Periostin promotes migration and osteogenic differentiation of human periodontal ligament mesenchymal stem cells *via* the Jun amino-terminal kinases (JNK) pathway under inflammatory conditions

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Abstract

Objectives: Mesenchymal stem cell (MSC)-mediated periodontal tissue regeneration is considered to be a promising method for periodontitis treatment. The molecular mechanism of functional regulation by MSCs remains unclear, thus limiting their application. Our previous study discovered that Periostin (POSTN) promoted the migration and osteogenic differentiation of periodontal ligament mesenchymal stem cells (PDLSCs), but it is still unclear whether POSTN is able to restore the regenerative potential of PDLSCs under inflammatory conditions. In this study, we investigated the effect of POSTN on PDLSCs under inflammatory conditions and its mechanism.

Materials and methods: PDLSCs were isolated from periodontal ligament tissue. TNF- α was used at 10 ng/mL to mimic inflammatory conditions. Lentivirus POSTN shRNA was used to knock down POSTN. Recombinant human POSTN (rhPOSTN) was used to stimulate PDLSCs. A scratch assay was used to analyse cell migration. Alkaline phosphatase (ALP) activity, Alizarin Red staining and expression of osteogenesis-related genes were used to investigate the osteogenic differentiation potential. Western blot analysis was used to detect the mitogen-activated protein kinases (MAPK) and AKT signalling pathways.

Results: After a 10 ng/mL TNF- α treatment, knockdown of POSTN impeded scratch closure, inhibited ALP activity and mineralization in vitro, and decreased expression of RUNX2, OSX, OPN and OCN in PDLSCs, while 75 ng/mL rhPOSTN significantly accelerated scratch closure, enhanced ALP activity and mineralization in vitro, and increased expression of RUNX2, OSX, OPN and OCN. In addition, knockdown of POSTN inhibited expression of phosphorylated c-Jun N-terminal kinase (p-JNK), while 75 ng/mL rhPOSTN increased expression of p-JNK in PDLSCs with TNF- α treatment. Furthermore, inhibition of JNK by its inhibitor SP600125 dramatically blocked POSTN-enhanced scratch closure, ALP activity and mineralization in PDLSCs.

Conclusions: Our results revealed that POSTN might promote the migration and osteogenic differentiation potential of PDLSCs *via* the JNK pathway, providing insight into the mechanism underlying MSC biology under inflammatory conditions and identifying a potential target for improving periodontal tissue regeneration.

Yi Tang and Lin Liu have contributed equally to this work and should be considered co-first authors.

1 | INTRODUCTION

Periodontitis is a prevalent oral chronic inflammatory disease that disrupts the functional and structural integrity of tooth-supporting tissues and has a significant impact on oral health as well as overall quality of life.^{1,2} MSCs have a self-renewal capacity, multidirectional differentiation ability and weak immunogenicity; they are also easy to collect.^{2,3} Thus, MSC-mediated regeneration of lost periodontium provides a new opportunity for periodontitis treatment.³ Inflammation is a significant challenge to tissue regeneration and is inevitable in periodontitis treatment; therefore, it is necessary to study tissue regeneration under inflammatory conditions. Periodontal ligament mesenchymal stem cells (PDLSCs) are an optimal choice for the regeneration of PDL due to their ability to generate mineral-forming cementoblasts, bone-forming osteoblasts and PDL-forming fibroblasts in vitro.⁴ However, poor adhesion, migration and differentiation of delivered cells limit the therapeutic efficiency when PDLSCs are transplanted in situ to periodontal defects.⁵ Previous studies revealed that extracellular matrix (ECM) substrates provide molecular signals, regulate the cell phenotype and are essential for regulating stem cell behaviours.^{6,7}

