Journal of Dental Sciences 18 (2023) 184–190

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

Original Article

Role of immune-related lncRNAs-PRKCQ-AS1 and EGOT in the regulation of IL-1β, IL-6 and IL-8 expression in human gingival fibroblasts with TNF-α stimulation

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Received 6 June 2022; Final revision received 13 June 2022
Available online 4 July 2022

KEYWORDS
Periodontitis;
lncRNAs;
CIBERSORT;
HGF;
Bioinformation

Abstract *Background/purpose:* It was reported that lncRNAs have an effect on immune-related diseases, however, their roles in periodontitis remain to be investigated. The aim of this study was to look for immune-related lncRNAs in periodontitis, and to preliminarily explore their function *in vitro*.
Materials and methods: CIBERSORT was used to analyze abundance of immune cell in the periodontal tissue. Correlation between the expression profile of lncRNAs and abundance of immune cell was calculated and immune-related lncRNAs were identified. The expressions of immune-related lncRNAs identified were validated by RT-qPCR with 15 periodontitis and 15 healthy gingival tissues. The expressions of PRKCQ-AS1 and EGOT in HGFs were detected under the stimulation of different concentrations of TNF-α (0, 10, 15, 20, 30 ng/mL) and different duration (0, 12, 24 and 48 h). Then, siRNA was used to silence PRKCQ-AS1 and EGOT in HGFs. The expression level of IL-1β, IL-6, IL-8 of the HGFs after stimulated by 15 ng/mL TNF-α, and the activation of NF-κB pathway was observed.
Results: PRKCQ-AS1 and EGOT were identified as top 2 immune-related lncRNAs in periodontal tissues. The expressions of PRKCQ-AS1 and EGOT were significantly up-regulated in inflamed periodontal tissue and in HGFs under TNF-α stimulation. After knock-down of PRKCQ-AS1 and EGOT, expression level of IL-1β, IL-6, and IL-8 in HGFs with TNF-α stimulation were decreased, and activation of NF-κB pathway was inhibited.
Conclusion: PRKCQ-AS1 and EGOT were firstly identified as immune-related lncRNAs in

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https://doi.org/10.1016/j.jds.2022.06.006
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periodontal tissue, and they regulate the expression of IL-1β, IL-6, and IL-8 of HGFs through the NF-κB pathway.
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Introduction

LncRNAs are a class of non-coding RNAs (ncRNAs) with more than 200 nucleotides in length. Although they do not encode proteins, they can regulate gene expression at different transcriptional and post-transcriptional level.[1] Recently, there has been increasing evidence linking lncRNAs to various inflammatory diseases like rheumatoid arthritis, inflammatory bowel diseases and periodontitis.[2–4]

Periodontitis is a chronic inflammatory disease occurring in periodontal tissue, accompanied with gingival inflammation, attachment loss, pocket formation, alveolar bone absorption, tooth mobility, and even tooth loss. As one of the most common oral diseases, periodontitis is the leading cause of tooth loss worldwide, which becomes a significant healthcare, social, and economic burden.[5] Plaque is the initiating factor in periodontitis. The colonization of subgingival pathogenic bacteria activates the host's excessive immune response, leading to the progression of periodontitis. The host immune response has an important role in the pathogenesis of periodontitis.[6] There have been many previous studies on mechanism of encoding-proteins genes in periodontitis,[7] only a few studies on ncRNAs, especially lncRNAs, have been found to be involved in periodontal immune regulation.[8,9]

Several researches had been undertaken in recent years utilizing transcriptome and bioinformatics analysis to screen for immune-related lncRNAs, particularly in cancer.[10] However, only a few studies have reported immune-related lncRNAs in non-tumour diseases such as periodontitis. Zhang et al.[11] identified two immune-related subtypes of periodontitis and discovered 291 immune-related lncRNAs which could have a role in periodontitis used the weighted correlation network analysis (WGCNA). Nevertheless, the role of how these immune-related lncRNAs in immune regulation remains unclear and need to be further investigated. The aim of this work was to look for lncRNAs related to periodontal immune modulation, and to preliminarily explore their function *in vitro*.

