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ORIGINAL ARTICLE

Effects of IL-1β on MMP-9 Expression in Cementoblast-Derived Cell Line and MMP-Mediated Degradation of Type I Collagen

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Abstract— It has been reported that matrix metalloproteinases (MMPs) are induced by many cytokines, and they are involved in various inflammatory processes, including periodontitis. However, the effects of interleukin-1β (IL-1β) on MMP-9 expression in cementoblasts, the cells responsible for cementum production, remain largely unknown. In this study, we used qPCR and gelatin zymogram analysis to show that IL-1β upregulated MMP-9 expression in cementoblast-derived cell line. Several signaling pathways, such as ERK1/2, JNK, p38, and AP-1 (c-Fos and ATF-2), were activated in response to IL-1β stimulation. Furthermore, enhancement of AP-1 activity by IL-1β was further confirmed by the AP-1 reporter assay and the electrophoretic mobility shift assay (EMSA). Pretreatment with specific inhibitors of ERK1/2 (U0126), JNK (SP600125), and AP-1 (tanshinone IIA) attenuated IL-1β-induced MMP-9 expression. In addition, inhibitors of ERK1/2 (U0126) and JNK (SP600125) attenuated IL-1β-enhanced AP-1 activity. This suggested that IL-1β stimulated AP-1 activation, at least partially, through ERK1/2 and JNK signaling pathways. Moreover, we found that IL-1β also upregulated the expression of MMP-13 and enhanced MMP-mediated degradation of type I collagen. Collectively, these results suggested that IL-1β induced MMP-9 expression by activation of AP-1 through the ERK1/2 and JNK signaling pathways in cementoblast-derived cell line and enhanced MMP-mediated collagen degradation possibly by MMP-13 and MMP-9.

KEY WORDS: interleukin-1β; MAPK signaling pathway; dental cementum; matrix metalloproteinases; cell-mediated collagen degradation.

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INTRODUCTION

Periodontitis is a chronic inflammatory oral disease, mainly caused by perturbed homeostasis between periodontopathic bacteria and host defenses [1, 2]. Derived from the plaque biofilm in supporting periodontal tissues, lipopolysaccharides and/or other molecules recruit host immune cells to the site, and these cells respond by releasing proinflammatory cytokines, such as IL-1β and TNF-α [3]. These cytokines further trigger a series of inflammatory reactions. During this inflammatory process, the host

proteolytic enzymes contribute to the destruction of the periodontal tissue [4], resulting in connective tissue degradation, bone resorption, and even tooth loss. Among the proteolytic enzymes, MMPs play an essential role in the progression of periodontal destruction [5].

MMPs, a group of highly homologous extracellular proteolytic enzymes, are essential for various physiological processes, such as embryogenesis, wound healing, and bone remodeling, and they play an important role in pathological processes, including inflammation, arthritis, and tumor invasion [6–9]. MMPs are zinc-dependent endopeptidases that are capable of degrading almost all extracellular matrix (ECM) components, and they are grouped into collagenases, gelatinases, stromelysins, matrilysins, and membrane type (MT) MMPs based on their substrate specificity. Moreover, tissue inhibitors of metalloproteinases (TIMPs) are endogenous matrix–degrading protease inhibitors, which inhibit the activity of most MMPs through binding with them [10]. Because type I collagen is the main component of the periodontal extracellular matrix, many studies on proteolytic enzymes in periodontitis have focused primarily on collagenases (MMP-1, MMP-8, and MMP-13) and gelatinases (MMP-2 and MMP-9) [11–13]. Previous studies have shown that MMP-9 is an important indicator of the severity and progression of periodontal disease [14, 15]. It was reported that MMP-9 levels in gingival crevicular fluid were higher in periodontitis patients than in healthy controls, and they had a significant correlation with the clinical parameters [15]. Studies conducted by Kubota *et al.* [16] demonstrated that gene expression of MMP-9/TIMP ratio was increased in periodontitis-affected gingival tissues. Moreover, many studies showed that MMP-9 expression was upregulated by proinflammatory cytokines, such as IL-1β, in various cell types, including osteoblasts, osteoclasts, neutrophils, and tumor cells, and it thereby contributed to degradation of the ECM and in turn led to bone resorption, tissue destruction, and/or tumor invasion [17–20]. However, Guan *et al.* [21] reported that MMP-9 expression was not induced by IL-1β stimulation in periodontal ligament cells. Therefore, the effects of IL-1β on MMP-9 expression may vary in different cell types.

Cementum is a mineralized connective tissue covering the root surface, and it anchors the tooth root to the surrounding alveolar bone by the periodontal ligament. Therefore, it is important to ensure the integrity of cementum for tooth stability. Cementoblasts, the cells lining the tooth root surface, are responsible for cementum formation, maintenance of the structural integrity, and normal function of the teeth. A recent study reported that MMP-3 expression was upregulated by TNF-α in cementoblasts *via* PEG2 signaling [22]. However, the expression of MMP-9 in cementoblasts is largely unknown.

In the present study, experiments were undertaken to investigate the effect of IL-1β on the expression of MMP-9 in cementoblast-derived cell line and to attempt to clarify the underlying mechanism. Meanwhile, we also examined the effect of IL-1β on MMP-mediated degradation of type I collagen.

MATERIALS AND METHODS

Chemicals and Antibodies

Recombinant murine IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant mouse IL-1 receptor antagonist was purchased from R&D Systems (Minneapolis, MN, USA). Specific chemical inhibitors, including SB203580, U0126, SP600125, tanshinone IIA, and batimastat (BB-94), were purchased from Selleck (Houston, TX, USA). Antibodies for phospho-p38 (Thr180/Tyr182), total p38, phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, phospho-JNK (Thr183/Tyr185), total JNK, phosphor-c-Fos (Ser32), phospho-c-Jun (Ser63), and phospho-ATF-2 (Thr71) were bought from Cell Signaling Technology (Danvers, MA, USA). Antibodies for TIMP-1 and MMP-13 were bought from Proteintech (Wuhan, Hubei, China). The TIMP-3 antibody was bought from ABclonal (Cambridge, MA, USA). Plasmin protein was purchased from Abcam (Cambridge, MA, USA).

