Article

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PAR1 Activation, via LMBR1/BMP Axis, Promotes the Osteogenesis of Periodontal Ligament Stem Cells (PDLSCs) and Alleviates the Inhibitory Effect of Sodium Butyrate on PDLSCs Osteogenesis

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Background: Periodontitis is the leading cause of tooth loss and can exacerbate various systemic inflammatory conditions. Periodontal ligament stem cells (PDLSCs) stand out as prominent and favorable candidates for promoting periodontal tissue regeneration. This study aimed to investigate whether the protease-activated receptor type 1 (PAR1) can mitigate the sodium butyrate (NaB)-induced PDLSCs osteogenesis inhibition and unravel the underlying mechanism.

Methods: Public datasets from the Gene Expression Omnibus (GEO) were utilized to analyze differentially expressed genes (DEGs) in periodontitis and subsequent Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. PDLSCs were cultured normally in control medium (CM) as the negative control or in osteogenic medium (OM) to induce osteogenesis. PAR1 was either activated or suppressed using a selective agonist or antagonist (OM+agonist and OM+antagonist). The evaluation of PDLSCs osteogenesis was based on the levels of osteogenesis-related markers, including runt-related transcription factor 2 (RUNX2), osterix (OSX), osteocalcin (OCN), and osteopontin (OPN), alkaline phosphatase (ALP) activity, and calcium concentration. Additionally, cell proliferation and osteogenic differentiation were measured through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Alizarin Red Staining. To determine the PAR1 targeting the limb development membrane protein 1 (LMBR1)/bone morphogenetic protein (BMP) pathway, LMBR1 was upregulated through cell transfection and BMP2 was inhibited using the selective inhibitor Noggin protein. Finally, NaB was introduced into PDLSCs to investigate the effect on NaB-induced inhibition of PDLSCs osteogenesis.

Results: PAR1, RUNX2, OSX, OCN, OPN, proliferation, ALP activity, calcium concentration, osteogenic differentiation, BMP2, and BMP4 exhibited significant increases in PDLSCs cultured in OM ($p < 0.01$). These parameters were further elevated by PAR1 agonist and conversely reduced by PAR1 antagonist ($p < 0.01$). Conversely, LMBR1 was decreased in PDLSCs cultured in OM ($p < 0.001$), with further reduction induced by PAR1 agonist and a reverse increase observed with PAR1 antagonist ($p < 0.001$). OE-LMBR1 transfection successfully elevated LMBR1 levels, subsequently inhibiting BMP2 and BMP4 ($p < 0.001$). Meanwhile, the Noggin protein effectively suppressed BMP2 and BMP4 ($p < 0.001$). All observed osteogenesis-related changes were reversed by the increased LMBR1 or inhibition of the BMP pathway ($p < 0.001$). Furthermore, NaB suppressed osteogenesis-related changes in OM-cultured PDLSCs ($p < 0.001$), and these effects were entirely reversed by PAR1 agonist ($p < 0.001$). Conversely, the increased LMBR1 or inhibited BMP pathway disrupted the osteogenesis reversion induced by PAR1 agonist ($p < 0.001$).

Conclusion: The activation of PAR1, through suppressing LMBR1 signaling and activating BMP pathway, demonstrates the ability to enhance the osteogenesis of PDLSCs and mitigate the inhibitory effects on PDLSCs osteogenesis caused by NaB.

Keywords: PDLSCs; osteogenesis; PAR1; LMBR1; BMP; sodium butyrate; periodontitis

Introduction

Periodontitis, recognized as a destructive gum disease, is a chronic inflammatory condition linked to microbial factors and mainly caused by polymicrobial infection, leading to the impairment of periodontal tissue integrity [1,2]. Periodontitis has gained recognition as one of the six most prevalent non-communicable diseases globally [3,4]. In China, more than 90% of adults grapple with periodontal diseases [4], while globally, 70% of adults aged 65 years or older are affected by periodontitis [5].

During periodontal diseases, microorganisms adhere to tooth surfaces, triggering an aggressive immune response and ultimately resulting in tooth loosening and loss [6]. The high and continually increasing incidence of periodontitis is a primary contributor to tooth loss [7]. More signifi-

cantly, periodontitis not only leads to tooth loss but also exacerbates various systemic inflammatory conditions such as diabetes, osteoporosis, rheumatoid arthritis, chronic obstructive pulmonary disease, cardiovascular disease, and more [5]. Beyond the health burden imposed by periodontal diseases, there is a substantial economic impact. Reports indicated that in 2018, the cost of periodontal diseases amounted to €2.5 billion directly and €158.64 billion indirectly in Europe, and $3.49 billion directly and $154.06 billion indirectly in the USA [8].

Given these implications, effective treatments for periodontitis are crucial not only for alleviating adverse impacts but also for enhancing the overall life quality of the population. Currently, the widely employed clinical treatments for periodontitis include the non-surgical approach of guided tissue regeneration (GTR) to eliminate the infection's cause and the surgical method of guided bone regeneration (GBR) to directly achieve regeneration [9]. However, these treatments have been proven insufficient in providing long-term stable and complete regeneration of both soft and hard periodontal structures, often resulting in incomplete periodontal structure regeneration or the formation of a long junctional epithelium [10].

In contrast, the use of cell sheets has emerged as a promising therapy, wherein stem cells facilitate contact between interconnected cells along with their matricellular matrix [11,12]. Among various applications of cell sheets, the utilization of periodontal ligament stem cells (PDLSCs) stands out, as they have demonstrated efficacy in stimulating periodontal tissue regeneration by simulating the natural environment of the periodontal ligament and enhancing signal communications [10]. Moreover, PDLSCs are preferred candidates in clinical therapies due to their potential for proliferation, self-renewal, homing, tissue repair, and most importantly, their superior differentiation ability [13].

During periodontal diseases, cytotoxins and metabolites produced by bacteria contribute to the defense response, leading to increased secretion of protein-rich gingival crevicular fluid. This, in turn, fosters the growth of pathogenic bacteria within periodontal tissues [14]. The short chain fatty acids (SCFAs) continue a major type of metabolic products secreted by pathogenic bacteria, with butyrate being found at elevated concentrations in the gingival crevicular fluid of periodontitis patients [15–17]. Previous studies have established that butyrate disrupts gingival epithelial cell homeostasis [18,19], induces gingival epithelial cell autophagy and death [20], promotes gingival fibroblast apoptosis, and amplifies inflammatory responses [21]. Furthermore, sodium butyrate (NaB) has been demonstrated to inhibit osteogenesis in human PDLCSs [22], thereby compromising the potential use of PDLSCs in cell-based treatment in mediating periodontal regeneration [23].

The protease-activated receptor type 1 (PAR1), a G protein-coupled cell membrane receptor, plays roles in enhancing osteogenic differentiation, increasing mineralized matrix deposition, and promoting cementogenic gene expression by mediating related signaling pathways [24]. Previous claims have indicated that the activation of PAR1 exerted effects on osteogenesis and bone regeneration [25]. Additionally, PAR1 has been associated with coagulatory and vascular processes during the early stages of bone healing [26]. Furthermore, PAR1 has been proved to be able to enhance osteogenic activity in human PDLSCs both *in vivo* [10] and *in vitro* [27].

The precise mechanisms underlying the promotion of PAR1 on PDLSCs osteogenesis are not fully understood, and whether it can rescue PDLSCs osteogenesis from suppression by NaB remains unclear. In the present study, we investigated whether PAR1 plays a role in ameliorating the inhibition of NaB on PDLSCs osteogenesis and further delved into the underlying mechanisms.

Materials and Methods

Bioinformatics

The datasets were sourced from the public functional genomics database Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) using search keywords "periodontitis" and "gingival tissue". The criteria for selecting suitable datasets were as follows: each dataset included a minimum of 30 samples, comprising at least 15 cases and 15 controls, and the data were obtained through microarray analysis. Eventually, the datasets GSE16134 and GSE10334 were chosen for analysis in the present study [28].

Differentially expressed genes (DEGs) were identified using the GEO online analysis tool GEO2R, with genes meeting the criteria of p-values < 0.05 and $|\log_2$ (fold change)$| > 1$ considered as DEGs [29]. The obtained DEGs were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the ClusterProfiler package (version 4.8.2) in the R computing environment (R Core Team, Boston, MA, USA), with significance determined by p-values < 0.05 [30]. The top 20 enriched pathways were visualized using the ggplot2 package (version 3.3.2) [31].

