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ORIGINAL ARTICLE

CKIP-1 mediates *P. gingivalis*-suppressed osteogenic/cementogenic differentiation of periodontal ligament cells partially via p38 signaling pathway

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ABSTRACT

Objectives: Casein kinase 2 interacting protein-1 (CKIP-1) is a versatile player involved in various biological processes. However, whether CKIP-1 mediates the osteogenic/cementogenic differentiation of periodontal ligament cells (PDLCs) under *Porphyromonas gingivalis* (Pg) stimulation remains unknown.

Material and Methods: The effect of Pg on PDLC differentiation was first verified. CKIP-1 expression in Pg-infected PDLCs or in PDL of apical periodontitis (AP) mice was detected. The changes of CKIP-1 during PDLC differentiation was also determined. PDLC differentiation capacity in *CKIP-1* knockout (KO) mice and CKIP-1-silenced PDLCs with or without Pg stimulation were further studied. Inhibitor was finally applied to verify the involvement of p38 signaling pathway in PDLC differentiation.

Results: The suppression effect of Pg on PDLC differentiation was demonstrated. CKIP-1 increased in the PDL of AP mice and Pg-induced PDLCs, and decreased gradually during PDLC differentiation. Increased OSX and RUNX2 expression in PDL were observed in *CKIP-1* KO mice. Also, CKIP-1 silencing facilitated and rescued Pg-inhibited PDLC differentiation. Inhibitor for p38 signaling pathway blocked CKIP-1 silencing-facilitated PDLC differentiation.

Conclusions: CKIP-1 mediated the osteogenic/cementogenic differentiation of PDLCs partially through p38 signaling pathway, which may provide evidence for the regeneration of periodontal hard tissues damaged by Pg.

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Introduction

The initiation and development of periodontitis are linked to periodontal pathogens, among which, *Porphyromonas gingivalis* (*P. gingivalis*, Pg) was demonstrated to be the keystone factor responsible for the destruction of periodontal hard tissues, such as cementum and bone [1–3]. Different from the hard tissues, the periodontal ligament (PDL) is a soft tissue between the cementum and bone, connecting the teeth to the alveolar bone by the fibers anchoring into the two mineralized tissues [4]. Within PDL, periodontal ligament cells (PDLCs) are the multipotent and heterogeneous cell populations that own the potential differentiating into osteoblasts, cementoblasts, adipocytes, chondroblasts and neural cells [5,6]. Based on the stem cell-like characteristics and anatomical position of PDLCs, they are regarded as a promising cell source for periodontal repair [6], including the bone regeneration and cementum regeneration [7]. On the other hand, it was reported that PDLCs infected with Pg can produce a large number of inflammatory cytokines like matrix metalloproteinase (MMP), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α), which also contribute to the collapse of periodontal hard tissues [8–10]. Taking the above points into consideration, how to facilitate and utilize the osteogenic/cementogenic differentiation of PDLCs are essential for mitigating the periodontal destruction caused by Pg.

Casein kinase 2 interacting protein-1 (CKIP-1), also known as pleckstrin homology domain containing protein O1 (PLEKHO1), is a scaffold protein that possesses a pleckstrin homology domain (N-terminus), a putative leucine zipper motif (C-terminus), and five proline-rich motifs [11,12]. Based on these structures, CKIP-1 can interact with various proteins and transduce signals (CK2α, Smurf1, CPα, PI3K/AKT, AP1/c-Jun, ATM/p53, IFP35/Nmi, TRAF6, PAK1, Arp2/3, HDAC1, Rpt6,

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REGγ, CARMA1) between the cytoplasm and nucleus [11–15]. Studies have verified that CKIP-1 is involved in numerous cell functions such as cell morphology, migration, proliferation, apoptosis, autophagy, polarization, differentiation [11,14,16–19], and different biological processes like bone formation, tumorigenesis, myoblast fusion, cardiac remodeling and immune regulation [11,20,21].

Further, CKIP-1 can negatively regulate bone formation by enhancing the affinity between Smad ubiquitination regulatory factor 1 (Smurf1) and its substrates, and degrading the substrates of BMP signaling pathway [22]. Also, *CKIP-1* knockout (KO) mice possessed higher bone mass due to the increased osteogenesis in an age-dependent manner [22]. Taking the similarities between bone and cementum into consideration, our previous work uncovered that CKIP-1 is also a negative regulator for cementoblast mineralization and cementum formation, by means of OCCM-30 cell line and *CKIP-1* KO mice [3]. Given that bone and cementum are the two similar hard tissues of periodontium, and the fact that PDLCs own differentiation capacities, we wonder whether CKIP-1 also regulates the osteogenic/cementogenic differentiation of PDLCs negatively.

In the present study, we aimed to study the regulating effect of CKIP-1 on the osteogenic/cementogenic differentiation of Pg-infected PDLCs, and also the underlying mechanism involved in it. First, the suppression of Pg on PDLC differentiation was verified. The role of CKIP-1 in inflammation and differentiation was also uncovered. Moreover, the rescue effect of CKIP-1 silencing on Pg-suppressed PDLC differentiation via p38 signaling pathway was also demonstrated, which may contribute to the repair and regeneration of the damaged periodontal hard tissues caused by Pg.

Materials and methods

PDLC isolation and culture

Healthy PDL tissues were collected from the permanent teeth of clinical donors (16 ~ 26 years old) with treatment reasons, and PDLCs were isolated as previously reported [23]. Written informed consent was provided by all donors who agreed to provide their teeth for research purposes. PDLCs were cultured in α-minimum essential medium (α-MEM; Hyclone) containing 10% fetal bovine serum (FBS; Gibco), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cells from passages 3 ~ 6 were used. Osteogenic/cementogenic medium prepared with α-MEM, 10% FBS, 50 μg/mL ascorbic acid, 10 mM sodium β-glycerophosphate, and 10 nM dexamethasone (Sigma) was applied to induce PDLC differentiation. Medium was changed every 2 days. All experiments in this study were approved by the Ethics Committee of School and Hospital of Stomatology, Wuhan University.

