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ORIGINAL RESEARCH

Long Non-Coding RNA EPB41L4A-AS1 Serves as a Diagnostic Marker for Chronic Periodontitis and Regulates Periodontal Ligament Injury and Osteogenic Differentiation by Targeting miR-214-3p/YAP1

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Background: Several long non-coding RNAs (lncRNAs) are dysregulated in chronic periodontitis (CP).

Purpose: The study aimed to elucidate the molecular mechanisms and clinical significance of lncRNA EPB41L4A antisense RNA 1 (EPB41L4A-AS1) in CP.

Patients and Methods: This study enrolled 101 patients with CP and 90 subjects with healthy periodontal tissues. Patients with CP were categorized according to severity. The expression of EPB41L4A-AS1 and osteogenic markers in the lipopolysaccharide (LPS)-induced human periodontal ligament cells (hPDLCs) was assessed using real-time quantitative reverse transcription PCR (RT-qPCR). The diagnostic significance of EPB41L4A-AS1 was evaluated using receiver operating characteristic (ROC) analysis. The levels of inflammatory factors were measured using an enzyme-linked immunosorbent assay. Cell proliferation and apoptosis were analyzed using cell counting kit −8 and flow cytometry, respectively. The interaction between EPB41L4A-AS1 and microRNAs was verified using dual luciferase reporter assays, RNA immunoprecipitation, and RNA pull-down assays.

Results: EPB41L4A-AS1 was downregulated in the gingival sulcus fluid of patients with CP and LPS-induced hPDLCs. Additionally, EPB41L4A-AS1 could distinguish patients with CP from control subjects with sensitivity (88.12%) and specificity (81.11%). The expression of EPB41L4A-AS1 was downregulated in patients with severe CP. EPB41L4A-AS1 downregulation was directly correlated with severe clinical indicators and inversely correlated with inflammatory indicators. The overexpression of EPB41L4A-AS1 promoted the proliferation and osteogenic differentiation of hPDLCs and mitigated LPS-induced inflammation. Mechanistically, EPB41L4A-AS1 directly targets and downregulates miR-214-3p expression, resulting in the upregulation of Yes1-associated transcriptional regulator (YAP1) levels. The overexpression of miR-214-3p partially suppressed the effects of EPB41L4A-AS1 on LPS-induced hPDLC injury and osteogenic differentiation.

Conclusion: The overexpression of EPB41L4A-AS1 suppressed LPS-induced hPDLC injury and enhanced osteogenic differentiation through the miR-214-3p/YAP1 axis. Thus, EPB41L4A-AS1 is a novel diagnostic marker and a therapeutic target for CP.

Keywords: EPB41L4A-AS1, miR-214-3p, osteogenic differentiation, chronic periodontitis

Introduction

Chronic periodontitis (CP), an osteolytic inflammatory condition, is characterized by an imbalance between the periodontal microbiome and host defense mechanism,[1] leading to inflammation of the periodontal tissues and damage to the

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tissues supporting the teeth, including the alveolar bone.[2] These changes cause tooth mobility, shifting, and even loss. CP is the most common form of periodontitis, affecting approximately 50% of adults and 60% of the elderly worldwide. In addition to affecting the oral tissues, CP is a significant risk factor for systemic diseases, such as coronary heart disease, stroke, diabetes mellitus, and Alzheimer's disease.[3,4] Therefore, there is a need to develop early diagnostic strategies and elucidate the pathogenetic mechanisms to devise preventive and therapeutic strategies for CP. Human periodontal ligament cells (hPDLCs) maintain periodontal tissue health and promote tissue restoration.[5] The alleviation of periodontal ligament injury and hPDLCs inflammation and the induction of osteogenic differentiation are potential strategies to induce periodontal tissue repair and regeneration.[6,7]

Long non-coding RNAs (lncRNAs) are non-coding RNAs with a size of less than 200 nt. The roles of lncRNAs, such as LINC01126,[8] SNHG5,[9] and GAS5,[10] in osteogenic differentiation and inflammation have piqued the interest of the scientific community. EPB41L4A antisense RNA 1 (EPB41L4A-AS1), which is a recently discovered lncRNA, is also known as C5orf26, NCRNA00219, and TIGA1. The expression of EPB41L4A-AS1 is reported to be dysregulated in carotid atherosclerotic plaques in patients with type 2 diabetes mellitus.[11] The downregulation of EPB41L4A-AS1 exacerbates diabetes-associated inflammation through the activation of the NF-κB pathway.[12] Additionally, EPB41L4A-AS1 expression is markedly downregulated in patients with myelodysplastic syndromes.[13] EPB41L4A-AS1 is also involved in the shared pathological mechanisms of CP and chronic obstructive pulmonary disease.[14] Furthermore, EPB41L4A-AS1 enhances the proliferation of bone marrow stromal cells (BMSCs).[15] A research reported 10 differentially expressed lncRNAs, including EPB41L4A-AS1, in periodontitis.[16] Similarly, microarray analysis reported that EPB41L4A-AS1 was one of the differentially expressed lncRNAs in differentiated and undifferentiated hPDLCs.[17] However, the clinical values and molecular functions of EPB41L4A-AS1 in CP have not been elucidated.

This study hypothesized that EPB41L4A-AS1 dysregulation influences periodontal ligament injury, inflammation, and osteogenic differentiation, regulating the clinical course of CP. To validate this hypothesis, this study analyzed the expression pattern and clinical significance of EPB41L4A-AS1 in patients with CP and subjects with healthy periodontal tissues. Additionally, an in vitro cell model was constructed using lipopolysaccharide (LPS)- induced hPDLCs to assess the molecular mechanisms through which EPB41L4A-AS1 regulates CP pathogenesis. The findings of this study can offer novel insights for diagnosing and treating CP.

Materials and Methods

Subjects and Severity

The study recruited 101 patients diagnosed with CP and 90 individuals with healthy periodontal tissues between June 2019 and December 2021 at the Affiliated Hospital of Nantong University. The inclusion criteria were as follows: 1) patients with CP and periodontal disease who met the staging criteria defined by the American Academy of Periodontology (AAP) and the European Federation of Periodontology in 2018;[18] 2) patients with ≥ 20 teeth; 3) patients aged ≥ 18 years; 4) patients who have not received immunosuppressive drugs, antibiotics, or periodontal treatment in the 3 months before admission; 5) patients with complete clinical information; and 6) patients who agreed to sign the informed consent form. The exclusion criteria were as follows: 1) taking specific medications in the past three months that could affect periodontal status; 2) pregnant, breastfeeding, or menopausal subjects; 3) patients with systemic diseases, such as obesity, diabetes mellitus, chronic renal disease, rheumatoid arthritis, and cardiovascular disease that affect the progression of periodontal disease; 4) patients with a history of orthodontic treatment. Subjects with healthy periodontal tissues were identified based on the following criteria: probing pocket depth (PD) ≤ 3 mm, clinical attachment loss (CAL) < 1 mm, bleeding in probing (BOP) site ≤ 10%, no tooth loss due to periodontitis, no history of periodontal disease, and no alveolar bone loss. These patients were age-matched and gender-matched to patients with CP. This study was performed according to the principles of the Declaration of Helsinki and was approved by the Affiliated Hospital of Nantong University. All subjects signed an informed consent to participate in the study. The age, gender, body mass index (BMI), smoking history, drinking history, and periodontal indicators (such as gingival index (GI), plaque index (PI), PD, CAL, BOP, and alveolar bone resorption length (ABL)) were recorded as described previously.[19] The average values of the periodontal indicators were calculated from six

buccal/lingual proximal-medial, central-medial, and distal-medial sites in the mouth. PD was defined as the distance from the gingival margin to the base of the pocket. The tip of the probe was used to probe the position of the enamel-osseous boundary along the root surface. CAL was defined as the distance from the base of the pocket to the enamel-osseous boundary along the longitudinal axis of the tooth. The GI is an indicator of gingival conditions, including gingival color and texture.