Cell Culture and Identification

Human PDLSCs (HUM-iCell-m002, iCell Bioscience Inc., Shanghai, China) were acquired and routinely cultured in alpha-modified Eagle's medium (α-MEM; iCell-0003, iCell Bioscience Inc., Shanghai, China) supplemented with fetal bovine serum (10%; iCell-0500, iCell Bioscience Inc., Shanghai, China), penicillin/streptomycin (100 µg/mL; iCell-15140-122, iCell Bioscience Inc., Shanghai, China), and Amphotericin B (0.5 mg/mL; HY-B0221, MedChemExpress, Monmouth Junction, NJ, USA). Culturing conditions were maintained at 37 °C in a 95% humidified atmosphere with 5% CO_2. Upon reaching 70% confluence, cells were trypsinized and subcultured. PDLSCs from passages

3–5 were utilized for subsequent experiments. Cell authentication was performed using a short tandem repeat (STR) test, and mycoplasma testing was conducted to detect cell contamination.

Cell Transfection

The empty hU6-MCS-CMV-Puromycin lentiviral vectors (OE-NC) and hU6-MCS-CMV-Puromycin lentiviral vectors containing the full-length cDNA of limb development membrane protein 1 (*LMBR1*) (OE-*LMBR1*; Gene ID: 64327) were procured from GeneChem (Order No. GOSE0586539; Shanghai, China). Lentivirus was added to the medium at a ratio of 1:125 and transfected into PDLSCs for 48 hours. Transfection efficacies were assessed before proceeding with subsequent experiments.

Experimental Groups and Treatments

PDLSCs cultured normally in control medium were designed as the group of CM [27]. The osteogenic medium consists of the control medium along with dexamethasone (100 nM; HY-14648, MedChemExpress, Monmouth Junction, NJ, USA), β-glycerophosphate (5 mM; ST637, Beyotime, Shanghai, China), and Vitamin C (50 µg/mL; ST1434, Beyotime, Shanghai, China). PDLSCs cultured in this osteogenic medium were labeled as the group of OM. PDLSCs cultured in osteogenic medium with the addition of the PAR1-selective agonist TFLLR-NH2 (100 nM; HY-P0226, MedChemExpress, Monmouth Junction, NJ, USA) were denoted as the OM+agonist group. Similarly, PDLSCs cultured in osteogenic medium with the supplementation of the PAR1-selective antagonist RWJ-56110 (100 nM; HY-108556, MedChemExpress, Monmouth Junction, NJ, USA) were termed the OM+antagonist group. To inhibit the activation of the bone morphogenetic protein (BMP) pathway, the BMP2 inhibitor Noggin protein (2 µg/mL; HY-P70558, MedChemExpress, Monmouth Junction, NJ, USA) was added to the medium. PDLSCs cultured in osteogenic medium with the further addition of sodium butyrate (1 mM; S1539, Beyotime, Shanghai, China) were designated as the OM+NaB group.

Cell Proliferation, Alkaline Phosphatase (ALP) and Calcium Concentration

Cell proliferation was assessed after 48 hours of culture with specific treatments using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (ab211091, Abcam, Cambridge, MA, USA) following the manufacturer's instructions. After 14 days of culturing cells with specific treatments, the supernatant was collected for the measurement of alkaline phosphatase (ALP) activity using the ALP enzyme-linked immunosorbent assay (ELISA) kit (ab263890, Abcam, Cambridge, MA, USA). Additionally, the calcium concentration in the medium supernatant was determined with the calcium colorimetric assay kit (E-BC-K103-M, Elabscience Biotechnology Co., Ltd., Wuhan, China). Absorbance at 405 nm during these measurements was recorded using a microplate reader (CMax Plus, Molecular Devices, Shanghai, China).

Alizarin Red Staining

The osteogenic differentiation of PDLSCs was assessed using the Alizarin Red Staining kit (C0148S, Beyotime, Shanghai, China) after 14 days of culture, following the manufacturer's instructions. The results were visualized using a microscope (CKX53, OLYMPUS, Tokyo, Japan), and the absorbance at 405 nm was measured using a microplate reader (CMax Plus, Molecular Devices, Shanghai, China).

Protein Levels Detected by Enzyme-Linked Immunosorbent Assay (ELISA)

After 14 days of culture with specific treatments, the protein levels in PDLSCs were quantified using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits for PAR1 (ab283544, Abcam, Cambridge, MA, USA), limb development membrane protein 1 (LMBR1; abx531171, Abbexa Ltd., Milton, Cambridge, UK), bone morphogenetic protein 2 (BMP2; ab119581, Abcam, Cambridge, MA, USA), bone morphogenetic protein 4 (BMP4; ab231930, Abcam, Cambridge, MA, USA), runt-related transcription factor 2 (RUNX2; JL20081, Jianglai, Shanghai, China), osterix (OSX; JL15982, Jianglai, Shanghai, China), osteocalcin (OCN; ab270202, Abcam, Cambridge, MA, USA), and osteopontin (OPN; PO809, Beyotime, Shanghai, China).

Briefly, after the 14-day culture period, cells were washed with phosphate-buffered saline (PBS; C0221A, Beyotime, Shanghai, China) and treated with trypsin (15090046, Gibco, Grand Island, NY, USA). Following centrifugation at 1000 $\times$g for 5 minutes, the cells were collected, washed with PBS three times, and re-suspended in PBS (1×10^6 cells/200 µL PBS) containing a protease inhibitor cocktail (HY-K0010, MedChemExpress, Monmouth Junction, NJ, USA). After centrifugation of the cell suspension at 1500 $\times$g for 10 minutes, the supernatant was collected, and the protein levels in the cells were measured according to the instructions of the corresponding ELISA kit.

Expression of Osteogenesis-Related Genes in PDLSCs

Gene expression levels were determined using real-time reverse transcriptase-polymerase chain reaction (RT-qPCR). After 14 days of culture, total RNA was extracted with TRIzol reagent (1 mL; R0016, Beyotime, Shanghai, China) and reverse transcribed into complementary DNA (cDNA) using the BeyoRT™ Q First Strand cDNA Synthesis kit (D7190M, Beyotime, Shanghai, China).

Table 1. Primer sequences used in RT-qPCR.

Name	Sequence (5′-3′)
PAR1-F	TGTGAACTGATCATGTTTATG
PAR1-R	TTCGTAAGATAAGAGATATGT
RUNX2-F	TCGCCAGGCTTCATAGCAAA
RUNX2-R	GGCCTTGGGTAAGGCAGATT
OSX-F	CCTCCTCAGCTCACCTTCTC
OSX-R	GTTGGGAGCCCAAATAGAAA
OCN-F	CCTGACTGCATTCTGCCTCT
OCN-R	TCGTCACAATTGGGGTTGAG
OPN-F	AGCCACATCGCTCAGACACC
OPN-R	TGAAATTCATGGCTGTGGAA
LMBR1-F	GCGGGAGTCCACGATATGTTT
LMBR1-R	GCTGACACTGCGAGAGTGAA
BMP2-F	ACCCGCTGTCTTCTAGTGTTG
BMP2-R	TTCTTCGTGATGGAAGCTGAG
BMP4-F	ACTTCGAGGCGACACTTCTG
BMP4-R	GTCCACCTGCTCCCGAAATA
GAPDH-F	TCAACAGCGACACCCACTC
GAPDH-R	GCTGTAGCCAAATTCGTTGTC

RT-qPCR, the real-time reverse transcriptase-polymerase chain reaction; *PAR1*, protease-activated receptor type 1; *LMBR1*, limb development membrane protein 1; *BMP2*, bone morphogenetic protein 2; *BMP4*, bone morphogenetic protein 4; *RUNX2*, runt-related transcription factor 2; *OSX*, osterix; *OCN*, osteocalcin; *OPN*, osteopontin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

RT-qPCR was carried out using the BeyoFast™ SYBR Green qPCR Mix (D7260, Beyotime, Shanghai, China) on a qPCR instrument (LightCycler96, Roche, Shanghai, China) with the following conditions: 95 °C (10 minutes), 40 cycles of 95 °C (15 seconds), and 60 °C (1 minute), followed by a final step at 95 °C (20 minutes). Gene expression was normalized to the *GAPDH* gene, and the $2^{-\Delta\Delta Cq}$ method was employed for data analysis. Table 1 provides the primer sequences used in RT-qPCR.

Western Blot

After 14 days of culture, total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (P0013, Beyotime, Shanghai, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF; ST505, Beyotime, Shanghai, China). The proteins were then loaded onto an SDS-PAGE gel (10%, NP0346BOX, Invitrogen, Carlsbad, CA, USA) for electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, Billerica, MA, USA).

