HSP90AA1 promotes the inflammation in human gingival fibroblasts induced by Porphyromonas gingivalis lipopolysaccharide via regulating of autophagy

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Research Article

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Abstract

Background

Peri-implantitis of tooth seriously affects the life quality of patients. This study aims to investigate the role of HSP90AA1 in the inflammatory of human gingival fibroblasts (HGFs) induced by *porphyromonas gingivalis* lipopolysaccharide (Pg-LPS), and to provide a potential therapeutic target for clinical treatment of peri-implantitis.

Methods

Used Pg-LPS (0.1, 1, 10 µg/mL) to construct an inflammatory model of HGFs, and transfected HSP90AA1-siRNA to construct HSP90AA1 low expression HGFs cell line, respectively. After that, cell viability, the contents of IL-1β, IL-6, TNF-α were detected by CCK-8, and ELISA assay. Intracellular ROS, the expressions of HSP90α, HSP90β were detected by immunofluorescence. Apoptosis was detected by flow cytometry. Western blot, RT-PCR detected the protein and gene level of HSP90AA1, and relative protein expressions of p-NF-κB p65/NF-κB p65, LC3 II/I, ATG5, and Beclin-1 was detected by western blot.

Results

In the inflammatory cell model, compared with the control group, Pg-LPS did not affect cell viability, increased the contents of IL-1β, IL-6, and TNF-α in cells, and increased protein, gene level of HSP90AA1, and p-NF-κB p65/NF-κB p65, LC3 II/I, ATG5, Beclin-1 protein levels. In HGFs cell line with low expression of HSP90AA1, compared with siNC group, the levels of inflammatory factors, ROS, apoptosis rate, HSP90α, HSP90β protein expression, and autophagy related protein levels in siHSP90AA1 group were significantly reduced.

Conclusions

This study confirmed that HSP90AA1 gene promotes Pg-LPS induced HGFs inflammation mediated by autophagy *in vitro*. It is suggested that HSP90AA1 is a key gene in the development of peri-implantitis.

1 Background

The application of dental implants has changed the way of prosthetics and treatment for some patients with completely or partially edentulous; due to its advantages of not damaging adjacent teeth and good retentive support, dental implants have gradually replaced the position of fixed bridge[^1^, ^2^]. Although dental implants have a high success rate, complications and failures are inevitable. Peri-implantitis has been described as a destructive inflammatory lesion that affects bone integration implant hard and soft
tissue, resulting in bone loss and peri-implant pocketing\[3\]. The occurrence of peri-implantitis is related to microbial infection. Due to the invasion of pathogenic bacteria, there is a continuous inflammatory reaction in the gingiva and combined alveolar bone around it. In the late stage of inflammation, there will be absorption of peri-implant pocketing and surrounding alveolar bone, which will eventually cause implant loosening and falling off, resulting in implant failure\[4\]. In addition, risk factors such as smoking, diabetes, poor oral hygiene, history of periodontitis, improper repair design will also promote the occurrence of peri-implantitis\[5, 6\]. According to the survey, the incidence rate of peri-implantitis is about 10%\[7\]. Therefore, in-depth study of the pathogenesis of peri-implantitis is very important to alleviate the tooth implant complications and improve the quality of life of implant patients.

Human gingival fibroblasts (HGFs) are the main cells of periodontal soft tissue\[8\]. HGFs play an active and key role in mechanisms of the host immune defense against multiple pathogens, and maintain tissue structure and function\[9\]. Studies have reported that bacterial colonization was found when the implant surface was exposed to the mouth for more than 30 min\[10\]. The main pathogenic bacteria of peri-implantitis are mainly Gram-negative bacteria, such as *Porphyromonas gingivalis*\[11\]. Compared with healthy sites, peri-implantitis sites have higher average colony forming units of bacteria. *Porphyromonas gingivalis* has many virulence factors, such as lipopolysaccharide (LPS), which can cause the metabolism and inflammatory response of HGFs\[12\]. Under the stimulation of *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS), HGFs can secrete a variety of inflammatory cytokines, such as proinflammatory mediator IL-1β, IL-6, IL-8, TNF-α, to activate the inflammation response\[13, 14\]. IL-1 β can promote the production of IL-6, and its excessive production may lead to the destruction of connective tissue\[15\]. IL-6 participates in the pathogenesis of periodontal disease by inducing osteoclastogenesis, tissue destruction and bone resorption\[16\]. The reduction of these inflammatory mediators may reduce oxidative burst and inhibit inflammatory response\[17\]. Therefore, the inflammatory response of HGFs induced by Pg-LPS may play an important role in the occurrence and development of tooth peri-implantitis.