Cell Culture

An immortalized cementoblast cell line, OCCM-30, was kindly provided by Dr. Martha J. Somerman (National Institutes of Health, Bethesda, MD, USA), and it was cultured as described previously [23]. Briefly, the cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin in 5% CO_2 at 37 °C. The medium was changed to DMEM containing 5% FBS when IL-1β or chemical inhibitors were applied. All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the medium was not more than 0.1%.

Gelatin Zymogram

Gelatin zymogram analysis was conducted as previously described [24, 25]. OCCM-30 cells were plated onto 6-well culture plates. The culture medium was changed to serum-free DMEM, when the cells were stimulated by IL-1β with or without chemical inhibitors. After treatment, the culture medium was collected and centrifuged at 10,000 rpm for 5 min at 4 °C to remove the cell debris. Samples of the culture medium were electrophoresed without reduction on an 8% SDS-PAGE gel containing 1% gelatin. After electrophoresis, gels were washed in 3% Triton X-100 for 1 h to remove SDS, rinsed with 50 mM Tris-HCl (pH 7.5), and then incubated for 24 h at 37 °C in a developing buffer (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij). After incubation, gels were stained in a solution of 0.5% (*w*/*v*) Coomassie Brilliant Blue, 30% methanol, and 10% acetic acid for 1 h. Gelatinolytic activity was manifested as clear white bands against a blue background.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated at the indicated time points using Trizol (Life Technologies Corporation, Carlsbad, CA, USA) according to the protocol of the manufacturer. The mRNA was converted into complementary DNA (cDNA) using a First-Strand cDNA Synthesis Kit (Takara, Tokyo, Japan). Quantitative real-time polymerase chain reaction (qPCR) was performed on a real-time PCR system with a 2× FastStart Universal SYBR Green master mix (Roche, Mannheim, Germany). Relative mRNA expression level was calculated by the delta-delta CT method [26], and the housekeeping gene GAPDH was used as an internal reference. The specificity of each product was tested by a melting curve analysis. The sequences of the primer pairs are listed in Supplementary Material Table 1.

Western Blot Analysis

OCCM-30 cells were seeded onto a 60-mm culture dish and then stimulated by IL-1β for indicated time with or without chemical inhibitors. Total protein of cells was extracted using a protein lysis buffer (Thermo Scientific, Pittsburgh, PA, USA) supplemented with protease inhibitor (Roche). Protein quantification was performed using a BCA kit purchased from Beyotime (Shanghai, China). The protein samples were denatured, electrophoresed on a 10% SDS–PAGE gel, and then transferred onto a PVDF membrane (Roche). The membrane was blocked with 5% nonfat milk and then incubated overnight at 4 °C with primary antibodies for p-ERK1/2, total ERK1/2, p-JNK, total JNK, p-p38, total p38, p-c-Fos, p-c-Jun, p-ATF-2, or GAPDH, followed by a peroxidase-conjugated secondary antibody. The membrane was then visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and then, signaling was detected by Odyssey instrument (LI-COR, USA).

Electrophoretic Mobility Shift Assay

OCCM-30 cells were plated onto a 60-mm culture dish and then stimulated by IL-1β. Nuclear protein was extracted at the indicated treatment time, and then it was quantified by the BCA method. The biotin-labeled AP-1 probe used for electrophoretic mobility shift assay (EMSA) was purchased from Beyotime, and the sequence of the consensus oligonucleotide was as follows: 5′-CGCT TGATGACTCAGCCGGAA-3′, 3′-GCGA ACTACTGAGTCGGCCTT-5′. Protein–DNA binding reactions were performed using a Chemiluminescent EMSA Kit (Beyotime). The mixtures were subjected to electrophoresis on a nondenaturing 4% polyacrylamide gel, transferred onto a nylon membrane, and then visualized by using a chemiluminescence procedure.

Transfection and Reporter Gene Assay

OCCM-30 cells were seeded onto a 6-well plate in DMEM without antibiotics. The plasmids containing pAP1-Luc and pRL-SV40 (Beyotime) were co-transfected into cells using Lipofectamine 3000 (Thermo Scientific) according to the instructions. After 24 h, cells were stimulated by IL-1β with or without chemical inhibitors for the indicated time period. Luciferase activities were measured using the Dual Luciferase Reporter Assay kit (Beyotime) with a GloMax® 20/20 Luminometer (Promega, Madison, WI, USA) according to the manufacturer's instructions. Transfection efficiency was normalized by dividing Firefly luciferase activity by Renilla luciferase activity.

Cell-Mediated Type I Collagen Fibril Dissolution

The method was modified based on that used in previous studies [27]. Briefly, a stock solution of acid-soluble type I collagen was diluted and mixed with a neutralizing phosphate buffer to a final concentration of 300 μg/ml. Aliquots of 1.5 ml/well were dispensed in 6-well culture plates, and collagen fibrils were formed by heat gelation at 37 °C for 2 h. The collagen gels were air-dried overnight in a laminar flow hood, and then, they were

washed extensively with sterile water to remove salt precipitates. OCCM-30 cells were resuspended in serum-free DMEM plus with 0.1% bovine serum albumin (DMEM/BSA), and then, they were seeded in collagen-coated plates in the form of a droplet (60,000 cells in 50 μl) in the central part of each well. The cells were incubated in a humidified chamber at 37 °C for 5 h, and then, 2 ml of DMEM/BSA was added to each well with or without IL-1β for the indicated time period. Cell-mediated collagen degradation was examined after removing the cells with 0.25% trypsin/1 mM EDTA at 37 °C for 10 min before the addition of Triton X-100 to a final concentration of 0.3%. Wells were then washed and stained with a solution of 0.5% (*w*/*v*) Coomassie Brilliant Blue, 30% methanol, and 10% acetic acid. The fibrillar collagen film was highly resistant to trypsin at 37 °C; therefore, cell-mediated collagen degradation was visualized as clear areas against a blue background. Collagen degradation was then photographed and analyzed by ImageJ software.