The severity of the periodontics was classified as follows: mild/moderate periodontitis (MCP)(CAL of 1–4 mm, ABL of one-third to one-half of the root length, PD ≤ 6 mm, no or mild tooth loosening, inflammation of the gingival, BOP, and possible pus); severe periodontitis (CAL≥ 5 mm, ABL > one-third to one-half of the root length, PD > 6 mm, significant tooth loosening, teeth appearing to be loose in multiple places, severe inflammation of the gums, or periodontal abscess).[20]

Gingival Crevicular Fluid (GCF) Samples

The samples were collected after the subjects were allowed to fast for 2 h. The calculus, plaque, and soft tartar were removed from the tooth surface. The tooth surface was dried. Whatman I filter paper strips were placed in the gingival sulcus at six sites. After 30s, the strips were removed and transferred to EP tubes containing 0.2 mL of phosphate-buffered saline (PBS) as described previously.[21]

Cell Culture and Lipopolysaccharide (LPS) Treatment

hPDLCs were obtained from the American Type Culture Collection (ATCC), and minimal essential medium supplemented with 10% fetal bovine serum, 5mM L-glutamine, and 1% penicillin/streptomycin, placed at 5% CO2 and 37°C with saturated humidity. The culture medium was replaced once every 3 days. hPDLCs passed 3–5 times were used for experiments. Additionally, hPDLCs were induced with 10 μg/mL of LPS for 0, 12, 24, and 48 h as reported previously.[22]

Cell Transfection

EPB41L4A-AS1 overexpression plasmids (oe-EPB41L4A-AS1) and negative control plasmids (oe-NC) were obtained from GenePharma. MicroRNA (miR)-214-3p mimic (miR-214-3p) and mimic negative control (miR-NC) oligonucleotides were procured from RiboBio Co. hPDLCs were seeded in 6-well plates, incubated for 20 min at room temperature, and transfected with oe-EPB41L4A-AS1, oe-NC, or miR-214-3p, miR-NC for 20 min using lipofectamine 3000 (#1656200, Invitrogen). The medium was replaced once after 6 h for subsequent studies.

Osteogenic Differentiation

To induce osteogenic differentiation induction, hPDLCs were seeded in 6-well plates and cultured in osteogenic medium (10 nM dexamethasone, 50 mg/mL ascorbic acid, 10 mm β-glycerophosphate) for 14 days as described previously.[23] The medium was replaced once every 3 days. RNA was extracted from hPDLCs.

Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

GCF samples preserved at −80°C were thawed at room temperature. hPDLCs were rinsed with PBS. RNA was extracted from the samples using a TRIzol reagent. The concentration and quality of RNA were evaluated. RNA (500 ng) was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT kit (for mRNA, #RR047A, Takara) and the miRCute Plus miRNA First-Strand cDNA Synthesis Kit (for miRNA, #KR211, Tiangen). RT-qPCR analysis was performed using the QuantStudio 7 Flex Real-time PCR System, with SuperReal PreMix Plus (SYBR Green, for mRNA, # FP205, Tiangen) and a miRcute Plus miRNA qPCR kit (for miRNA, #FP411, Tiangen) along with primers and cDNA. U6 and GAPDH were utilized as internal reference genes to examine the expression levels of miRNAs, EPB41L4A-AS1, osteopontin (OPN), related transcription factor 2 (RUNX2), and bone morphogenetic 2 (BMP2). The relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method as described previously.[24] Three biological replicates were used.

Cell Proliferation Assay

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8, #CK04, Dojindo Molecular Technologies). hPDLCs (2×10^3 cells/well) were seeded in 96-well plates and cultured for 24 h and 48 h. Next, the cells were incubated with 90 μL of the medium supplemented with 10 μL of CCK-8 reagent in a CO_2 incubator for 1 h. The optical density (OD) of the samples at 450 nm was recorded.

Cell Apoptosis Assay

Annexin V-fluorescein isothiocyanate (FITC)/ propidium Iodide (PI) double-staining cell apoptosis assay was conducted to examine the apoptosis of hPDLCs. The cells were washed with pre-cooled PBS, digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin, and hPDLCs were resuspended in 200 μL of binding buffer. Next, the cells were stained with 5 μL of FITC-coupled Annexin V and 5 μL of PI for 20 min in the dark. The apoptosis rate was analyzed using flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of inflammatory markers tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β were examined using commercial ELISA kits. Briefly, the GCFs and the supernatant of hPDLCs were diluted, transferred to the plate, and incubated at 37 °C for 1.5 h. The plate was washed thrice with PBS containing Tween-20 (PBST) for 5 min, and incubated with enzyme-conjugated secondary antibody at 37°C for 1 h. After washing with PBST, the samples were incubated with 50 μL of the substrate solution in the dark for the chromogenic reaction, followed by incubation with 50 μL of stop solution. The OD value of samples of 450 nm was recorded.

Alkaline Phosphatase (ALP) Activity Assay

ALP activity was examined on day 7 days post-osteogenic induction. The cells were seeded in 24-well plates (2×10^4 cells/well), washed thrice with PBS, and lysed with 1% Triton ×100 for 10 min. The lysates were centrifuged at 10,000 rpm for 10 min. ALP activity was measured at 680 nm using an ALP assay kit (Nanjing Institute of Bioengineering).

Subcellular Fractionation

Nuclear and cytosolic fractions were prepared using the commercial PARIS kit (#AM1921, Life Technologies) as reported previously.[25] hPDLCs were washed with pre-cooled PBS and lysed on ice for 10 min. The samples were incubated with ice-cold cell fractionation buffer for 1 min at 4°C. The upper cytoplasmic extract was collected by centrifugation at 500 g for 5 min. The residual fraction was mixed with ice-cold cell disruption buffer, vortexed for 15s, and incubated for 10 min at 4°C. The nuclear extracts were obtained via centrifugation at 500 g for 5 min. RNA was isolated and subjected to RT-qPCR analysis to measure cytoplasmic and nuclear levels of EPB41L4A-AS1, GAPDH, and U6 were used as the internal controls for the cytoplasmic and nuclear expression levels of target genes, respectively.

Dual Luciferase Reporter (DLR) Assay

The miRNA targets of EPB41L4A-AS1 were predicted using ENCORI, DINAN, LncBook 2.0, TRlnc, and LncRNASNP. The target mRNA of miR-214-3p was predicted using Targetscan, which revealed Yes1-associated transcriptional regulator (YAP1) as the target. EPB41L4A-AS1-wild type (WT), YAP1-WT, EBB41L4A-AS1-mutant (MT), and YAP1-MT recombinant luciferase plasmids were constructed by introducing the 3' untranslated region (UTR) fragment of EPB41L4A-AS1 or YAP1 containing the miR-214-3p binding site into the pmirGLO plasmid. Next, hPDLCs were co-transfected with these constructed and miR-214-3p mimic or miR-NC using lipofectamine 3000. The relative luciferase activity was assessed after 48 h using a dual-luciferase reporter assay kit.