Periostin (POSTN), a 90-kDa adhesion molecule that is secreted in the extracellular matrix, is highly expressed in various fibrous connective tissues and organs, as well as in fibroblasts in the PDL, and its role in collagen fibrillogenesis has been extensively studied.⁸⁻¹⁵ POSTN is closely correlated to survival and angiogenesis, is highly expressed in tissue trauma and wound repair as well as participates in repairing tissue damage, suggesting that its primary function is tissue repair rather than acting as a structural protein.¹⁶⁻²⁰ Previous studies have demonstrated its key role in extracellular matrix maturation and stabilization, morphogenesis and maintenance of bone and tooth.^{21,22} Previous studies have reported that POSTN promotes cell mobility, adhesion and survival in a number of cell types by binding to integrin $\alpha\beta3$ and $\alpha\beta5$ via the Integrin/FAK/AKT signalling pathway.²³⁻²⁵ Our previous study confirmed that POSTN effectively promotes the migration and osteogenic differentiation of PDLSCs via the integrin signalling pathway.⁵ The phosphoinositide 3-kinase (PI3K)/AKT signalling pathway plays central regulatory roles in MSC survival, proliferation, migration, angiogenesis, cytokine production and differentiation.²⁶ The PI3K/AKT and generic mitogen-activated protein kinases (MAPK) signalling pathways are often involved in complex cross-talk on multiple levels.²⁷ Signalling by these pathways governs fundamental physiological processes, such as cell proliferation, differentiation, migration, senescence and apoptosis, and the co-ordination between these pathways determines the cell's fate.²⁸⁻³⁰ The MAPK signalling pathway includes ERK1/2, Jun amino-terminal kinases (JNK1/2/3) and P38-MAPK, and there are at least 284 proteins of endogenous ERK1 complexes that interact with the PI3K-mediated signalling pathway.²⁷ According to previous research, the differentiation and inflammatory response of PDLSCs or PDL cells could be affected by the ERK1/2 and P38 signalling pathways as well as by many other stimulatory factors.³¹⁻³⁴ However, it is unknown whether POSTN has a function similar to that shown in our previous under inflammatory

conditions. The mechanism of POSTN under inflammatory conditions is also unknown.

Here, we used human PDLSCs to investigate the role and mechanism of POSTN under inflammatory conditions. The results revealed that POSTN promoted both the migration and osteogenic differentiation potential of PDLSCs via the JNK signalling pathway with TNF- α treatment. Our findings provide useful information for understanding the biology of MSCs under inflammatory conditions and periodontal tissue regeneration.

2 | MATERIALS AND METHODS

2.1 | Cell isolation, cultures and characterization

All research involving human stem cells was approved by the Ethics Committee of the Affiliated Stomatological Hospital of Nanjing Medical University. Premolars were collected from six healthy orthodontic patients (12-16 years old) under approved guidelines set by the Jiangsu provincial dental hospital with informed consent. Primary PDLSCs were isolated and cultured as previously described.³⁵ Briefly, primary PDLSCs were separated from the periodontal ligament in the middle third of the root. Subsequently, periodontal ligament tissue was digested in a solution of 3 mg/mL collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) and 4 mg/mL dispase (Roche Diagnostics Corp., Indianapolis, IN, USA) for 1 hour at 37°C. Primary PDLSCs were grown in a humidified incubator under 5% CO₂ at 37°C in Gibco® MEM α (with GlutaMAX™) supplemented with 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen). Passaged cells of primary PDLSCs were cultured in mesenchymal stem cell medium (MSCM, Sciencell, Sandiego, CA, USA) and changed every 3 days. The obtained PDLSCs were characterized as in our previous study.⁵ Briefly, cells were immunostained with fluorochrome-conjugated rabbit anti-human antibodies against surface markers for 20 minutes at room temperature in the dark. The stained cells were washed twice with 0.01 M phosphate-buffered saline (PBS) and analysed by a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

TNF- α at 10 ng/mL (Peprotech, USA) and rhPOSTN (R&D Systems, Inc., Minneapolis, MN, USA) was used to treat PDLSCs. SP600125 (Cell Signaling Technology, Beverly, MA, USA) was used to block the JNK signalling pathway.