Statistical Analysis

All of the experiments were performed at least three times, and data are expressed as the mean ± standard deviation of independent experiments. Statistical significance was analyzed by one-way analysis of variance, followed by the Bonferroni test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

RESULTS

IL-1β Induced MMP-9 Expression in OCCM-30 Cells

To investigate whether MMP-9 expression could be induced by IL-1β in OCCM-30 cells, gelatin zymogram analysis was performed after treatment of OCCM-30 cells with different concentrations of IL-1β (0–40 ng/ml) for 24 h. As shown in Fig. 1a, IL-1β increased MMP-9 enzyme activity at different concentrations, and the apparent increase occurred in the 92 kDa pro-form zymogen of MMP-9. The activated form of MMP-9 was also observed, but no significant increase was observed. Besides, the pro and activated forms of MMP-2 were also observed on the gel, but levels of both these forms were not affected by IL-1β stimulation. This finding was similar to the results of other studies which showed that MMP-2 was constitutively expressed in various cell types and thereby served as an internal reference. To identify the time course character, OCCM-30 cells were stimulated with IL-1β (20 ng/ml) for 0–24 h. As shown in Fig. 1b, IL-1β increased the MMP-9 enzyme activity in a time-dependent manner with a significant increase from 12 h onwards.

To further examine whether IL-1β regulated the mRNA expression levels of MMP-9 and MMP-2, qPCR was performed after incubation of OCCM-30 cells with IL-1β (20 ng/ml) for 0–6 h. As shown in Fig. 1c, the mRNA expression of MMP-9 was significantly increased from 1 h after IL-1β (20 ng/ml) stimulation and there was no effect on the mRNA expression of MMP-2. Besides, to confirm a direct effect of IL-1β on MMP-9 expression, IL-1 receptor antagonist (IL-1 Ra) was used. As shown in Fig. 1d, gelatin zymogram analysis showed that the increased MMP-9 expression was blocked by IL-1 Ra (1000 ng/ml).

IL-1β Induced MMP-9 Expression *via* ERK1/2 and JNK Signaling Pathways

To investigate whether the three well-characterized members of the MAPK family, ERK1/2, JNK, and P38, were involved in IL-1β-induced MMP-9 expression in OCCM-30 cells, these protein kinases were examined by western blot analysis. As shown in Fig. 2a–c, IL-1β phosphorylated ERK1/2, JNK, and P38 protein in a time-dependent manner in OCCM-30 cells. To determine whether these signaling pathways were involved in the upregulation of MMP-9 by IL-1β in these cells, specific inhibitors were used. As shown in Fig. 2d, qPCR analysis confirmed that the ERK1/2 inhibitor (U0126) and the JNK inhibitor (SP600125) attenuated IL-1β-induced MMP-9 mRNA expression. However, the p38 inhibitor (SB203580) did not attenuate this stimulating effect. On gelatin zymogram analysis, data presented in Fig. 2e–g showed that inhibition of ERK1/2 and JNK also reduced the IL-1β-induced MMP-9 enzyme activity, whereas inhibition of p38 did not affect the increased enzyme activity level. These data suggested that IL-1β induced MMP-9 expression, at least partially, *via* ERK1/2 and JNK signaling pathways.

AP-1 Was Involved in IL-1β-Induced MMP-9 Expression

It is well documented that the MMP-9 promoter region contains several AP-1 binding sites [28]. Thus, in this study, we attempted to verify whether AP-1 was involved in IL-1β-induced MMP-9 expression in cementoblast-derived cell line.

Firstly, to examine whether AP-1 activity was enhanced by IL-1β stimulation, EMSA and AP-1 promoter reporter assay were performed. EMSA data presented in Fig. 3a showed that the binding of protein–DNA

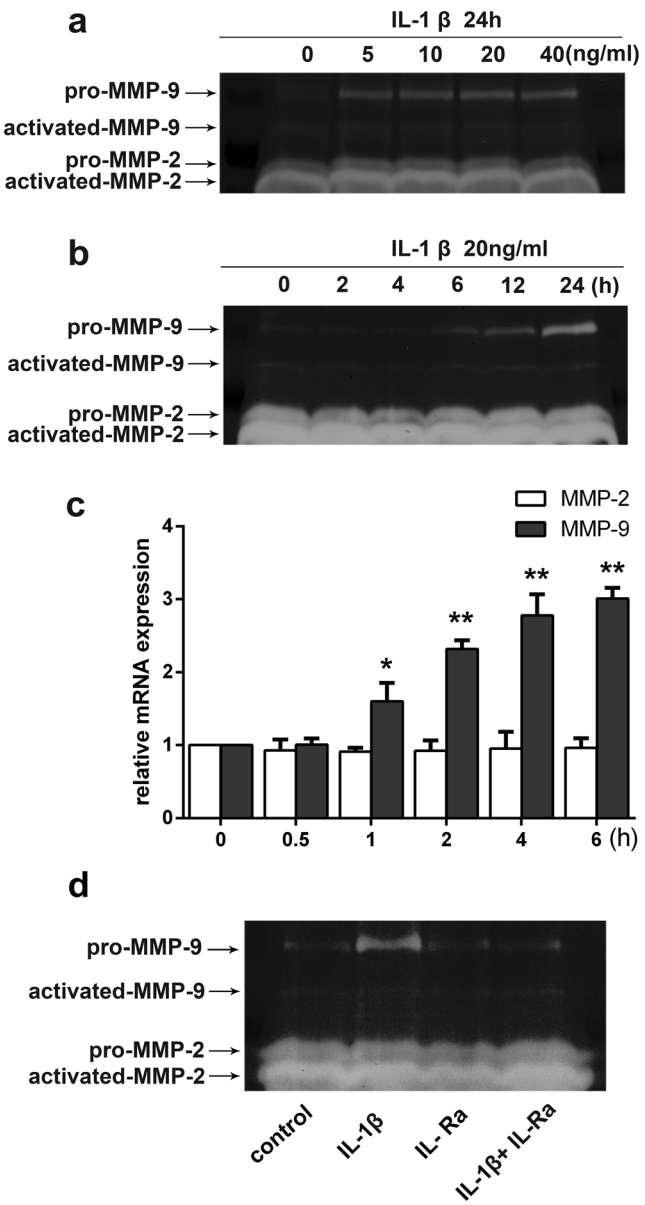

Fig. 1. Effect of IL-1β on MMP-9 expression in OCCM-30 cells. **a** Cells were incubated with IL-1β by different concentrations (0, 5, 10, 20, 40 ng/ml) for 24 h, and conditioned media were collected and analyzed by gelatin zymogram. **b** Cells were incubated with IL-1β (20 ng/ml) for the indicated time period, and conditioned media were collected and analyzed by gelatin zymogram. **c** qPCR analysis of MMP-9 and MMP-2 in OCCM-30 cells was performed after stimulation with IL-1β (20 ng/ml) for indicated time period. **d** Cells were pretreated with IL-1 Ra (1000 ng/ml) for 1 h and then incubated with 20 ng/ml IL-1β for 24 h. Conditioned media were collected and analyzed by gelatin zymogram. $*P < 0.05$ and $**P < 0.01$ compared with the control group.

