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Original Article

Long noncoding RNA GAS5 alleviates the inflammatory response of human periodontal ligament stem cells by regulating the NF-κB signalling pathway

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Summary

Objectives: This study investigated the role of lncRNA growth arrest-specific transcript 5 (GAS5) in the inflammatory response of periodontal ligament stem cells (PDLSCs) during periodontitis with attempts to its possible mechanisms.

Materials and methods: Gingiva samples were collected from healthy people and patients with periodontitis. The ligature-induced periodontitis model was established in mice. Cell transfection was utilized to knock down and overexpress GAS5 in PDLSCs. Quantitative real-time polymerase chain reaction (qRT-PCR) and fluorescence *in situ* hybridization were performed to detect the GAS5 expression. In combination with high-throughput sequencing technology, qRT-PCR, Western blotting, and immunofluorescence were performed to detect the effects of GAS5 on cytokines and proteins in the NF-κB pathway.

Results: GAS5 expression decreased in PDLSCs subjected to compressive force. GAS5 expression was downregulated in the gingiva tissues from patients with periodontitis. Consistent with the results of clinical samples, GAS5 expression decreased in the mouse ligature-induced periodontitis model. GAS5 expression was downregulated in PDLSCs under tumour necrosis factor (TNF)-α stimulation. Knockdown and overexpression of GAS5 increased and decreased the expression of cytokines induced by TNF-α in PDLSCs, respectively. The sequencing results showed that overexpressing GAS5 was related to genes in the NF-κB pathway. Overexpressing GAS5 alleviated p65 phosphorylation and inhibited the entry of p65 into the nucleus in the TNF-α activated NF-κB pathway, whereas GAS5 knockdown resulted in contrasting results.

Conclusions: GAS5 alleviated the expression of cytokines in PDLSCs by inhibiting activation of the TNF-α-mediated NF-κB pathway. These findings provide new insight into the regulation of the PDLSCs inflammation response.

Introduction

Orthodontic tooth movement is strongly related to the remodelling of periodontal tissue (1). Under the mechanical force, the periodontal tissue surrounding the root of the tooth is in a state of aseptic inflammation, which is characterized by elevated expression of a series of inflammatory cytokines and chemokines, promoting periodontal tissue remodelling and preparing for tooth movement (2). Due to easy access to bacteria in the mouth, periodontal tissue is easily attacked by periodontopathogens, where periodontitis occurs (3). The control of periodontal inflammation is very important in the whole process of orthodontic treatment, especially for people with periodontitis (4). However, the specific mechanism of periodontitis remains a mystery. Furthermore, studies are needed to uncover the inflammatory regulation mechanism.

Periodontal ligament stem cells (PDLSCs) possess strong self-renewal ability and can differentiate into multiple types of cells in periodontal tissue, such as fibroblasts and osteoblasts, which maintain periodontal homeostasis and repair tissue damage (5–7). As mechanosensitive cells, PDLSCs play an important role in the force-induced inflammatory periodontal tissue remodelling during orthodontic movement (2,8). Under the force stimulus, PDLSCs produce a variety of cytokines [such as interleukin [IL]-1β and tumour necrosis factor (TNF)-α] and chemokines (IL-8) that participate in the activation of immune cells and destruction of periodontal tissue and bone (9). A better understanding of the key molecules and underlying mechanisms that regulate the inflammatory response of PDLSCs may help in maintaining periodontal tissue homeostasis.

Long noncoding RNAs (lncRNAs), RNAs with a length of more than 200 nucleotides, are key regulators in various biological processes (10). They play a critical role in the regulation of cell growth, differentiation, and apoptosis (11–13). Previous studies have indicated the regulatory role lncRNAs may play in periodontitis (14–17). LncRNA growth arrest-specific transcript 5 (GAS5) is widely involved in multiple life activities such as cell proliferation, differentiation,

migration, and apoptosis (18,19). Studies have suggested that it serves as a regulator in inflammatory and infectious diseases such as rheumatoid arthritis and systemic lupus erythematosus (20). However, researches on the role of GAS5 in the regulation of periodontal inflammation are scarce. We attempt, therefore, to determine how GAS5 modulates the inflammatory response of PDLSCs and explore the possible mechanism.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee (PKUSSIRB-201950166, LA2021133, and PKUSSIRB-201837096).

Application of mechanical force *in vitro*

PDLSCs were seeded onto six-well cell culture plates at a density of 2.0 × 10^5 cells per well. After cells reached 80 per cent confluence, a thin glass plate was placed on the cell layer to make the cell layer evenly stressed, and the force (2 g/cm^2) was applied through metal balls on the glass plate as illustrated in Figure 1 for 0, 6, 12, or 24 hours (21,22). The cells in the control group were not exposed to compression.

Clinical sample collection

Gingiva samples were collected from patients undergoing periodontal surgery in the periodontal department of XXX Hospital of Stomatology, including eight patients with periodontitis and seven healthy individuals. Informed consent was obtained from all the patients who provided the samples. The healthy group included patients who required crown lengthening surgery with no redness or swelling of the gingiva, no bleeding or other inflammatory manifestations upon probing, and no loss of attachment or a periodontal probing depth ≤ 3 mm. The periodontitis group enrolled patients who were clinically diagnosed with periodontitis and needed periodontal flap surgery. The periodontal probing depth of the operation area was still ≥5 mm after basic periodontal treatment, accompanied by bleeding during probing. Smokers or those taking certain drugs (such as calcium channel blockers) or who had other systemic diseases were excluded. For real-time polymerase chain reaction (qRT-PCR) analyses, the collected samples were separately frozen immediately and stored at −80°C. For histological analysis, the tissues were separately fixed in 4 per cent paraformaldehyde preparing for subsequent haematoxylin and eosin (H&E) staining (Solarbio, Beijing, China) and IL-1β immunohistochemical staining (Servicebio, GB11113, Wuhan, China).

Ligature-induced periodontitis model in mice

Male, 8-week-old, C57BL/6 mice were obtained from WeiTong LiHua Co. (Beijing, China) and used in the experiments. After 1 week of adaptive feeding, the mice were intraperitoneally anaesthetized and fixed in a supine position on the operating table. In the experimental group, a 5-0 silk thread was ligated on the neck of the bilateral maxillary second molars of the mouse, and a knot was fixed on the buccal side (23). The experimenter regularly checked the condition of the silk suture and re-ligated if it came off. The mice in the control group were left untreated. The mice were sacrificed 2 weeks later, and the maxilla and gingiva were preserved.