Bacteria culture

P. gingivalis (Pg; ATCC 33,277) was cultured in trypticase soy broth containing 5 μg/mL hemin, 1 μg/mL vitamin K1, 0.1% yeast extract (Sigma) in an anaerobic atmosphere (10% H_2, 10% CO_2, 80% N_2) at 37°C. Concentration for Pg was measured by the optical density (OD) value at 600 nm (1 OD = 10^9 Pg/mL) [3]. Pg suspension (10^9) was centrifuged and collected at a speed of 10,000 g for 10 min. The precipitate was resuspended with 1 mL 10% α-MEM or induction medium. Resuspended Pg was added to stimulate PDLCs when necessary. PDLCs were seeded in 6-well plates at a density of 2.7×10^5/well, and cultured or induced with or without Pg/inhibitor stimulation for different preset days. Then, the samples were collected for reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blotting.

Chemical inhibitor and antibodies

SB203580 (SB; Selleck; 5 μM) was used for p38 pathway inhibition. Antibodies for CKIP-1 (24883–1-AP), β-actin (66009–1-AP) and horseradish peroxidase (HRP)-conjugated goat *anti*-rabbit (SA00001–2) or *anti*-mouse (SA00001–1) secondary antibodies was purchased from Proteintech. Antibodies for Osterix (OSX, ab22552), Runt-related transcription factor 2 (RUNX2, ab23981) were purchased from Abcam. Antibody for osteocalcin (OCN, sc390877) was purchased from Santa Cruz. Antibody for bone sialoprotein (BSP, DF7738) was purchased from Affinity. Antibodies for p-p38 (4511S), total-p38 (9212S) were purchased from CST.

Lentiviral transfection

Lentiviruses containing a CKIP-1-specific short hairpin RNA (sh-CKIP-1; sense, 5'-GAGAAGGAATCGTGGATCAAT-3') as well as the control (sh-NC; sense, 5'-TTCTCCGAACGTGTCACGT-3') were constructed by GenePharma. PDLCs at passage 3 was infected with the lentiviruses at a MOI of 40 together with 5 ng/mL polybrene. The transduction medium was changed with fresh medium 24 hours after the infection. Puromycin (2 μg/mL) was used for screening when necessary.

RT-qPCR

500 ng RNA extracted with TRIzol (TaKaRa) was reverse transcribed to cDNA by PrimeScript RT Reagent (TaKaRa) following the manufacturer's protocol. qPCR was conducted with SYBR qPCR Master Mix (Vazyme) and specific primers (Sangon) by Applied Biosystems 7500. Thermocycling conditions are shown below: 95°C for 30 s; 40 cycles at 95°C for 10 s, 62°C for 34 s; 72°C for 30 s. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and quantified by $2^{\Delta\Delta Ct}$ method. Primers for *OCN*, *BSP*, cementum attachment protein (*CAP*), cementum protein-1 (*CEMP-1*), *IL-6*, *IL-8*, *IL-1β*, *MMP-1*, *MMP-3*, *CKIP-1* and *GADPH* are shown in Table S1. *OCN* and *BSP* were used to indicate both osteogenesis and cementogenesis, while *CAP* and *CEMP-1* were used to indicate cementogenesis only.

Western blotting

Protein was extracted by M-PER reagent (Thermo Scientific), and quantified by BCA protein assay (Beyotime). 30 μg protein of each sample were electrophoresed by 10% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat milk at room temperature (RT) for 1 h, and incubated at 4°C overnight with *anti*-OCN (1:500, Santa Cruz), *anti*-OSX (1:1000, Abcam), *anti*-RUNX2 (1:1000, Abcam), *anti*-BSP (1:1000, Affinity), *anti*-p-p38 (1:1000, CST), *anti*-total-p38 (1:1000, CST), *anti*-β-actin (1:15000, Proteintech). After incubating with HRP-conjugated goat *anti*-rabbit (1:8000, Proteintech) or goat *anti*-mouse (1:10000, Proteintech) secondary antibodies at RT for 1 h, bands were visualized with the SuperSignal™ West Femto Reagent (Thermo Scientific) by the Odyssey LI-COR scanner.

Alkaline phosphatase (ALP) staining

Cells were seeded in 6-well plates at a density of 2.5×10^5/well. After 7 days of induction, cells were fixed by 4% paraformaldehyde for 15 min. ALP staining was performed by the BCIP/NBT ALP Color Development Kit (Beyotime) according to the manufacturer's instructions. Images were taken and ALP activity was quantified by Image J.

Sirius red staining (SRS)

Cells were seeded in 6-well plates at a density of 2.5×10^5/well. Collagen secretion was evaluated 7 or 14 days after the induction. After fixation, cells were stained with 0.1 wt% Sirius Red in saturated picric acid (Boerfu biotechnology) at RT overnight. 0.5% acetic acid was used to wash off the unbound stain, and images were taken. The absorbance of the stain dissolved in 0.2 M NaOH/methanol (1:1) at 540 nm was detected.

Alizarin red staining (ARS)

Cells were seeded in 6-well plates at a density of 2.5×10^5/well. After inducing for 14 days, cells were fixed and stained with 1% Alizarin Red (OriCell) at 37°C for 15 min. Excess dye was removed by double distilled H_2O. Mineral nodules were imaged, and then dissolved in 10% cetylpyridinium chloride (Yuanye biotechnology). The absorbance at 562 nm was measured.

Animals, micro-CT, hematoxylin and eosin (HE) staining and immunohistochemistry (IHC)

