

## —原著—

## 歯肉組織における Wnt5a と sFRP5 の発現機能解析

前川知樹

新潟大学大学院医歯学総合研究科 高度口腔機能教育研究センター (センター長：前田健康 教授)

Investigation of Wnt5a and sFRP5 Expressions in Healthy and Chronic  
Periodontitis Tissues

Tomoki Maekawa

Research Center for Advanced Oral Science (director of the center: Prof. Takeyasu Maeda), Niigata University Graduate School of Medical and Dental Sciences

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**Abstract**

Periodontitis is a biofilm-induced chronic inflammatory disease that causes gingival inflammation and attachment loss. Destruction of periodontal tissue is a consequence of host immune inflammatory responses induced by periodontal microorganisms. Wnt5a is strongly associated with inflammatory responses and shown by several studies to be involved in inflammatory diseases including periodontitis. Soluble frizzled related protein-5 (sFRP5) is a homolog of Wnt5a receptor, frizzled protein. sFRP5 is a known inhibitor of Wnt5a signaling. To investigate the correlation of Wnt5a and sFRP5 in periodontitis and healthy tissues, experiments were set up using both clinical specimens and further investigation with stimulations of human gingival epithelial cells (HGECs). Wnt5a and sFRP5 mRNA expressions and IL-8 mRNA and chemokine levels in HGECs were determined by quantitative real-time PCR and enzyme-linked immunosorbent assay, respectively. Results showed higher level of Wnt5a expression in periodontitis group than in healthy group and vice versa for sFRP5 expression. Lipopolysaccharide from *Porphyromonas gingivalis* or *Escherichia coli* induced Wnt5a expression and reduced sFRP5 expression. Moreover, sFRP5 mediated anti-inflammatory effects by inhibiting interleukin-8 production in HGECs. In conclusion, inverse correlation of Wnt5a and sFRP5 expression was shown for the first time and human gingival epithelial cells were also proved to express both Wnt5a and sFRP5. Wnt5a appears to an important target for intervention in periodontitis and sFRP5 is likely a promising therapeutic compound.

**抄録**

歯周病は細菌のバイオフィルムによって引き起こされる感染症であり、宿主との免疫反応によって歯周組織の破壊と歯槽骨の吸収を引き起こす。Wnt5a は、歯周病を含む炎症反応と強い関連が報告されている。Soluble frizzled related protein-5 (sFRP5) は Wnt5a の受容体の相同体であり、Wnt5a 経路を阻害する。歯周炎組織と健常歯肉組織において Wnt5a と sFRP5 の関連性については不明な点が多い。そこで本研究では、ヒト歯肉サンプルでの Wnt5a と sFRP5 の mRNA 発現、ヒト歯肉上皮細胞 (HGECs) における interleukin-8 (IL-8) mRNA とケモカインのレベルを定量的 PCR および ELISA にて各々解析した。健常組織と比べて歯周病罹患組織において Wnt5a 遺伝子発現は高く、sFRP5 発現は抑制されていることが明らかになった。同様に、HGECs においても *Porphyromonas gingivalis* リポ多糖により Wnt5a 発現の上昇と sFRP5 発現の抑制が認められた。加えて、sFRP5 の HGECs における IL-8 の産生抑制効果が認められた。以上のことから、HGECs では Wnt5a と sFRP5 は双方発現し負の相関関係にあることが初めて示された。また、Wnt5a は歯周病治療の対象となること、sFRP5 が歯周病治療化合物として使用可能であることが示唆された。

## Introduction

The inflammatory process is important in host immune defense and occurs in many parts of the body. Inflammation helps defend against pathogens and takes part in wound healing. As soon as the body encounters injury or infection, acute inflammation arises. However, if the infection persists, chronic inflammation will ensue. A good example is the oral cavity. Microorganisms are always present which can cause inflammatory response in the form of gingivitis. Gingivitis is a reversible condition and affects only the gingival tissue, whereas the underlying alveolar bone remains intact. In cases that there are other contributing factors, for example, compromised host responses or intense microbial challenge, then gingivitis may transition to periodontitis. Periodontitis is a biofilm-induced chronic inflammatory disease that causes gingival inflammation and attachment loss<sup>1</sup>. Destruction of periodontal tissue is a consequence of host immune inflammatory responses induced by periodontal microorganisms. In the past, researchers showed that bacteria have a major role in the pathogenesis of periodontitis<sup>2, 3</sup>. Later, the important role of host immune responses contributing to disease progression has been investigated and confirmed<sup>4, 5</sup>. Hereditary factors, systemic conditions and environmental factors were also shown to be involved<sup>6</sup>.