RNA Immunoprecipitation (RIP) Assay

RIP analysis was performed using the Magna RIP kit (#17700, Millipore) as described previously.[26] hPDLCs were lysed in RIP lysis buffer. The lysate was incubated with magnetic beads coupled with anti-Ago2 or negative control IgG

antibodies in RIP buffer for 4 h (at 4°C). The samples were then incubated with proteinase K at 55°C for 30 min. RNA was extracted from the immunoprecipitates and subjected to RT-qPCR analysis.

RNA Pull-Down Assay

Biotin-labeled EPB41L4A-AS1 probe (bio-EPB41L4A-AS1) and negative control (bio-NC) were purchased from RiboBio. Probe-coated beads were constructed by incubating the biotinylated probes with M-280 streptavidin Dyn bead as reported previously.[27] Cells were lysed, and the lysates were incubated with probe-coated beads at 4°C for 24 h. The coupled miRNAs were eluted, extracted, and subjected to RT-qPCR analysis to examine miR-214-3p levels.

Statistical Analysis

SPSS 23.0 and GraphPad Prism 9.0 were used for statistical analysis and visualization. Data are expressed as mean ± standard deviation (SD) and obtained from at least three replications. Means between two groups were compared using the Student's *t*-test, whereas those between more than two groups were compared using one-way or two-way analysis of variance, followed by Tukey's post-hoc tests. The diagnostic value of EPB41L4A-AS1 was examined using the receiver operating characteristic curve. Pearson correlation coefficient was calculated for linear correlation between the indicators. Differences were considered significant at $P < 0.05$.

Results

Expression of EPB41L4A-AS1 in Patients with Chronic Periodontitis

The clinical baseline characteristics were not significantly different between control subjects and patients (Table 1). However, the levels of periodontal indicators (PD, CAL, ABL, BOP, GI, and PI) and inflammatory factors (IL-6, IL-1β, and TNF-α) in patients with CP were markedly higher than those in the controls ($P < 0.05$). Additionally, the GCF levels of EPB41L4A-AS1 in CP patients were significantly lower than those in control subjects ($P < 0.0001$, Figure 1A). Based on the cut-off value of 0.765, the area under the receiver operating characteristic curve value for EPB41L4A-AS1 was 0.899, indicating that EPB41L4A-AS1 levels can distinguish patients with CP from control subjects with high sensitivity (88.12%) and specificity (81.11%) (95% CI: 0.854–0.946, Figure 1B).

Table 1 Demographic and Clinical Characteristics of the Subjects

Indicators	Controls (n = 90)	CP (n = 101)	*P* value
Age (years)	45.03 ± 15.29	47.00 ± 13.98	0.357
BMI (kg/m^2)	23.40 ± 3.18	23.15±3.72	0.625
Gender (Male/Female)	49/41	66/35	0.140
Smoking history (no/yes)	47/43	47/54	0.470
Drinking history (no/yes)	45/45	48/53	0.773
Dietary favor (light/heavy)	43/47	45/56	0.666
PD (mm)	1.70 ± 0.48	5.15 ± 1.55	<0.001
CAL (mm)	0.73 ± 0.12	3.52 ± 1.49	<0.001
ABL (%)	0.24 ± 0.07	29.00 ± 6.88	<0.001
BOP (%)	8.86 ± 1.08	49.41 ± 7.17	<0.001
GI	0.45 ± 0.09	2.46 ± 0.53	<0.001
PI	0.80 ± 0.35	2.38 ± 0.55	<0.001
IL-6 (pg/mL)	4.17 ± 0.53	10.04 ± 4.39	<0.001
IL-1β (pg/mL)	2.82 ± 0.38	5.77 ± 1.77	<0.001
TNF-α (pg/mL)	3.92 ± 1.37	6.08 ± 2.36	<0.001

Abbreviations: CP, Chronic periodontitis; BMI, body mass index; PD, probing pocket depth; CAL, clinical attachment level; GI, gingival index; PI, plaque index; ABL, alveolar bone resorption length; BOP, bleeding in probing; IL-6, interleukin-6; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α.

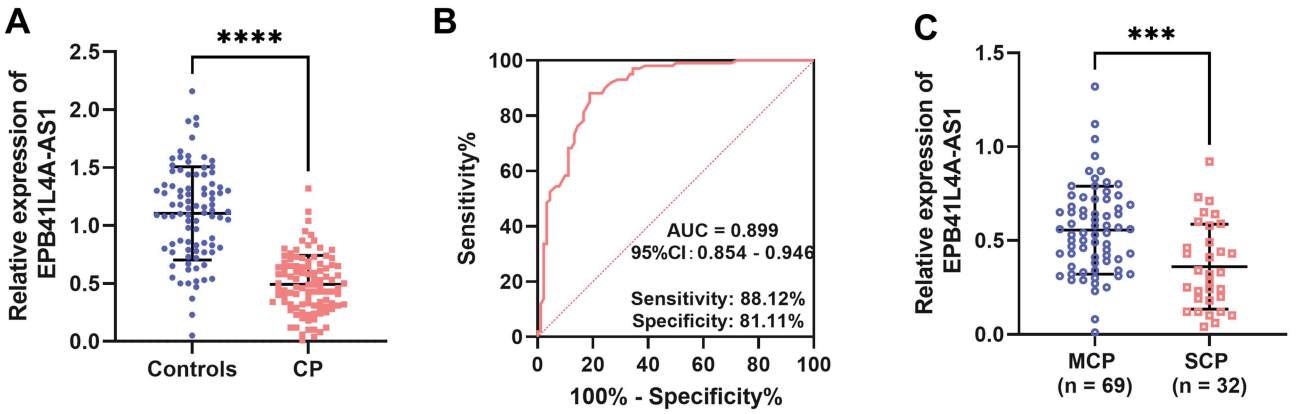

Figure 1 Expression of EPB41L4A-AS1 in the gingival sulcus fluid (GCF) samples of patients with chronic periodontitis (CP). (**A**) The expression of EPB41L4A-AS1 in GCF samples of control subjects with healthy periodontal tissues and patients with CP. (**B**) Receiver operating characteristic (ROC) analysis was performed to examine the diagnostic value of EPB41L4A-AS1 in CP. (**C**) Real-time quantitative reverse transcription PCR analysis of the levels of EPB41L4A-AS1 in patients with mild/moderate periodontitis (MCP) and severe periodontitis (SCP). ***$P < 0.001$, ****$P < 0.0001$.

Correlation Between EPB41L4A-AS1 Expression and Severity of Chronic Periodontitis

Of the 101 patients with CP, 69 and 32 were mild to moderate CP (MCP) and severe CP (SCP) cases, respectively. The GCF EPB41L4A-AS1 levels in patients with SCP were significantly downregulated when compared to those in patients with MCP ($P < 0.05$, Figure 1C). Additionally, patients were stratified into high-EPB41L4A-AS1 expression and low-EPB41L4A-AS1 expression groups based on the mean values. EPB41L4A-AS1 downregulation was positively correlated with severe clinical indicators (deeper PD, higher CAL, ABL, and BOP) and inflammatory marker production ($P < 0.05$, Table 2). This is consistent with the downregulation of EPB41L4A-AS1 in SCP cases.