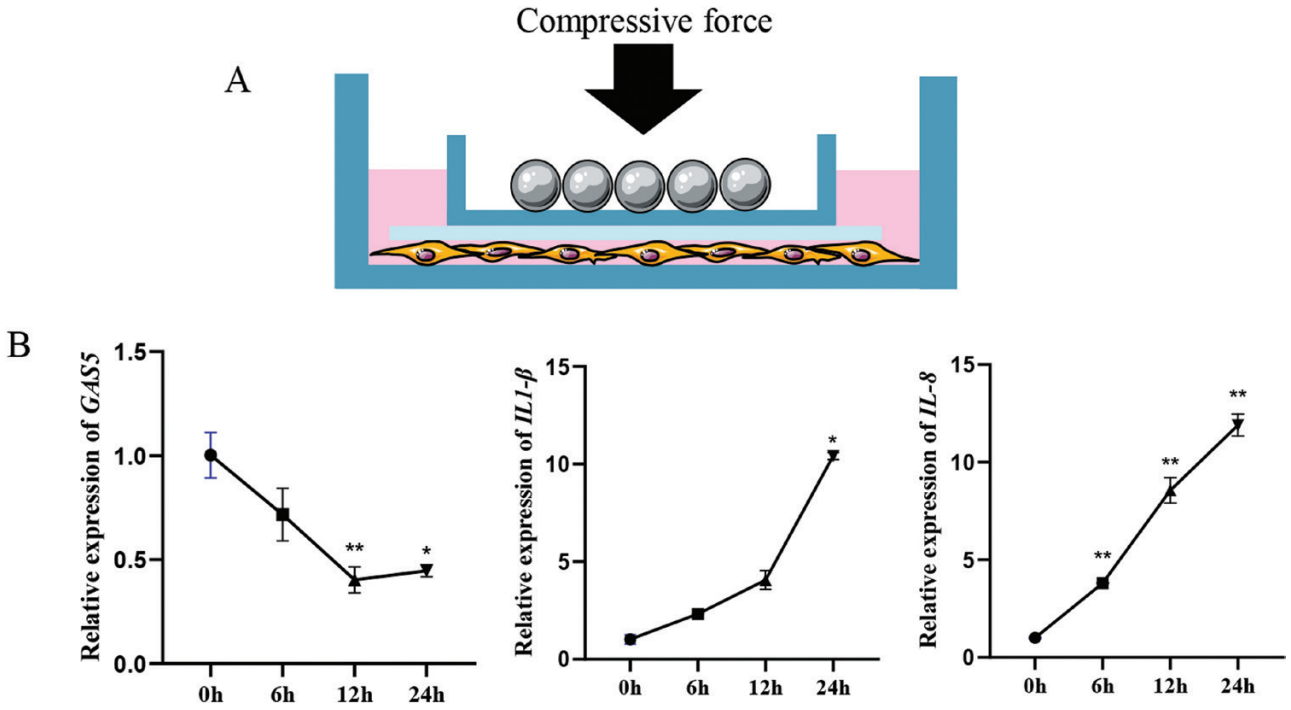

Figure 1. Growth arrest-specific transcript 5 (GAS5) is downregulated in periodontal ligament stem cells (PDLSCs) subjected to compressive force. (A) Schematic diagram showing periodontal ligament stem cells (PDLSCs) exposed to compressive mechanical force at 2 g/cm^2. (B) The messenger RNA (mRNA) expression levels of *GAS5* and the inflammatory factors interleukin (*IL-1*β) and chemokines (*IL-8*) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). One-way ANOVA analysis was performed (*$P < 0.05$; **$P < 0.01$).

Micro-computed tomography and histology

To detect the alveolar bone resorption that occurs in periodontitis, the maxilla of the mice was fixed in 4 per cent paraformaldehyde and scanned at a resolution of 10.8 μm by micro-computed tomography (micro-CT) (Skyscan1174; Bruker, Kontich, Belgium). NRecon and CTvox software were used to reconstruct the three-dimensional images. Then the maxillary bone was placed in 10 per cent EDTA solution for decalcification for about 2 weeks, followed by paraffin embedding and sectioning at a thickness of 4 μm. The sections were stained with H&E (Solarbio).

Cell isolation, culture, and treatment

The methods of PDLSCs isolation are described in detail in our previous study (24). The periodontal ligament in middle third of the root was gently scraped and digested with collagenase and trypsin (Gibco) for 60 minutes, then the tissue pieces were incubated into culture flasks containing α-MEM (Gibco, Grand Island, New York, USA) containing 10 per cent fetal bovine serum (Gibco) and 1 per cent penicillin and streptomycin (Gibco) in the presence of 5 per cent CO_2 at 37°C. P3–P6 generation cells were utilized for subsequent experiments. These cells were identified and positive for mesenchymal stem cell markers CD73, CD105, and CD90 (25). TNF-α (100 ng/ml; Proteintech, Rocky Hill, New Jersy, USA; Cat. No. HZ-1014) was used to stimulate PDLSCs and establish the *in vitro* inflammatory environment (26,27).

Cell transfection

Small-interfering RNAs (si-RNAs) against GAS5 (si-GAS5), the siRNA control (si-NC), recombinant lentivirus containing full-length GAS5 (GAS5), and the control (NC) were all designed by GenePharma Co. (Shanghai, China). si-NC and si-GAS5 were transfected into PDLSCs separately using 100 nM Lipofectamine3000 (Invitrogen, Carlsbad, California, USA) when the cells have reached 70–80 per cent of confluence according to the manufacturer's protocol. The cells for GAS5 and NC lentivirus transfection were cultured in a medium containing lentivirus particles (multiplicity of infection = 20) for 24 hours and then exposed to a medium containing puromycin (10 ng/ml) to select stably transfected cells.

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

The total RNA from tissue samples and cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocols and quantified using a Nanodrop 8000 UV-visible spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Total RNA (2 μg) was then reverse transcribed into cDNA by utilizing the PrimeScriptTM RT Reagent Kit (Takara Bio, Shiga, Japan). The real-time PCR conditions were as follows: predenaturation at 95 °C for 10 minutes, then followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute. The sequences of each primer were listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization and the $2^{-\Delta\Delta CT}$ method was used to calculate relative RNA expression.

Enzyme-linked immunosorbent assay

The protein level of IL-1β and IL-8 in the supernatant of PDLSCs treated with 100 ng/ml human TNF-α for 12 hours was detected by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Tiangen Biotech Company, Beijing, China).

Fluorescence *in situ* hybridization

The GAS5 probe applied for RNA fluorescence in situ hybridization (FISH) was purchased from RIOBIO Co. (Guangzhou, China). The cells were seeded onto a cell slide. The cells in the experimental group were stimulated with 100 ng/ml of human TNF-α for 12 hours and those in the control group were left treated. Then the cells were fixed in 4 per cent paraformaldehyde for 10 minutes and treated with 0.1 per cent TritonX-100 for 10 minutes to increase cell permeability. After three washes, the cells were incubated with a pre-hybridization solution for 30 minutes followed by a hybridization solution containing the GAS5 probe overnight at 37°C. After washing, the cells were stained with DAPI and images were captured using a confocal imaging system (LSM 5 EXCITER, Carl Zeiss).

Table 1. Primer sequence

Gene name	Forward primer	Reverse primer
Human		
GAPDH	CGACAGCAGCCGCATCTT	CCAATACGACCAAATCCGTTG
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC
IL-6	CAGUACUUUUGUGUAGUACAA	GTCATGTCCTGCAGCCACTG
IL-8	TGCAGCTCTGTGTGAAGG	AATTTCTGTGTTGGCGCAGT
TNF-α	CGAGTGACAAGCCTGTAGC	GGTGTGGGTGAGGAGCACAT
GAS5	GTGTGGCTCTGGATAGCAC	ACCCAAGCAAGTCATCCATG
Mouse		
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Il-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnf-α	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
Gas5	GGAAGCTGGATAACAGAGCGA	GGTATTCCTTGTAATGGGACCAC

Immunofluorescence staining

5.0×10^4 cells per well were plated on sterile glass coverslips (Solarbio, Cat. No. YA0350, Beijing, China), and 4 per cent paraformaldehyde was utilized to fix the cells for 20 minutes at room temperature. The cells were permeabilized with 1 per cent Triton X-100 and blocked with 5 per cent goat serum (ZLI-9022, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 hour. Primary antibody p65 (1:200; Proteintech; Cat. No. 10745-1-AP) and anti-rabbit secondary antibody (1:500; ZF-0317, Zhongshan Golden Bridge Biotechnology) were used. DAPI (Solarbio, S2110, Beijing, China) was used to stain the nuclei, and the morphology was observed and photographed using a confocal fluorescent microscope (LSM 5 EXCITER, Carl Zeiss, Jena, Germany).

