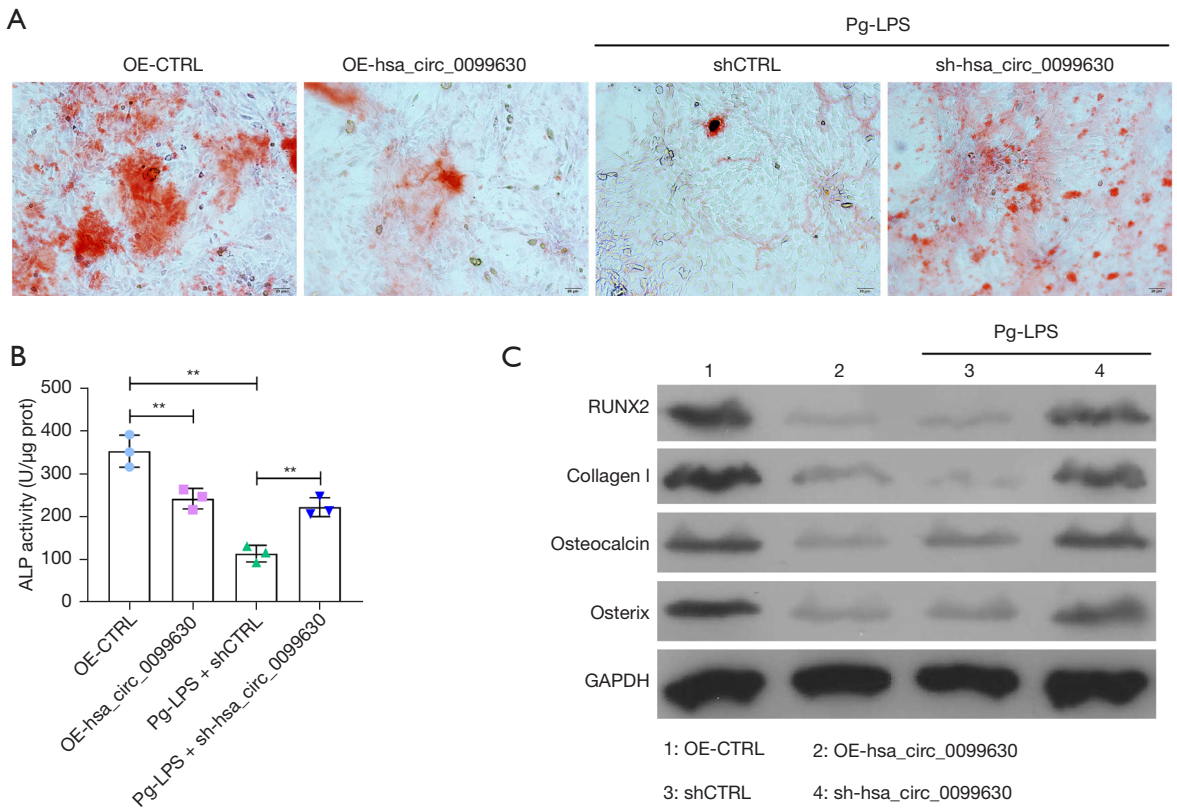

Figure 4 Hsa_circ_0099630 knockdown induces osteogenic differentiation of Pg-LPS-induced HPLFs. Hsa_circ_0099630 was overexpressed in the HPLFs, or hsa_circ_0099630 was silenced in the HPLFs after Pg-LPS stimulation. (A) Alizarin red staining was performed to identify the osteogenic differentiation capacity. Magnification, 200×. (B) A kit was used to analyze the ALP activity. (C) Western blot was used to examine the expressions of osteogenic differentiation-related proteins. **, P<0.01. Pg-LPS, porphyromonas gingivalis–lipopolysaccharide; HPLFs, human periodontal ligament fibroblasts; ALP, alkaline phosphatase; OE, overexpression; CTRL, control.