complexes was stronger within 2–4 h than that in the control group, and then, it weakened slightly within 6 h. AP-1 promoter reporter assay confirmed that the transcriptional activity of AP-1 was increased in a time-dependent manner with a peak at 2 h, indicating that AP-1 activity was enhanced by IL-1β stimulation in OCCM-30 cells (Fig. 3b). We further examined the activity of three major proteins of AP-1 by western blot analysis and the mRNA expression of c-Fos and c-Jun by qPCR. Western blot analysis showed that c-Fos and ATF-

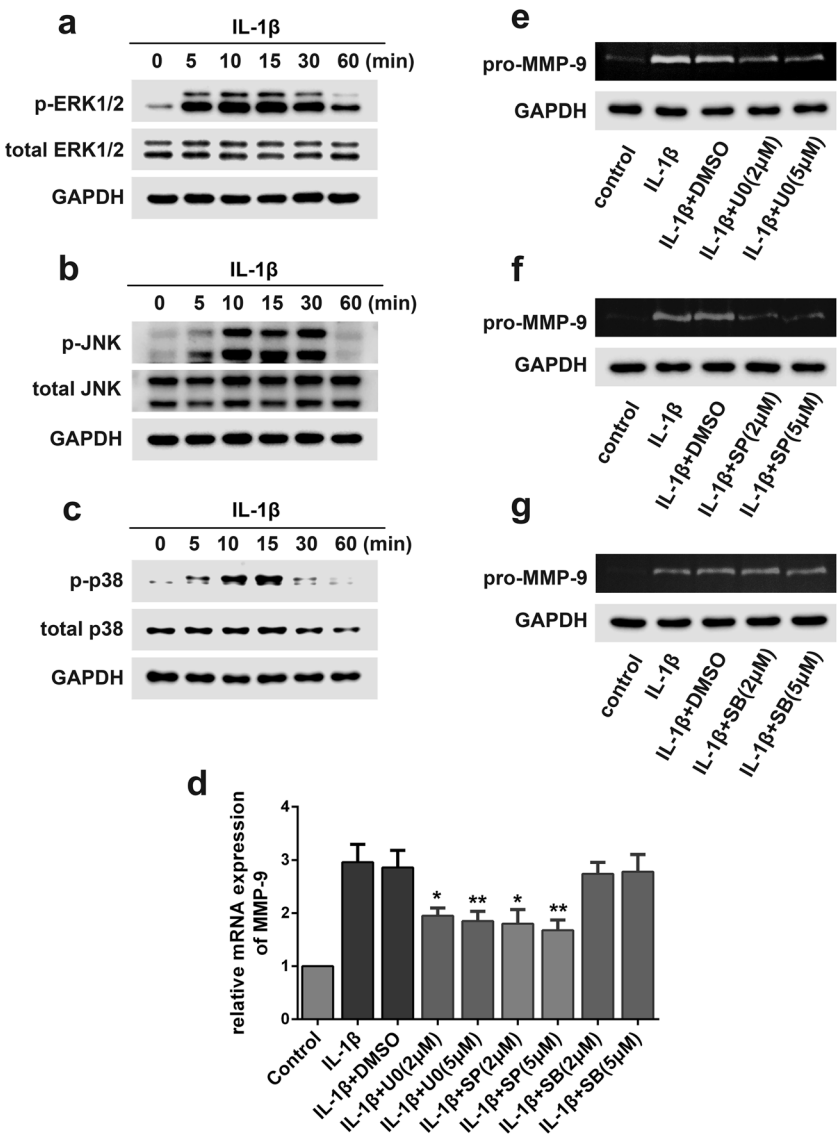

Fig. 2. Effects of MAPK signaling pathways on IL-1β-induced MMP-9 expression in OCCM-30 cells. **a**–**c** Western blot analysis was performed to test the level of phosphorylated form of ERK1/2, JNK, and P38 and their corresponding total amounts after IL-1β (20 ng/ml) stimulation. **d** Cells were pretreated with U0126 (U0), SP60012 (SP), SB203580 (SB), or DMSO for 1 h and then incubated with 20 ng/ml IL-1β for 6 h. qPCR analysis was performed to determine relative mRNA expression of MMP-9. **e**–**g** Cells were pretreated with U0126 (U0), SP60012 (SP), SB203580 (SB), or DMSO for 1 h and then incubated with 20 ng/ml IL-1β for 12 h. Conditioned media were collected and analyzed by gelatin zymogram. The cell lysates were analyzed by western blot. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle control group.

2, but not c-Jun, were phosphorylated in a time-dependent manner by IL-1β stimulation (Fig. 3c). Data presented in Fig. 3d demonstrated that the mRNA expression of c-Fos and c-Jun was also upregulated by IL-1β stimulation. To confirm the role of AP-1 in IL-1β-induced MMP-9 expression, we used an AP-1 inhibitor (tanshinone IIA). Gelatin zymogram and qPCR analysis demonstrated that inhibition of AP-1 activity with tanshinone IIA attenuated IL-1β-induced MMP-9 mRNA expression and enzyme activity (Fig. 3e, f). Thus, these results indicated that AP-1 was required for IL-1β-induced MMP9 expression in OCCM-30 cells.