This study conformed to the updated ARRIVE 2.0 guidelines for animal studies. Mice were housed under light-, temperature-, and humidity-controlled conditions (12-h light/dark cycle; 22 ± 2°C; 55 ± 5% humidity) with free access to food and water. Apical periodontitis (AP) model was constructed by a pulp chamber unfolding and infection method as previously described [24]. Briefly, the pulp chambers of the left first molars of 8-week-old male mice were unfolded by a fissure bur under a stereomicroscope. The unfolded molars were then injected with *P. gingivalis* (in 2% carboxymethylcellulose) and left open for 2 weeks. The right first molars were set as the healthy controls. Micro-CT (Quantum GX2, PerkinElmer) was used to verify the successful construction of the AP model with the following parameters: 70 kV, 100 μA, 0.5 Al filter, 5 mm field of view, 20 μm pixel size. The sagittal sections of the first molars were viewed and collected by the SimpleViewer. After excessive anesthesia, mandibles from the whole-body *CKIP-1* KO mice and WT controls (6/group, 7 ~ 8 weeks old, 22 ± 1.5 g, male), and mandibles from healthy controls and apical periodontitis mice (6/group, 8 weeks old, 22 ± 1 g, male) were fixed, decalcified, and paraffin embedded. After cutting, the sections were used for HE staining and IHC. IHC was performed by using the UltraSensitive SP IHC Kit (MAIXIN Biotech) following the manufacturer's instructions and incubating the primary antibodies (*anti*-CKIP-1, 1:200, Proteintech; *anti*-Osterix, 1:200, Abcam; *anti*-RUNX2, 1:400, Abcam) overnight at 4°C. Images were taken, and quantified by Image J.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 8, and presented as the mean ± SD of at least three independent experiments. Shapiro-Wilk was used to determine the normal distribution. The Student's t-test was used to compare the differences between two groups. One-way ANOVA was used for

comparisons of multiple groups, followed by Bonferroni correction. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. $P > 0.05$ was considered not significant (ns).

Results

P. gingivalis *obstructs the osteogenic/cementogenic differentiation of PDLCs*

To investigate the effect of *P. gingivalis* (Pg) on the osteogenic/cementogenic differentiation of PDLCs, we incubated PDLCs in the induction medium with Pg for 7 days. RT-qPCR results showed decreased mRNA expression of *OCN*, *BSP*, *CAP*, *CEMP-1* in PDLCs after Pg infection (Figure 1a). Besides, protein expression of mineralization-related markers in PDLCs like OCN, BSP, OSX, RUNX2 all decreased in the Pg-stimulated group (Figure 1b). Similarly, after culturing with Pg, ALP staining on day 7 (Figure 1c,d), SRS on day 7 (Figure 1e,f) and ARS on day 14 (Figure 1g,h) all revealed a suppressive effect of Pg on the osteogenic/cementogenic differentiation of PDLCs.

CKIP-1 increases in inflammation and decreases during the osteogenic/cementogenic differentiation of PDLCs

Then, we uncovered the role of CKIP-1 in PDLC inflammation and differentiation, respectively. We cultured PDLCs with Pg for different time points. The results showed increased *IL-6*, *IL-8*, *IL-1β*, *MMP-1*, *MMP-3* in all Pg-stimulated groups when