2.2 | Oil red O staining and alcian blue staining

To prove that the obtained mesenchymal stem cells retained multipotency to differentiate into adipocytes or chondrocytes, the StemPro™ Adipogenesis Differentiation Kit and StemPro™ Chondrogenesis Differentiation Kit (Gibco™, Thermo Scientific, USA) were used to induce PDLSCs. After 3 weeks of adipogenic induction, cells were fixed with 10% formalin, dissolved in 60% isopropanol and stained in Oil red O solution (Sigma-Aldrich). After 3 weeks of chondrogenic induction, cells were fixed with 4% paraformaldehyde and stained in an alcian blue solution (Sigma-Aldrich).

2.3 | Plasmid construction, viral infection and cell identification after viral infection

All recombinant vector and lentiviral packaging procedures were performed by GeneChem Co., Ltd. (Shanghai, China). Short-hairpin RNAs (shRNAs) with complementary sequences of POSTN were subcloned into the GV248 lentiviral vector. The target sequences for the shRNAs were as follows: POSTN shRNA (POSTNsh), 5'-GTGACAGTATAACAGTAAA-3'; control shRNA (CONTsh), 5'-TTCTCCGAACGTGTCACGT-3'.

PDLSCs were infected with POSTNsh and CONTsh lentiviruses according to the manufacturer's instructions. Briefly, PDLSCs were plated overnight and then infected with lentiviruses in the presence of polybrene (6 µg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 12 hours. After 48 hours, infected cells were selected with 1 µg/mL puromycin (Sigma, USA) for 72 hours. The knockdown efficiency of the transduced PDLSCs was confirmed by Western blot and real-time RT-PCR analysis.

To identify transduced PDLSCs, isolated cells were immunostained with the CD34 Monoclonal Antibody-APC (Catalog#: 17-0349-42) and CD146 Monoclonal Antibody (Catalog#: 12-1469-42) (eBioscience™, Thermo Fisher Scientific, San Diego, CA, USA) for 30 minutes at room temperature in the dark. Stained cells were washed twice with 0.01 M PBS and analysed by a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.4 | Scratch migration assay

PDLSCs were seeded onto six-well plates at a density of 2×10^5 cell/well and allowed to grow close to 95% confluence. After treatment with TNF- α for 2 hours, a scratch was made along the diameter of the well with a 1000 µL pipette tip (Axygen® Corning, NY, USA), and the scraped cells were gently removed by washing with PBS. Fresh culture medium was added and supplemented with each one of the treatment groups as described above. After that, images from the same view (a circle drawn on the bottom of each well was used as a reference; the view was right inside of the circle) were taken at baseline (0 hour) and 24 hours. The scraped width measured by ImageJ 1.42q (National Institutes of Health, USA) was used to calculate the void area (VA), and the extent of wound closure was evaluated (Relative Width (%)=VA/baseline area) in each group.

2.5 | Alkaline phosphatase and alizarin red detection

Mesenchymal stem cell osteogenic differentiation medium (MODM, ScienCell, Sandiego, CA, USA) was used to induce osteogenic differentiation of PDLSCs. After 3 days of induction, alkaline phosphatase (ALP) activity was assayed with an ALP activity kit according to the manufacturer's protocol (Sigma-Aldrich). Signals were normalized based on the protein concentrations. To detect mineralization, cells were induced for 2 weeks, fixed with 70% ethanol and stained with 2% Alizarin Red (Sigma-Aldrich). To quantify the calcium mineral density, Alizarin Red was destained with 10% cetylpyridinium chloride (CPC) in 10 mmol/L sodium phosphate for 30 minutes at room

temperature. The calcium concentrations were determined by an absorbance measurement at 562 nm on a multiplate reader and compared to a standard calcium curve with calcium dilutions in the same solution. The final calcium level in each group was normalized to the total protein concentration detected in a duplicate plate.