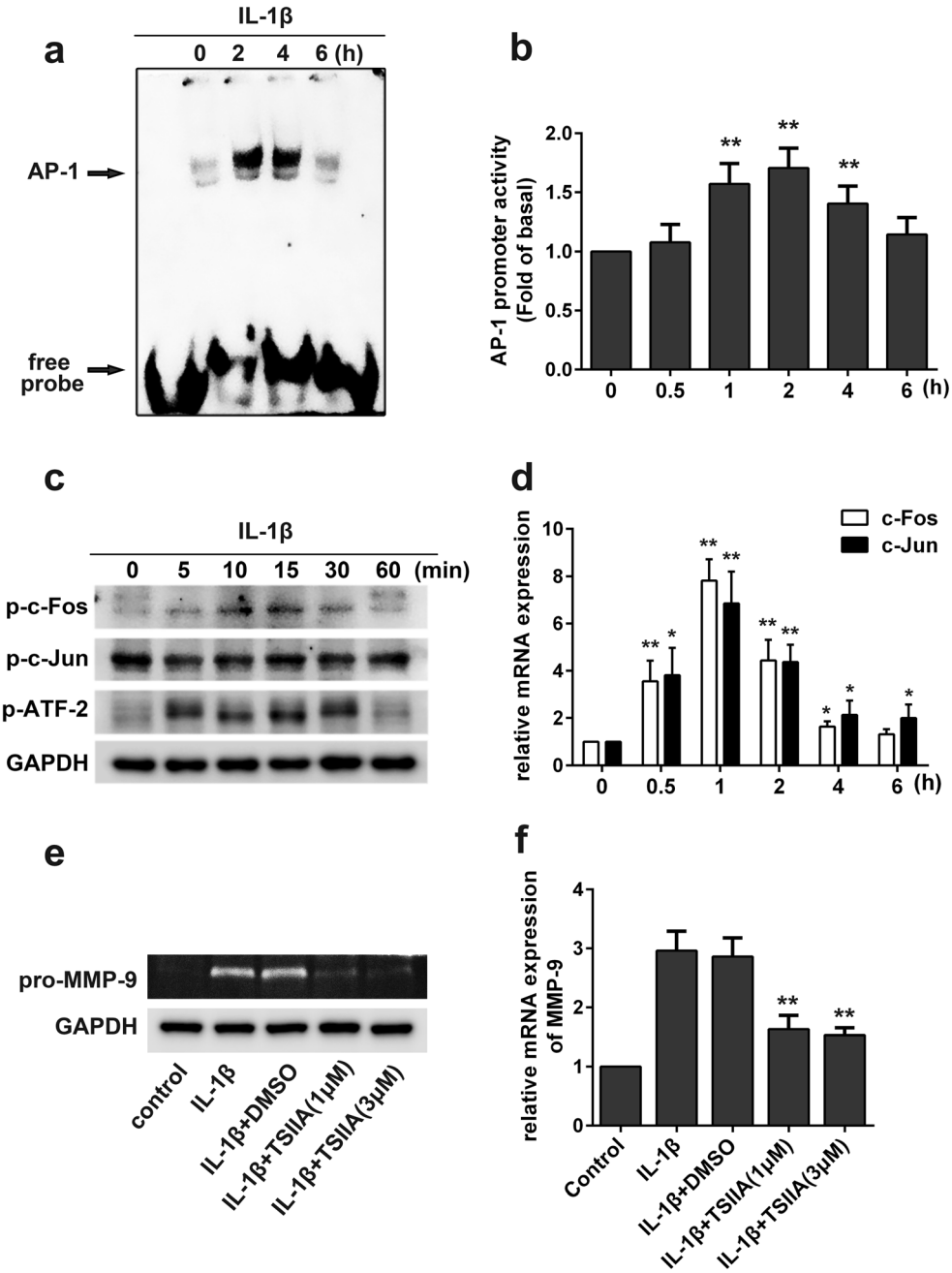

Fig. 3. AP-1 activity was enhanced by IL-1β stimulation and involved in IL-1β-MMP-9 expression in OCCM-30 cells. **a** Cells were incubated with IL-1β (20 ng/ml) for indicated time period, and nuclear protein was prepared and then examined by EMSA. **b** Cells were transfected with a reporter vector containing AP-1 promoter, and AP-1 activity was measured after incubation of cells with IL-1β (20 ng/ml). To adjust for the transfection efficiency, Renilla luciferase-positive control vector (pRL-SV40) was co-transfected. **c** Total protein was extracted after treatment of cells with IL-1β (20 ng/ml). Western blot analysis was performed to test the protein level of phosphorylated form of c-Fos, c-Jun, and ATF-2. **d** qPCR analysis of c-Fos and c-Jun was performed in cells after stimulation with IL-1β (20 ng/ml) for the indicated time period. $*P < 0.05$ and $**P < 0.01$ compared with the control group. Cells were pretreated with tanshinone IIA (TSIIA) or DMSO for 1 h and then incubated with 20 ng/ml IL-1β. **e** Conditioned media were collected and analyzed by gelatin zymogram. The cell lysates were analyzed by western blot. **f** qPCR analysis was performed to determine relative mRNA expression of MMP-9. $**P < 0.01$ compared with the vehicle control group.

IL-1β Enhanced AP-1 Activity Through ERK1/2 and JNK Signaling Pathways

We showed that ERK1/2, JNK, and AP-1 signaling molecules were involved in IL-1β-induced MMP-9 expression. In addition, it has been demonstrated that AP-1 is mediated by various signaling molecules, including ERK1/2 and JNK [29]. Thus, we investigated whether IL-1β-stimulated ERK1/2 and JNK activation mediated AP-1 activity in OCCM-30 cells. As shown in Fig. 4a, AP-1 promoter reporter assay revealed that inhibition of ERK1/2 and JNK attenuated IL-1β-enhanced AP-1 transcriptional activity. Furthermore, pretreatment of the cells with inhibitors of ERK1/2 and JNK abrogated IL-1β-stimulated phosphorylation of c-Fos and ATF-2, respectively (Fig. 4b). Moreover, we also found that inhibition of ERK1/2 and JNK decreased IL-1β-induced c-Fos and c-Jun mRNA expression, respectively (Fig. 4c, d). These results suggested that IL-1β-stimulated AP-1 activity was mediated, at least partially, through ERK1/2 and JNK signaling pathways.