EPB41L4A-AS1 Alleviates Apoptosis and Inflammatory Response in hPDLCs

To investigate the molecular mechanisms of EPB41L4A-AS1 in CP, in vitro experiments were performed using LPS-induced hPDLCs. LPS time-dependently downregulated the levels of EPB41L4A-AS1 ($P < 0.001$, Figure 2A). Therefore, hPDLCs

Table 2 Relationship Between EPB41L4A-AS1 and Periodontal Markers and Inflammatory Factors

Indicators	High EPB41L4A-AS1 Expression (n = 48)	Low EPB41L4A-AS1 Expression (n = 53)	*P* value
PD (mm)			
≤5	27	14	0.003
>5	21	39	
CAL (mm)			
≤5	38	31	0.033
>5	10	22	
ABL (%)			
≤29	31	19	0.005
>29	17	34	
BOP (%)			
≤49	27	13	0.002
>49	21	40	
GI			
≤2	13	6	0.073
>2	35	47	

(*Continued*)

Table 2 (Continued).

Indicators	High EPB41L4A-AS1 Expression (n = 48)	Low EPB41L4A-AS1 Expression (n = 53)	*P* value
PI			
≤2	15	9	0.106
>2	33	44	
IL-6 (pg/mL)			
≤10.04	30	21	0.029
>10.04	18	32	
IL-1β (pg/mL)			
≤5.77	33	15	0.003
>5.77	20	33	
TNF-α (pg/mL)			
≤6.08	30	19	0.010
>6.08	18	34	

Abbreviations: PD, probing pocket depth; CAL, clinical attachment loss; GI, gingival index; PI, plaque index; ABL, alveolar bone resorption length; BOP, bleeding in probing; IL-6, interleukin-6; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α.

induced for 24 h were followed up. Furthermore, transfection with oe-EPB41L4A-AS1 upregulated EPB41L4A-AS1 expression and diminished LPS-induced downregulation of EPB41L4A-AS1 levels ($P < 0.001$, Figure 2B and C). Additionally, EPB41L4A-AS1 overexpression significantly mitigated the LPS-induced downregulation of proliferation and upregulation of apoptosis in hPDLCs ($P < 0.05$, Figure 2D and E). Furthermore, EPB41L4A-AS1 overexpression suppressed the LPS-induced upregulation of inflammatory factors (TNF-α, IL-6, and IL-1β) ($P < 0.05$, Figure 2F).

EPB41L4A-AS1 Overexpression Promotes the Osteogenic Differentiation of Lipopolysaccharide (LPS) Induced hPDLCs

Next, the effect of EPB41L4A-AS1 on osteogenic differentiation was examined. Osteogenic differentiation induction time-dependently upregulated the EPB41L4A-AS1 levels ($P < 0.05$, Figure 3A). LPS downregulated the ALP activity and the levels of osteogenic differentiation markers (OPN, RUNX2, and BMP2) ($P < 0.05$, Figure 3B and C). However, EPB41L4A-AS1 overexpression mitigated the LPS-induced downregulation of ALP activity and osteogenic differentiation markers ($P < 0.05$, Figure 3D and E).

EPB41L4A-AS1 Directly Targets miR-214-3p

To investigate the specific molecular mechanisms underlying EPB41L4A-AS1 in LPS-induced hPDLCs, the subcellular localization of EPB41L4A-AS1 was examined. As shown in Figure 4A, EPB41L4A-AS1 was predominantly localized to the cytoplasm, suggesting that it can potentially function as a miRNA molecular sponge. The potential target miRNAs of EPB41L4A-AS1 were predicted using five databases. The Venn diagram revealed that miR-214-3p was the only overlapping target miRNA for EPB41L4A-AS1 (Figure 4B). The predicted binding between EPB41L4A-AS1 and miR-214-3p is illustrated in Figure 4C. Furthermore, the luciferase activity of EPB41L4A-AS1-WT, but not that of EPB41L4A-AS1-MT, in miR-214-3p mimic-transfected cells was lower than that in miR-NC-transfected cells ($P < 0.05$, Figure 4D). Furthermore, the RIP assay results demonstrated the enrichment of EPB41L4A-AS1 and miR-214-3p in anti-Ago2 immunoprecipitates ($P < 0.05$, Figure 4E). Treatment with bio-EPB41L4A-AS1 effectively upregulated the levels of miR-214-3p ($P < 0.05$, Figure 4F). Furthermore, the GCF miR-214-3p levels in patients with CP were significantly higher than those in control subjects ($P < 0.05$, Figure 4G). Additionally, the GCF miR-214-3p levels were significantly negatively correlated with the GCF EPB41L4A-AS1 levels ($P < 0.05$, Figure 4H). EPB41L4A-AS1 overexpression significantly downregulated the miR-214-3p levels in hPDLCs ($P < 0.05$, Figure 4I).