2.6 | Western blot analysis

Cells were lysed in RIPA Radio-Immunoprecipitation Assay buffer (10 mmol/L Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulphate [SDS], 1% NP-40, 1:100 proteinase inhibitor cocktail, 50 mmol/L β -glycerophosphate and 50 mmol sodium fluoride). Twenty-five micrograms of protein of each sample was used for Western blot analysis. The samples were separated on a 15% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes with a semi-dry transfer apparatus (BioRad, Hercules, CA, USA). The membranes were blotted with 5% dehydrated milk for 1 hour and then incubated with primary antibodies overnight. The immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI, USA) and visualized with SuperSignal reagents (Pierce, Rockford, IL, USA). Primary antibodies, a monoclonal antibody against SAPK/JNK (Cat No. 9253), a monoclonal antibody against phospho-SAPK/JNK (Cat No. 4668), a monoclonal antibody against AKT (Cat No. 4685), a polyclonal antibody against phospho-AKT (Cat No. 9271), a monoclonal antibody against extracellular regulated protein kinase 1/2 (ERK1/2) MAPK (Cat No. 4695), a monoclonal antibody against phospho-ERK1/2 MAPK (Cat No. 4377), a monoclonal antibody against P38 MAPK (Cat No. 8690) and a monoclonal antibody against phospho-P38 MAPK (Cat No. 4631) were purchased from Cell Signaling Technology. Other primary antibodies were purchased commercially, including Anti-Periostin antibody (ab14041), Anti-beta Actin antibody (ab8226), Anti-Sp7/Osterix (OSX) antibody (ab22552), Anti-RUNX2 antibody (ab76956), Anti-Osteocalcin (OCN) antibody (ab13418) and Anti-Osteopontin (OPN) antibody (ab8448) from Abcam® (Cambridge, UK).

2.7 | Quantitative real-time reverse transcription PCR (real-time RT-PCR)

Total RNA was isolated using the TRIzol® Reagent (Invitrogen, USA). Two micrograms of total RNA was reverse-transcribed into cDNA using a PrimeScript RTreagent Kit (Takara). A Roche Synthesis Kit was used for amplification of the target genes (Roche, Basel, Switzerland). The thermal cycling conditions were as follows: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes and 95°C for 15 seconds, 60°C for 1 minutes. Primer sequences are provided in the Table 1.

2.8 | Statistics

All statistical calculations were performed using Statistical Product and Service Solutions (version 22.0; SPSS, Inc., Chicago, IL, USA). Student's *t* test or one-way ANOVA was utilized to determine the statistical significance; a *P* ≤ .05 was considered significant.

TABLE 1 The primer sequences used in this study

Gene	Primer pairs	Primer sequence (5'~3')	GenBank accession no.
GAPDH	Sense	gcaccgtcaaggctgagaac	NG_007073
	Antisense	tggtgaagacgccagtga	
Periostin	Sense	tgccagcagttttgcccat	NM_006475
	Antisense	cgttgctctccaaacctcta	
RUNX2	Sense	tcttagaacaattctgcccttt	NM_001024630
	Antisense	tgcttggtcttgaaatcaca	
OSX	Sense	ctcctgcgactgccctaa	NM_152860
	Antisense	gcgaagccttgccataca	
OPN	Sense	atgatggccgaggtgatagt	NM_001040058
	Antisense	accattcaactcctcgcttt	
OCN	Sense	agcaaaggtgcagcctttgt	NM_199173
	Antisense	gcgcctgggtctcttctact	

3 | RESULTS

3.1 | Identification of PDLSCs and transduced PDLSCs

Our previous flow cytometer findings revealed that the obtained PDLSCs were positive for the typical mesenchymal cell surface markers. After 3 weeks of adipogenic induction, the PDLSCs differentiated into adipocytes, as determined by Oil red O staining (Figure S1a). At 3 weeks after the chondrogenesis induction, the PDLSCs differentiated into chondrocytes, as determined by alcian blue staining (Figure S1b). The flow cytometer results showed that the PDLSCs infected with CONT and POSTN shRNA were positive for CD146 and negative for CD34, the depletion of POSTN reduced the percentage of CD146+ cells and increased the percentage of CD34+ cells (Figure S2a-d).