IL-1β Induced MMP-13 Expression and Enhanced MMP-Mediated Degradation of Type I Collagen

Besides MMP-9, we found that MMP-13 expression was also induced by IL-1β in OCCM-30 cells at mRNA and protein level (Fig. 5a, b). Thus, we attempted to examine the effect of IL-1β on MMP-mediated dissolution of type I collagen. As described in "Materials and Methods" section, OCCM-30 cells were cultured as a central cell plug on type I collagen films in BSA-containing medium with or without IL-1β, and the degradation was assessed by Coomassie Blue assay at 48, 72, and 96 h after cell seeding. As shown in Fig. 5c, the area of degradation was not beyond the boundaries of the cell plug, and the degradation was visible at 48 h in both control group and IL-1β-stimulated group, whereas it was not visible within 24 h (data not shown). Furthermore, collagen degradation in the both groups was increased in a time-dependent manner, and it increased 2–3-fold between 48 h and 96 h. However, collagen degradation in the IL-1β-stimulated group was not enhanced than that in the control group within the same time period (Fig. 5c). Next, taking into account that MMPs are mostly secreted as inactive zymogens (pro-MMPs), we used plasmin, an activator for pro-MMPs, in collagen degradation assay. As shown in Fig. 5d, when plasmin was added into cell medium, collagen degradation in the IL-1β-stimulated group was significantly enhanced than the group without IL-1β stimulation. Besides, to verify that the collagen degradation was mediated by cell-secreted MMPs, a synthetic broad-spectrum MMP inhibitor (BB-94) was used. As shown in Fig. 5e, collagen degradation by plasmin-treated OCCM-30 cells with IL-1β stimulation was almost completely inhibited by BB-94 (20 nM/ml). Therefore, these data suggested that in the presence of plasmin, collagen degradation by OCCM-30 cells was enhanced by IL-1β and was MMP-mediated.

DISCUSSION

In periodontitis, MMP-9 has been considered to be an important proteolytic enzyme that contributes to periodontal tissue destruction [15]. It has been reported that MMP-9 can be induced in many cell types by various extracellular stimuli [30]. However, to our knowledge, the expression of MMP-9 remains unknown in cementoblasts, cells that are indispensable for cementum formation and tooth function. IL-1β, a major inflammatory cytokine in periodontal inflammation, has been shown to induce MMP-9 expression through different signaling pathways, such as s-Src, PI3K/Akt, MAPKs, AP-1, SP-1, and NF-κB, in various cell types [31–33]. Therefore, in this study, we investigated the underlying mechanisms of IL-1β-induced MMP-9 expression in OCCM-30 cells, and we tested the effects of IL-1β on MMP-mediated type I collagen degradation. Our results demonstrated that IL-1β induced MMP-9 expression by activation of AP-1 *via* ERK1/2 and JNK signaling pathways in OCCM-30 cells and enhanced MMP-mediated type I collagen degradation.

MMP-2 and MMP-9 are members of the gelatinase subfamily of MMPs since their best-known substrate is gelatin (denatured collagen). Furthermore, MMP-2 can cleave native type I collagen, but MMP-9 only cleaves denatured type I collagen [34–36]. The expression of most MMPs is low in normal tissues, and it is regulated when remodeling of the ECM is required. The regulation of MMP-2 and MMP-9 showed different responses to an extracellular stimulus, which is largely related to their promoter sequences. The human *MMP-9* promoter region harbors several consensus motifs for regulatory elements, including SP1, AP-1, TIE, and NF-κB binding sites [37]. However, in contrast to *MMP-9*, the *MMP-2* gene promoter structure does not contain binding elements for AP-1, NF-κB, TIE, and other transcription factors [38, 39]. Accumulating evidence has shown that MMP-2 is constitutively expressed in most cell types, and it appears to be moderately regulated, or not responsive at all. Indeed, our data demonstrated that in OCCM-30 cells, IL-1β increased the enzyme activity and mRNA expression of MMP-9 in a time-dependent manner, but it did not exert any effects on