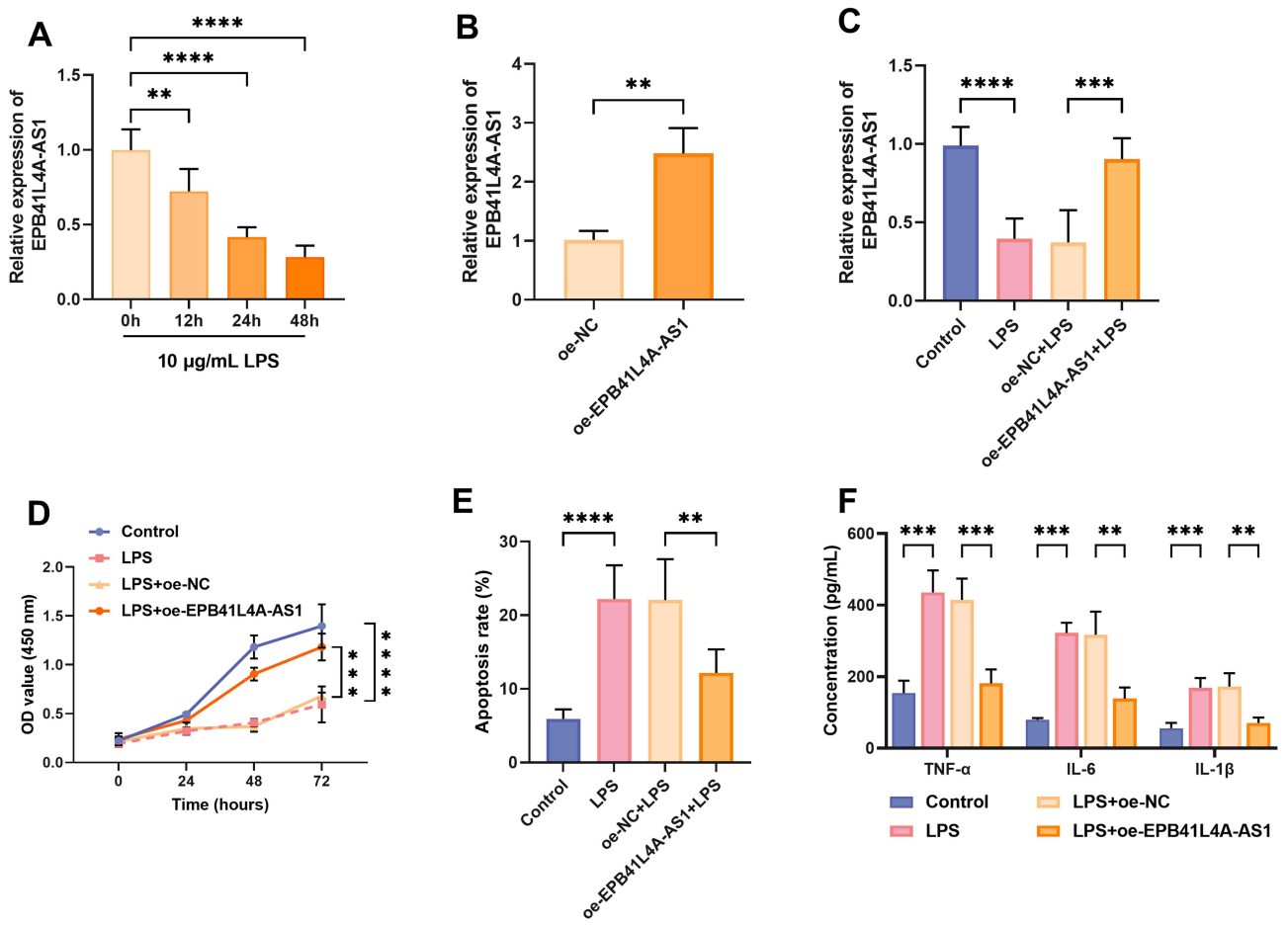

Figure 2 Effects of EPB41L4A-AS1 on lipopolysaccharide (LPS)-induced human periodontal ligament cell (hPDLC) proliferation, apoptosis, and inflammation. (**A**) LPS time-dependently altered the levels of EPB41L4A-AS1. Real-time quantitative reverse transcription PCR analysis of EPB41L4A-AS1 levels in EPB41L4A-AS1 overexpression plasmid-transfected hPDLCs (**B**) and LPS-induced hPDLCs (**C**). (**D**) Effect of EPB41L4A-AS1 overexpression on cell proliferation were determined using the cell counting kit-8. (**E**) Effects of EPB41L4A-AS1 overexpression on cell apoptosis were examined using flow cytometry. (**F**) Effects of EPB41L4A-AS1 on the secretion of inflammatory factors in hPDLCs were examined using enzyme-linked immunosorbent assay. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

miR-214-3p Suppresses the Effects of EPB41L4A-AS1 on hPDLC Injury and Osteogenic Differentiation

LPS time-dependently upregulated the levels of miR-214-3p in hPDLCs ($P < 0.05$, Figure 5A). Additionally, miR-214-3p overexpression upregulated the expression of miR-214-3p in hPDLCs and LPS-induced hPDLCs ($P < 0.05$, Figure 5B and C). Transfection with miR-214-3p mimic mitigated the EPB41L4A-AS1 overexpression-induced upregulation of cell proliferation ($P < 0.05$, Figure 5D), downregulation of apoptosis ($P < 0.05$, Figure 5E), and suppression of inflammatory factor production in LPS-induced hPDLCs ($P < 0.05$, Figure 5F). The levels of miR-214-3p increased with the osteogenic differentiation of hPDLCs ($P < 0.05$, Figure 5G). Furthermore, transfection with miR-214-3p mimic suppressed the EPB41L4A-AS1 overexpression-induced upregulation of ALP activity and osteogenic differentiation markers expression levels ($P < 0.05$, Figure 5H and I).

YAP1 is a Target of miR-214-3p

Next, the targets of miR-214-3p were examined. YAP1 harbored the binding site for miR-214-3p ($P < 0.05$, Figure 6A). Additionally, transfection with miR-214-3p mimic suppressed the luciferase activity of YAP1-WT but not that of YAP1-MT ($P < 0.05$, Figure 6B). Compared with those in anti-IgG, EPB41L4A-AS1, miR-214-3p, and YAP1 were enriched in anti-Ago2 ($P < 0.05$, Figure 6C). The GCF YAP1 levels in patients with CP were significantly lower than those in control

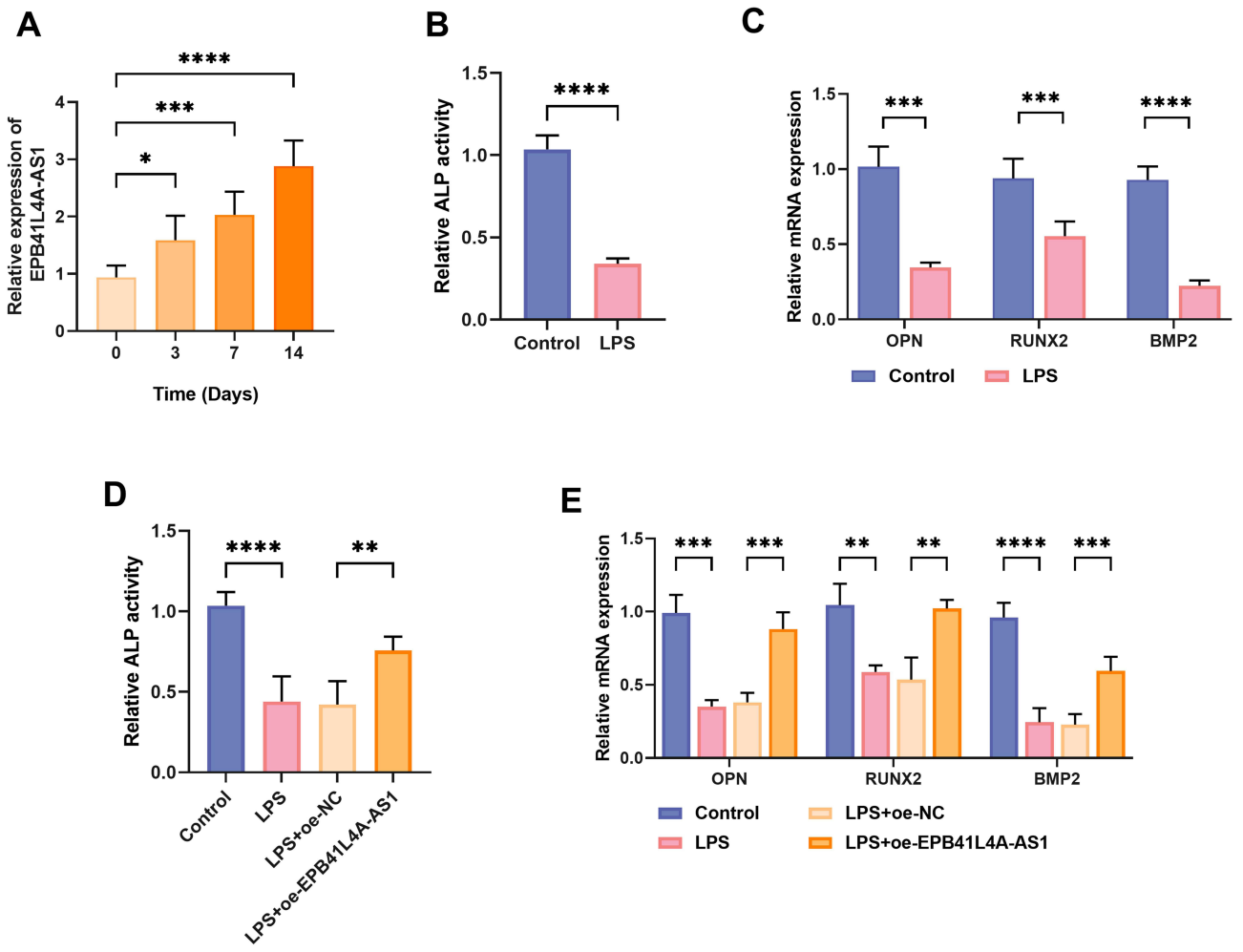

Figure 3 Effects of EPB41L4A-AS1 on osteogenic differentiation. (**A**) Real-time quantitative reverse transcription PCR analysis of the effect of osteogenic differentiation induction on the expression levels of EPB41L4A-AS1. (**B**) Effect of lipopolysaccharide (LPS) induction duration on LPS activity. (**C**) Effects of LPS on the levels of OPN, RUNX2, and BMP2 (osteogenic differentiation markers). Effects of EPB41L4A-AS1 overexpression on alkaline phosphatase (ALP) activity (**D**) and the levels of osteogenic differentiation markers (**E**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