in the osteogenic differentiation of HPLFs is crucial for periodontal therapy, and it has extremely broad clinical application prospects. The identification of osteogenic markers is the basis for studying the osteogenic differentiation of HPLFs. ALP is one of the early marker enzymes of osteoblast differentiation and a key enzyme in the mineralization process (39). To some extent, ALP activity reflects the osteogenic differentiation capacity of the cells (40). RUNX2, as a bone differentiation-specific transcription factor, is involved in activating and initiating osteoblast differentiation and maturation (41). RUNX2 also promotes the synthesis and expression of Collagen I. Collagen I is the main component of the organic matter of bone tissue (42). Osteocalcin, an inducer of matrix mineralization, plays a key role in adaptive responses that alter energy homeostasis (43). According to one report, circ_0076906 can induce the osteogenic differentiation of bone marrow mesenchymal stem cells (BM-MSCs) and reduce osteoporosis by binding miR-1305 to regulate osteocalcin (44). Osterix (OSX) is a transcription factor that determines the differentiation of osteoblasts (45). It was found that changes in *OSX* gene expression affect the osteogenic differentiation of stem cells, such as dental pulp stem cells, periodontal cells, and adipose stem cells (46,47). In our study, we found that Pg-LPS stimulation notably weakened ALP activity, and downregulated RUNX2, Collagen I, osteocalcin, and Osterix in the HPLFs. Thus, we observed that osteogenic differentiation ability was reduced in Pg-LPS-induced HPLFs.

Currently, most studies on circRNAs are directed at cancer (48). However, some studies have shown that circRNAs affect tissue regeneration and stem cell

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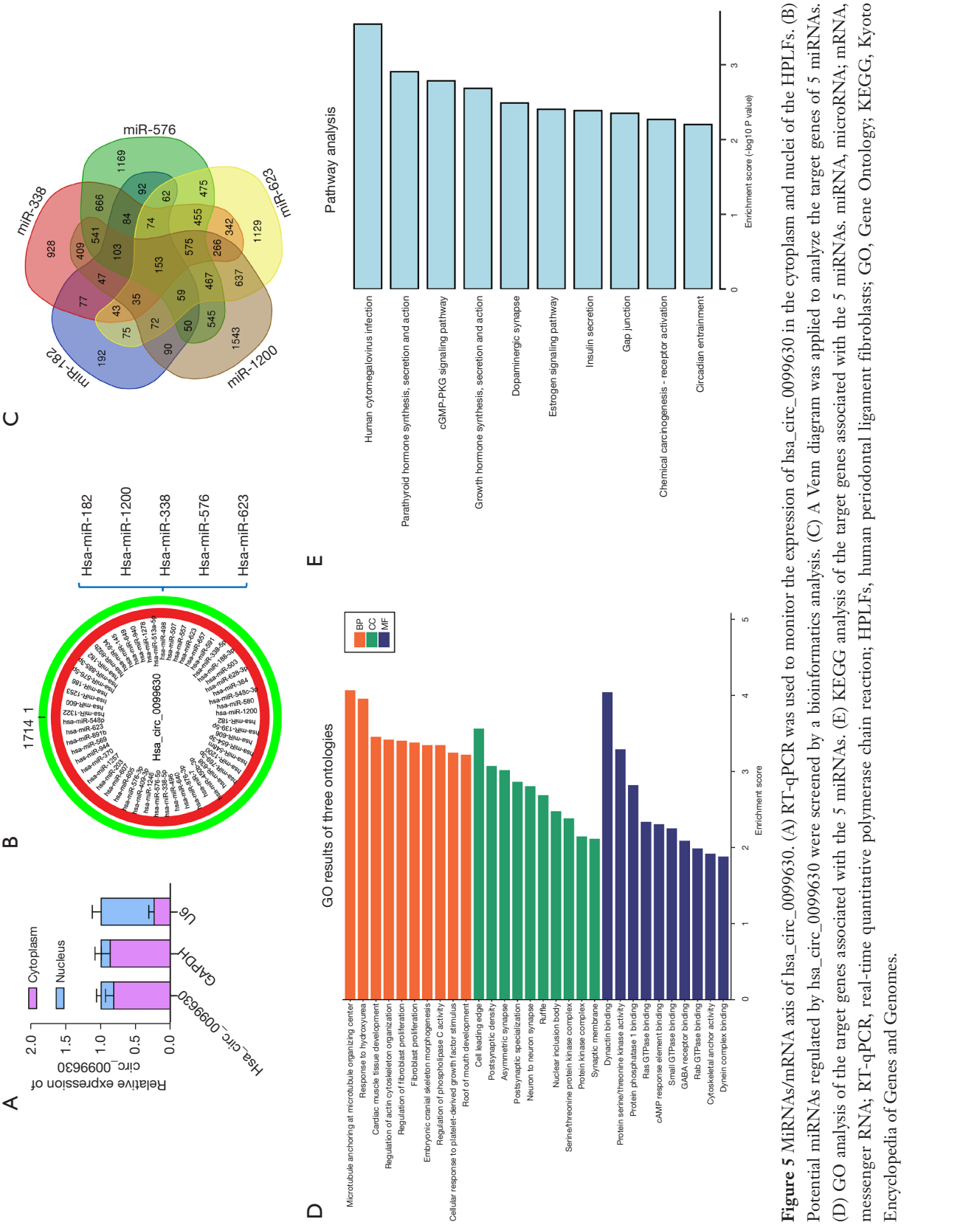