RNA sequencing

RNA sequencing was used to analyse the gene expression profiles of the PDLSCs transfected with GAS5 and NC. Total RNA was extracted using the Trizol method. Sequencing was completed by Boao Biological Co., Ltd. (Beijing, China). Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the DAVID Bioinformatic Resources. Adjusted *P*-values < 0.05 between the two groups were considered significantly enriched.

Preparation of the cytoplasmic and nuclei fractions

PDLSCs were stimulated with 100 ng/ml human TNF-α for 1 hour and those in the control group were left treated. The Cytoplasmic and Nuclei Fractionation kit (Invent Biotechnologies, Minnesota, USA) was used to collect the cytoplasmic and nuclear proteins from PDLSCs according to the kit's manuals. GAPDH (Proteintech) is used as internal control in cellular extracts and histone H3 (Abcam, Cambridge, UK) as an internal control in nuclear extracts for the detection of p65 protein.

Western blotting analysis

Cells were lysed with RIPA lysis buffer, and the protein concentrations were determined by the BCA assay (Thermo Scientific, Waltham, Massachusetts, USA). A total of 30-μg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). Bovine serum albumin (5 per cent) was used as the block at room temperature for 1 hour, followed by phosphorylated-p65 (1:1000; Cell Signaling Technology, Danvers, Massachusetts, USA; #3033S), p65(1:1000; Proteintech; Cat. No. 10745-1-AP), inhibitory κB protein (IκB)-α (1:1000; Proteintech; Cat. No. 10268-1-AP), β-actin (1:2000; ZSGB-Bio; TA-09), GAPDH (1:2000; Proteintech; Cat. No. 60004-1-Ig), and histone H3 (1:1000; Abcam, Cambridge, UK; ab1791) incubations overnight. Then the membrane was washed and incubated with corresponding secondary antibodies (1:10 000; ZSGB-Bio; ZB-2305; ZB-2306) at room temperature for 1 hour. The bands were visualized with enhanced chemiluminescence using the Bio-Rad system for detection (ChemiDocTM MP Imaging System, Bio-Rad Laboratories, Hercules, California, USA), and band intensity was measured using Image J (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software 8.0 (GraphPad Software Inc., La Jolla, CA, USA), and the data are presented as mean ± standard deviation. Three repeated experiments were performed. The Student's *t*-test was employed to compare two groups, and one-way analysis of the variance was applied to compare three or more groups. A *P*-value < 0.05 was considered significant.

Results

GAS5 is downregulated in PDLSCs subjected to compressive force

Given the static compression pressure (Figure 1A), the expression of inflammatory cytokines *IL-1β* and *IL-8* was significantly upregulated with time and reached a peak at 24 hours in response to the compression force (Figure 1B). In contrast, the *GAS5* expression was decreased, which implies that GAS5 may be a response to the mechanical force and play a role in the periodontal homeostasis. Therefore, we decided to study the changes of GAS5 in the inflammatory microenvironment of PDLSCs and its regulatory role in inflammation.

GAS5 is downregulated in the gingival tissues of patients with periodontitis

H&E staining showed that the gingival tissue of patients with periodontitis had more plasma cells and infiltrated lymphocytes than the gingival tissues from healthy people (Figure 2A). The immunohistochemistry results showed more IL-1β-positive cells in the gingival tissue of patients with periodontitis than those in healthy people (Figure 2B). qRT-PCR revealed that the expression of inflammatory cytokines *IL-1β*, *IL-8*, and *TNF-α* was significantly upregulated in the gingival tissues of patients with periodontitis compared to healthy human gingival tissues, and GAS5 expression was downregulated by about 50 per cent (Figure 2C) ($P < 0.01$), indicating that GAS5 may play an important regulatory role in periodontitis.

GAS5 expression is downregulated in mice with ligature-induced periodontitis

A 5-0 silk thread was ligated on the neck of the bilateral maxillary second molars of the mice to induce periodontitis (Figure 3A). Micro-CT showed that compared with the control group, the level of alveolar bone absorption was stronger in the mouse experimental periodontitis (Figure 3B). Consistent with the CT results, H&E staining showed that the alveolar bone was absorbed, destroyed, and infiltrated with a large number of inflammatory cells (Figure 3C), suggesting that the ligature-induced periodontitis model was successfully established in mice. qRT-PCR was used to detect the expression of *Gas5* in the gingival tissues. The results showed that compared with the control group, the expression of inflammatory factors *Il-1β*, *Il-6*, and *Tnf-α* was significantly upregulated, and the expression level of *Gas5* was downregulated in inflamed gingival tissues (Figure 3D). This result is consistent with the downregulation of GAS5 expression in the gingival tissues of patients with periodontitis, suggesting that GAS5 is involved in the regulation of periodontal inflammation.

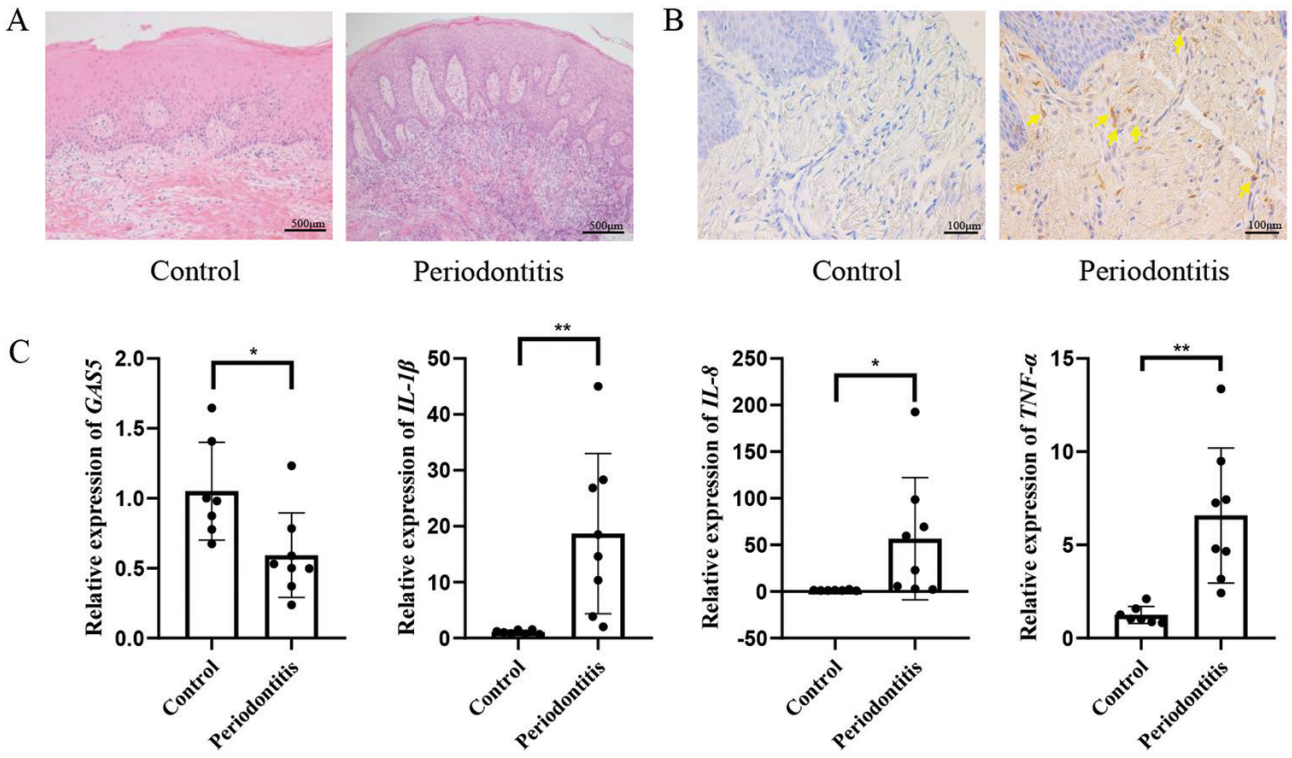

Figure 2. Growth arrest-specific transcript 5 (GAS5) is downregulated in the gingival tissues of patients with periodontitis. (A) H&E staining of the gingival tissues of healthy people (control) and patients with periodontitis (periodontitis). (B) Interleukin (IL-1β) immunohistochemical staining of the gingival tissues of healthy people (control) and patients with periodontitis (periodontitis). The arrow shows the interleukin (IL-1β)-positive cells. (C) The expression levels of *GAS5* and the inflammatory factors interleukin (*IL-1β*), chemokines (*IL-8*), and tumour necrosis factor (*TNF-α*) were detected by quantitative real-time polymerase chain reaction (qRT-PCR) in the gingival tissues of healthy people (control, $n = 7$) and patients with periodontitis (periodontitis, $n = 8$). Student's *t*-test was performed (*$P < 0.05$; **$P < 0.01$).