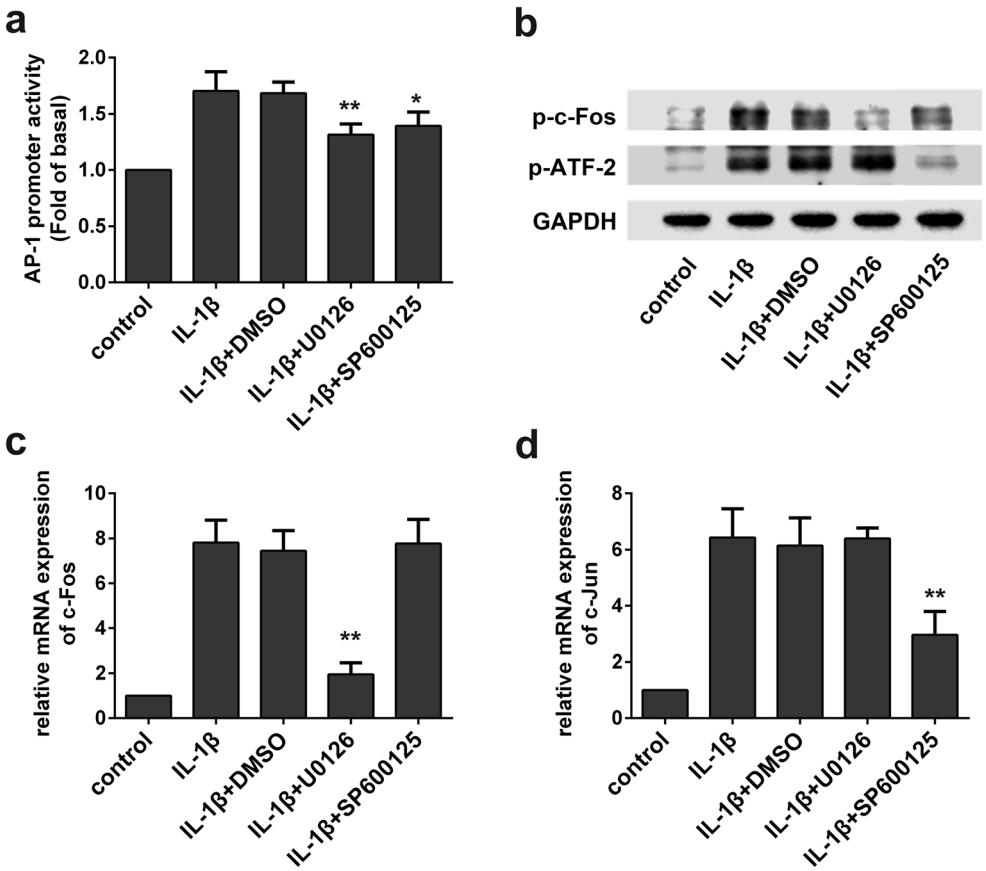

Fig. 4. Inhibition of ERK1/2 and JNK attenuated IL-1β-enhanced AP-1 activity in OCCM-30 cells. **a** Cells were transfected with a reporter vector containing AP-1 promoter. After pretreatment with U0126 (5 μM), SP600125 (5 μM), or DMSO for 1 h, cells were stimulated with 20 ng/ml IL-1β for 2 h, and AP-1 activity was then measured. To adjust for the transfection efficiency, Renilla luciferase-positive control vector (pRL-SV40) was co-transfected. **b**–**d** Cells were pretreated with U0126 (5 μM), SP600125 (5 μM), or DMSO for 1 h and then incubated with 20 ng/ml IL-1β. Total protein was prepared and analyzed by western blot analysis (**b**) to test the protein level of phosphorylated form of c-Fos and ATF-2 after incubation of cells with IL-1β for 15 min. qPCR analysis of relative mRNA expression of c-Fos (**c**) and c-Jun (**d**) was performed after incubation of cells with IL-1β for 1 h. $*P < 0.05$ and $**P < 0.01$ compared with the vehicle control group.

the enzyme activity or the mRNA expression of MMP-2. These results are similar to those in many previous studies [31, 40]. Furthermore, inhibition of gene transcription using actinomycin D (an inhibitor of RNA polymerase) abrogated the IL-1β-induced MMP-9 expression (data not shown). This suggested that IL-1β-induced MMP-9 expression is mediated at the transcriptional level in OCCM-30 cells.

The MAPK superfamily is serine-threonine protein kinases that transmit signals from extracellular stimuli to a wide range of intracellular responses. In mammals, three well-characterized groups of MAPKs—ERK1/2, JNK, and P38—are widely involved in cell proliferation, apoptosis, differentiation, and motility [41]. A number of studies have demonstrated that MAPK signaling pathways are involved in IL-1β-induced MMP-9 expression in various cell types [42, 43]. This is confirmed by our findings, which showed that inhibition of ERK1/2 and JNK using specific chemical inhibitors decreased IL-1β-induced MMP-9 expression. Interestingly, although IL-1β stimulated phosphorylation of p38 in OCCM-30 cells, inhibition of p38 did not affect IL-1β-induced MMP-9 expression. This observation was different from that in several studies performed using other cell types, in which all three MAPKs were involved in IL-1β-induced MMP-9 expression. However, Tseng *et al.* [40] reported that IL-1β induced MMP-9 expression *via* ERK1/2 and JNK, but not *via* p38, in rabbit corneal cells. The findings of that study were similar to our results.