Figure 5 MiRNAs/mRNA axis of hsa_circ_0099630. (A) RT-qPCR was used to monitor the expression of hsa_circ_0099630 in the cytoplasm and nuclei of the HPLFs. (B) Potential miRNAs regulated by hsa_circ_0099630 were screened by a bioinformatics analysis. (C) A Venn diagram was applied to analyze the target genes of 5 miRNAs. (D) GO analysis of the target genes associated with the 5 miRNAs. (E) KEGG analysis of the target genes associated with the 5 miRNAs. miRNA, microRNA; mRNA, messenger RNA; RT-qPCR, real-time quantitative polymerase chain reaction; HPLFs, human periodontal ligament fibroblasts; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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differentiation processes; for example, studies have shown that circRNAs participate in liver regeneration in rats and promote the osteogenic differentiation of maxillary sinus membrane stem cells (49,50). Further, research has shown that BMP2 may induce osteoblast differentiation by targeting the miRNA/mRNA axis through circRNAs (51). Several circRNAs, including circ_0062491, circCDK8, circ_0095812, circ_0081572, and circCDR1as, were found aberrant expression in periodontal ligament (PDL) or gingival tissues from sites with periodontitis. The circRNA-miRNA interactions play important roles during osteogenic differentiation with an inhibitory mechanism, where circRNAs suppresses miRNAs expression, as shown by the gain-of-function or loss-of-function experiments. This suggests that circRNAs are able to modulate several critical biological pathways in the periodontium (49). In our study, we first found that hsa_circ_0099630 was upregulated in patients with periodontitis, indicating that hsa_circ_0099630 has an inductive role on the progression of periodontitis. Our data also revealed that hsa_circ_0099630 overexpression, like Pg-LPS, led to the inhibition of proliferation and osteogenic differentiation and the induction of apoptosis in HPLFs; the knockdown of hsa_circ_0099630 also accelerated proliferation and osteogenic differentiation prevented apoptosis in the Pg-LPS-induced HPLFs. Overall, the silencing of hsa_circ_0099630 may be a target for periodontal therapy. Nevertheless, the problems and challenges in the clinical application of circRNA containing two sides. On the one hand, the main challenge of circRNA targeting is the off-target effect of circRNA knockdown technology. At least half of the sequences of siRNA targeting circRNA are paired with the parent gene mRNA, which may have miRNA-like non-specific effects. This effect may affect cell function to varying degrees and produce non-specific effects. On the other hand, the challenge in circRNA therapy is to improve the translation efficiency of circRNA. We will focus on the problems and challenges to improve the clinical application of circRNA.

Many circRNAs have been reported to have rich and stable potential functions as competing endogenous RNAs (48). CircRNAs have potential functions in the regulation of gene expression (52). A recent study has shown that endogenous circRNAs act as miRNA sponges to attenuate the function of miRNAs (53). A study has also confirmed that the decrease in osteogenic potential in chronic inflammatory environments is associated with miRNA dysregulation (54). In the current study, vast miRNAs, especially miR-182, miR-1200, miR-338, miR-576, miR-623, were identified as possibly interacting with hsa_circ_0099630 based on a bioinformatics analysis. Additionally, we predicted that 153 mRNAs might interact with these 5 miRNAs.

Conclusions

In summary, our findings suggested that hsa_circ_0099630 regulates the proliferation, osteogenic differentiation, and apoptosis of Pg-LPS-induced HPLFs. We also preliminarily screened out a large number of miRNA/mRNA axis related to hsa_circ_0099630, which is also the main direction of our future research. Furthermore, how to judge the prognostic characteristics of periodontitis based on the expression of hsa_circ_0099630 and provide hsa_circ_0099630 as a reliable target for the treatment of periodontitis need to be further investigated.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-4209/rc

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Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4209/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of the Hospital of Stomatology, Sun Yat-sen University (No. 2020-07-162). All the participants

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signed a written informed consent form.

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