subjects ($P < 0.05$, Figure 6D). In patients with CP patients, YAP1 expression was significantly and negatively correlated with miR-214-3p expression ($P < 0.05$, Figure 6E) and significantly and positively correlated with EPB41L4A-AS1 expression ($P < 0.05$, Figure 6F). Furthermore, transfection with miR-214-3p mimic mitigated the EPB41L4A-AS1 overexpression-induced upregulation of YAP1 in LPS-induced hPDLCs ($P < 0.05$, Figure 6G).

Discussion

This study aimed to elucidate the regulatory mechanism of lncRNA EPB41L4A-AS1 in CP. Previous studies have reported that lncRNAs are potential diagnostic and therapeutic targets for various diseases. However, limited studies have examined the potential lncRNAs as the therapeutic targets for CP. This study, for the first time, demonstrated that EPB41L4A-AS1 is downregulated in the GCF of patients with CP, as well as in LPS-induced hPDLCs. Furthermore, EPB41L4A-AS1 overexpression significantly alleviated LPS-induced hPDLC injury and promoted osteogenic differentiation. Additionally, EPB41L4A-AS1 expression was negatively correlated with miR-214-3p expression. The overexpression of miR-214-3p mitigated the effect of EPB41L4A-AS1 overexpression and suppressed osteogenic differentiation by targeting *YAP1*. EPB41L4A-AS1 could distinguish patients with CP from subjects with healthy periodontal tissues with high sensitivity and specificity, indicating its potential application as a novel biomarker for

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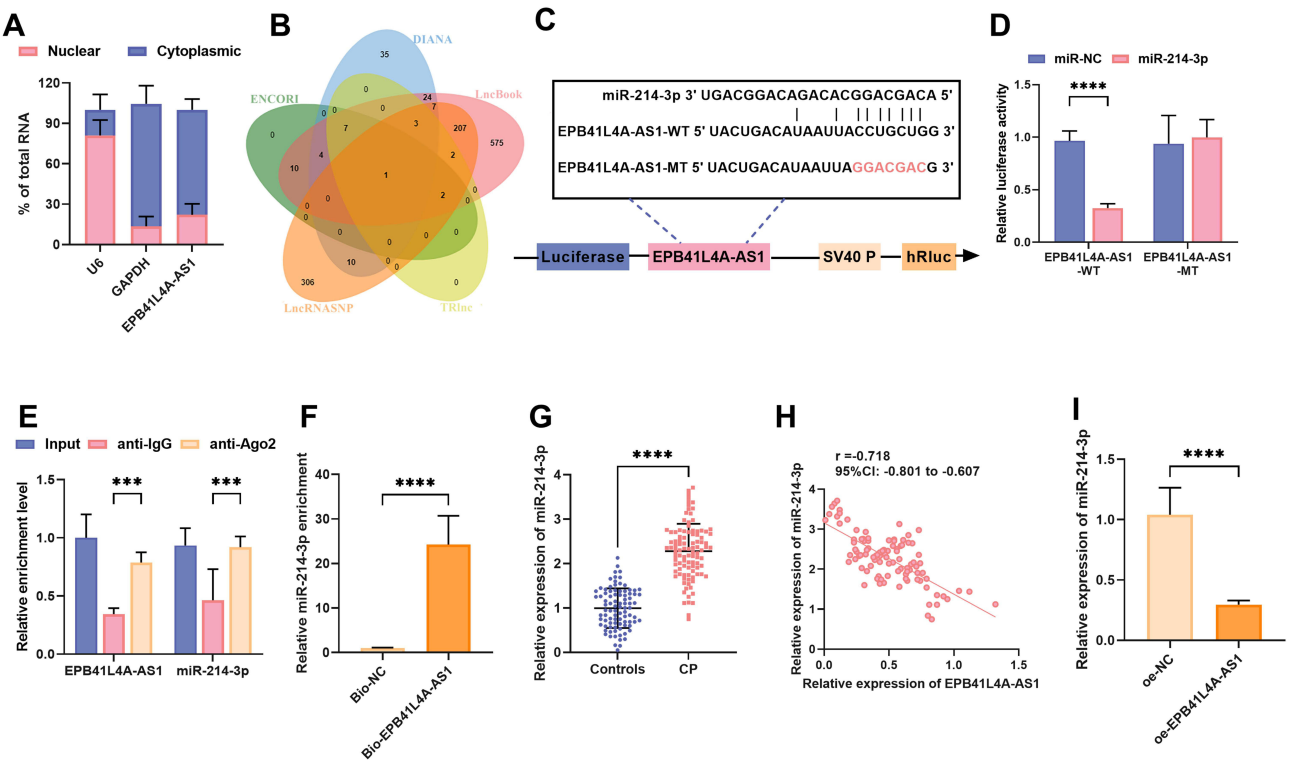

Figure 4 EPB41L4A-AS1 directly targets miR-214-3p. (**A**) The subcellular localization of EPB41L4A-AS1 was examined by using a commercial kit. (**B**) Venn diagram of overlapping target miRNA of EPB41L4A-AS1 predicted using five databases. (**C**) The predicted binding of miR-214-3p with EPB41L4A-AS1 3' untranslated region (UTR). The interaction of EPB41L4A-AS1 and miR-214-3p was analyzed using the dual luciferase reporter (**D**), radioimmunoprecipitation (RIP) (**E**), and RNA pull-down assays (**F**). (**G**) The miR-214-3p levels in the gingival crevicular fluid (GCF) of control subjects and patients with CP. (**H**) Pearson correlation coefficient was calculated for the linear correlation between miR-214-3p and EPB41L4A-AS1 in patients with CP. (**I**) Real-time quantitative reverse transcription PCR analysis of the levels of miR-214-3p in cells transfected with EPB41L4A-AS1 overexpression plasmid. ***$P < 0.001$, ****$P < 0.0001$.

CP. Thus, this study demonstrated that EPB41L4A-AS1 downregulation is a potential diagnostic biomarker for CP and that EPB41L4A-AS1 upregulation is a potential therapeutic strategy to delay CP progression.