Materials and methods

Sample collection and RNA-sequencing (RNA-seq)

Inflamed periodontal tissue and healthy gingivae were collected during periodontal surgery at Peking University Hospital of Stomatology from May 2020 to April 2021. All the participants gave written informed consent. The study protocol was approved by the ethical committee of the Peking University Hospital of Stomatology (PKUSSIRB-202055070). In total, 20 periodontitis and 20 healthy samples were collected, 5 samples of each group were used for RNA-seq, the other for RT-qPCR experiment.

For RNA-seq, total RNA was extracted and sequenced on the DNBSEQ platform (Huada Gene Technology, Wuhan, China). Sequencing data were filtered using SOAP nuke, and then, clean reads were mapped to reference genome using HISAT2. Stringtie was used to assemble and quantify transcripts. DESeq2 was used for differential expression analysis, genes with $P \leq 0.05$, FDR ≤ 0.05, and $|\log_2 FC$ (fold change)$| \geq 1$ were identified as differentially expressed.

Identification of immune-related lncRNAs

The LM22 of CIBERSORT is a matrix file consisting of 547 characteristic genes of 22 types of immune cells. It had been widely validated to analyze the level of immune cell in tissues.[12] Spearman correlation analysis was used to analyze the correlation between the differentially expressed lncRNAs and the relative abundance of immune cell. Genes with a correlation greater than 0.8 and $P < 0.05$ were selected as immune-related lncRNAs.

Primary cell culture

Primary human gingival fibroblasts (HGFs) were extracted from healthy gingival tissue. Gingival tissue was de-epithelized and washed in phosphate-buffered saline (PBS) containing 5% antibiotics for 5 mins. The connective tissue was then minced into small pieces (1–2 mm^2) and plated onto a culture dish in α-Minimum Essential Medium (α-MEM; Gibco, NY, USA) supplemented with 20% fetal bovine serum (FBS, Biosera, Miami, FL, USA) and 1% antibiotic-antimycotic mixture (Gibco, Grand Island, NY, USA). The cells were then incubated at 37 °C with 5% CO_2. HGFs from passages 3–6 were used for experiments.

Small interfering RNA transfection

EGOT-specific small interfering RNA (siRNA), PRKCQ-AS1-specific siRNA and non-targeting control siRNA were synthesized by Genepharma Biotech (Suzhou, China). 1×10^6 HGF were seeded into 6-well plates for 24 h, and the siRNAs were transfected into HGFs grown to 70% using Rfect (Baidai, Changzhou, China). After 48 h, RT-qPCR was applied to validate the effectiveness of RNA interference (RNAi).

Quantitative real-time PCR (qRT-qPCR)

Total RNA was extracted using TRIzol® Reagent (Thermo Fisher, Waltham, MA, USA). The ABScript II cDNA Fist-Strand Synthesis Kit (ABclonal, Wuhan, China) was used for reverse

transcription to synthesize cDNA. RT—qPCR was conducted as described previously using qPCR SYBR® Green Master Mix (Roche, Basel, Switzerland).[13] GAPDH was used as endogenous reference. All primer sequences were synthesized by Sangon Biotech (Shanghai, China). Relative expression of lncRNAs or mRNAs was determined using the ΔΔCT method.

Enzyme linked immunosorbent assay

Cell supernatant was collected and the concentration of IL-1β, IL-6, IL-8 was analyzed using enzyme-linked immunosorbent assay according to the manufacturer's instructions (ABclonal).

Western blot analysis

The HGFs were lysed in RIPA lysis buffer with PMSF (Beyotime, Shanghai, China), protease inhibitor cocktail and phosphatase inhibitor cocktail on ice for 30 min and quantified with a BCA kit (Beyotime). An equal amount of protein (30 μg) from each sample was subjected to SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher). After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with primary antibodies (anti-p65, anti-p—p65 (1:1000, Abcam, Cambridge, United Kingdom) and anti-GADPH (1:1000, Protein, Abclonal)) at 4 °C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, ZSGB-Bio, Beijing, China) for 1 h at room temperature. Finally, protein expression was detected by chemiluminescence.

Statistics

All data represent the results of at least three independent experiments. Statistical analyses were performed with SPSS 22.0 (IBM, Armonk, NY, USA). Data were evaluated by ANOVA and two-tailed t tests were also performed where appropriate (comparison of two groups only). Differences were considered statistically significant at the $P < 0.05$ level.