GAS5 expression decreases in TNF-α-induced PDLSCs

To verify the effects of inflammation on GAS5, we used 100 ng/ml TNF-α to stimulate PDLSCs to mimic the *in vitro* inflammatory environment. Changes in the expression of inflammatory cytokines *IL-1β*, *IL-6*, *IL-8*, and *GAS5* were detected at different time points (0, 6, 12, and 24 hours). The three cell inflammatory factors were significantly upregulated ($P < 0.01$) under the stimulation condition of 100 ng/ml TNF-α. GAS5 expression in the TNF-α stimulated group decreased in a time- and dose-dependent manner compared with that in the control group (Figure 4A). We further tested the GAS5 intracellular localization and expression-level changes in FISH experiments. GAS5 was distributed in both the cytoplasm and the nucleus. After stimulating with 100 ng/ml TNF-α for 12 hours, GAS5 expression in PDLSCs had decreased significantly (Figure 4B). These results indicate that GAS5 may play an important regulatory role in the PDLSC inflammatory response.

GAS5 reduces the expression of inflammatory factors in TNF-α-induced PDLSCs

We use the Lipofectamine3000 transfection reagent and lentivirus to knockdown and overexpress GAS5 in PDLSCs, respectively, and we used qRT-PCR to test the transfection efficiency 48 hours after transfection. The expression levels of the inflammatory factors *IL-1*, *IL-6*, and *IL-8* were detected after stimulating with 100 ng/ml TNF-α for 12 hours. The results showed that after knocking down GAS5, its expression was downregulated by about 80 per cent. The *GAS5* expression level in the overexpressing group increased about three times compared with that in the control group, indicating that the transfection efficiency was qualified. The expression of the inflammatory factors *IL-1*, *IL-6*, and *IL-8* increased after knocking down GAS5 (Figure 5A). However, after overexpressing GAS5, the expression of these inflammatory factors decreased ($P < 0.05$) (Figure 5B). Then, we used ELISA to further determine the protein level of the inflammatory factors IL-1 and IL-8 in the cell-free supernatants. The ELISA results were consistent with the qRT-PCR results. (Figure 5C). These results indicate that GAS5 reduces the expression of inflammatory factors in TNF-α-induced PDLSCs.

Changes in the transcriptome after overexpressing of GAS5

To further clarify the downstream effects caused by GAS5 overexpression, we performed transcriptome sequencing on the cells of the GAS5-overexpressing group and the control group. The volcano blot showed the downregulated and upregulated genes (Figure 6A). GO (Figure 6B) and KEGG pathway (Figure 6C) analyses revealed that different genes were enriched in the NF-κB pathway, indicating that the expression of NF-κB pathway-related molecules was affected after overexpressing GAS5. The NF-κB pathway is an important signalling pathway that mediates inflammatory stimulation, and it plays an important regulatory role in the pathophysiology of periodontitis (28).

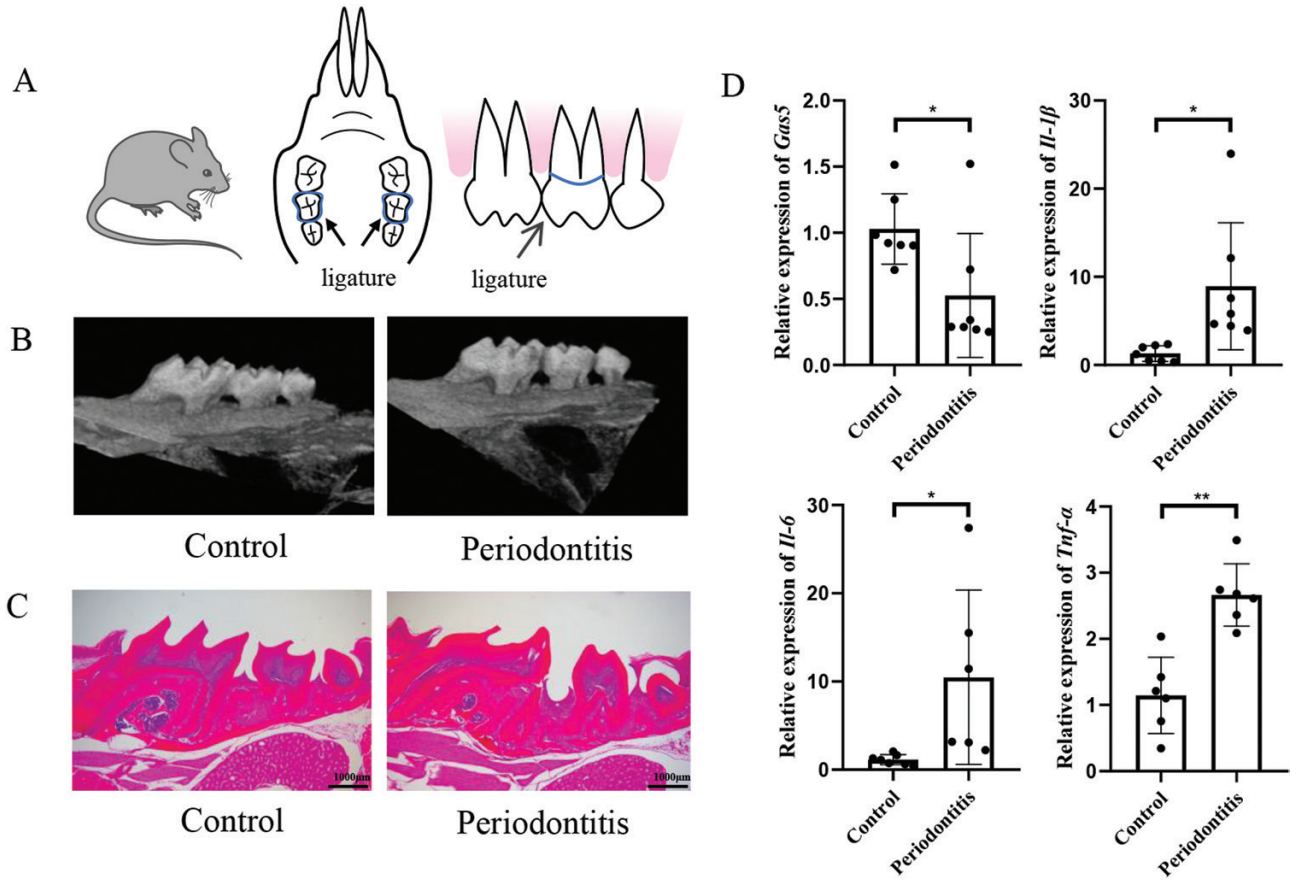

Figure 3. Growth arrest-specific transcript 5 (GAS5) expression is downregulated in mice with ligature-induced periodontitis. (A) 5-0 silk thread was ligated on the neck of the bilateral maxillary second molars of the mice to establish the ligatured-induced periodontitis model. (B) Micro-CT shows three-dimensional reconstructed images of the teeth and alveolar bone of mice with periodontitis and the control group. (C) The H&E-stained images of teeth in mice with periodontitis and the control group. (D) The expression of *Gas5*, interleukin (*Il-1*β), *Il-6*, and tumour necrosis factor (*Tnf*-α) was detected by quantitative real-time polymerase chain reaction (qRT-PCR) in gingival tissues of mice with periodontitis ($n = 6$) and the control group ($n = 6$). Student's *t*-test was performed (*$P < 0.05$; **$P < 0.01$).