Autophagy is a physiological process of programmed cell death, in which excess protein and subcellular components are wrapped in autophagosomes and are directed to lysosomes to digest and degrade\[18\]. The formation of these autophagosomes depends on autophagy associated protein 5 (ATG5) and microtubule-associated protein light chain 3 (LC3)\[19\]. Beclin-1 is the first protein identified to be associated with autophagy. It usually regulates autophagy in the form of cytoplasmic solute\[20\]. Under normal physiological conditions, all cells undergo physiological autophagy to maintain the balance of physiological activities. Heat shock protein 90 (HSP90) is a highly conserved and very important chaperone protein in eukaryotes\[21\]. The most widely studied is HSP90β continuously expressed in cells, and HSP90α induced by cell stress. These two proteins are the result of gene replication, and 86% of their amino acid sequences are the same. HSP90α is encoded by HSP90AA1 gene. Research shown that HSP90α is closely related to the occurrence and development of autophagy. Xiao\[22\] *et al.* found that high expression of HSP90AA1 gene could enhance the tolerance of osteosarcoma cells to chemotherapeutic
drugs by inducing autophagy. Hu[23] et al. discovered that HSP90AA1 can promote the autophagy by directly interacting with AKT/mTOR signaling pathway. Autophagy can be mediated by NF-κB signaling pathways induce inflammation[24]. Our previous study found that HSP90AA1 may be a key gene in the development of peri-implantitis. Therefore, we hypothesized that HSP90AA1 promotes the inflammatory response of HGFs induced by Pg-LPS by regulating autophagy. This study will further explore the role of HSP90AA1 gene in the development of peri-implantitis, and provide a potential target for clinical treatment of peri-implantitis.

2 Materials And Methods

2.1 Cell culture and treatment

HGFs cells (HUM-iCell-m005, iCell Bioscience Inc, Shanghai, China) were cultured in DMEM medium containing 10% fetal bovine serum, at 5% CO₂, 95% air and 37 °C constant temperature. HGFs were incubated with different concentrations of Lipopolysaccharide of Porphyromonas gingivalis (Pg-LPS) for 24 h to construct an inflammatory model of HGFs in vitro. Cells were divided into different groups: control group, no processing; Pg-LPS (0.1, 1, 10 µg/mL) treatment group.

2.2 HSP90AA1-siRNA transfection

Selecting 1 µg/mL as an appropriate concentration of Pg-LPS to infect HGFs. On this basis, siRNA targeting HSP90AA1 gene was designed and transfected into HGFs with transfection reagent to construct HSP90AA1 HGFs cell line with low expression. The cells were are processed in several ways: HGFs + Pg-LPS, HGFs + Pg-LPS + siRNA empty carrier, HGFs + Pg-LPS + HSP90AA1-siRNA 1, 2, respectively.

2.3 CCK-8 assay

Logarithmic growth phase HGFs was treated with different concentrations of Pg-LPS and cultured in 96 well plates for 24 h. Then add 10 µL per hole CCK-8 solution incubated in the incubator. At last, measured the absorbance at 490 nm wavelength, calculated the cell survival rate. Six replicate cells were measured in parallel for each group.

2.4 ELISA kit detection

Take 200 µL cell suspension (5 × 10⁵/mL) was added to 96 well plate for 24 h. After centrifugation, the supernatant was taken. using 0.22 µM microporous membrane filtration to obtain the sample to be tested. Enzyme labeled plates were first added to the tested samples, followed by enzyme-linked antibody (HRP) 100 µL incubated for 1 h. After washing the plate, add the chromogenic solution for chromogenic reaction, and add the termination solution. Finally, determine the OD value of each hole with an enzyme labeling instrument. The content of the factors (IL-1β, IL-6, TNF-α) in the sample was calculated by comparing with the standard curve.