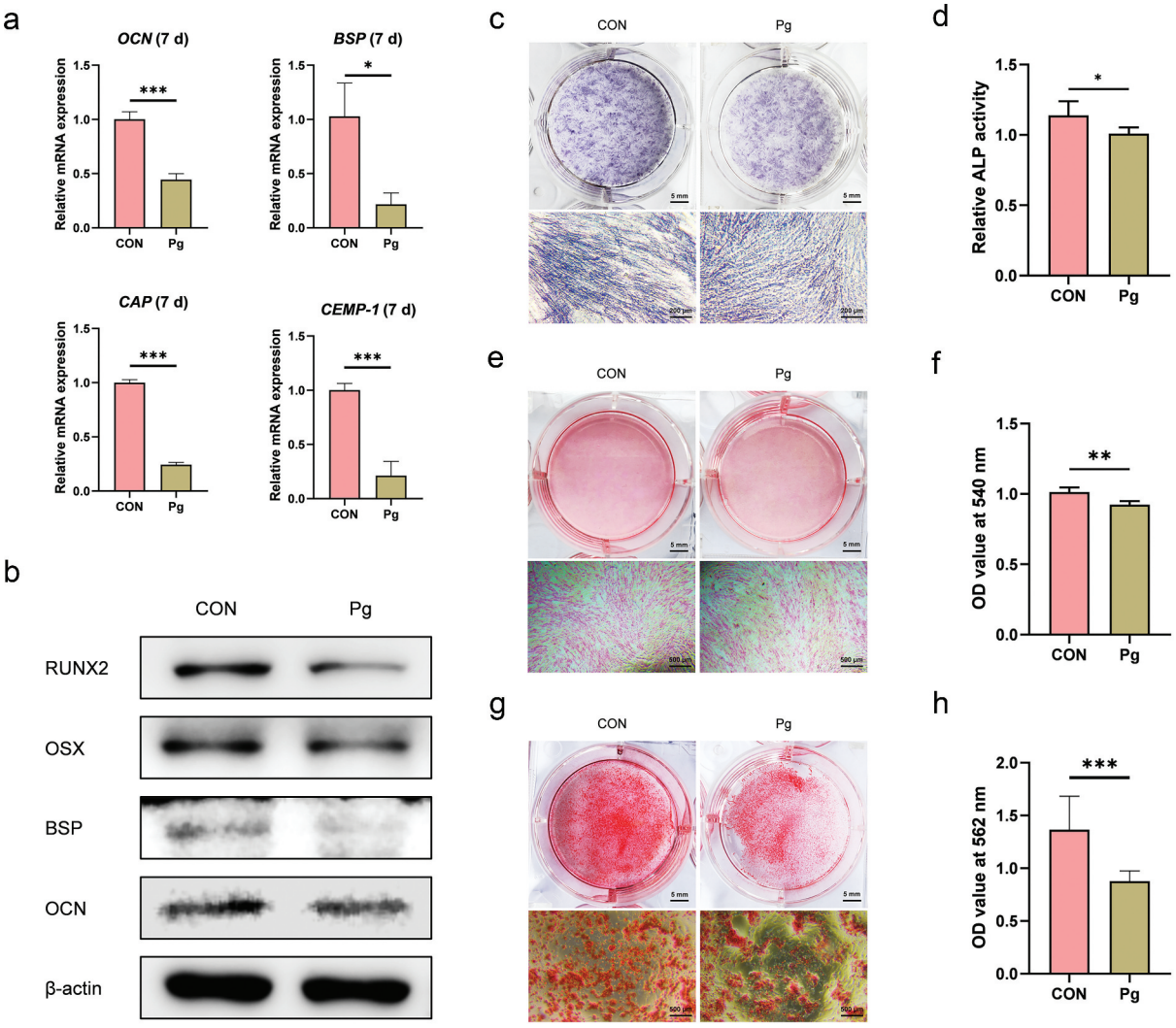

Figure 1.*P. gingivalis* obstructs the osteogenic/cementogenic differentiation of PDLCs. PDLCs were cultured with Pg at a MOI of 100 for 7 days, mRNA expressions of *OCN*, *OSX*, *CAP*, *CEMP-1* were measured by RT-qPCR (a), and protein expressions of OCN, BSP, OSX, RUNX2 in PDLCs were detected by western blotting (b). PDLCs were incubated with Pg at a MOI of 200 for 7 or 14 days, and ALP staining on day 7 (c), SRS on day 7 (e) and ARS (g) on day 14 were conducted and quantified (d, f, h). Pg: *P. gingivalis*; PDLCs: periodontal ligament cells; MOI: multiplicity of infection; OCN, osteocalcin; BSP, sialoprotein; CAP, cementum attachment protein; CEMP-1, cementum protein-1; OSX: osterix; RUNX2: runt-related transcription factor 2; RT-qPCR:: reverse transcription quantitative polymerase chain reaction; ALP: alkaline phosphatase; SRS: sirius red staining; ARS: alizarin red staining. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

compared to the 0 h group, almost with a peak value at 6 h. Interestingly, *CKIP-1* also increased in all Pg-stimulated groups and showed a similar expression pattern to the inflammation factors (Figure 2a). Then, we also incubated PDLCs with different MOIs of Pg for 6 hours. The results revealed an upregulated expression of *CKIP-1* together with *IL-8*, *IL-1β* in PDLCs as the MOI value increased (Figure 2b). To further confirm the relationship between PDLC inflammation and CKIP-1, we constructed an AP model (Figure 2c), and verified it successfully by micro-CT (Figure 2d). As shown in the figure (Figure 2e), a large amount of inflammatory cells were observed within the PDL adjacent to the apical area, also suggesting the successful establishment of AP model. IHC results also showed higher expression of CKIP-1 in the inflammatory PDL when compared to the healthy control (Figure 2e,f). On the contrary, after inducing PDLCs for 0, 4, 7 days, increased expression of mineralization-related markers and decreased expression of CKIP-1 as the induction time goes was found (Figure 2g,h).