Primary antibodies used included anti-PAR1 (1:1000 dilution; ab117749, Abcam, Cambridge, MA, USA), anti-LMBR1 (1:1000 dilution; PA5-43411, Invitrogen, Carlsbad, CA, USA), and anti-GAPDH (1:1000 dilution; ab8245, Abcam, Cambridge, MA, USA). Subsequently, a horseradish peroxidase (HRP) conjugated secondary antibody (1:2000 dilution; ab205719, Abcam, Cambridge, MA, USA) was employed for further incubation. Reactive bands were visualized using enhanced chemiluminescence (ECL) reagent (P0018S, Beyotime, Shanghai, China), and grayscale analysis was performed using ImageJ software (1.48, National Institutes of Health, Rockville, MD, USA).

Statistical Analysis

All data were analyzed using GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA, USA) and are presented as the mean ± standard deviation (mean ± SD). Group comparisons were performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A significance level of $p < 0.05$ was considered statistically significant.

Results

Osteoclast Differentiation is Closely Related to Periodontitis

The results of KEGG pathway enrichment based on the DEGs in GSE16134 (Fig. 1A) and GSE10334 (Fig. 1B) demonstrated that osteoclast differentiation was enriched by DEGs in both datasets, highlighting the significance of osteoclastogenesis in periodontitis. This finding indicates the relevance of periodontitis to bone homeostasis, which depends on the interplay between osteoblast differentiation supporting osteogenesis and osteoclast differentiation supporting osteoclastogenesis. Additionally, the enrichment of the Interleukin (IL)-17 signaling pathway further supports the association of periodontitis with inflammatory responses.

PAR1 is Related to the Osteogenesis of PDLSCs

As illustrated, upon culturing PDLSCs in osteogenic medium (OM) to induce osteogenesis, the expression of *PAR1* significantly increased at both the mRNA level (Fig. 2A; $p < 0.001$) and the protein level (Fig. 2B; $p < 0.001$). This preliminary finding suggests a potential association between PAR1 and the osteogenesis of PDLSCs.

PAR1 Activation Promotes the Osteogenesis of PDLSCs

In Fig. 3A,B, it is evident that when PDLSCs were cultured in osteogenic medium, both *PAR1* mRNA level ($p < 0.001$) and PAR1 protein level ($p < 0.001$) increased, consistent with the observations in Fig. 2. Additionally, within the context of the osteogenic medium, further treatment with the PAR1 selective agonist led to additional increases in *PAR1* mRNA (Fig. 3A; $p < 0.01$) and PAR1 protein (Fig. 3B; $p < 0.001$) compared to the OM group. Conversely, compared to the OM group, treatment with the PAR1 selective antagonist significantly decreased *PAR1* mRNA ($p < 0.001$) and PAR1 protein ($p < 0.001$) levels.

Furthermore, the alterations in PAR1 levels were accompanied by changes in the levels of osteogenesis-related

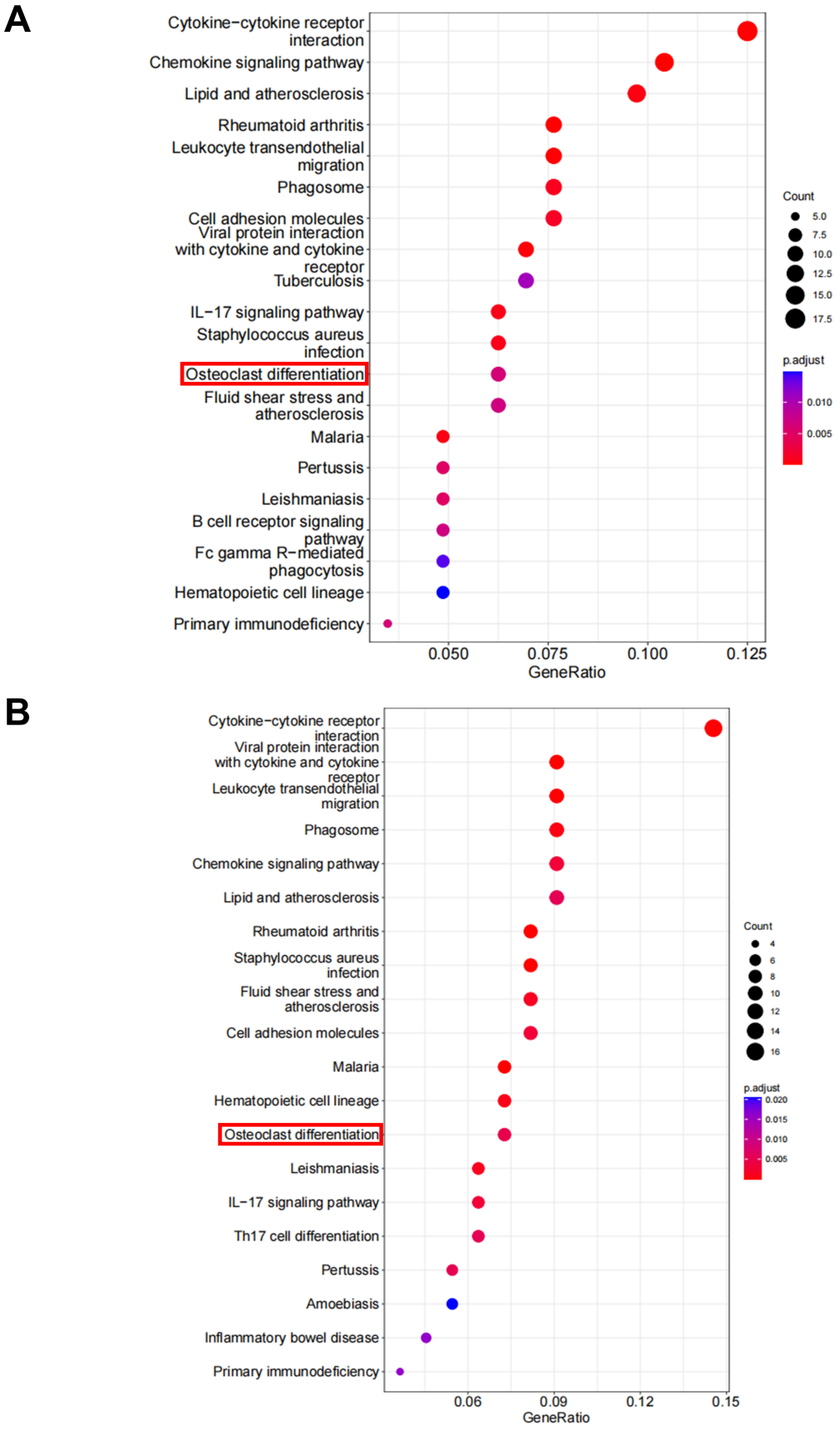

Fig. 1. Osteoclast differentiation is closely related to periodontitis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed on differentially expressed genes (DEGs) obtained by (A) GSE16134 and (B) GSE10334.

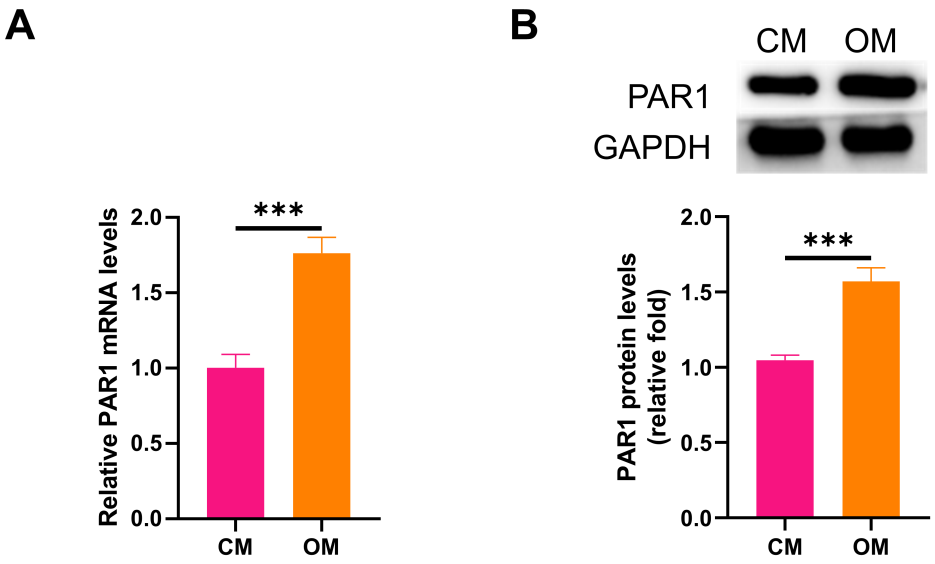

Fig. 2. PAR1 is related to the osteogenesis of PDLSCs. The levels of (A) *PAR1* mRNA and (B) PAR1 protein in PDLSCs cultured in different medium (n = 5). PDLSCs, periodontal ligament stem cells; PAR1, protease-activated receptor type 1; CM, PDLSCs cultured normally in control medium; OM, PDLSCs cultured in osteogenic medium. ***$p < 0.001$.