2.5 Immunofluorescence assay
Cells were planted on the plate and treated with drugs. The cells were fixed with 4% paraformaldehyde, and permeabilized with Triton X-100. PBS was rinsed and sealed with 5% BSA. Then add anti-HSP90α (abcam, ab79849, 1:100), anti-HSP90β (abcam, ab53497, 1:100) antibody incubated at 37 °C for 3 h. After washing with PBS, incubate with secondary antibody IgG H & L (abcam, ab150080, 1:500) for 30 min. DAPI was added dropwise to dye the nucleus under dark conditions, and sealed slices, then the collected image was observed under the fluorescence microscope.

2.6 ROS detection

HGFs cells (5×10^5/ml) were cultured on a 6 well-plate. After drug treatment, the cells were washed with PBS solution. Incubated in a medium containing 10 µM DCFH-DA for 45 min at 37 °C in dark environment. Then, DCFH-DA loaded cells were washed with PBS, and observed by fluorescence microscope. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

2.7 Flow cytometry

The cell density was adjusted and incubate in a constant temperature incubator for 24 h. Cells were collected, centrifuged and resuspended with buffer. Next, 5, 10 µL PI, Annexin V reagent were added to each tube, and incubated at room temperature for 10 min. The apoptosis rate was detected and three parallel assays were performed.

2.8 Western blotting analysis

The cells were treated with lysate, centrifuged to obtain cell samples, and the total protein concentration was determined by BCA method. Separation by 15% SDS-PAGE electrophoresis 30 µg protein, transfer the protein to PVDF membrane. Then, it was sealed at room temperature with 5% skimmed milk powder for 2 h. Add anti-HSP90AA1 (Affinity, BF0084, 1:1000), anti-LC3AB (Affinity, AF6139, 1:1000), anti-ATG5 (Affinity, DF6010, 1:1000), anti-Beclin1 (Affinity, AF0120, 1:2000), anti-NF-κB p65 (Affinity, AF5006, 1:1000), anti-Phospho-NF-kB p65 (Ser536) (Affinity, AF2006, 1:1000), β-actin (Affinity, AF7018, 1:5000) antibody at 4 °C overnight. Then, the secondary antibody was added and incubated at room temperature for 1.5 h. Finally, the chromogenic reaction was carried out, and the gray values of the bands were counted and the results were analyzed.

2.9 RT-PCR analysis

Trizol reagent was used to extract total RNA from cells. CDNA was synthesized according to reverse transcription kit. Then the cDNA was used as the template and amplified by real-time fluorescence quantitative PCR to detect the miRNA gene expression (2- ΔΔCt value) in each group. GAPDH was the internal reference of the sample. The primer gene sequence is as Table 1.
Table 1
qPCR Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Human HSP90AA1</td>
<td>CACAGGTGAGACCAAGGACC</td>
<td>TTCCCCTAGTTTTCATGCCACA</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>CATGTACGTGTGCTATCCAGGC</td>
<td>CTCCCTTAATGTACGCAACGAT</td>
</tr>
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2.10. Statistical analysis

By using SPSS 18.0 software, datas were analysed. One-way-ANOVA was used to analyze the multi-group difference, and then SNK test was used. Kruskal-Wallis H was used when the variance was uneven. The data were expressed mean ± SD, P < 0.05, with statistically significant.

3 Results

3.1 Pg-LPS did not affect the cell viability of HGFs, while increased the levels of cytokines

Different concentrations of Pg-LPS (0.1, 1, 10 µg/mL) were used to treat HGFs. It was found that Pg-LPS at all concentrations did not inhibit the cell viability (Fig. 1a). We also measured the changes of inflammatory factors after cell infection. As shown in Fig. 1b, compared with the normal control group, all concentrations of Pg-LPS significantly increased the cytokine levels of IL-1β, IL-6, and TNF-α. It showed that there is a certain inflammatory reaction in the cells. We observed that the effect of Pg-LPS (1, 10 µg/mL) is the same, indicating that when the concentration is greater than 1 µg/mL, the infection effect of Pg-LPS does not increase with increasing concentration.