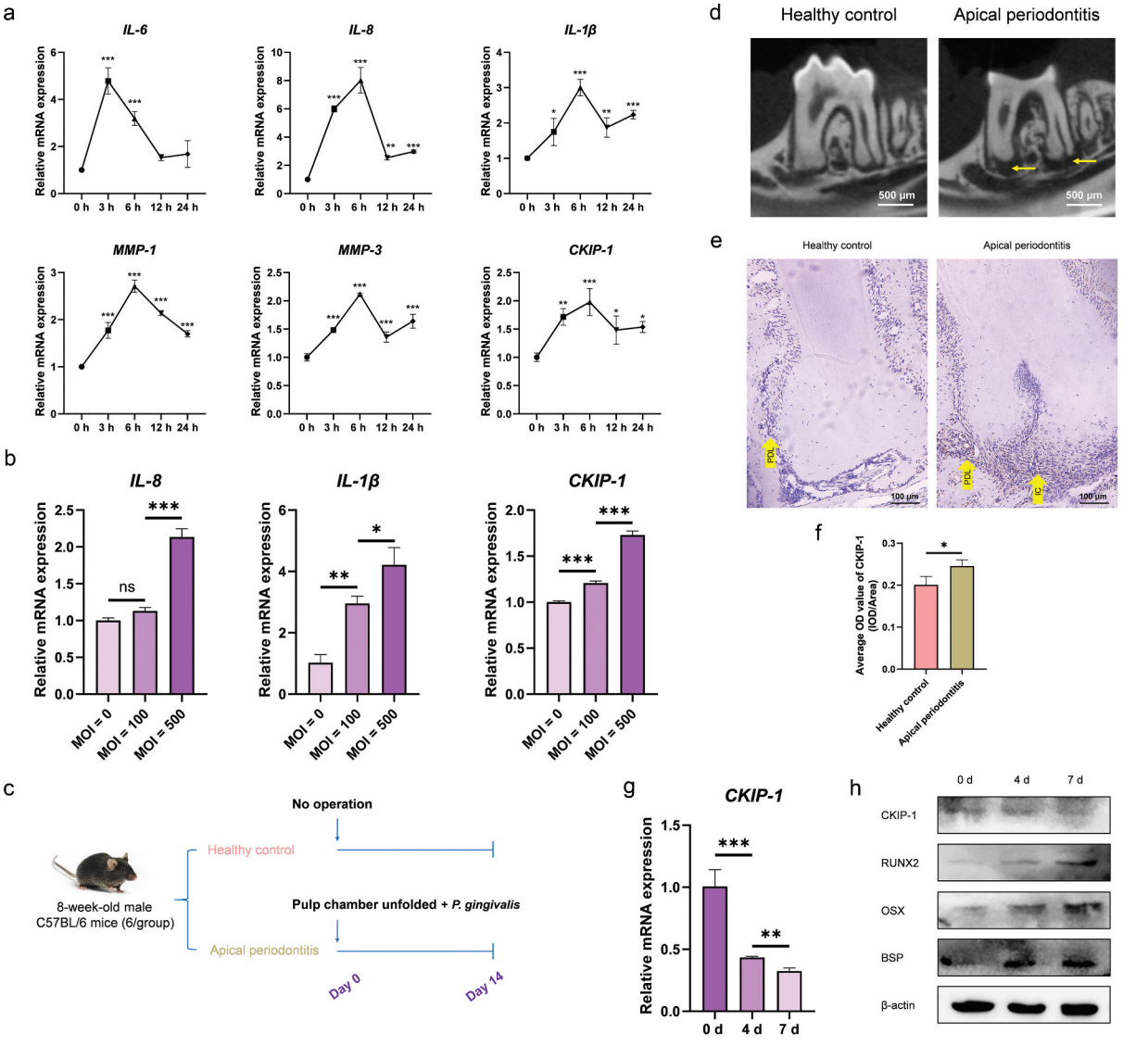

Figure 2. CKIP-1 increases in inflammation, and decreases during the osteogenic/cementogenic differentiation of PDLCs. The change role of CKIP-1 in inflammatory PDLCs stimulated with Pg was detected *in vitro*. (a) PDLCs were incubated with Pg at a MOI of 500 for 0, 3, 6, 12, 24 hours, and mRNA expressions of *IL-6*, *IL-8*, *IL-1β*, *MMP-1*, *MMP-3*, *CKIP-1* were measured by RT-qPCR. (b) PDLCs were cultured with Pg at MOIs of 0, 100, 500 for 6 hours, and mRNA expressions of *IL-8*, *IL-1β*, *CKIP-1* were detected by RT-qPCR. The change role of CKIP-1 in inflammatory PDL of AP model was also evaluated *in vivo*. (c) Schematic diagram of the mouse AP model. (d) Validation of the AP model by micro-CT. (e, f) the CKIP-1 expression and inflammation in the PDL area of AP model were confirmed by IHC and H&E staining. PDLCs were induced for 0, 4, 7 days, the expression of CKIP-1 during the osteogenic/cementogenic differentiation of PDLCs was detected by RT-qPCR (g) and western blotting (h). IL-(6, 8, 1β), interleukin-(6, 8, 1β); MMP-(1, 3), matrix metalloproteinase-(1, 3); CKIP-1, casein kinase-2 interaction protein-1; AP: apical periodontitis; IHC: immunohistochemistry; H&E: hematoxylin and eosin; PDL: periodontal ligament; IC: inflammatory cell. Yellow arrows indicate areas of bone resorption. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. $P > 0.05$ was considered not significant (ns).

CKIP-1 silencing promotes the osteogenic/cementogenic differentiation of PDLCs in vivo and in vitro

To figure out whether CKIP-1 silencing promoted the osteogenic/cementogenic differentiation of PDLCs, the *CKIP-1* KO mice were applied. Higher expression of OSX (Figure 3a) and RUNX2 (Figure 3b) in PDL, osteoblasts, cementoblasts, and immature cementocytes was found in *CKIP-1* KO mice when compared to the wild type mice. Then, CKIP-1-silenced PDLCs and their controls were constructed (Figure 3c) and identified (Figure 3d,e). After a 7-day induction, higher expression of mineralization-related markers in the sh-CKIP-1 group were verified when compared with the sh-NC group (Figure 3f,g). Meanwhile, ALP staining on day 7 (Figure 3h,i), SRS on day 14 (Figure 3j,k) and ARS (Figure 3i,m) on day 14 all confirmed the above findings.

CKIP-1 silencing rescues the osteogenic/cementogenic differentiation of PDLCs suppressed by P. gingivalis

Then, we asked whether CKIP-1 silencing could rescue Pg-suppressed osteogenic/cementogenic differentiation of PDLCs. We induced CKIP-1-silenced PDLCs and the control cells with or without Pg for 7 days. Higher mRNA and protein expression of mineralization-related markers were found in (sh-CKIP-1 + Pg) group when compared with the (sh-NC + Pg) group (Figure 4a,b). Similarly, ALP staining on day 7 (Figure 4c,d), SRS on day 7 (Figure 4e,f) and ARS (Figure 4g,h) on day 14 all verified the rescue effects of CKIP-1 on the osteogenic/cementogenic differentiation of PDLCs suppressed Pg.