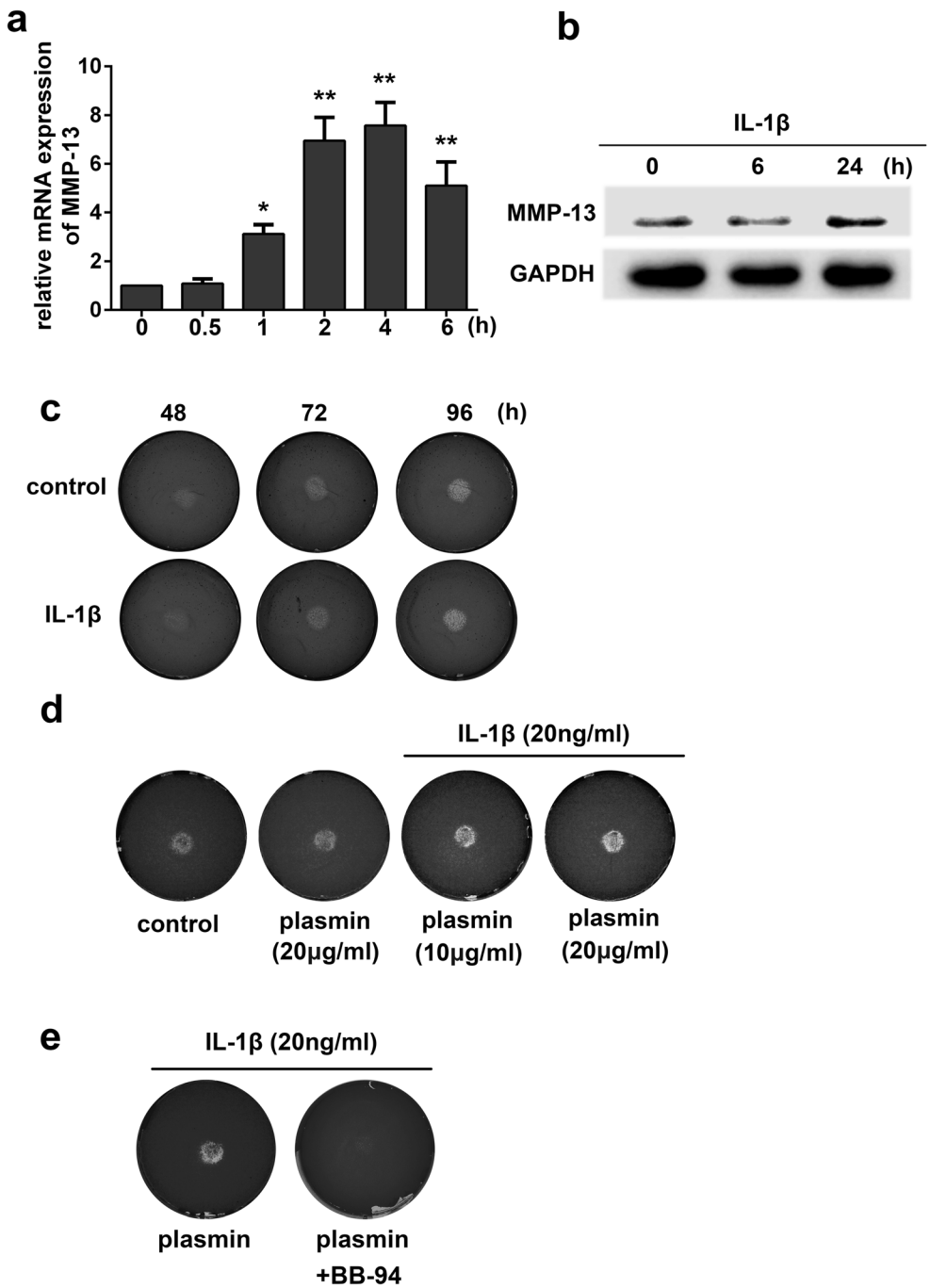

Fig. 5. IL-1β upregulated MMP-13 expression and enhanced MMP-mediated degradation of type I collagen. **a**, **b** Cells were incubated with IL-1β (20 ng/ml) for indicated time period, qPCR analysis was performed to determine relative mRNA expression of MMP-13 (**a**), and western blot analysis was performed to examine the protein expression of MMP-13 (**b**). **c** Collagen degradation was visualized after 48 h, 72 h, and 96 h by removal of cells and then staining of residual collagen with Coomassie Blue. **d** Cell medium was added with plasmin in assay for collagen degradation as in **c**, and collagen degradation was visualized after 72 h by removal of cells. **e** Cell medium was added with or without BB-94 in assay for collagen degradation, and collagen degradation was visualized after 72 h by removal of cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group.

The regulation of cell functions by MAPKs is mediated mainly through phosphorylation of their downstream substrates, including several transcription factors, such as AP-1 [44]. AP-1 is a dimeric transcription factor composed of the Jun, Fos, and ATF subunits, which form a variety of homodimers and heterodimers that bind to a common DNA site, the AP-1 binding site. It is established that the MMP-9 gene promoter region contains the AP-1 binding site in different mammalian species [37], and many studies have demonstrated that AP-1 was essential for IL-1β-induced MMP-9 expression in many cell types [33]. This is consistent with our observation that inhibition of AP-1 activity by tanshinone IIA attenuated IL-1β-induced MMP-9 expression in OCCM-30 cells. In addition, the findings of the AP-1 promoter assay confirmed that AP-1 activity was enhanced by IL-1β stimulation through ERK1/2 and JNK signaling pathways in this cell type. Furthermore, our results showed that inhibition of ERK1/2 signaling pathway abrogated IL-1β-phosphorylated c-Fos and attenuated IL-1β-induced c-Fos mRNA expression and inhibition of JNK signaling pathway abrogated IL-1β-phosphorylated ATF-2 and attenuated IL-1β-induced c-Jun mRNA expression. This suggested that ERK1/2 and JNK mediated AP-1 activity not only by modulation of the AP-1 protein but also by increasing the abundance of c-Fos and c-Jun proteins in the AP-1 components. Taken together, the results show that in OCCM-30 cells, IL-1β upregulates MMP-9 expression by activation of AP-1 through the ERK1/2 and JNK signaling pathways.

Due to its native triple helical structure, interstitial type I collagen is highly resistant to proteolytic attack, but it can be cleaved by collagenases (MMP-1, MMP-8, and MMP-13) and gelatinases (MMP-2 and MMP-9). Collagen fragments produced by collagenases are susceptible to gelatinases which rapidly degrade denatured collagen and collagen fragments [45]. Previous studies demonstrated that IL-1β promoted MMP-mediated degradation of type I collagen by gingival fibroblasts and that TNF-α induced degradation of type I collagen by upregulation of MMP-9 in osteoblasts [46, 47]. In this study, we found that MMP-13 and MMP-9 expressions were upregulated by IL-1β stimulation in OCCM-30 cells. MMP-1 and MMP-8 were not affected significantly by IL-1β (data not shown). Since MMPs are secreted mostly as inactive zymogens, IL-1β did not enhance collagen degradation by OCCM-30 cells in the absence of plasmin. Plasmin, a serine protease, derives from its inactive zymogen form (plasminogen) and can activate different pro-MMPs [48]. In our experiments, when plasmin was added into cell medium, collagen degradation was significantly increased by IL-1β-stimulated cells. Furthermore, cell-mediated collagen degradation was inhibited by BB-94, an inhibitor of MMPs. These data indicated that collagen degradation by OCCM-30 cells in the presence of plasmin was enhanced by IL-1β and was MMP-mediated. Interestingly, we also found that the expression of TIMP-1 and TIMP-3 was increased in OCCM-30 cells upon IL-1β stimulation (Supplementary Material Fig. 1). As natural inhibitors of MMPs, TIMPs inhibit MMP activity and thereby restrict ECM degradation, and the balance between MMPs and TIMPs plays an important role in therapy for inflammatory diseases [49]. In this study, the IL-1β-upregulated TIMP-1 and TIMP-3 implied that there might be a type of self-protection mechanism to weaken negative effects by acting as balancing players to attenuate collagen degradation by IL-1β.

In summary, our results showed that in OCCM-30 cells, IL-1β induced MMP-9 expression by activation of AP-1 through ERK1/2 and JNK signaling pathways and enhanced MMP-mediated collagen degradation possibly by MMP-13 and MMP-9. These results provide insights into the effects of IL-β in cementoblasts on the expression of MMP-9 and MMP-mediated collagen degradation.

AUTHOR'S CONTRIBUTION

Mingyuan Du contributed to conception and design, contributed to data acquisition, analysis, and interpretation, and drafted the manuscript. Yunlong Wang, Zhingjian Liu, Leilei Wang, Zhengguo Cao contributed to conception and design; Chen Zhang and Hong He contributed to data analysis and interpretation; Yunlong Wang and Yunru Hao contributed to acquisition. All authors critically revised the manuscript, gave final approval, and agree to be accountable for all aspects of the work, ensuring integrity and accuracy.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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