3.2 Pg-LPS treatment increased HSP90AA1 protein and gene level, and elevated the expressions of autophagy related protein

As shown in Fig. 2a, b, compared with the control group, when concentration was less than 1, Pg-LPS dose dependently increased HSP90AA1 protein and gene level. Similarly, 1, 10 µg/mL Pg-LPS has the same effect, even at the gene level, 1 µg/mL Pg-LPS works better. After PG LPS treatment, we measured the expression of autophagy related proteins. The results showed that compared with the control group, PG LPS treatment could significantly increase the protein levels of p-NF-κB p65/NF-κB p65, LC3 II/I, ATG5, and Beclin-1 (Fig. 2c, d, e).

3.3 Construction and screening HGFs cell lines with stable, and low expression of HSP90AA1

Based on the above results, we selected the highest expression of HSP90AA1 in inflammatory cell lines (1 µg/mL Pg-LPS treatment group) as the follow-up research object. As shown in Fig. 3a, b, after transfection of HSP90AA1-siRNA, the protein and gene level of HSP90AA1 decreased significantly as
expected. Accordingly, we screened HSP90AA1 cell line (siHSP90AA1-1) with stable, and low expression to perform the the subsequent confirmatory experiments. As shown in Fig. 3c, by detecting the level of inflammatory factors in the cell group with low expression of HSP90AA1, it was found that, after transfection HSP90AA1-siRNA, the levels of IL-1β, IL-6, and TNF-α were obviously reduced. These results indicate that HSP90AA1 could regulate the levels of inflammatory cytokines.

3.4 HSP90α, HSP90β expression were decreased by HSP90AA1-siRNA

We detected the changes of HSP90α, HSP90β related protein expression in Pg-LPS stimulated HGFs regulated by HSP90AA1 gene, after HSP90AA1 siRNA was transfected into cells. Obviously, the results showed that the fluorescence intensity of HSP90α, HSP90β decreased significantly in HSP90AA1-siRNA transfection group (Fig. 4a, b).

3.5 HSP90AA1-siRNA transfection decreased cellular ROS generation, and inhibited the apoptosis

We also detected the ROS level in cells transfected with HSP90Aa1-SiRNA, and it was evident that the ROS level in siHSP90AA1 group was decreased (Fig. 5a). According to the results of above, we have observed the important role of HSP90AA1 gene in regulating inflammation in HGFs, and we further explored its potential mechanism. As shown in Fig. 5b, by detecting apoptosis, we found that the number of apoptotic cells, and apoptosis rate in siHSP90AA1 group were significantly lower than those in siRNA group.

3.6 Low expression of HSP90AA1 reduced autophagy related protein levels

After the cells were transfected with HSP90AA1-siRNA, we detected the changes of autophagy related proteins, and found that the protein levels of LC3 II/I, ATG5, and Beclin-1 were evidently decreased (Fig. 6a, b). Furthermore, in response to autophagy-induced inflammation, we also found the protein expression of p-NF-κB p65/NF-κB p65 was reduced (Fig. 6c). These fully verified that the HSP90AA1 gene could regulate the level of autophagy related proteins.

4 Discussion

Peri-implantitis as a late complication usually occurs after planting superstructure or dental prosthesis[25]. Although the survival rate of implant is very high (92.8–97.1%)[1], biological complications such as peri implant inflammation are easy to occur[26]. The occurrence of peri-implantitis has seriously affected the quality of life of dental implant patients. In this study, we investigated the effects of HSP90AA1 gene on autophagy, apoptosis and inflammatory state of HGFs after Pg-LPS infection. We
confirmed that HSP90AA1 could promote the Pg-LPS induced HGFs inflammatory response mainly by mediating cell autophagy. This study provides an effective target gene for the treatment of peri-implantitis.