genes. In comparison to PDLSCs in the control medium (CM) group, PDLSCs in the OM group (with increased PAR1 levels) exhibited higher levels of *RUNX2* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3C, $p < 0.001$), *OSX* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3D, $p < 0.001$), *OCN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3E, $p < 0.001$), and *OPN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3F, $p < 0.001$), indicating that PAR1 activation promotes the expression of these osteogenesis-related genes. This observation was further supported by the comparison of PDLSCs in the OM group with those in the OM+agonist group (where PDLSCs had further increased levels of PAR1), revealing additional increases in the levels of *RUNX2* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3C, $p < 0.001$), *OSX* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3D, $p < 0.001$), *OCN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3E, $p < 0.001$), and *OPN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3F, $p < 0.001$). Conversely, compared with the OM group, the decreased level of PAR1 in the OM+antagonist group resulted in decreased levels of *RUNX2* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3C, $p < 0.001$), *OSX* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3D, $p < 0.001$), *OCN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3E, $p < 0.001$), and *OPN* (mRNA: Fig. 3A, $p < 0.001$; protein: Fig. 3F, $p < 0.001$).

These collective results demonstrate that PAR1 activation, which promotes the expression of osteogenesis-related genes (*RUNX2*, *OSX*, *OCN*, and *OPN*), enhances the osteogenesis of PDLSCs. Moreover, the results in Fig. 3G illustrate that, compared with PDLSCs in the CM group, PDLSCs in the OM group exhibited increased proliferation (as indicated by increased absorbance; Fig. 3G, $p < 0.001$). Furthermore, compared with PDLSCs in the OM group, cell proliferation was further increased in the OM+agonist group ($p < 0.001$) while decreased in the OM+antagonist group ($p < 0.001$). The results, along with the observed alterations in PAR1 levels among groups, underscore the role of PAR1 in promoting the differentiation of PDLSCs.

The results in Fig. 4 further substantiate the effect of PAR1 activation in promoting PDLSCs osteogenesis. When compared to the CM group, the ALP activity (Fig. 4A, $p < 0.001$), calcium concentration (Fig. 4B, $p < 0.001$), and osteogenic differentiation (Fig. 4C,D, $p < 0.001$) were all increased in the OM group. Furthermore, these parameters were further heightened in the OM+agonist group (ALP activity, $p < 0.001$; calcium concentration, $p < 0.001$; osteogenic differentiation, $p < 0.001$) and reduced in the OM+antagonist group (ALP activity, $p < 0.01$; calcium concentration, $p < 0.01$; osteogenic differentiation, $p < 0.001$) when compared to the OM group. These findings provide additional confirmation that PAR1 activation enhances the osteogenesis of PDLSCs.

PAR1 Activation Promotes PDLSCs Osteogenesis by Inhibiting LMBR1 Pathway and Activating BMP Pathway

In Fig. 5A, the *LMBR1* mRNA was significantly decreased in the OM group ($p < 0.001$) and further reduced in the OM+agonist group ($p < 0.001$), while increased in the OM+antagonist group ($p < 0.001$). Simultaneously, *BMP2* and *BMP4* mRNA levels were elevated in the OM group (BMP2, $p < 0.001$; BMP4, $p < 0.001$) and further increased in the OM+agonist group (*BMP2*, $p < 0.001$; *BMP4*, $p < 0.001$), while decreased in the OM+antagonist group

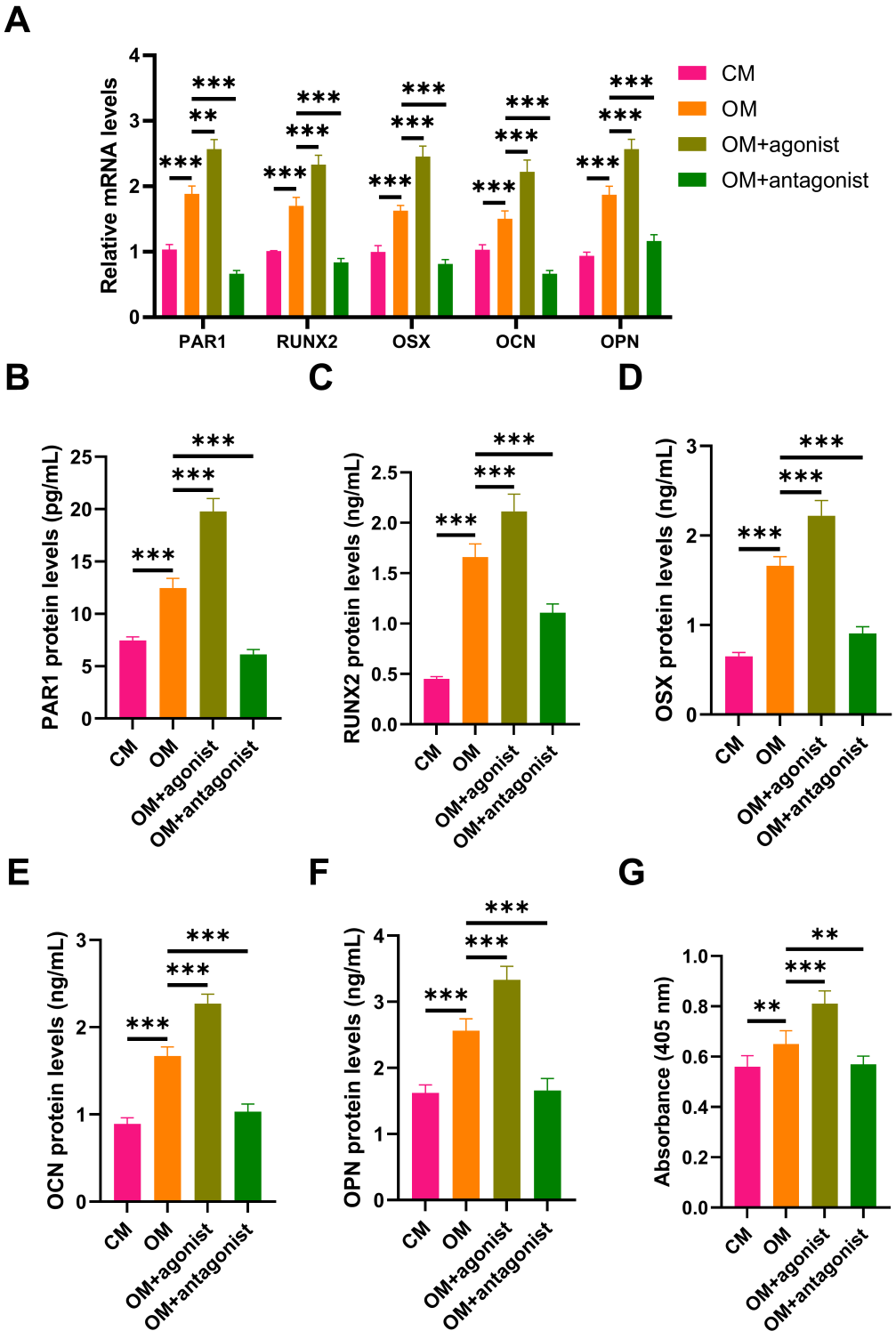

Fig. 3. PAR1 activation promotes the osteogenesis of PDLSCs. (A) The mRNA levels of *PAR1* and osteogenesis-related genes (n = 5). The protein levels of (B) PAR1, (C) RUNX2, (D) OSX, (E) OCN and (F) OPN (n = 5). (G) The proliferation of PDLSCs determined by the absorbance at 405 nm (n = 5). *RUNX2*, runt-related transcription factor 2; *OSX*, osterix; *OCN*, osteocalcin; *OPN*, osteopontin; OM+agonist, PDLSCs cultured in osteogenic medium supplemented with PAR1-selective agonist TFLLR-NH2 (100 nM); OM+antagonist, PDLSCs cultured in osteogenic medium supplemented with PAR1-selective antagonist RWJ-56110 (100 nM). $^{**}p < 0.01$, $^{***}p < 0.001$.