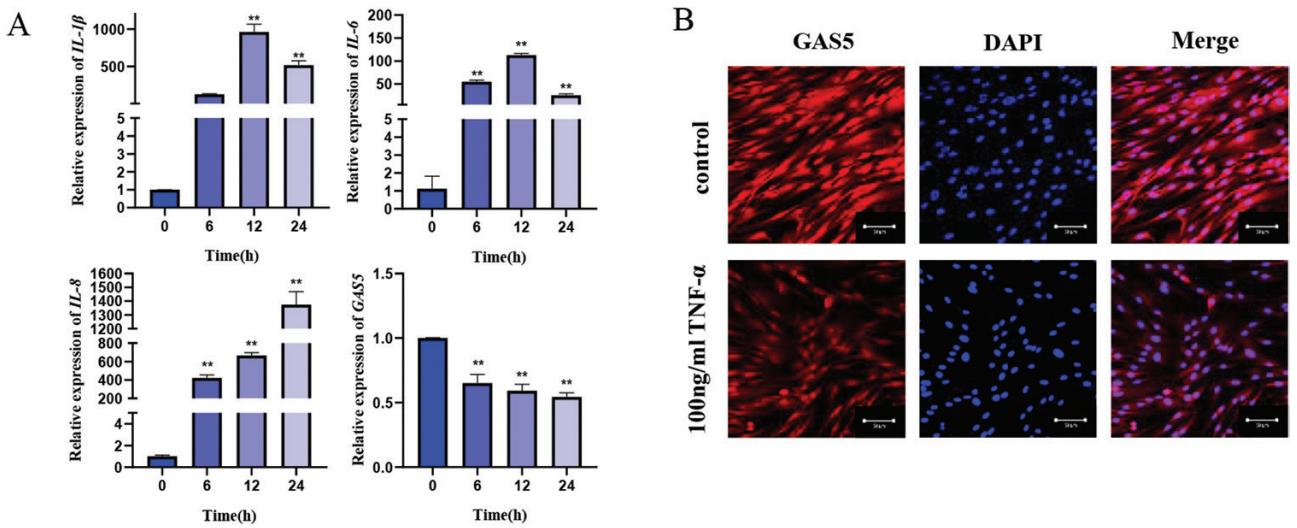

Figure 4. Growth arrest-specific transcript 5 (GAS5) expression decreased in tumour necrosis factor (TNF-α)-induced periodontal ligament stem cells (PDLSCs). (A) The relative expression of interleukin (*IL-1*β), *IL-6*, chemokines (*IL-8*), and *GAS5* was determined by quantitative real-time polymerase chain reaction (qRT-PCR) at different time points (6, 12, and 24 hours) of tumour necrosis factor (TNF-α) stimulation. Ribonucleicacid (RNA) expression at these time points was normalized to 0 hour. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. One-way ANOVA was performed (*$P < 0.05$; **$P < 0.01$). (B) The intracellular distribution and expression changes in GAS5 after 100 ng/ml TNF-α stimulation for 12 hours were detected by fluorescence *in situ* hybridization (FISH).

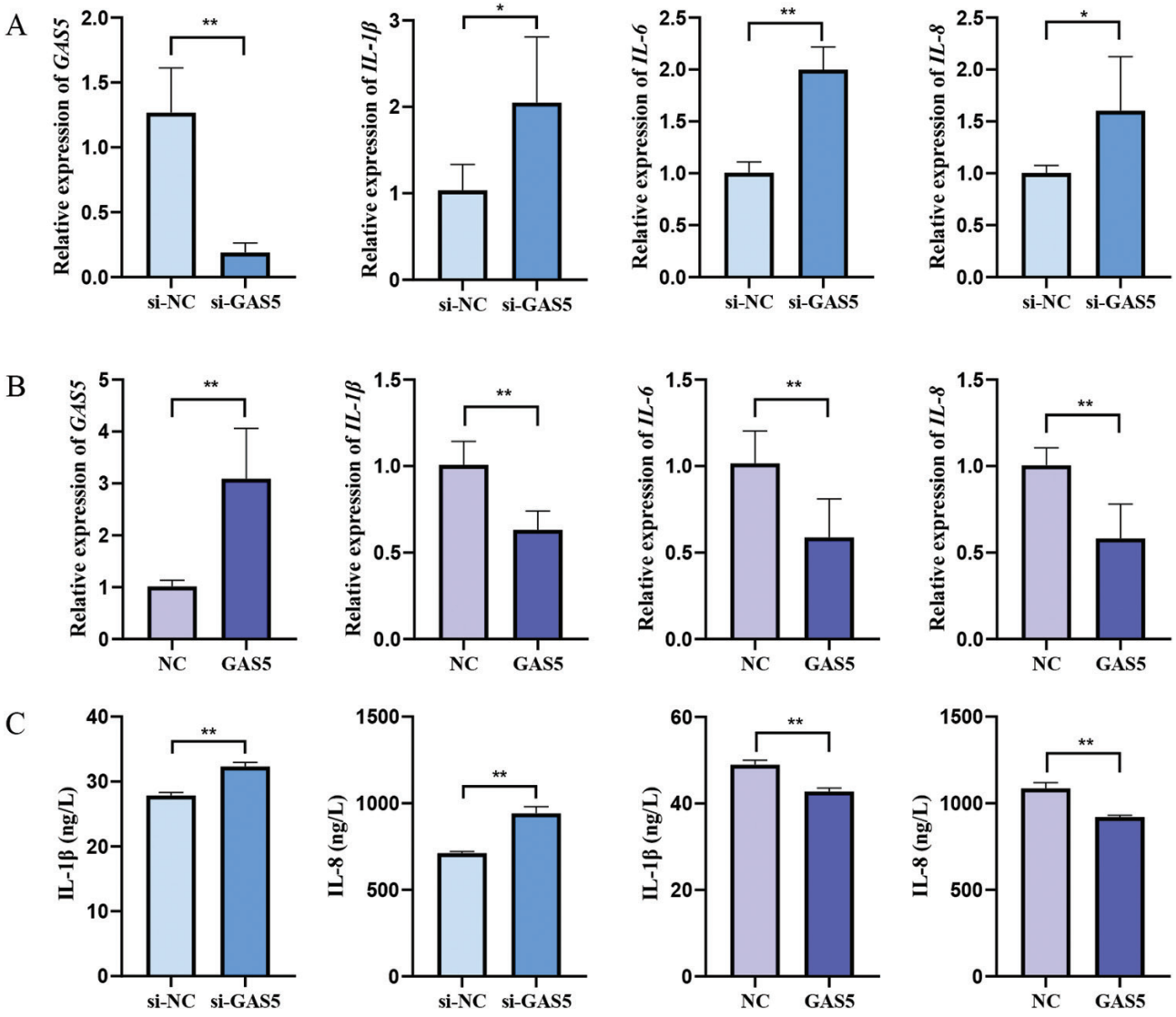

Figure 5. Growth arrest-specific transcript 5 (GAS5) reduces the expression of inflammatory factors in tumour necrosis factor (TNF-α)-induced periodontal ligament stem cells (PDLSCs). (A) The messenger RNA (mRNA) expression of *GAS5*, interleukin (*IL-1*β), *IL-6*, and chemokines (*IL-8*) was measured in the si-NC, and si-GAS5 groups after 12 hours of tumour necrosis factor (TNF-α) stimulation (*$P < 0.05$; **$P < 0.01$). (B) The messenger RNA (mRNA) expression of *GAS5*, interleukin (*IL-1*β), *IL-6*, and chemokines (*IL-8*) was measured in the NC, and GAS5 groups after tumour necrosis factor (TNF-α) stimulation for 12 hours (*$P < 0.05$; **$P < 0.01$). (C) The supernatant protein expression of interleukin (IL-1β) and chemokines (IL-8) was detected by enzyme-linked immunosorbent assay (ELISA) in the si-NC, si-GAS5 groups, NC, and GAS5 groups after TNF-α stimulation for 12 hours. Student's *t*-test was performed (A–C) (*$P < 0.05$; **$P < 0.01$).