Results

Identification of immune-related lncRNAs

A total of 90 lncRNAs were found differently expressed in periodontitis compared to healthy gingivae through RNA-seq. Then, we obtained 16 kinds of immune cells in periodontal tissue by CIBERSORT, including B cells naive, B cells memory, plasma cells, T cells CD8, T cells CD4 naive, T cells CD4 memory, T cells follicular helper, NK cells resting, monocytes, macrophages M0, macrophages M1, macrophages M2, dendritic cells activated, mast cells resting, mast cells activated, and neutrophils. Correlation analysis was conducted and a total of 23 lncRNAs were screened as immune-related lncRNAs with correlation coefficient >0.8 and $P < 0.05$ (Table 1). Among them, the top two lncRNAs with the highest correlation are PRKCQ-AS1 and EGOT.

Upregulated expression of PRKCQ-AS1 and EGOT in inflamed periodontal tissue

Rt-qPCR was used to detect expression level of PRKCQ-AS1 and EGOT in 15 periodontitis tissues and 15 healthy tissue samples, and the results showed that the expression of PRKCQ-AS1 and EGOT in periodontitis tissues was significantly up-regulated (Fig. 1).

Expression of EGOT and PRKCQ-AS1 is upregulated in HGFs with TNF-α stimulation

HGFs were stimulated with 0, 10, 15, 20, and 30 ng/mL of TNF-α. The RT-qPCR findings revealed that the expression levels of PRKCQ-AS1 and EGOT in HGFs were all up-regulated to varying degrees as shown in Fig. 2(a). All groups except 10 ng/mL group showed significant different from control group, with the highest elevation of PRKCQ-AS1 and EGOT occurring at a concentration of 15 ng/mL.

Then, HGFs was stimulated with 15 ng/mL TNF-α. The cells were collected after 0, 12, 24, and 48 h, respectively. TNF-α stimulation at different time increased the expression of PRKCQ-AS1 and EGOT in HGFs in time dependence, as shown in Fig. 2(b). At 12 h, the difference was not statistically significant ($P > 0.05$). However, the significant up-regulated expression were found at 24 and 48 h, with the highest increase of expression occurring at 48 h ($P < 0.001$).

PRKCQ-AS1 and EGOT affect the expression of IL-1β, IL-6 and IL8 in HGFs with TNF-α stimulation

PRKCQ-AS1 and EGOT knockdown models were constructed using three different siRNA sequences, with siRNAs transfected with random sequences serving as negative controls. All three siRNA sequences were able to diminish PRKCQ-AS1 and EGOT expression when compared to the negative control. Two sequences, si-EGOT-2 and si-PRKCQ-AS1-1, had the most substantial knockdown effect (Fig. 3), so these two sequences were chosen for the further tests.

HGFs were transfected with si-PRKCQ-AS1-1, si-EGOT-2, and random sequences, and then stimulated with 15 ng/mL TNF-α. After 48 h, the expression levels of IL-1, IL-6, and IL-8 mRNAs were measured by RT-qPCR, and the cell culture supernatants were analyzed by ELISA for IL-1, IL-6, and IL-8 levels. After knocking down PRKCQ-AS1 and EGOT, the expression levels of IL-1, IL-6, and IL-8 mRNAs (Fig. 4(a)) and protein secretion levels of IL-1, IL-6, and IL-8 (Fig. 4(b)) were considerably down-regulated in HGFs generated by TNF-α stimulation.

Role of NF-κB pathway in PRKCQ-AS1, EGOT regulation of HGFs expression of IL-1β, IL-6, IL-8

The cells were transfected with si-PRKCQ-AS1-1, si-EGOT-2, and random sequences, and HGFs was stimulated with 15 ng/mL of TNF- at 48 h after transfection, with TNF-α stimulation lasting 48 h. After that, total cell protein was extracted, and changes in protein expression of phosphorylated p65 (p-p65) and p65 were detected to reflect NF-κB pathway activity. The results showed that when PRKCQ-AS1

Table 1 Correlation between differential expression of lncRNAs and score of immune cells.