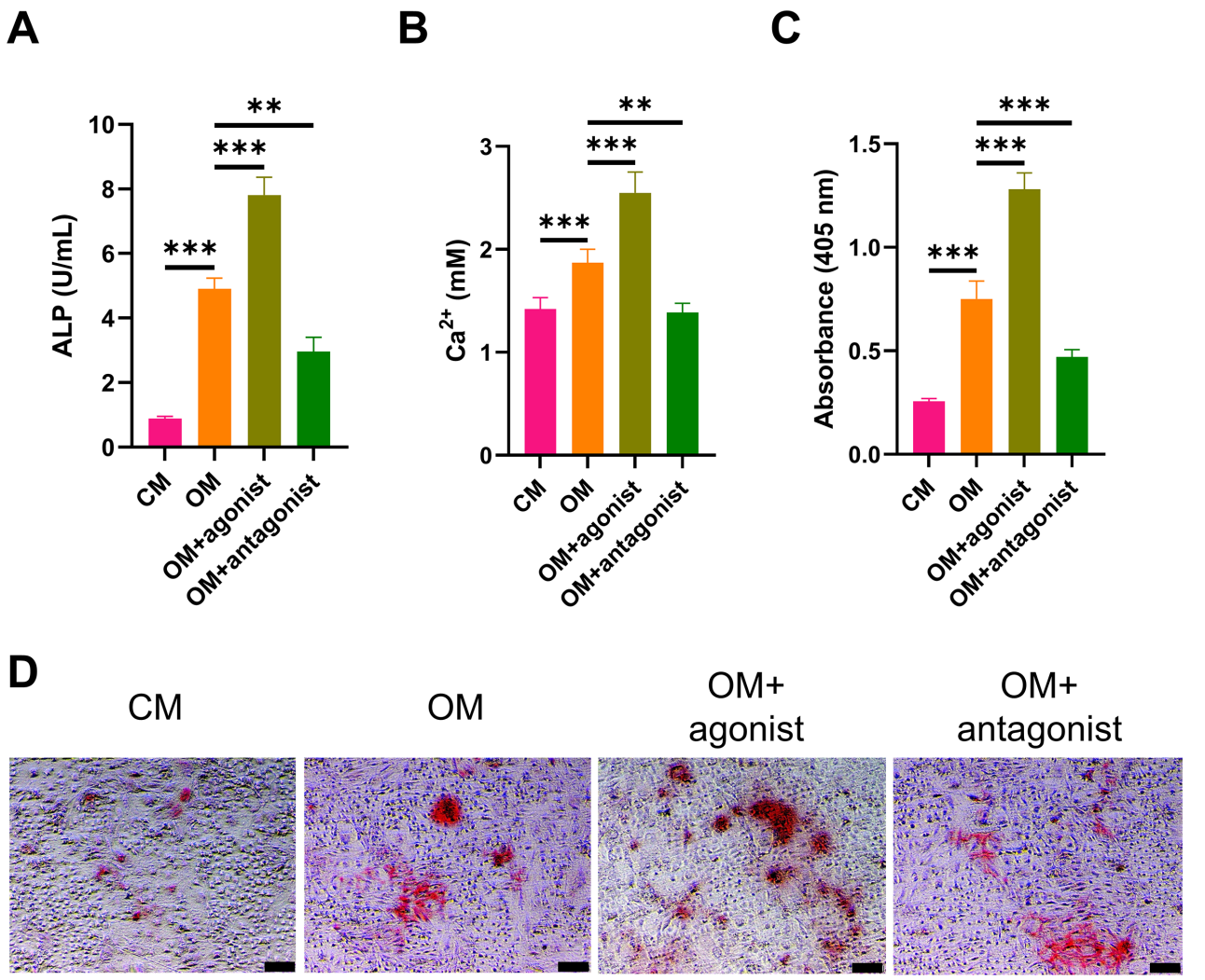

Fig. 4. PAR1 activation promotes the osteogenesis of PDLSCs. The (A) ALP activity and (B) the deposited calcium concentration different groups (n = 5). The (C) quantification (n = 5) and (D) representative images of PDLSCs osteogenic differentiation detected by Alizarin Red Staining (scale bar: 100 µm). ALP, alkaline phosphatase. $^{**}p < 0.01$, $^{***}p < 0.001$.

(*BMP2*, $p < 0.001$; *BMP4*, $p < 0.001$). These findings indicate that PAR1 activation inhibits the LMBR1 pathway while promoting the BMP pathway in PDLSCs.

Fig. 5B confirms the regulation of PAR1 on the LMBR1/BMP axis. The LMBR1 protein was decreased in the OM group ($p < 0.001$) and further reduced in the OM+agonist group ($p < 0.001$), while increased in the OM+antagonist group ($p < 0.001$). Additionally, BMP2 and BMP4 proteins were elevated in the OM group (BMP2, $p < 0.001$; BMP4, $p < 0.001$) and further increased in the OM+agonist group (BMP2, $p < 0.001$; BMP4, $p < 0.001$), while decreased in the OM+antagonist group (BMP2, $p < 0.001$; BMP4, $p < 0.001$).

For activating the LMBR1 pathway, transfection was utilized to increase the expression and level of LMBR1. The transfection of OE-LMBR1 significantly increased the levels of *LMBR1* mRNA (Fig. 5C, $p < 0.001$) and LMBR1 protein (Fig. 5D, $p < 0.001$), indicating the successful transfection of OE-*LMBR1* and the activation of the LMBR1 pathway.

Furthermore, PAR1 was promoted by its selective agonist (Fig. 5E, $p < 0.001$), and this effect was not further affected by the transfection of OE-*LMBR1* (Fig. 5E, $p > 0.05$). The Noggin protein (Fig. 5E, $p > 0.05$) did not significantly impact PAR1 expression. LMBR1 was inhibited by increased PAR1 (Fig. 5F, $p < 0.001$), promoted by the transfection of OE-*LMBR1* (Fig. 5F, $p < 0.001$), but not affected by Noggin (Fig. 5F, $p > 0.05$). BMP2 (Fig. 5G) and BMP4 (Fig. 5H) were promoted by increased PAR1 (BMP2, $p < 0.001$; BMP4, $p < 0.001$), inhibited by the transfection of OE-*LMBR1* (BMP2, $p < 0.001$; BMP4, $p < 0.001$), and suppressed by Noggin (BMP2, $p < 0.001$; BMP4, $p < 0.001$).

Collectively, Fig. 5E–H demonstrate that compared to the OM group, the OM+agonist group exhibited promotion of PAR1 and BMP signaling, while the LMBR1 pathway was inhibited. When compared to the OM+agonist/OM+agonist+OE-NC group, the OM+agonist+OE-*LMBR1* group maintained unchanged PAR1 levels, activated the LMBR1 pathway, but inhibited the BMP pathway. Conversely, in the OM+agonist+Noggin group, PAR1 and LMBR1 pathways remained unchanged, while the BMP pathway was inhibited. These results collectively indicate that the BMP pathway is downstream of the LMBR1 pathway and is negatively regulated by the LMBR1 pathway.

In comparison to the OM group, the increased PAR1, along with induced LMBR1 inhibition and BMP activation in OM+agonist, led to an elevation in osteogenesis-related genes (Fig. 6A; all $p < 0.001$), PDLSC proliferation (Fig. 6B; $p < 0.001$), ALP activity (Fig. 6C; $p < 0.001$), calcium concentration (Fig. 6D; $p < 0.001$), and osteogenic differentiation (Fig. 6E,F; $p < 0.001$). These effects were reversely decreased in the OM+agonist+OE-LMBR1 group (where LMBR1 was activated and BMP was inhibited; $p < 0.001$) and in the OM+agonist+Noggin group (where BMP was inhibited; $p < 0.001$). These results provide evidence that PAR1 activation promotes PDLSC osteogenesis by successfully inhibiting the LMBR1 pathway and activating the BMP pathway.

PAR1 Activation Alleviates the NaB-Induced Suppression on PDLSCs Osteogenesis by Inhibiting LMBR1 Pathway and Activating BMP Pathway

Compared to the OM group, the addition of NaB resulted in a significant decrease in PAR1 levels (Fig. 7A, $p < 0.001$), subsequently leading to an increase in LMBR1 levels (Fig. 7B, $p < 0.001$), and a decrease in levels of BMP2 (Fig. 7C, $p < 0.001$) and BMP4 (Fig. 7D, $p < 0.001$). These effects were all reversed by the additional treatment of the PAR1 agonist (increased PAR1, $p < 0.01$; decreased LMBR1, $p < 0.001$; increased BMP2, $p < 0.001$; increased BMP4, $p < 0.001$).