Current diagnostic approaches are dependent on clinical assessments and imaging techniques. However, the high costs of these approaches and the severity of CP diagnosis impose significant burdens on patients. CP is often not diagnosed at early stages due to the absence of distinct early symptoms. Patients already present serious complications at the time of manifestation of clinical signs or tooth loss. Thus, there is an urgent need to identify effective biomarkers for CP diagnosis. Recently, EPB41L4A-AS1 was reported to be a potential biomarker for osteosarcoma,[28] gastric cancer,[29] and psoriasis.[30] The EPB41L4A-AS1, levels were reported to be downregulated in myelodysplastic syndromes.[13] Furthermore, He et al identified various differentially expressed lncRNAs, including EPB41L4A-AS1 in patients with periodontitis.[16] A research identified various differentially expressed lncRNAs, including EPB41L4A-AS1, in differentiated and undifferentiated periodontal ligament stem cells using microarray technology and ranked them.[17] Consistent with these findings, the EPB41L4A-AS1 levels in patients with CP were lower than those in healthy subjects in this study. The study, for the first time, demonstrated that EPB41L4A-AS1 levels were correlated with the severity of CP. Further analyses revealed that EPB41L4A-AS1 levels can identify patients with CP with high specificity and sensitivity. Previous studies have reported that CP is associated with an increased risk of coronary heart disease, stroke, and peripheral vascular disease.[4] Furthermore, periodontal therapy mitigates cardiovascular disease and endothelial dysfunction. Thus, the findings of this study provide a potential strategy to enhance the quality of life of patients. The outcomes of patients with CP can be improved by identifying patients with EPB41L4A-AS1 downregulation and implementing early management and prophylactic treatment in clinical settings.

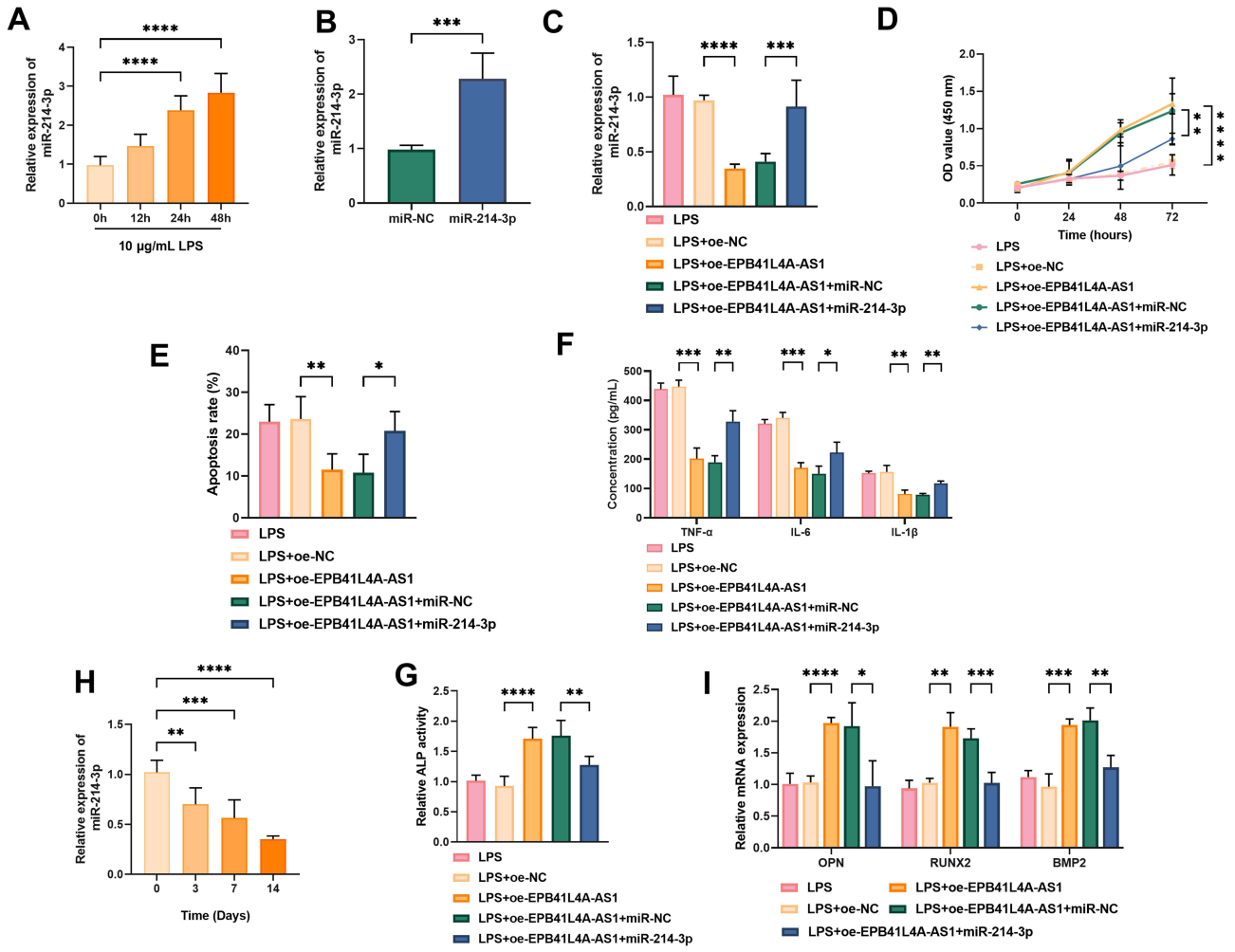

Figure 5 miR-214-3p suppresses the effect of EPB41L4A-AS1 overexpression on periodontal ligament injury and osteogenic differentiation. (**A**) Real-time quantitative reverse transcription PCR analysis of the levels of miR-214-3p in lipopolysaccharide (LPS) induced human periodontal ligament cells (hPDLCs). RT-qPCR analysis of miR-214-3p levels in miR-214-3p mimic-transfected hPDLCs (**B**) and LPS-induced hPDLCs (**C**). (**D**) The cell counting kit-8 assay was performed to examine the proliferation. (**E**) Flow cytometry was performed to evaluate apoptosis. (**F**) Effects of miR-214-3p on the secretion of inflammatory factors in hPDLCs were determined using enzyme-linked immunosorbent assay. (**H**) RT-qPCR analysis of miR-214-3p levels and osteogenic differentiation markers. (**G**) The alkaline phosphatase (ALP) activity was determined using a commercial kit. (**I**) The expression levels of osteogenic markers were examined using RT-qPCR analysis. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

The virulence of *Porphyromonas gingivalis*, which is the etiological factor for CP, can be attributed to LPS. The pathological features of CP include increased subgingival pathogenic bacteria and periodontal connective tissue damage.[31] EPB41L4A-AS1 regulates inflammatory responses in diabetes and COPD. This study examined the function of EPB41L4A-AS1 in CP using LPS-induced hPDLCs. EPB41L4A-AS1 overexpression significantly mitigated LPS-induced apoptosis, enhanced cell proliferation, and suppressed excessive inflammatory mediator production, alleviating hPDLC damage. Furthermore, the role of EPB41L4A-AS1 in bone repair was examined. Enhancing the osteogenic ability of hPDLCs represents one of the most promising treatment options for CP. This study, for the first time, demonstrated that EPB41L4A-AS1 overexpression significantly promotes osteogenic differentiation, indicating its role in bone repair.