LncRNAs	Correlation	p-value	LncRNAs	Correlation	*P*-value
PRKCQ-AS1	0.9476	3.10E-05	LINC01561	0.8329	0.002774
EGOT	0.9183	0.000177	LINC00411	0.8303	0.002937
AC005532.1	0.8879	0.000603	AL354733.2	−0.8292	0.003014
LINC02242	−0.8813	0.000752	AC008011.2	0.8286	0.003055
AC082651.3	0.8596	0.001429	AC064805.1	−0.8274	0.003134
LINC02243	0.8483	0.001923	AC019080.4	0.8211	0.003593
NALCN-AS1	0.8585	0.001474	AP000866.2	0.819	0.003751
NR4A1AS	0.8498	0.001849	AC099684.3	0.8171	0.003899
AC010719.1	0.8493	0.001873	THBS3-AS1	0.815	0.00407
TFAP2A-AS2	0.8455	0.002059	AC067863.1	0.8102	0.004487
AC079174.1	0.8453	0.002071	B3GALT5-AS1	0.8081	0.004674
AC078883.2	0.8379	0.002469			

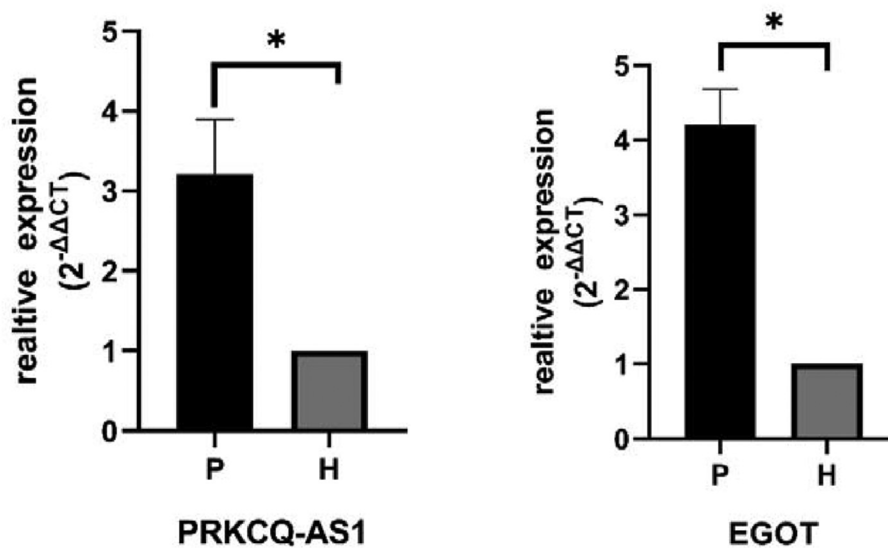

Figure 1 Expression levels of PRKCQ-AS1 and EGOT in 15 periodontal inflammatory tissues and 15 healthy tissues. The relative expression levels were expressed by2-ΔΔCT *:P < 0.05, **:P < 0.01.

and EGOT were knocked down, p-p65 expression was downregulated, but p65 was not (Fig. 5), indicating that TNF-stimulated NF-κB pathway activation was blocked.

Discussion

Our study identified 23 immune-related lncRNAs through correlation analysis between lncRNAs expression profiles and abundance of immune cell infiltration from CIBERSORT. Based on our analysis, PRKCQ-AS1 and EGOT were identified as top two immune-related lncRNAs in periodontal tissues, while their roles and levels of expression in periodontitis had yet to be documented in research.

It had been reported that expression of PRKCQ-AS1 was increased in tissues and cells of colorectal cancer.[14] PKCθ, which is encoded by PRKCQ (the PRKCQ-AS1 host gene), plays a crucial role in T-cell activation,[15–17] and the NF-κB transcription factor is the major target of PKCθ:[15] PKCθ translocation to the immunological synapse in T-cell is required for activation of the NF-κB signalling pathway.[16]

Previous studies had found that after infection with human hepatitis C virus (HCV), EGOT expression is upregulated in liver tissue and HuH7 cells, and that TNF-α may be the main driver of EGOT expression.[18] THP-1 macrophages, as well as activated CD4 and CD8 lymphocytes, have considerably higher EGOT expression levels in response to TNF-α and LPS stimulation.[19] EGOT plays a role in the activation of cell inflammation via the PI3K/AKT, MAPK, and NF-κB pathways.[19]

In this study, we first reported the increased expression of PRKCQ-AS1 and EGOT in inflamed periodontal tissues, as well as increased PRKCQ-AS1 and EGOT expression in HGFs in response to TNF-α stimulation. It suggested that PRKCQ-AS1 and EGOT involved in periodontal immune regulation.