3.2 | TNF- α decreased expression of POSTN in PDLSCs

PDLSCs were treated with 10 ng/mL TNF- α for 1, 2, 4 and 8 hours, the real-time RT-PCR results revealed that TNF- α decreased the POSTN mRNA levels at 1, 2, 4 and 8 hours (Figure 1A). Then, PDLSCs were treated with 10 ng/mL TNF- α for 72 hours, and the Western blot results showed that TNF- α repressed expression of POSTN at the protein level (Figure 1B,C).

3.3 | Knockdown of POSTN attenuated the migration and osteogenic differentiation function of PDLSCs after TNF- α treatment

We designed a shRNA to target POSTN and introduced it into PDLSCs via lentiviral infection. After selection, the knockdown efficiency was confirmed by Western blot and real-time RT-PCR analysis (Figure 1D-F). Then, PDLSCs were treated with 10 ng/mL TNF- α . The scratch

assay and quantitative analysis results showed that depletion of POSTN decreased cell migration compared with the control group at 24 hours with TNF- α treatment (Figure 1G,H). Moreover, depletion of POSTN significantly reduced ALP activity at 3 days after osteogenic induction compared to the control group with TNF- α treatment (Figure 2A). After osteogenic induction for 2 weeks, Alizarin Red staining and quantitative calcium measurements showed that mineralization was markedly inhibited in POSTN-knockdown PDLSCs compared to the control group with TNF- α treatment (Figure 2B,C). Additionally, the real-time RT-PCR results showed that depletion of POSTN significantly decreased expression of RUNX2 and OSX at 7 and 14 days after induction (Figure 2D,E) as well as that of OCN and OPN at 14 days after induction (Figure 2F,G) in PDLSCs compared to the control group with TNF- α treatment. The Western blot findings also showed that depletion of POSTN significantly decreased expression of RUNX2, OSX, OCN and OPN at 14 days after induction (Figure 2H,I) in PDLSCs compared to the control group with TNF- α treatment.

3.4 | POSTN enhanced the osteogenic differentiation and migration function in PDLSCs after TNF- α treatment

To further investigate the effect of POSTN, we used rhPOSTN to stimulate PDLSCs. TNF- α at 10 ng/mL was used to treat PDLSCs. First, we measured the ALP activity of PDLSCs after treatment with 0, 15, 25, 50, 75, 100, 150 and 200 ng/mL of rhPOSTN, at 3 days after osteogenic induction with TNF- α treatment. The results showed that PDLSCs treated with 75 ng/mL rhPOSTN had the highest ALP activity compared with the other groups (Figure 3A). Then, 75 ng/mL rhPOSTN was used to stimulate PDLSCs, and 0 ng/mL rhPOSTN was used as the control. Consistent with this finding, 2 weeks after osteogenic induction with TNF- α treatment, mineralization was markedly enhanced in PDLSCs treated with 75 ng/mL rhPOSTN compared to the control group as determined by Alizarin Red staining and quantitative calcium measurements (Figure 3B,C). Moreover, the real-time RT-PCR results showed that 75 ng/mL rhPOSTN increased expression of RUNX2 and OCN at 7 and 14 days after induction (Figure 3D,F) as well as that of OSX and OPN at 14 days after induction (Figure 3E,G) in PDLSCs compared to the control group with TNF- α treatment. The Western blot findings showed that 75 ng/mL rhPOSTN significantly increased expression of RUNX2, OSX, OPN and OCN at 14 days after induction (Figure 3H,I) in PDLSCs compared to the control group with TNF- α treatment. Further investigation determined that PDLSC migration was significantly accelerated by the addition of 75 ng/mL rhPOSTN compared to the control group with TNF- α treatment (Figure 3J,K).