Cytoplasmic lncRNAs can function as competing endogenous RNA by sponging miRNAs, resulting in the down-regulation of miRNA levels and the upregulation of mRNA expression.[32,33] EPB41L4A-AS1 was mainly localized to the cytoplasm. Analysis of multiple databases revealed that miR-214-3p is the target of EPB41L4A-AS1. miR-214-3p, which promotes inflammation, exacerbates the progression of hyperlipidemic pancreatitis[34] and is involved in the inflammatory response and degenerative processes in osteoarthritis.[35] Additionally, miR-214-3p is associated with isoflurane-induced

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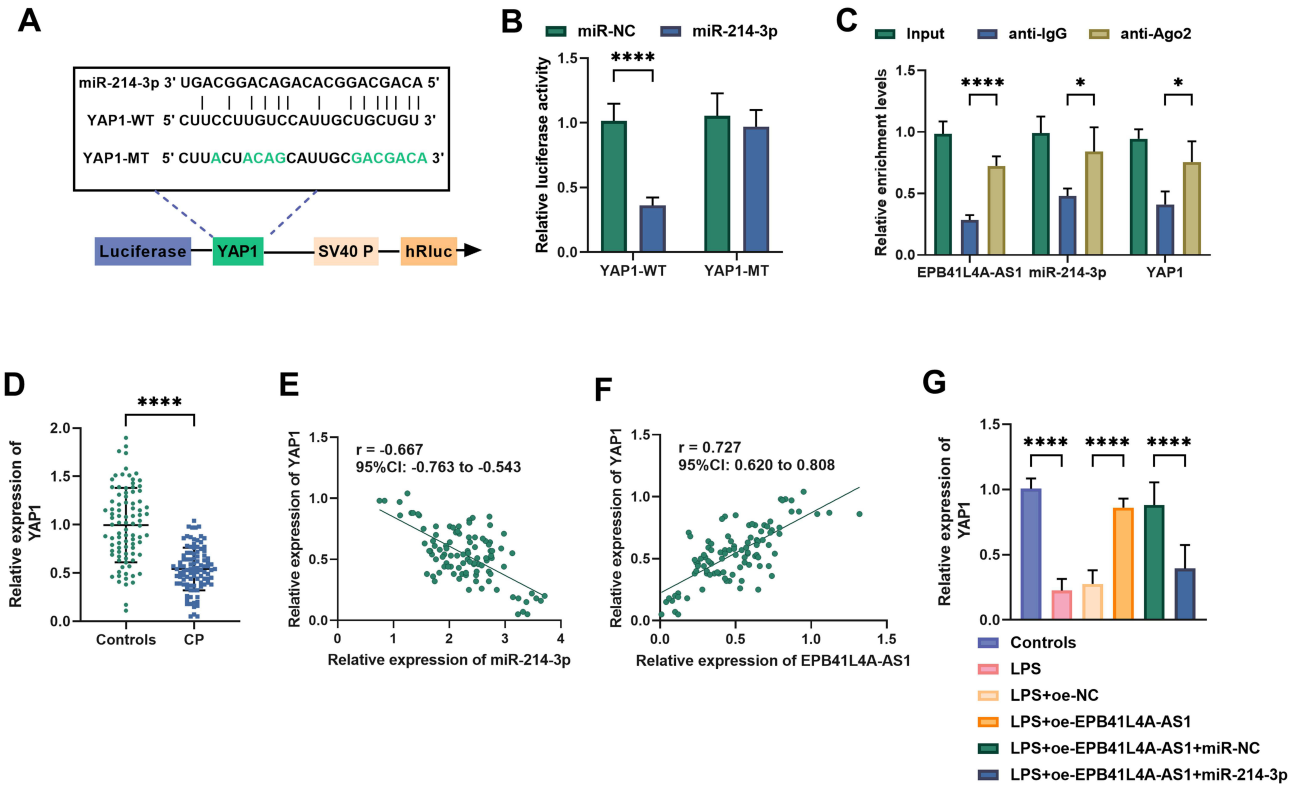

Figure 6 *YAP1* is a target of miR-214-3p. (**A**) The predicted binding of miR-214-3p with YAP1 3' untranslated region (UTR). The interaction of *YAP1* with miR-214-3p was analyzed using the dual luciferase reporter (**B**) and radioimmunoprecipitation (RIP) (**C**) assays. (**D**) The YAP1 levels in the gingival crevicular fluid (GCF) of control subjects and patients with chronic periodontitis (CP). Pearson correlation coefficient was calculated for linear correlation between YAP1 (**E**) or EPB41L4A-AS1 (**F**) and miR-214-3p in patients with CP. (**G**) Real-time quantitative reverse transcription PCR analysis of the levels of *YAP1* in EPB41L4A-AS1 overexpression plasmid-transfected and miR-214-3p mimic-transfected cells. *$P < 0.05$, ****$P < 0.0001$.

neuroinflammation and cognitive dysfunction.[36] High glucose levels stimulate miR-214-3p, which plays a role in the osteogenic differentiation of BMSCs in patients with diabetes.[37] In a three-dimensional mechanical microenvironment, miR-214-3p overexpression mitigated the suppressed osteogenic differentiation in jawbone BMSCs derived from elderly patients.[38] The expression of miR-214-3p was significantly downregulated during the osteogenic induction of periodontal ligament stem cells, leading to suppressed ALP activity and downregulated expression of osteogenic differentiation markers.[39] In this study, miR-214-3p expression was downregulated during osteogenic differentiation. Meanwhile, the expression of miR-214-3p was upregulated in patients with CP. Additionally, miR-214-3p suppressed the beneficial effects of EPB41L4A-AS1 overexpression on periodontal ligament injury and suppressed osteogenic differentiation. These findings indicate that the EPB41L4A-AS1/miR-214-3p axis mediates the progression of CP.

YAP1, which is associated with osteogenesis, was upregulated in hPDLCs.[40] Furthermore, YAP1 levels were reported to be significantly downregulated in a TNF-α-induced periodontitis cell model. Recent studies have suggested that periodic lamellae poly (ε-caprolactone) electrospun nanofibers can mitigate inflammatory bone loss and enhance bone regeneration in periodontitis by activating YAP1.[41] Aging increases susceptibility to periodontitis. YAP1 expression is downregulated in gingival tissues during human aging. The overexpression of YAP1 promoted the regeneration of gingival tissues.[42] YAP1 regulates the proliferation, osteogenic differentiation, and apoptosis of periodontal ligament stem cells.[43] miR-214-3p is reported to target *YAP1* in pancreatic cancer and lung squamous cell cancer.[44,45] This is consistent with the findings of this study, which demonstrated that miR-214-3p targets *YAP1* in CP. The YAP1 levels were significantly downregulated in patients with CP. Additionally, the GCF YAP1 levels were negatively correlated with miR-214-3p levels and positively correlated with EPB41L4A-AS1 levels. In LPS-induced hPDLCs, miR-214-3p mimic

transfection mitigated the EPB41L4A-AS1 overexpression-induced upregulation of YAP1. Therefore, EPB41L4A-AS1 and miR-214-3p mediate the progression of CP by regulating YAP1.

In summary, this study revealed that EPB41L4A-AS1 downregulation is a potential diagnostic biomarker for CP. The upregulation of EPB41L4A-AS1 expression can exert productive effects on periodontal tissue and promote bone regeneration by inhibiting the miR-214-3p/YAP1 axis, potentially improving patient outcomes.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Affiliated Hospital of Nantong University before the study began. The participants' right to be informed about the study was ensured and agreed to participate in the study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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