GAS5 inhibits the activation of the TNF-α induced NF-κB pathway

The NF-κB pathway in PDLSCs is activated under the activation of TNF-α, and a series of changes occur (29). With IκB subunit degraded, the dimer formed by p65, and p50 is transferred from the cytoplasm to the nucleus, where it binds to the promoter regions of regulatory genes to affect pro-inflammatory factors and the transcription of related target genes (30). Among the changes, phosphorylation of p65 and IκB, as well as nuclear translocation of p65 are important evidence for activation of the NF-κB pathway (31).

To further clarify the regulatory effect of GAS5 on the NF-κB pathway, we tested the effects of knockdown and overexpression of GAS5 on the TNF-α-induced of NF-κB signalling pathway. After TNF-α stimulation, the NF-κB pathway was activated with increased expression of NF-κB pathway-related protein p-p65 and decreased expression of IκBα. After knocking down GAS5, p-p65 expression increased and IκBα expression decreased. Whereas, overexpressing GAS5 ended up in the contrary results (Figure 7A). The immunofluorescence results showed that the NF-κB pathway was activated after stimulating with 100 ng/ml TNF-α for 12 h; p65 was translocated to the nucleus, and the nuclear translocation of p65 was inhibited after overexpressing GAS5 (Figure 7B). Furthermore, cytoplasm and nuclear separation followed by western blotting was performed to detect the effect of overexpression of GAS5 on p65 nuclear translocation in PDLSCs. The western blot results showed that nuclear translocation of p65 was inhibited after overexpressing GAS5 (Figure 7B). These results indicate that GAS5 inhibits activation of the TNF-α-induced NF-κB signalling pathway.

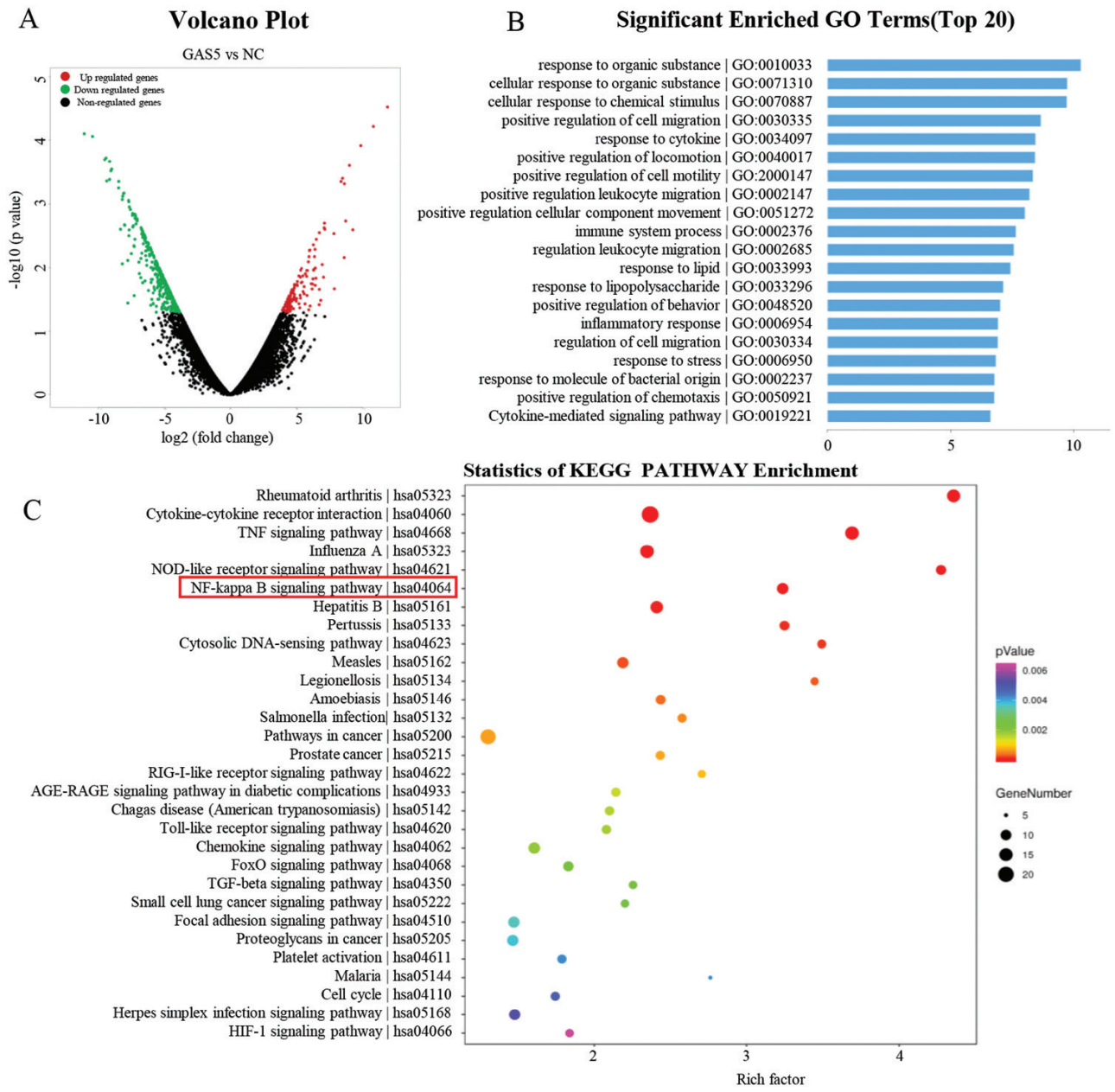

Figure 6. Changes in the transcriptome after overexpressing growth arrest-specific transcript 5 (GAS5). (A) Volcano map showing the number of differentially expressed messenger RNA (mRNAs) in the GAS5-overexpressing group compared with the control group (509 genes downregulated and 156 genes upregulated). (B) Top 20 enriched Gene Ontology (GO) terms of differentially expressed genes. (C) Enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways of the differentially expressed genes.

Discussion

We found that GAS5 expression in the gingival tissues of patients with periodontitis is downregulated, suggesting that it may have a regulatory effect in periodontitis. To further verify the role of GAS5 in periodontitis, we successfully constructed a ligature-induced periodontitis model in mice and discovered decreased GAS5 expression in their gingival tissues, consistent with the results of clinical samples. This further demonstrates that GAS5 is involved in the pathophysiology of periodontitis. TNF-α is considered an upstream inflammatory factor in the pathological process of periodontitis, which is closely related to the course of periodontitis (32,33). Therefore, we used TNF-α to induce PDLSCs to mimic the *in vitro* inflammatory environment. Under the stimulation of TNF-α, the expression of GAS5 decreased in time- and dose-dependent manners. This trend is consistent with the results of clinical and animal samples and suggests that GAS5 is involved in the regulation of the TNF-α-induced PDLSC inflammatory response. We also revealed the regulatory role of GAS5 in the PDLSC inflammatory response by overexpressing and knocking down GAS5. The expression of inflammatory factors increased after knocking down GAS5 and the expression of inflammatory factors decreased after overexpressing GAS5. We conclude that GAS5 inhibits the expression of inflammatory factors in PDLSCs induced by TNF-α. So what is the role of GAS5 in regulating the PDLSC inflammatory response? To further clarify the downstream effects caused by overexpressing GAS5, we performed transcriptome sequencing on the cells in the GAS5-overexpressing and control groups. According to KEGG analysis, the differentially expressed genes were mainly enriched in the NF-κB pathway.