The present study first demonstrated that PRKCQ-AS1 and EGOT play a role in regulating the expression of IL-1β, IL-6 and IL-8 in HGFs under TNF-α stimulation. TNF-α stimulation represents inflammatory microenvironment. In this environment, the expression of IL-1β, IL-6 and IL-8 in HGFs increased, the expression level of PRKCQ-AS1 and EGOT in HGFs were also up-expressed. When knockdown of

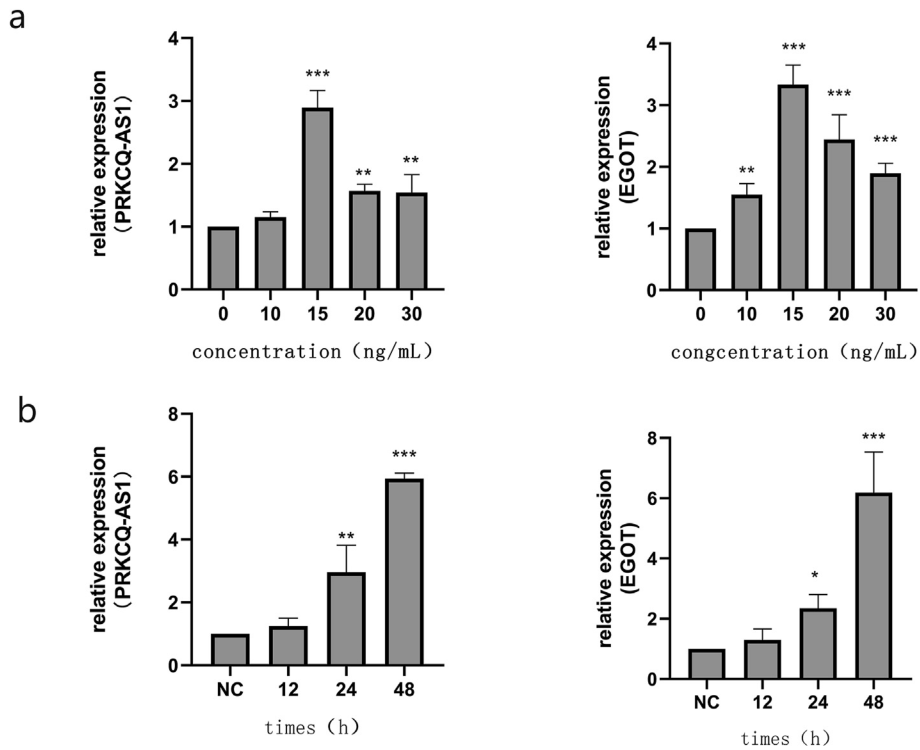

Figure 2 (a) Effect of different concentrations of TNF-α stimulation on HGFs expression of PRKCQ-AS1, EGOT. (b) Effect of different durations of TNF-α stimulation on HGFs expression of PRKCQ-AS1, EGOT. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

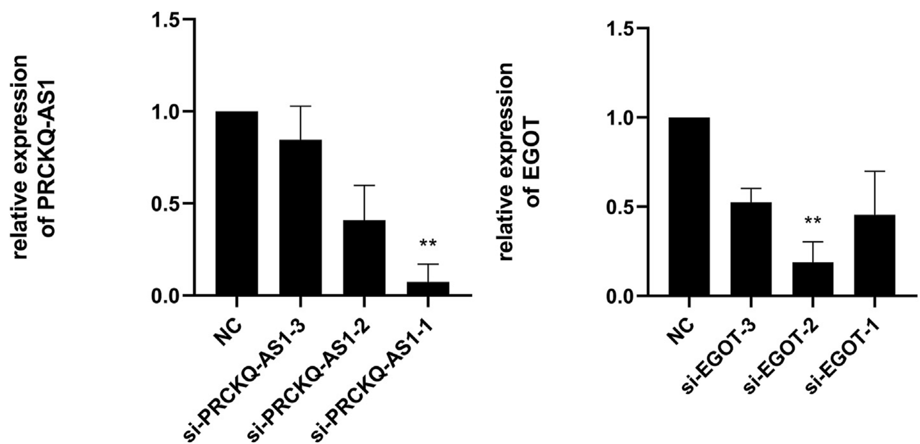

Figure 3 PRKCQ-AS1, EGOT knockdown model construction. *: $P < 0.05$; **: $P < 0.01$; NS: $P > 0.05$.