Based on the treatments of OM, NaB, and PAR1 agonist, further OE-LMBR1 transfection did not alter the PAR1 level ($p > 0.5$) but increased LMBR1 ($p < 0.001$), and decreased BMP2 ($p < 0.001$) and BMP4 ($p < 0.001$). Similarly, further Noggin treatment did not affect the levels of PAR1 ($p > 0.5$) and LMBR1 ($p > 0.5$), but decreased BMP2 ($p < 0.001$) and BMP4 ($p < 0.001$).

In summary, in the OM+NaB group, PAR1 was decreased, the LMBR1 pathway was activated, and the BMP pathway was inhibited compared to the OM group. These effects were reversed in the OM+NaB+agonist group. Moreover, compared to the OM+NaB+agonist group, PAR1 remained unchanged, the LMBR1 pathway was activated, and the BMP pathway was inhibited in the OM+NaB+agonist+OE-*LMBR1* group, while PAR1 and the LMBR1 pathway remained unchanged, and the BMP pathway was inhibited in the OM+NaB+agonist+Noggin group.

The treatment with NaB led to a significant decrease in protein levels of osteogenesis-related genes (Fig. 7E–H, $p < 0.001$), all of which were subsequently increased in a reverse manner by the additional administration of PAR1 agonist ($p < 0.001$). The osteogenesis-related genes, which were elevated by PAR1 agonist, were all decreased following OE-LMBR1 transfection ($p < 0.001$) or Noggin treatment ($p < 0.001$).

Likewise, NaB treatment resulted in decreased proliferation (Fig. 8A, $p < 0.001$), ALP activity (Fig. 8B, $p < 0.001$), calcium concentration (Fig. 8C, $p < 0.001$), and osteogenic differentiation (Fig. 8D, $p < 0.001$) of PDLSCs, all of which were reversed upon the additional administration of PAR1 agonist ($p < 0.001$). The osteogenesis promoted by PAR1 agonist was diminished by both OE-*LMBR1* transfection ($p < 0.001$) and Noggin treatment ($p < 0.001$).

Considering the changes in PAR1 activation, LMBR1/BMP axis, and the consequent alterations in osteogenesis in PDLSCs as described above, it can be concluded that PAR1 activation has the potential to reversely promote osteogenesis, which is suppressed by NaB, by successfully inhibiting the LMBR1 pathway and activating the BMP pathway.

Discussion

The findings of this study provide evidence that the activation of PAR1 enhances the osteogenic activity of PDLSCs and can alleviate the suppression of PDLSCs osteogenesis induced by NaB. Furthermore, the significance of the LMBR1/BMP pathway in mediating the functions of PAR1 has been verified and demonstrated.

Bone is a dynamic tissue undergoing continuous remodeling, where osteoclasts remove old or damaged bone, and osteoblasts replace it with the new bone [32]. The communication between osteoblasts and osteoclast occurs at various stages of differentiation [33]. The constant state of bone remodeling is crucial for bone homeostasis, ensuring the maintenance and preservation of the normal function and structure of the skeleton through a balanced activity between osteogenesis and osteoclastogenesis [34].

By conducting bioinformatics analysis on public datasets, we identified that the DEGs in periodontitis are closely associated with osteoclast differentiation. This observation aligns with previous reports highlighting excessive osteoclastic bone resorption as a prominent feature of bone diseases, including periodontitis [35]. Considering the correlation between osteoclasts and osteoblasts, it has been demonstrated that macrophage colony-stimulating factor (M-CSF), essential for osteoclast proliferation, primarily originates from osteoblasts within the bone environment [36]. Therefore, this finding further supports the critical role of achieving a healthy balance between osteogenesis

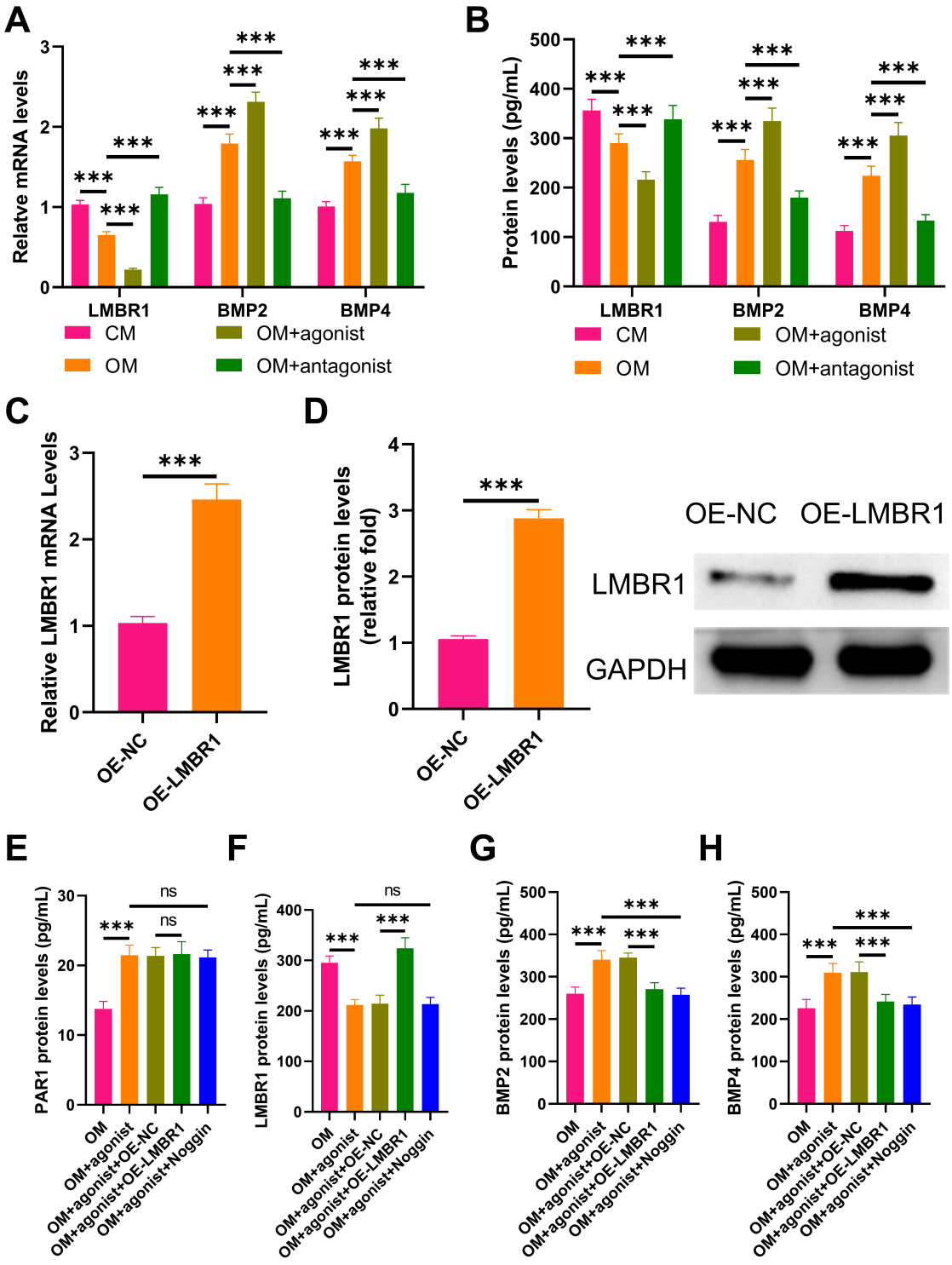

Fig. 5. PAR1 regulates the LMBR1/BMP axis. The (A) mRNA levels and (B) protein levels of *LMBR1*, *BMP2* and *BMP4* (n = 5). The levels of (C) *LMBR1* mRNA and (D) LMBR1 protein in transfected PDLSCs (n = 5). The protein levels of (E) PAR1, (F) LMBR1, (G) BMP2 and (H) BMP4 in different groups (n = 5). *LMBR1*, limb development membrane protein 1; *BMP2*, bone morphogenetic protein 2; *BMP4*, bone morphogenetic protein 4; OE-NC, blank lentiviral vector; OE-*LMBR1*, lentiviral vector inserted by full-length cDNA of *LMBR1*; OM+agonist+OE-NC, PDLSCs transfected with OE-NC and cultured in osteogenic medium supplemented with PAR1-selective agonist TFLLR-NH2 (100 nM); OM+agonist+OE-*LMBR1*, PDLSCs transfected with OE-*LMBR1* and cultured in osteogenic medium supplemented with PAR1-selective agonist TFLLR-NH2 (100 nM); OM+agonist+Noggin, PDLSCs cultured in osteogenic medium supplemented with PAR1-selective agonist TFLLR-NH2 (100 nM) and BMP pathway inhibitor Noggin protein (2 µg/mL). ***$p < 0.001$; ns, no significance ($p > 0.05$).