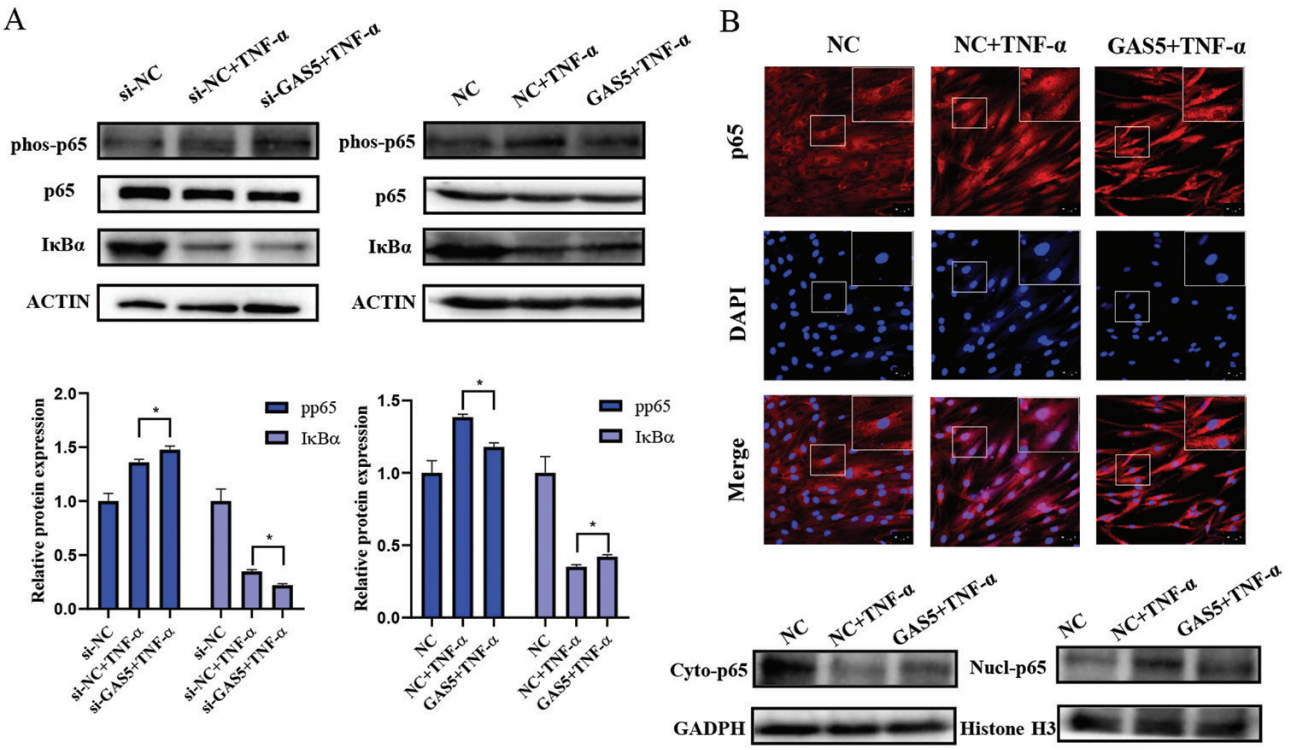

Figure 7. Growth arrest-specific transcript 5 (GAS5) inhibits the activation of the tumour necrosis factor (TNF-α)-induced NF-κB pathway. (A) Protein expression and relative quantitative analysis of the NF-κB pathway-related proteins p65, p-p65, IκBα, and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in si-NC, si-NC+TNFα, si-GAS5+TNF-α, NC, NC+TNF-α, and GAS5+TNF-α groups of periodontal ligament stem cells (PDLSCs). Student's *t*-test was performed. (*$P < 0.05$; **$P < 0.01$). (B) Immunofluorescence staining and western blots of p65 in the NC, NC+TNF-α, and GAS5+TNF-α groups.

Periodontal inflammation is closely related to the activation of NF-κB pathway (34,35). *In vitro* studies have shown that periodontal pathogens induce inflammation in periodontal tissues by activating the NF-κB pathway (28). Using inflammatory factors, such as TNF-α, to stimulate periodontal cells activates the NF-κB pathway to produce inflammatory mediators, such as prostaglandins and matrix metalloproteinases, which damage periodontal tissues (36). Inhibitors of the NF-κB pathway have been used in experimental periodontitis and other similar chronic inflammatory diseases (37). p65 expression increased after knocking down GAS5, and IκBα expression decreased; overexpressing GAS5 obtained the opposite result. TNF-α induced an increase in translocation of p65 into the nucleus, and overexpression of GAS5 reduced this translocation process. Therefore, we conclude that overexpressing GAS5 inhibits activation of the TNF-α-induced NF-κB signalling pathway. However, this study lacked an in-depth exploration of specific mechanisms; that is, how does GAS5 act on the NF-κB pathway? What are the inflammatory factors that play a key role? These issues need to be clarified through further in-depth research.

There are few reports about GAS5 and the NF-κB pathway, and the GAS5 regulatory mechanism in the NF-κB pathway is still unclear. GAS5 directly binds to IKK in the NF-κB pathway and positively regulates the expression of IKK, thereby inhibiting NF-κB activity (38). KLF2 is a zinc finger-like transcriptional regulator involved in mediating the monocyte inflammatory response. Studies have reported that GAS5 inhibits activation of the NF-κB pathway by upregulating the expression of KLF2, thereby inhibiting the chondrocyte inflammatory response (39). The specific mode of action of GAS5 and KLF2 is unknown. In addition, GAS5 also regulates the NF-κB pathway through the ceRNA mechanism. GAS5 knockdown reduces inflammation of human cardiomyocyte-like AC16 cells induced by high levels of glucose through the miR-21-mediated TLR4/NF-κB signalling pathway (40). It regulates the expression of the miR-26a target gene HMGB1 by binding miR-26a, thereby inhibiting activation of the NF-κB pathway (41). Although this study did not thoroughly explore the GAS5 regulatory mechanism of the NF-κB pathway during periodontal inflammation, the three GAS5 regulation mechanisms proposed here for the NF-κB pathway provide a direction for future research.

In conclusion, in this study, GAS5 alleviated the expression of cytokines in PDLSCs by inhibiting the activation of the TNF-α-mediated NF-κB pathway. These findings provide new insight into the regulation of the PDLSCs inflammatory response and provide a theoretical basis for molecular-targeted inflammation control during orthodontic treatment.

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Conflicts of interest

None to declare.

Data Availability

The raw data underlying this article are available in Sequence Read Archive (SRA) of the NCBI (SRR11093247 and SRR11093246).

Supplementary material

Supplementary data are available at *European Journal of Orthodontics* online.