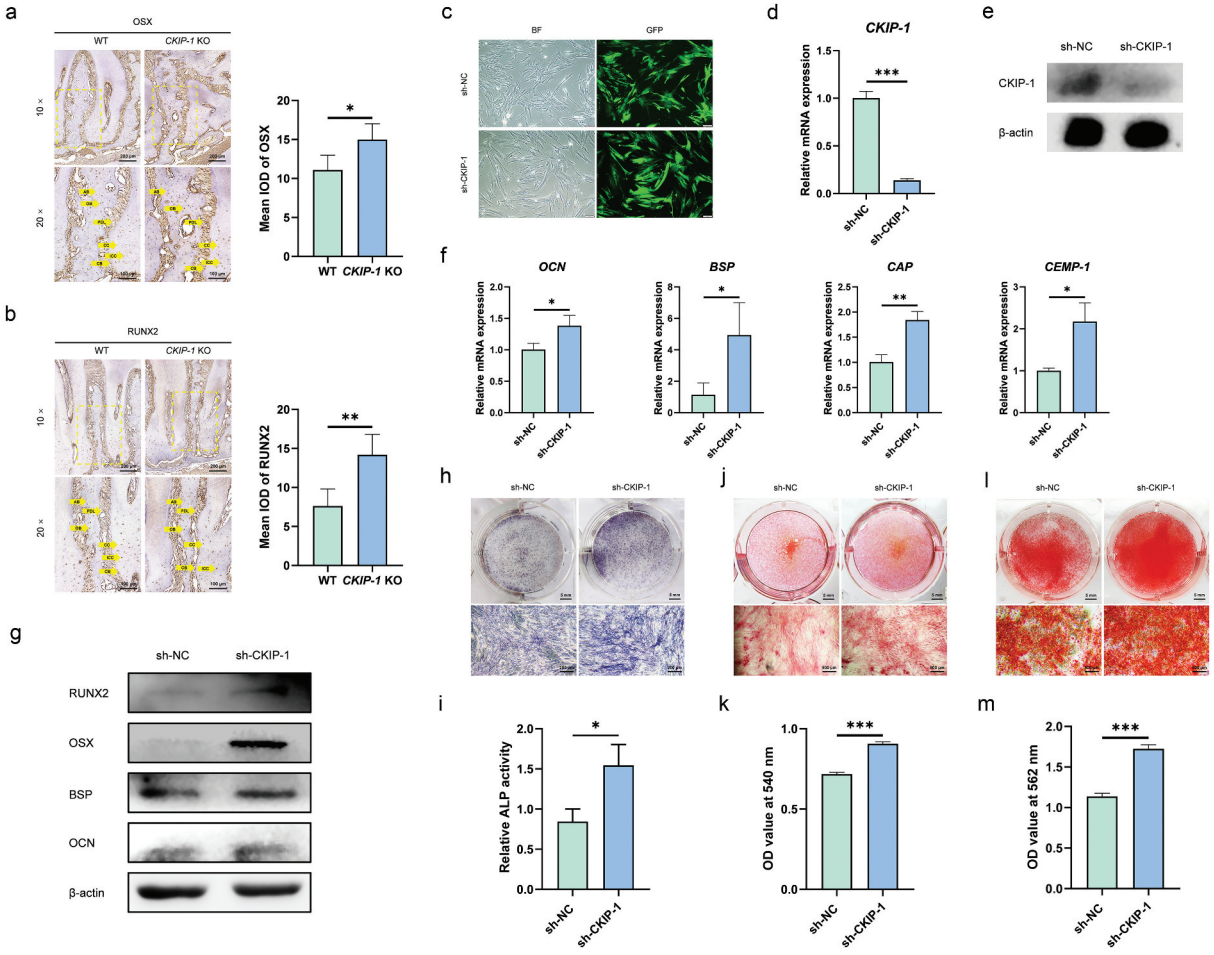

Figure 3.CKIP-1 negatively regulates the osteogenic/cementogenic differentiation of PDLCs in vivo and in vitro. Expression of OSX (a) and RUNX2 (b) in PDL, osteoblasts, cementoblasts, and immature cementocytes in *CKIP-1* KO mice and wild type mice were detected by IHC. CKIP-1 knockdown in PDLCs by lentivirus infection was observed by the expression of green fluorescent protein (c), and identified by RT-qPCR (d) and western blotting (e). CKIP-1-silenced PDLCs and the control cells were induced for 7 days, mRNA expressions of *OCN*, *OSX*, *CAP*, *CEMP-1* were measured by RT-qPCR (f), and protein expressions of OCN, BSP, OSX, RUNX2 were detected by western blotting (g). CKIP-1-silenced PDLCs and the control cells were induced for 7 or 14 days, and ALP staining on day 7 (h, i), SRS on day 14 (j, k) and ARS (i, m) on day 14 were performed. KO: knockout; AB: alveolar bone; CC: cellular cementum; OB: osteoblast, CB: cementoblast; ICC: immature cementocytes. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

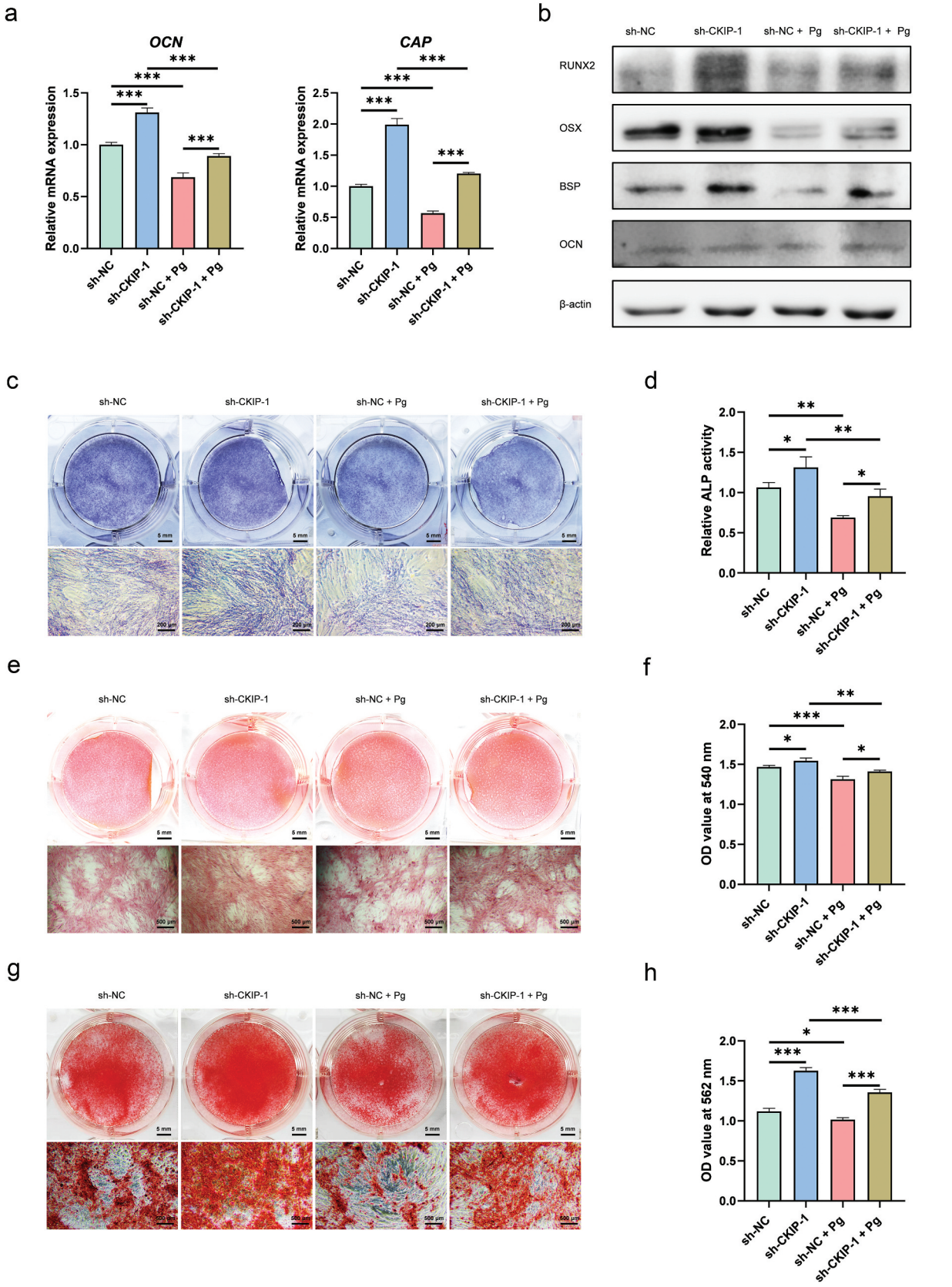

Figure 4. CKIP-1 silencing rescues the osteogenic/cementogenic differentiation of PDLCs suppressed by *P. gingivalis*. (a) CKIP-1-silenced PDLCs and the control cells were induced with or without Pg (MOI = 100) for 7 days, mRNA expressions of *OCN* and *CAP* were detected by RT-qPCR.. (b) CKIP-1-silenced PDLCs and the control cells were induced with or without Pg (MOI = 100) for 4 days, protein expressions of OCN, BSP, OSX, RUNX2 were determined by western blotting. CKIP-1-silenced PDLCs and the control cells were induced with or without Pg (MOI = 200) for 7 or 14 days, and ALP staining on day 7 (c, d), SRS on day 7 (e, f) and ARS (g, h) on day 14 were conducted. Significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