PRKCQ-AS1 and EGOT using siRNA, both mRNA and secreted protein levels of IL-1β, IL-6 and IL-8 were significantly down-regulated, indicates that PRKCQ-AS1 and EGOT play a role in TNF-α promoting the expression of pro-inflammatory factors IL-1β, IL-6 and chemokines IL-8 in HGFs.

Human gingival fibroblasts (HGFs) is a stromal cell in periodontal tissues. In response to stimulation of LPS from periodontal pathogenic bacteria, HGFs express a variety of pattern recognition receptors (PRR),[20,21] and release inflammatory factors IL-1β, IL-6, and the chemokine IL-8.[21] Both IL-1β and IL-6 are significant pro-inflammatory cytokines involved in host immune response of periodontal tissue,[22] as well as initiating osteoclast development and speeding alveolar bone resorption due to inflammation.[23] IL-8 is a powerful chemokine that plays a crucial role in the innate immune response by chemotactic immune cells (neutrophils, mononuclear macrophages) releasing pro-inflammatory mediators and therefore boosting the immune response. The gingival epithelium expresses IL-8 and forms a chemotactic gradient of IL-8, which controls the migration of neutrophils into the gingival sulcus. The levels of IL-1β, IL-6 and IL-8 in the gingival sulcus and saliva of patients with periodontitis significantly correlated with the severity and progression of periodontitis.[24,25] In one word, the secretion levels of IL-1β, IL-6, and IL-8 in HGFs are closely related to destruction of periodontal tissue and