3.5 | POSTN increased phosphorylation of JNK in PDLSCs after TNF- α treatment

To investigate how POSTN improved the migration and osteogenic differentiation of PDLSCs with TNF- α application, we performed Western blot analysis to examine the levels of proteins, including P38 MAPK, ERK1/2, AKT and JNK. The results showed that the

FIGURE 1 The knockdown of POSTN attenuated the migration in PDLSCs after TNF- α treatment. (A, B) Real-time RT-PCR (A) and Western blot (B) results revealed that 10 ng/mL TNF- α decreased the expression of POSTN. (C) Quantitative analysis of POSTN based on Western blot results for PDLSCs. (D-F) PDLSCs were infected with POSTN shRNA, Western blot (D), quantitative analysis results (E) and real-time RT-PCR (F) results showed the decreased POSTN expression after depletion of POSTN. (G, H) PDLSCs were treated with 10 ng/mL TNF- α , Scratch migration assay (G) and quantitative analysis (H) results revealed that the POSTNsh group showed a significantly decreased rate of wound closure compared with the CONTsh group. GAPDH was used as an internal control. The Student's *t* test was performed to determine statistical significance. The error bars represent the SD (*n* = 3). **P* $\leq$.05. ***P* $\leq$.01. Bar: 200 μ m

knockdown of POSTN inhibited phosphorylation of JNK in PDLSCs at 2 hours after treatment with 10 ng/mL TNF- α , while phosphorylation of P38 MAPK, ERK1/2 and AKT as well as the total protein levels of

JNK, ERK1/2, P38 MAPK and AKT were not affected (Figure 4A,C). To further confirm the signalling pathway involved in POSTN function, PDLSCs were pre-treated with 75 ng/mL rhPOSTN for

FIGURE 2 POSTN knockdown inhibited the osteogenic differentiation in PDLSCs after TNF- α treatment. PDLSCs were treated with 10 ng/mL TNF- α . (A) Alkaline phosphatase (ALP) activity. (B) Alizarin Red staining. (C) Calcium quantitative analysis. (D-G) Real-time RT-PCR results showed the expressions of RUNX2 (D), OSX (E), OCN (F) and OPN (G). GAPDH was used as an internal control. (H) Western blot results and (I) quantitative analysis showed the expressions of RUNX2, OSX, OCN and OPN. β -Actin was used as an internal control. Student's *t* test was performed to determine statistical significance. The error bars represent the SD ($n = 3$). * $P \leq .05$. ** $P \leq .01$

FIGURE 3 POSTN promoted the osteogenic differentiation and migration in PDLSCs after TNF- α treatment. PDLSCs were treated with 10 ng/mL TNF- α . (A) The results of the ALP activity by treated with different concentrations of rhPOSTN. (B) Alizarin Red staining. (C) Calcium quantitative analysis. (D-G) Real-time RT-PCR results showed the expressions of RUNX2 (D), OSX (E), OCN (F) and OPN (G). GAPDH was used as an internal control. (H) Western blot results and (I) quantitative analysis showed the expressions of RUNX2, OSX, OCN and OPN. β -Actin was used as an internal control. (J, K) Scratch migration assay (J) and quantitative analysis (K) results showed that 75 ng/mL rhPOSTN displayed a statistically significant enhancement in cell migration. The Student's *t* test or one-way ANOVA was performed to determine statistical significance. The error bars represent the SD (*n* = 3). **P* $\leq$.05. ***P* $\leq$.01. Bar: 200 μ m

FIGURE 4 POSTN activated Jun amino-terminal kinases (JNK) signalling pathway in PDLSCs after TNF- α treatment. (A) Western blot results showed that knockdown of POSTN inhibited phosphorylation of JNK at 2 h after treatment with 10 ng/mL TNF- α in PDLSCs. (B) PDLSCs were pre-treated with 75 ng/mL rhPOSTN for 30 min, and then treated with 10 ng/mL TNF- α . Western blot results showed that 75 ng/mL rhPOSTN increased phosphorylation of JNK at 1 h after treated with 10 ng/mL TNF- α . β -Actin was used as an internal control. (C, D) Quantitative analysis of p-JNK based on Western blot results for PDLSCs. The Student's *t* test was performed to determine statistical significance. The error bars represent the SD (*n* = 3). **P* $\leq$.05. ***P* $\leq$.01