CKIP-1 silencing promotes the osteogenic/cementogenic differentiation of PDLCs partially via p38 signaling pathway

Finally, the underlying mechanism was explored, and activated p38 signaling in sh-CKIP-1 PDLCs was found after inducing for 7 days (Figure 5a). To figure out whether CKIP-1 silencing promoted PDLC differentiation through the p38 signaling pathway, the specific inhibitor SB was applied and induced for 7 days. Western blotting (Figure 5b) and ALP staining (Figure 5c,d) both showed that the CKIP-1 silencing-facilitated differentiation of PDLCs was blocked when SB was added, which implied that CKIP-1 negatively regulated PDLC osteogenic/cementogenic differentiation partially through activating the p38 signaling pathway.

Discussion

As shown in the schematic diagram (Figure 5e), we confirmed the suppressive role of Pg in the osteogenic/cementogenic differentiation of PDLCs. The upregulation of inflammatory cytokines like *IL-6*, *IL-8*, *IL-1β*, *MMP-1*, *MMP-3* in PDLCs was observed. Interestingly, the upregulated expression pattern of CKIP-1 shares a similar trend to these indicators. On the contrary, CKIP-1 decreased gradually during PDLC differentiation. By means of lentivirus transfection and *CKIP-1* KO mice, the negative regulating effect of CKIP-1 on PDLC differentiation was demonstrated, with the increase of *CAP*, *CEMP-1*, OCN, BSP, OSX and RUNX2. Altogether, our study uncovered that CKIP-1 silencing could promote the osteogenic/cementogenic differentiation of PDLCs partially through activating the p38 signaling pathway, and thus rescued the Pg-inhibited PDLC differentiation, which may contribute to the repair of the destroyed bone and cementum caused by Pg.

Studies have confirmed that Pg could induce PDLC inflammation, and produce a number of inflammatory cytokines that result in periodontal resorption [8–10]. So far, many studies have demonstrated that the lipopolysaccharide, protein extract from Pg could impede the osteogenic differentiation of PDLCs [25,26]. In consistent with this, our previous work demonstrated the direct suppression effect of Pg on PDLC differentiation [27]. Different from the above studies, ALP production, extracellular matrix mineralization and also collagen secretion of PDLCs were all measured in this study to evaluate the effects of Pg on both the osteogenic and the cementogenic differentiation of PDLCs, and the final results verified the inhibition role of Pg.

CKIP-1 possesses many functional structures throughout the protein, mediating the interaction between CKIP-1 and many other proteins to be involved in different biological processes such as osteogenesis, cementogenesis, tumorigenesis, and immune regulation [3,11,20,21]. Besides these, it is worth mentioning that CKIP-1 may also positively associated with inflammation. For example, it was reported that the increase of inflammation with aging can aggravated the high expression of CKIP-1 in mesenchymal stem cells (MSCs), both in vivo and in vitro [28]. CKIP-1 was upregulated in C3H/10T1/2 MSC cells stimulated with lipopolysaccharide (LPS) in a concentration-dependent manner, and CKIP-1 knockdown led to enhanced immunosuppressive capacity with higher expression of IL-10 [29]. Similarly, our previous work also showed a MOI-dependent manner of the expression of CKIP-1 and IL-6 in OCCM-30 cells cultured with Pg, which also implied the positive correlation between CKIP-1 and inflammation [3]. Consistent with the above, CKIP-1 was also demonstrated to inhibit the anti-inflammatory gene expression by negatively controlling JAK1/STAT6 activation in macrophages. CKIP-1-overexpressed macrophages were more likely to be the pro-inflammatory M1 subtype after LPS or interferon (IFN)-γ stimulation, and CKIP-1-silenced macrophages showed higher tendency to be the anti-inflammatory M2 subtype after IL-4 or IL-13 stimulation [17]. In this study, we used an AP model to verify the upregulation of CKIP-1 in inflammatory PDL in vivo. The in vitro experiments also showed increased *CKIP-1* expression in Pg-infected PDLCs. More importantly, it shared a similar upregulation pattern to Pg-induced inflammation and changed up and down in accordance with the inflammation cytokines. A MOI-dependent manner of CKIP-1 expression was also detected in PDLCs. In all, CKIP-1 may positively correlated with inflammation, and more cell types and animal models should be used to confirm it.

Considering the role of CKIP-1 in osteogenesis and cementogenesis [3,22,30,31], we wonder whether it also regulates the osteogenic/cementogenic differentiation of PDLCs negatively. Strikingly, higher expression of OSX and RUNX2 in PDL area were found in *CKIP-1* KO mice when compared to the wild type controls, indicating a superior PDLC osteogenic/cementogenic differentiation capacity in *CKIP-1* KO mice. Moreover, osteoblasts, cementoblasts and immature cementocytes *CKIP-1* KO mice all exhibited higher expression of OSX and RUNX2, which in consistent with the previous work [3]. Thus, it is more likely that various kinds of cells with CKIP-1 knockdown altogether contributed to the increased bone and cementum formation. By means of lentivirus transfection method, we found that CKIP-1 regulated the osteogenic/cementogenic differentiation of PDLCs negatively partially via the p38 signaling pathway. By parity of reasoning, the differentiation