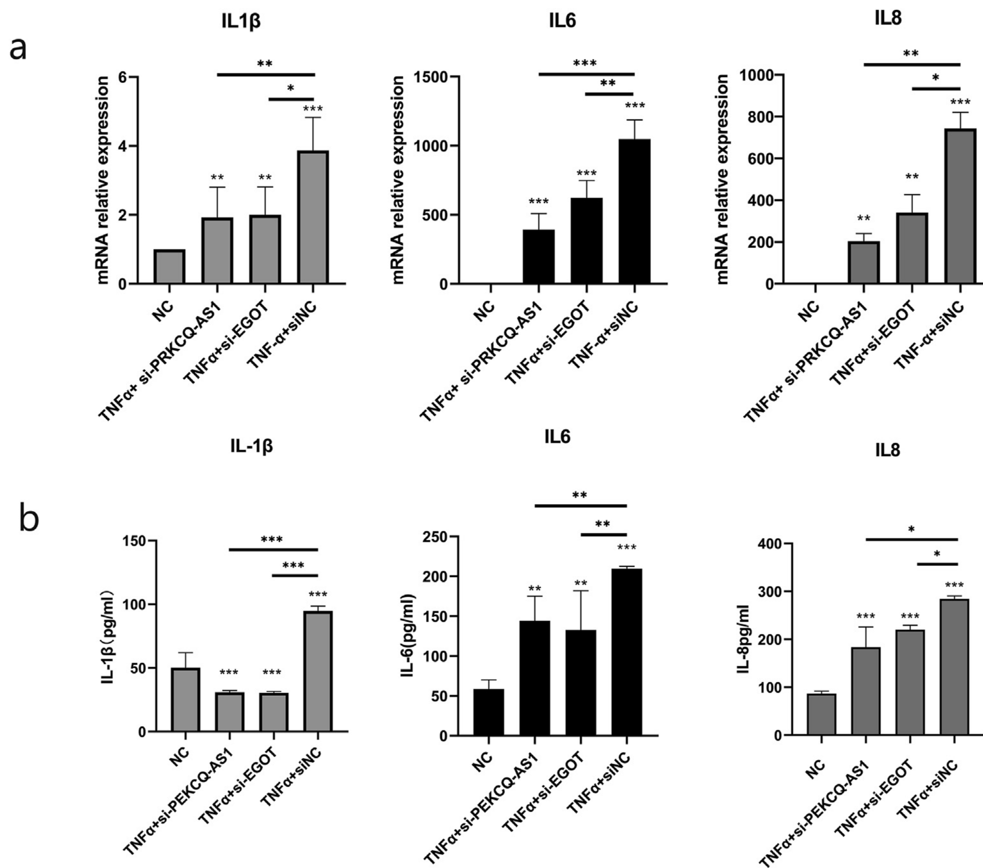

Figure 4 (a) Changes in mRNAs expression levels and (b) secretion levels of IL-1β, IL-6 and IL-8 in HGFs under TNF-α stimulation after knockdown of PRKCQ-AS1 and EGOT. *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$.

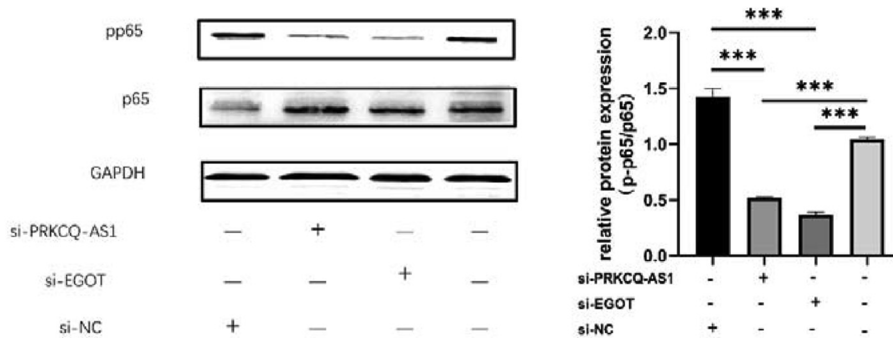

Figure 5 Changes in p-p65 and p65 protein expression levels in HGFs after knockdown of PRKCQ-AS1 and EGOT, ***: $P < 0.001$.

progression of periodontitis. Our study suggested that PRKCQ-AS1 and EGOT may be involved in the destruction of periodontal tissue and periodontitis by regulating the expression of IL-1β, IL-6, and IL-8 in HGF.

On this basis, our study also found that PRKCQ-AS1 and EGOT may exert their regulatory role through the NF-κB signaling pathway. As a key regulator of the periodontal inflammatory response, the activation of NF-κB is implicated in expression of inflammatory factors and chemokine.[26] Synergistic activation of NF-κB and AP-1 or NF-κB and NF-IL6 is necessary for transcription of the IL-8, and activation of NF-κB is also required for transcription of IL-1β and IL-6.[27] In the present study, knockdown of PRKCQ-AS1 and EGOT resulted in significantly lower phosphorylation levels of p65 in the NF-κB pathway under TNF-α stimulation compared to transfected random sequences, indicating that activation of the NF-κB pathway was inhibited by knockdown of PRKCQ-AS1 and EGOT under TNF-α stimulation, suggesting that in HGFs, PRKCQ-AS1 and EGOT may regulate the expression of IL-1β, IL-6 and IL-8 through the NF-κB signaling pathway.

In conclusion, our study firstly identified 23 immune-related lncRNA in periodontal tissue through CIBERSORT algorithm. Then, we selected top 2 immune-related

lncRNAs: PRKCQ-AS1 and EGOT, and found they could regulate the expression of IL-1β, IL-6, and IL-8 of HGFs through the NF-κB pathway. However, there are some limitations: we only constructed a knockdown model of PRKCQ-AS1 and EGOT by siRNA, as a way to observe the immunomodulatory functions of PRKCQ-AS1 and EGOT in HGFs under TNF-α stimulation, and further overexpression vectors should be constructed in subsequent studies. In addition, we observed cell behaviour *in vitro* with TNF-stimulation mimicked in the inflammatory microenvironment, and in future research, LPS stimulation or bacterial invasion may be used to refine its functional investigation in the pathogenic mechanism of periodontitis.

Declaration of competing interest

The authors declare that there are no competing interests in this study.

Acknowledgements

This work was funded by the National Science Foundation of China (No 81991500, No 81991502, No. 82071118, No 81870772, No 81570986).

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