30 minutes and then treated with 10 ng/mL TNF- α . The Western blot results showed that 75 ng/mL rhPOSTN increased phosphorylation of JNK at 1 hour after the addition of TNF- α , while phosphorylation of P38 MAPK, ERK1/2 and AKT as well as the total protein levels of JNK, ERK1/2, P38 MAPK and AKT were not affected (Figure 4B,D). Interestingly, long-term rhPOSTN treatment with the addition of TNF- α increased phosphorylation of JNK at days 7 and 14, while the total protein levels of JNK were not affected (Figure S3a,b).

3.6 | POSTN-enhanced migration and osteogenic differentiation were repressed by a JNK inhibitor in PDLSCs after TNF- α treatment

PDLSCs were treated with 20 μ mol/L SP600125, a specific inhibitor of JNK, for 48 hours to block JNK signalling, and Western blot analysis confirmed the block's efficiency (Figure 5A,B). Then, 75 ng/mL rhPOSTN was used to treat PDLSCs for 30 minutes, after which the PDLSCs were cultured in MSCM with 20 μ mol/L SP600125, 75 ng/mL rhPOSTN and 10 ng/mL TNF- α . The scratch assay revealed that 20 μ mol/L SP600125 significantly restrained the POSTN-enhanced scratch closure (Figure 5C,D). Similarly, after 3 days of osteogenic

induction, the results showed that 20 μ mol/L SP600125 strongly suppressed ALP activity in PDLSCs (Figure 5E). At 2 weeks after induction, the Alizarin Red staining results revealed that mineralization was significantly decreased after treatment with 20 μ mol/L SP600125 in PDLSCs (Figure 5F,G).

4 | DISCUSSION

Microbially induced inflammatory periodontal diseases affect virtually the entire population, constituting a major public health concern.³⁶ Regeneration of periodontal tissue has become the most promising method for periodontitis treatment. The foremost challenge of MSC-mediated periodontitis treatment is inflammation, and MSCs need to migrate to the exact area and then differentiate. ECM is a dynamic microenvironment for stem cell niche; several ECM proteins can directly or indirectly modulate the maintenance, proliferation, self-renewal and differentiation of stem cells.³⁷ POSTN, is an ECM protein, promotes cell mobility, adhesion and survival.⁵ In present study, we found that the depletion of POSTN reduced the percentage of CD146+ cells and increased the percentage of CD34+ cells in PDLSCs, which suggested

FIGURE 5 JNK inhibition repressed the POSTN-enhanced migration and osteogenic differentiation of PDLSCs after TNF- α treatment. (A) PDLSCs were treated with the JNK inhibitor, 20 μ mol/L SP600125 for 48 h, and then treated with 75 ng/mL rhPOSTN for 30 min. Western blot showed reduction in the phospho-JNK in POSTN-stimulated PDLSCs. β -Actin was used as an internal control. (B) Quantitative analysis of p-JNK based on Western blot results for PDLSCs. (C, D) PDLSCs were pre-treated with 20 μ mol/L SP600125 for 48 h, and then treated with 75 ng/mL rhPOSTN for 30 min, and then PDLSCs were cultured in routine medium with 20 μ mol/L SP600125, 75 ng/mL rhPOSTN and 10 ng/mL TNF- α . The scratch migration assay (C) and quantitative analysis (D) results revealed that SP600125 significantly restrained POSTN-enhanced scratch closure. (E-G) PDLSCs were pre-treated with 20 μ mol/L SP600125 for 48 h, next treated with 75 ng/mL rhPOSTN for 30 min, then PDLSCs were cultured in osteogenic-inducing medium with 20 μ mol/L SP600125, 75 ng/mL rhPOSTN and 10 ng/mL TNF- α . ALP activity (E), Alizarin Red staining (F) and quantitative analysis (G). The one-way ANOVA was performed to determine statistical significance. The error bars represent the SD (n = 3). * P $\leq$.05. ** P $\leq$.01. Bar: 200 μ m