HGFs can actively participate in immune and inflammatory responses[27]. The balance of cytokines regulated by immune response plays an important role in the stabilization and development of inflammation[28]. Studies have shown that Pg-LPS can stimulate the expression of TNF-α, IL-1β, IL-6, and macrophage inflammatory protein (MIP)-1α in monocytes and macrophages[29, 30], and then induce osteoclast formation leading to alveolar bone loss[31]. Many studies have shown that ROS plays an important role in inflammatory response. Bullon[32] et al. found that periodontal bacterial LPS stimulated HGFs to increase mitochondrial ROS. Liu[33] et al. considered that the secretion of IL-1β, IL-6, TNF-α induced by LPS in HGFs is mediated by the interaction between P53 regulating ROS and ROS stimulating P53. Li[34] et al. found that ROS over produced after LPS treatment of HGF could induce HGFs to increase the levels of TNF-α, IL-1β, and IL-6 through MAPK and NF-κB pathway. In this study, we found that after treatment of HGFs with different concentrations of Pg-LPS, the proinflammatory factors levels of IL-6, IL-1β, TNF-α increased in a dose-dependent manner. We also confirmed that after cells transfection of HSP90AA1-siRNA, these inflammatory factors and intracellular ROS levels were significantly reduced. It is suggested that HSP90AA1 gene could regulate the inflammatory reaction in cells.

Accumulating evidences shown that autophagy is an important part of the innate and adaptive immunity of the host, and is associated with many inflammatory diseases[35]. Oxidative stress induced ROS has been shown to induce apoptosis and autophagy[36]. Bullon[32] et al. found that Pg-LPS leads to reactive oxygen species (ROS) mediated autophagy, suggesting that there is a link between autophagy and ROS in HGFs. This result is consistent with Park's research, and the production of ROS promoted the transformation of LC3-I to LC3-II[37]. Both ATG5 and Beclin1 participate in the formation of autophagosome in autophagy signaling pathway[38]. It has been reported that the level of RNA and protein expression of ATG5 is increased in HGF stimulated by LPS[39]. El-Gowily have confirmed that up regulation of Beclin-1 could produce disruptive autophagy[40]. Our research is consistent with the above description. Pg-LPS significantly increased the proteins levels of ATG5, Beclin1, and p-NF-κB p65/NF-κB p65 in HGFs, and the ratio of LC3-II/LC3-I was increased in dose-dependent manner. After transfection of HSP90AA1-siRNA, the response of these indicators was also reversed, and accompanied with the decreased cell apoptotic rate. This indicates that HSP90AA1 is involved in regulating the levels of autophagy related proteins and cell apoptosis. Therefore, targeting the HSP90AA1 gene may be a potential target for regulating Pg-LPS induced HGFs inflammatory response.

Autophagy can sense and detect pathogens by recognizing pathogen related molecular patterns by pattern recognition receptor (PRRs)[41]. HSP90AA1 is the main pathogen receptor of bacterial LPS, and also a PRR component[42], it can bind with LPS, dengue virus[43], and avibirnavirus[44]. In recent years, some reports suggested that HSP90AA1 also exists on the cell surface[45]. Increased studies have used pathogen recognition to reveal the effect of pathogen infection on autophagy. It has been reported that
influenza A virus (IAV) induced autophagy through the hemagglutinin (HA) combinated with HSP90AA1 on the cell surface\textsuperscript{[41]}. Hu\textsuperscript{[23]} \textit{et al.} found that HSP90AA1 binded to avibirnavirus VP2 induced autophagy by inactivating the AKT-MTOR pathway. Therefore, HSP90AA1 as a target for regulating autophagy in Pg-LPS stimulated HGFs should be emphasized.

In summary, this study confirmed that HSP90AA1 could participate in regulating autophagy, and promote Pg-LPS induced HGFs to produce inflammatory injury. This will provide a theoretical basis for us to explore the mechanism of HSP90AA1 affecting peri-implantitis. A limitation of this study is that we only established an inflammatory model of Pg-LPS induced HGFs to observe the role of HSP90AA1 in autophagy \textit{in vitro}, and the establishment of \textit{in vivo} peri-implantitis model may be involved in our follow-up studies.