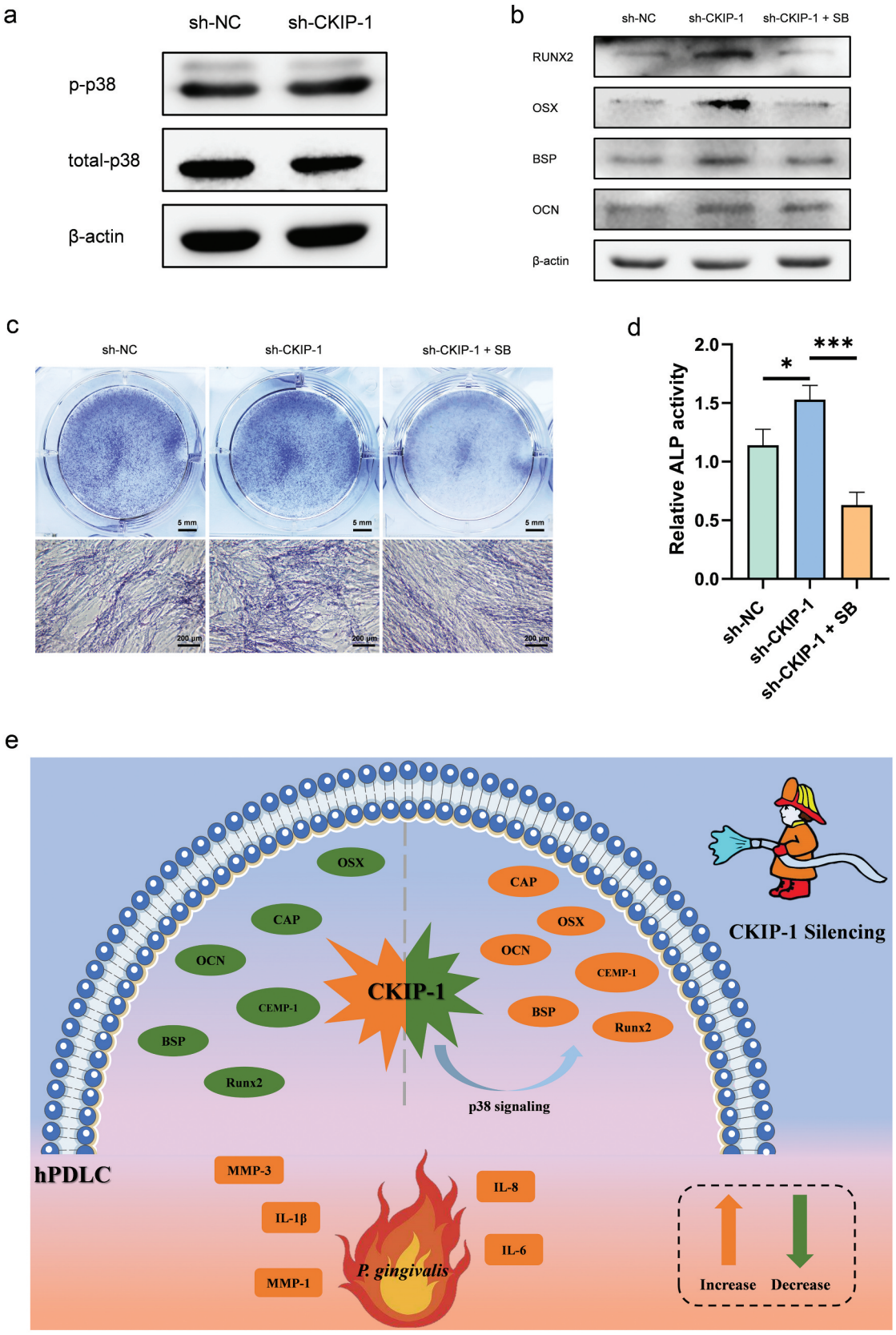

Figure 5.CKIP-1 mediates the osteogenic/cementogenic differentiation of PDLCs partially via p38 signaling pathway. (a) CKIP-1-silenced PDLCs and the control cells were induced for 7 days, and p38 signaling pathway was detected by western blotting. Specific inhibitor SB was applied to induce CKIP-1-silenced PDLCs and the control cells for 7 days, then protein expressions of OCN, BSP, OSX, RUNX2 and ALP activity were determined by western blotting (b) and ALP staining (c, d), respectively. (e) Schematic diagram of the work. SB: SB203580, specific inhibitor for p38 pathway. Significance was defined as $^{*}P < 0.05$ and $^{***}P < 0.001$.

capacities of other dental stem cells like pulp stem cells [32], dental follicle stem cells could also be regulated by CKIP-1.

There also exist some limitations in this study. For example, besides the AP model, the periodontitis model can also be conducted to observe the expression of CKIP-1 in inflammatory PDL. Also, though the negative regulating effect of CKIP-1 on PDLC osteogenic/cementogenic differentiation was demonstrated in vivo and in vitro, it would be better to isolate the PDLCs from the *CKIP-1* KO mice and the wild type mice for further in vitro study. Due to the technological difficulty, we do not perform the relevant experiments. Though p38 MAPK signaling was proved to be involved in CKIP-1 silencing-accelerated osteogenic/cementogenic differentiation, the upstream and downstream factors of CKIP-1 like miRNAs and many other CKIP-1-interacting proteins may also take part in the mechanism. For example, CKIP-1 was reported to be targeted by *Let-7i-5p* and *miR-98-5p*, the promotors for the osteogenic differentiation of bone marrow stromal cells and MC3T3-E1 cells [33,34]. Also, CKIP-1 could negatively regulate BMP pathway and bone formation by binding Smurf1 to enhance its affinity with substrate Smad1/5, and degrade it [22]. Moreover, given that CKIP-1 has a role in immune regulation [17,35,36], we can also take the immune microenvironment into consideration to explain the in-depth mechanism in the future.

In conclusion, our study clarified the role of CKIP-1 in PDLC inflammation and osteogenic/cementogenic differentiation. We demonstrated that CKIP-1 expression in PDLCs was increased responding to the Pg-induce inflammation. CKIP-1 knockdown facilitated PDLC differentiation partially through activating p38 signaling pathway, and further rescue Pg-suppressed PDLC differentiation, which may contribute to the repair and regeneration of the damaged periodontal hard tissues caused by Pg.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

Xin Huang contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Junhong Xiao, Huiyi Wang and Yan Peng contributed to data acquisition and interpretation, critically revised the manuscript; Heyu Liu, Li Ma and Xiaoxuan Wang contributed to analysis and interpretation, critically revised the manuscript; Zhengguo Cao contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors have approved the final manuscript.

Data availability statement

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

Ethics statement

All procedures performed in studies involving human biological materials were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All experiments in this study were approved by the Ethics Committee of School and Hospital of Stomatology, Wuhan University.

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