that endogenous POSTN may relate to the maintenance of stem cell biological characteristics. Then we studied the migration and osteogenic differentiation of PDLSCs under inflammatory conditions and we used TNF- α to mimic the inflammatory environment. Previous studies revealed that TNF- α reduces migration and POSTN expression in PDL cells.^{38,39} A former study reported that exogenous POSTN restores TNF- α -suppressed migration of PDL cells.³⁶ These results indicated that suppression of PDL cell migration by TNF- α may be caused by a decrease of POSTN. In the present study, we discovered that TNF- α decreased POSTN expression in PDLSCs at both the mRNA and protein levels, which indicates that POSTN may play an important role in PDLSCs under inflammatory conditions. What is more, the knockdown of POSTN led to a reduction of the migration and osteogenic differentiation functions in PDLSCs with the addition of TNF- α . By contrast, rhPOSTN dramatically increased the migration and osteogenic differentiation potential of PDLSCs under TNF- α treatment. These findings strongly suggest that POSTN played a positive role in regulating the migration and osteogenic differentiation of PDLSCs under inflammatory conditions.

Then, we investigated how POSTN improves migration and osteogenic differentiation of PDLSCs under inflammatory conditions. Previous studies have reported that POSTN promotes cell mobility, adhesion and survival in a number of cell types by binding to integrin $\alpha\beta 3$ and $\alpha\beta 5$ via focal adhesion kinase (FAK) phosphorylation and AKT phosphorylation in the integrin-mediated signalling pathway.²³⁻²⁵ According to previous research, the differentiation and inflammatory response of PDLSCs or PDL cells could be affected through the P38 and ERK1/2 signalling pathways by a number of stimulatory factors.³¹⁻³⁴ Additionally, our previous study found that POSTN acted on PDLSCs via the integrin signalling pathway; the integrin inhibitor Cilengitide represses POSTN-enhanced cell migration.⁵ Therefore, we wondered whether MAPK and AKT are involved in POSTN-mediated PDLSC migration and osteogenic differentiation under inflammatory conditions. Our results revealed that under TNF- α treatment, POSTN-knockdown inhibited phospho-JNK in PDLSCs, while rhPOSTN increased the expression of phospho-JNK in PDLSCs. To further confirm the role of JNK signalling in the effect of POSTN on PDLSCs, the inhibitor SP600125 was used to block the JNK signalling pathway. The results showed that inhibition of JNK repressed POSTN-enhanced scratch closure, ALP activity and mineralization in PDLSCs under TNF- α treatment. These discoveries indicated that POSTN enhances the migration and osteogenic differentiation of PDLSCs via the JNK signalling pathway under inflammatory conditions. However, further investigation will be carried out to elucidate the precise molecular mechanism. It is possible that the observed in vitro changes may not correlate with corresponding changes in vivo, so further study will be performed in vivo to investigate the POSTN function and possible heterogeneity between in vivo and in vitro experiments.

In conclusion, our results demonstrated that POSTN promoted the migration and osteogenic differentiation of PDLSCs and activated the JNK signalling pathway in response to TNF- α challenge. Importantly, application of a specific JNK inhibitor dramatically

weakened the POSTN-enhanced migration and osteogenic differentiation of PDLSCs. This study elucidated the molecular mechanisms underlying the migration and osteogenic differentiation of MSCs, showing that POSTN prompted the migration and osteogenic differentiation potential of PDLSCs via the JNK signalling pathway, revealing the underlying mechanism of POSTN-enhanced functions in MSCs and identifying a potential target for improving periodontal tissue regeneration based on MSCs under inflammatory conditions.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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