5 Conclusion

In conclusion, in this study, we found that the levels of IL-1\(\beta\), IL-6, TNF-\(\alpha\), p-NF-\(\kappa\)B p65/NF-\(\kappa\)B p65, LC3 II/I, ATG5, Beclin-1, and HSP90AA1 increased in Pg-LPS induced HGFs inflammation model, while these mediators were reversed after transfection with HSP90AA1-siRNA. It indicates that HSP90AA1 promotes Pg-LPS induced HGFs inflammatory response by regulating autophagy. It is suggested that HSP90AA1 plays an important role in the development of peri-implantitis inflammation and may provide a potential therapeutic target for clinical treatment.

Declarations

Ethics statement

This study was approved by Animal Experimentation Ethics Committee of Hangzhou Eyong Biotechnological Co., Ltd. Animal Experiment Center (SYXK\(\text{Zhe}\)2020-0024)

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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**Author Contributions**

Conception and design of the research: Huang Zhang and Yong Wu Wang; Acquisition and statistical analysis of data: Jie Huang, Xu Sheng Fan and Rui Jing Miao; manuscript drafting: Huang Zhang, Jie Huang, Xu Sheng Fan and Rui Jing Miao; Manuscript Revision: Yong Wu Wang; Huang Zhang provided funding support.

**Acknowledgments**

Not applicable

**References**


**Figures**
Figure 1

Pg-LPS did not affect the cell viability of HGFs, while increased the level of cytokines. HGFs was treated with different concentrations of PG-LPS (0.1, 1, 10 μg/mL) for 24 h, and detected the changes of cell viability (a) and inflammatory factors (b). Data was expressed as mean ± SD, n=6. Compared to the control, **) P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS, Pg-LPS.

Figure 2

Pg-LPS treatment increased HSP90AA1 protein and gene level, and elevated the expressions of autophagy related protein in HGFs. a and b: The changes of HSP90AA1 protein and gene level. c: The expression of p-NF-κB p65/NF-κB p65 was increased. Pg-LPS (0.1, 1, 10 μg/mL) treatment increased the expression of LC3 II/I (d), ATG5 and Beclin-1 (e) in HGFs. Data was expressed as mean ± SD, n=3. Compared to the control, *P<0.05, **) P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS, Pg-LPS.

Figure 3

Construction and screening HGFs cell lines with stable, and low expression of HSP90AA1 after HSP90AA1-siRNA transfection. a and b: The protein, mRNA level of HSP90AA1 in HGFs after transfection of HSP90AA1-siRNA. c: Effect of HSP90AA1-siRNA transfection on the contents of IL-1β, IL-6, and TNF-α. Data was expressed as mean ± SD, n=3, 6. Compared to the siRNA group, **) P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS (Pg-LPS, 1 μg/mL); HSP90AA1-siRNA, siHSP90AA1.
Figure 4

Immunofluorescence detected the protein content of HSP90α (a), HSP90β (b) after transfection of HSP90AA1-siRNA in HGF. The left is the immunofluorescence images (×200), and the right is the fluorescence intensity analysis. Data was expressed as mean ± SD, n=3. Compared with the siRNA group, **P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS (Pg-LPS, 1 μg/mL); HSP90AA1-siRNA, siHSP90AA1.

Figure 5

HSP90AA1-siRNA transfection decreased HGFs cellular ROS generation and inhibited apoptosis. a: The Fluorescent ROS images (×200), and fluorescence intensity analysis. b: The apoptosis of HGFs was analyzed after transfection of HSP90AA1-siRNA. Data was expressed as mean ± SD, n=3. Compared with the siRNA group, **P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS (Pg-LPS, 1 μg/mL); HSP90AA1-siRNA, siHSP90AA1.

Figure 6

Low expression of HSP90AA1 reduced autophagy related protein levels in HGFs. a: The protein levels of LC3 II/I, Beclin-1, ATG5, and p-NF-κB p65/NF-κB p65, respectively. b, c: statistical chart of corresponding protein bands. Data was expressed as mean ± SD, n=3. Compared with the siRNA group, **P<0.01. Note: human gingival fibroblasts, HGFs; P. gingivalis LPS (Pg-LPS, 1 μg/mL); HSP90AA1-siRNA, siHSP90AA1.

Supplementary Files

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