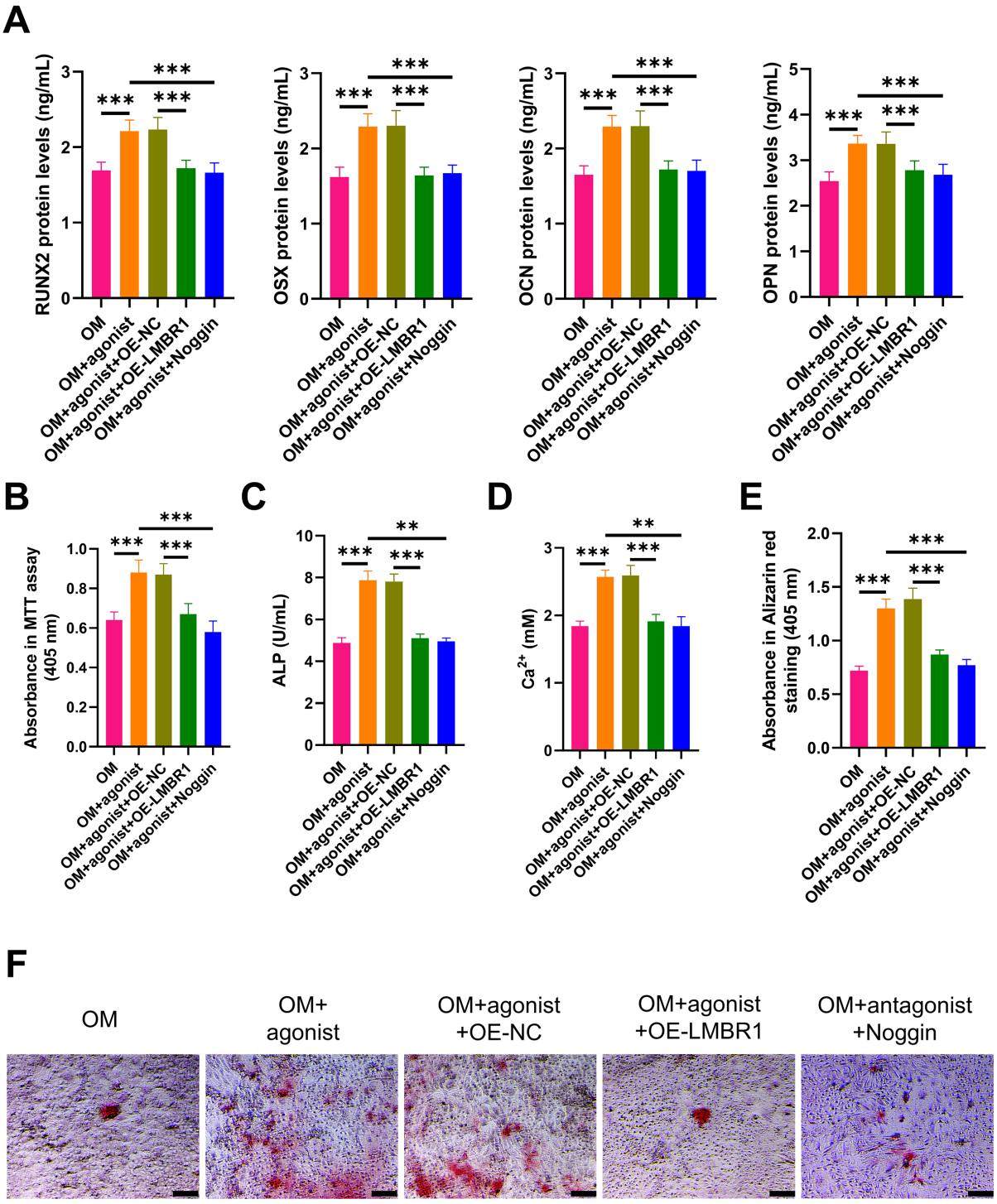

Fig. 6. PAR1 activation promotes PDLSCs osteogenesis by inhibiting LMBR1 pathway and activating BMP pathway. (A) Protein levels of osteogenesis-related genes (n = 5). The (B) proliferation, (C) ALP activity, and (D) calcium concentration of PDLSCs in different groups (n = 5). The (E) quantification (n = 5) and (F) representative images of osteogenic differentiation of PDLSCs in different groups (scale bar: 100 µm). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. $^{**}p < 0.01$, $^{***}p < 0.001$.

and osteoclastogenesis for the restoration of bone homeostasis, essential in the treatment of periodontitis.

It has been previously reported that *in vivo*, the absence of PAR1 was associated with impaired migration of derived bone marrow cells, decreased deposition of mineralized bone, increased colonization of osteoclasts, and enhanced osteoclastogenesis [37]. Additionally, PAR1 activation has been shown to upregulate skeleton formation by