References

1. Henneman, S., Von den Hoff, J.W. and Maltha, J.C. (2008) Mechanobiology of tooth movement. *European Journal of Orthodontics*, 30, 299–306.
2. Huang, H., Yang, R. and Zhou, Y.H. (2018) Mechanobiology of periodontal ligament stem cells in orthodontic tooth movement. *Stem Cells International Journal*, 2018, 6531216.
3. Slots, J. (2017) Periodontitis: facts, fallacies and the future. *Periodontology 2000*, 75, 7–23.
4. Rath-Deschner, B., *et al*. (2022) Interaction of periodontitis and orthodontic tooth movement-an in vitro and in vivo study. *Clinical Oral Investigations*, 26, 171–181.
5. Seo, B.M., *et al*. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*, 364, 149–155.
6. Nagatomo, K., *et al*. (2006) Stem cell properties of human periodontal ligament cells. *Journal of Periodontal Research*, 41, 303–310.
7. Kadar, K., *et al*. (2009) Differentiation potential of stem cells from human dental origin - promise for tissue engineering. *Journal of Physiology and Pharmacology*, 60, 167–175.
8. Feng, L., *et al*. (2016) PDL progenitor-mediated PDL recovery contributes to orthodontic relapse. *Journal of Dental Research*, 95, 1049–1056.
9. Jin, S.S., *et al*. (2020) Mechanical force modulates periodontal ligament stem cell characteristics during bone remodelling via TRPV4. *Cell Proliferation*, 53, e12912.
10. Ghosal, S., Das, S. and Chakrabarti, J. (2013) Long noncoding RNAs: new players in the molecular mechanism for maintenance and differentiation of pluripotent stem cells. *Stem Cells and Development*, 22, 2240–2253.
11. Yang, Q., *et al*. (2018) Long noncoding RNAs: new players in the osteogenic differentiation of bone marrow- and adipose-derived mesenchymal stem cells. *Stem Cell Reviews*, 14, 297–308.
12. Ponting, C.P., Oliver, P.L. and Reik, W. (2009) Evolution and functions of long noncoding RNAs. *Cell Journal*, 136, 629–641.
13. Wapinski, O. and Chang, H.Y. (2011) Long noncoding RNAs and human disease. *Trends in Cell Biology*, 21, 354–361.
14. Li, J.S., Xie, Y.F., Song, L.T., Wang, X.P. and Jiang, S.Y. (2018) Expression profile of long non-coding RNA in gingival tissues from the patients with aggressive periodontitis. *Zhonghua Kouqiang Yixue Zazhi.*, 53, 635–639.
15. Li, S., *et al*. (2018) Integrated analysis of long noncoding RNA-associated competing endogenous RNA network in periodontitis. *Journal of Periodontal Research*, 53, 495–505.
16. Sanchez-Munoz, F., *et al*. (2018) Periodontitis may modulate long-non coding RNA expression. *Archives of Oral Biology*, 95, 95–99.
17. Zou, Y., *et al*. (2015) lncRNA expression signatures in periodontitis revealed by microarray: the potential role of lncRNAs in periodontitis pathogenesis. *Journal of Cellular Biochemistry*, 116, 640–647.
18. Pickard, M.R. and Williams, G.T. (2015) Molecular and cellular mechanisms of action of tumour suppressor GAS5 LncRNA. *Genes (Basel)*, 6, 484–499.
19. Ma, C., *et al*. (2016) The growth arrest-specific transcript 5 (GAS5): a pivotal tumour suppressor long noncoding RNA in human cancers. *Tumour Biology*, 37, 1437–1444.
20. Mayama, T., Marr, A.K. and Kino, T. (2016) Differential expression of glucocorticoid receptor noncoding RNA repressor gas5 in autoimmune and nflammatory diseases. *Hormone and Metabolic Research*, 48, 550–557.
21. Kanzaki, H., Chiba, M., Shimizu, Y. and Mitani, H. (2002) Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. *Journal of Bone and Mineral Research*, 17, 210–220.
22. Cao, H., *et al*. (2014) Force-induced Adrb2 in periodontal ligament cells promotes tooth movement. *Journal of Dental Research*, 93, 1163–1169.
23. Abe, T. and Hajishengallis, G. (2013) Optimization of the ligature-induced periodontitis model in mice. *Journal of Immunological Methods*, 394, 49–54.
24. Li, X., *et al*. (2018) Circular RNA CDR1as regulates osteoblastic differentiation of periodontal ligament stem cells via the miR-7/GDF5/SMAD and p38 MAPK signaling pathway. *Stem Cell Research and Therapy*, 9, 232.
25. Zheng, Y., Li, X., Huang, Y., Jia, L. and Li, W. (2017) The circular RNA landscape of periodontal ligament stem cells during osteogenesis. *Journal of Periodontology*, 88, 906–914.
26. Lee, H.H., *et al*. (2017) TNF-alpha-induced inflammation stimulates apolipoprotein-A4 via activation of TNFR2 and NF-kappaB signaling in kidney tubular cells. *Scientific Reports*, 7, 8856.
27. Dutt, V., *et al*. (2018) S-allyl cysteine inhibits TNFalpha-induced skeletal muscle wasting through suppressing proteolysis and expression of inflammatory molecules. *Biochimica et Biophysica Acta, General Subjects*, 1862, 895–906.
28. Golz, L., *et al*. (2015) Hypoxia and P. gingivalis synergistically induce HIF-1 and NF-kappaB activation in PDL cells and periodontal diseases. *Mediators of Inflammation*, 2015, 438085.
29. Napetschnig, J. and Wu, H. (2013) Molecular basis of NF-kappaB signaling. *Annual Review of Biophysics*, 42, 443–468.
30. Lawrence, T. (2009) The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology*, 1, a001651.
31. Viatour, P., Merville, M.P., Bours, V. and Chariot, A. (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends in Biochemical Sciences*, 30, 43–52.
32. Page, R.C. (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease. *Journal of Periodontal Research*, 26, 230–242.
33. Graves, D.T. and Cochran, D. (2003) The contribution of interleukin-1 and tumour necrosis factor to periodontal tissue destruction. *Journal of Periodontology*, 74, 391–401.
34. Pacios, S., *et al*. (2015) Osteoblast lineage cells play an essential role in periodontal bone loss through activation of nuclear factor-kappa B. *Scientific Reports*, 5, 16694.
35. Arabaci, T., *et al*. (2010) Inflammatory and immune pathways in the pathogenesis of periodontal disease. *European Journal of Dental and Oral Health*, 4, 454–461.
36. Kida, Y., *et al*. (2005) Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. *Cytokine*, 29, 159–168.
37. Yip, K.H., Zheng, M.H., Feng, H.T., Steer, J.H., Joyce, D.A. and Xu, J. (2004) Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. *Journal of Bone and Mineral Research*, 19, 1905–1916.
38. Zhang, R., Han, X., Huang, T. and Wang, X. (2019) Danggui buxue tang inhibited mesangial cell proliferation and extracellular matrix accumulation through GAS5/NF-kappaB pathway. *Bioscience Reports*, 39, BSR20181740. doi:10.1042/BSR20181740
39. Li, F., Sun, J., Huang, S., Su, G. and Pi, G. (2018) LncRNA GAS5 overexpression reverses LPS-Induced inflammatory injury and apoptosis through up-regulating KLF2 expression in ATDC5 chondrocytes. *Cellular Physiology and Biochemistry*, 45, 1241–1251.
40. Zhao, J., Liu, B. and Li, C. (2020) Knockdown of long noncoding RNA GAS5 protects human cardiomyocyte-like AC16 cells against high glucose-induced inflammation by inhibiting miR-21-5p-mediated TLR4/NF-kappaB signaling. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 393, 1541–1547. doi:10.1007/s00210-019-01795-z
41. Yue, Q., *et al*. (2018) Downregulation of growth arrestspecific transcript 5 alleviates palmitic acidinduced myocardial inflammatory injury through the miR26a/HMGB1/NFkappaB axis. *Molecular Medicine Reports*, 18, 5742–5750.