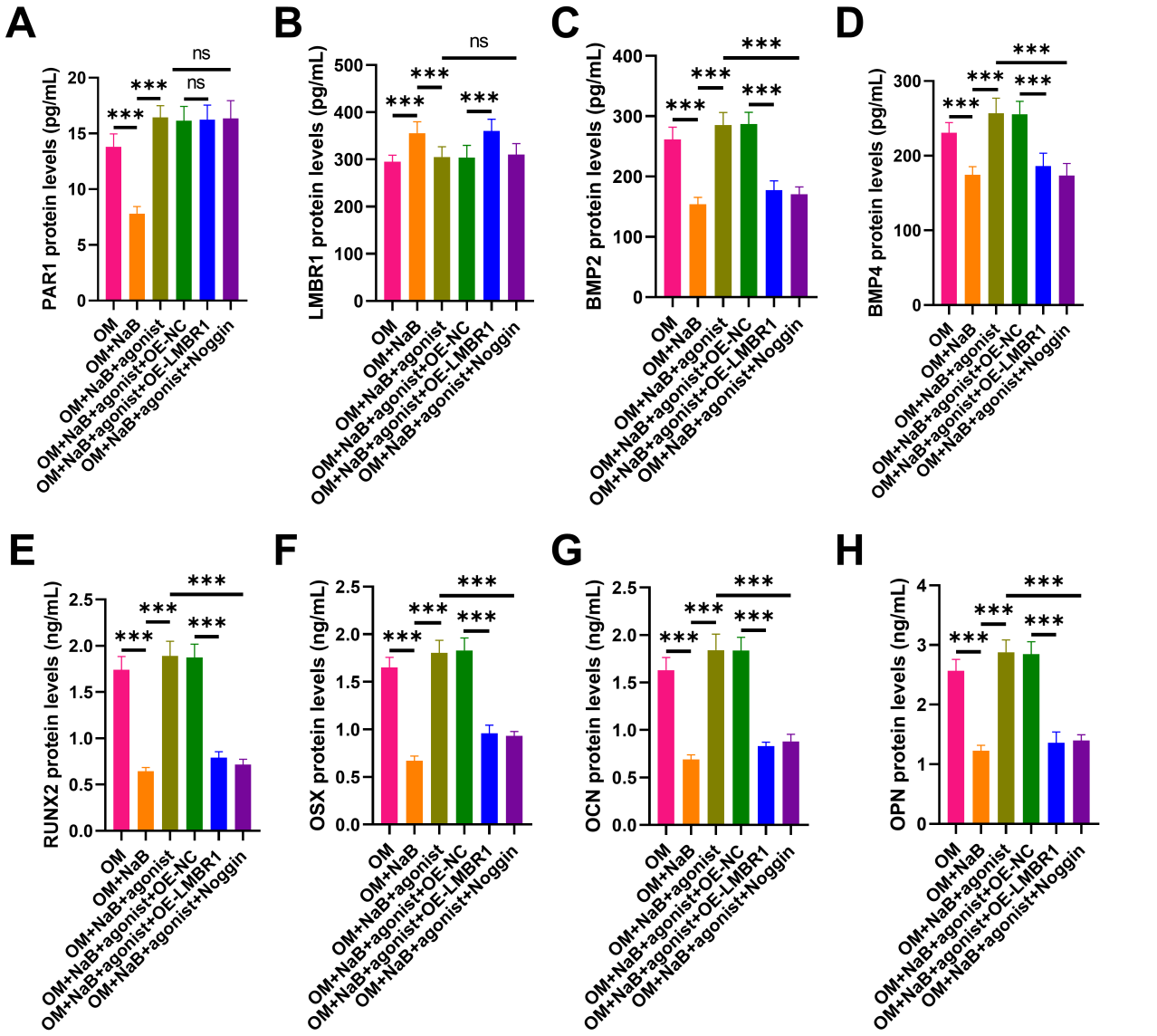

Fig. 7. PAR1 activation alleviates the NaB-induced suppression on PDLSCs osteogenesis by regulating the LMBR1/BMP axis. The protein levels of (A) PAR1, (B) LMBR1, (C) BMP2, (D) BMP4, (E) RUNX2, (F) OSX, (G) OCN and (H) OPN in different groups (n = 5). NaB, sodium butyrate; OM+NaB, PDLSCs cultured in osteogenic medium further supplemented with NaB (1 mM); OM+NaB+agonist, PDLSCs cultured in osteogenic medium further supplemented with NaB and PAR1-selective agonist TFLLR-NH2; OM+NaB+agonist+OE-NC, PDLSCs transfected with OE-NC and cultured in osteogenic medium supplemented with NaB and PAR1-selective agonist TFLLR-NH2; OM+NaB+agonist+OE-*LMBR1*, PDLSCs transfected with OE-*LMBR1* and cultured in osteogenic medium supplemented with NaB and PAR1-selective agonist TFLLR-NH2; OM+NaB+agonist+Noggin, PDLSCs cultured in osteogenic medium supplemented with NaB, PAR1-selective agonist TFLLR-NH2 and BMP pathway inhibitor Noggin protein. ***$p < 0.001$; ns, no significance ($p > 0.05$).

mediating osteoblast proliferation [25]. Conversely, blocking PAR1 has been demonstrated to decrease calcium deposition, inducing suppressed mineralization and differentiation of PDLSCs [27]. ALP activity is a critical marker for osteoblast differentiation [38] and in this study, activation of PAR1 by its selective agonist significantly increased the ALP activity, while the suppression of PAR1 by its selective antagonist significantly decreased ALP activity.

RUNX2, essential for osteoblast differentiation, is the earliest determinant in this process [39]. OSX is also crucial for osteoblast differentiation and has been shown to promote the osteoblast function *in vivo* [40,41]. OCN and OPN, significant bone matrix proteins for osteogenesis, have been reported to regulate cell migration, bone mineralization, induce stem cell proliferation, and promote osteogenesis [42]. This study found that PAR1 activation in-

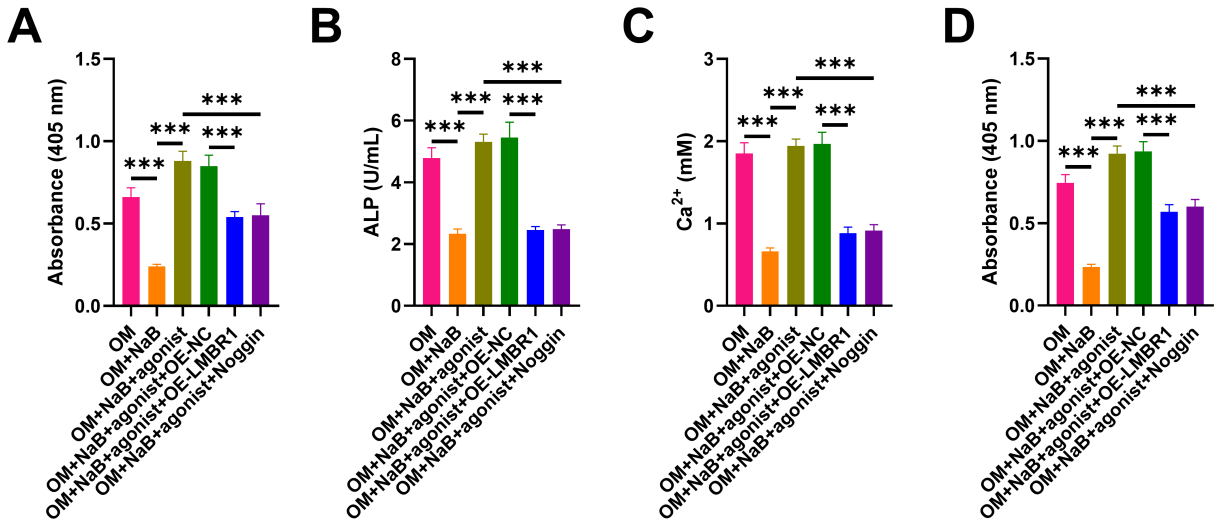

Fig. 8. PAR1 alleviates the NaB-induced suppression on PDLSCs osteogenesis by regulating the LMBR1/BMP axis. The (A) proliferation, (B) ALP activity, and (C) calcium concentration of PDLSCs in different groups (n = 5). (D) The quantification of osteogenic differentiation of PDLSCs in different groups (n = 5). ***$p < 0.001$.

creased the expression levels of these osteogenesis-related genes, while PAR1 suppression decreased their expression indicating that PAR1 promotes osteoblast differentiation and subsequent osteogenesis. Furthermore, PAR1 activation was found to enhance the proliferation of PDLSCs, potentially promoting maturation and mineralization of bone matrix when combined with simultaneously enhanced osteoblast differentiation [43].

The results of calcium concentration and Alizarin Red Staining clearly demonstrated the promoted mineralization and osteogenesis induced by the activated PAR1 and the suppressed effects when PAR1 was inhibited. These findings collectively provide evidence and validation for the promotive effect of PAR1 on osteoblast differentiation and subsequent osteogenesis.

LMBR1 potentially serves as a mediator in the development of the skeletal system and stemness [44]. Previous evidence has shown that LMBR1 is negatively correlated with odontogenesis and osteogenesis [45,46]. The BMP signaling pathway, a complex gene-signaling system essential for osteoblast differentiation from stem cells, consists of 16 members primarily associated with bone repair, growth, and skeletal disorders [47]. BMP2 is recognized as a promoter for mineralization and is crucial for repair [48]. Increased BMP2 levels confirm BMP pathway activation [49], and BMP4 has been proven to play roles in skeletal progression [50].

In this study, it was observed that PAR1 activation/suppression could suppress/activate the LMBR1 pathway while activating/suppressing the BMP pathway. Using a systematic approach, we identified that BMP signaling was downstream of the LMBR1 pathway, and successful inhibition of the LMBR1 pathway and activation of the BMP pathway were critical and essential for PAR1 activation in promoting the osteogenesis of PDLSCs. This finding further underscores the significance of the LMBR1/BMP pathway in PDLSCs as an underlying mechanism, consistent with previous study demonstrating the association of the LMBR1/BMP2/4 axis with the promotive effect on odontogenic and osteogenic differentiation of PDLSCs [46].

Within the periodontal tissue, the gingival epithelium facilitates the easy penetration and residence of butyrate, thanks to the squamous epithelial cells and thin mucosal layers [19]. It has been demonstrated that NaB has an inhibitory effect on the viability and osteogenic differentiation capability of human PDLSCs [22], a finding further verified in this study. However, our innovative discovery in this study reveals that PAR1 activation can enhance the osteogenesis of PDLSCs, even when inhibited by NaB, thus confirming the crucial roles of the LMBR1/BMP pathway in mediating this effect.

Considering that NaB has been shown to induce periodontitis and compromise treatment effectiveness [51], the findings in this study regarding PAR1 activation ameliorating NaB-inhibited PDLSCs osteogenesis by inhibiting the LMBR1 pathway and activating BMP signaling underscore the potential of PAR1 and the LMBR1/BMP axis as therapeutic targets in the treatment of periodontitis.

Conclusion

In summary, PAR1 activation demonstrates the ability to promote the osteogenesis of PDLSCs by suppressing LMBR1 signaling and activating the BMP pathway. This effect has been shown to be effective in ameliorating the inhibition of PDLSCs osteogenesis caused by NaB. The

findings in this study contribute to a more in-depth understanding of how PAR1 influences osteogenesis and highlight PAR1, along with the LMBR1/BMP axis, as novel therapeutic targets for treating periodontal diseases.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the corresponding author if needed.

Author Contributions

LX and ZY performed the research. CH provided help and advice on the experiments. LX, ZY and CH contributed to the analysis and interpretation of the data. LX and ZY wrote the manuscript. All authors contributed to significant editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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