The pathogenesis of periodontitis involves complex interactions between the microbial challenge and host response that alters connective tissue and bone metabolism. In biofilm, numerous bacterial strains reside. In the past, employing culturing techniques, a few bacteria have been designated as periodontal pathogens. These bacteria constitute the so-called “red complex” and include *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola*<sup>7</sup>. These three major strains of bacteria were thought to contribute to progression of periodontitis. However, the advent of culture-independent, molecular-based methods of bacterial identification has changed our understanding of the bacterial composition of the periodontal region<sup>1</sup>. Nowadays, it is widely accepted that periodontitis is not caused by single or a limited group of pathogens, but rather caused by polymicrobial synergy and dysbiosis<sup>8</sup>.

According to the polymicrobial synergy and dysbiosis (PSD) model, several kinds of bacteria exhibit synergistic interactions that promote colonization, persistence, or virulence orchestrated by keystone pathogens. *P. gingivalis* is a keystone pathogen that modulates the host response and tips the balance from homeostasis to dysbiosis which enables only certain kinds of bacteria to thrive and persist in the host. Inflammation has a major role that selects members of bacterial community in the way that only inflammophilic bacteria can survive. These bacteria are not destroyed by inflammation but feed on inflammatory breakdown products as a source of nutrients. Therefore, this selective mechanism causes the flourishing of inflammophilic bacteria which helps perpetuate tissue destruction that finally loops back to serve as nutrient source for these bacteria, thereby generating a vicious cycle<sup>9</sup>.

Bacterial antigens, lipopolysaccharide (LPS), and toxins from biofilm stimulate host immune response. Once stimulated, immune cells such as neutrophils, and humoral and cell-mediated response are directed to reduce the bacterial challenge. This results in the production of inflammatory mediators such as cytokines, chemokines, prostaglandins and proteolytic enzymes that alter connective tissue and bone metabolism. If host immune system cannot cause resolution of these inflammatory processes, then periodontal inflammation and periodontal damage will follow<sup>6</sup>.

The Wnt signaling protein family has a significant role in the developmental events of multicellular organisms. Wnts is a large family of cysteine-rich secreted ligands that are essential in the regulation of embryogenesis, development, proliferation, polarity, migration, and invasion. Historically, there are two groups of Wnt proteins; the group that activates the beta-catenin-dependent pathway (canonical) and the group that activates beta-catenin-independent pathway (non-canonical). Wnt1, Wnt3, Wnt3a, and Wnt7a are the canonical Wnts which stabilize beta-catenin, while Wnt5a, Wnt5b, Wnt6, Wnt7b and Wnt11 are the non-canonical Wnts<sup>10</sup>. However, some Wnt proteins have both canonical and non-canonical properties – for example, Wnt5a, which is considered a non-canonical Wnt, was found to be able to induce secondary axis formation when the receptor frizzled 5 (Fz5) was present<sup>11</sup>. Therefore, some non-canonical Wnts may

initiate canonical Wnt signaling when bound to specific Wnt receptors.

Of all the wingless proteins, Wnt5a is particularly interesting. It is known to have significant roles in the development process of various organs and postnatal functions. Recent reports demonstrated that abnormalities in Wnt5a signaling are involved in cancers, inflammatory diseases, and metabolic diseases<sup>12)</sup>.

Wnt5a is strongly associated with inflammatory responses. As stated above, Wnt5a induces inflammatory process through non-canonical pathway. There are several studies supporting this statement. An important study in *Drosophila* by Gordon's group is considered to be the first direct evidence of the function of Wnt pathway in inflammation and immunity. They demonstrated that the *Drosophila* Wnt protein family member WntD is upregulated in the fly via Toll/nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling and shown to be involved in antibacterial defense against *Listeria monocytogenes* in a septic fly model<sup>13)</sup>. Later on, Blumenthal's study in humans also demonstrated that Toll/NF- $\kappa$ B is required for the upregulation of Wnt5a in response to microbial challenge<sup>14)</sup>. Furthermore, this study also demonstrated that Wnt5a upregulates the microbially-induced IL-12 response of antigen-presenting cells and interferon- $\gamma$  production by mycobacterial antigen-stimulated T-cells, illustrating a functional involvement in the antimicrobial defense. This suggested the involvement of Wnt signaling in innate and adaptive immunity to infections.

Recent study by Divaris et al also suggested significance of Wnt5a in pathology of chronic periodontitis. They presented the results of a genome-wide association study of chronic periodontitis and detected association of Wnt5a with severe chronic periodontitis<sup>15)</sup>. These findings indicate that wnt5a may be involved in inflammatory diseases and thus might be an attractive candidate for therapeutic intervention.

Fz protein is a surface receptor for Wnt proteins. From 10 Fzs, Wnt5a signaling can be mediated through at least Fz3, Fz4, Fz5 and Fz8<sup>16)</sup>. It is suggested that Wnt5a regulates cellular functions by binding to several Fzs. The soluble Fz related protein is a family of soluble proteins that are structurally related to Fz proteins, the serpentine receptors that mediate the extensively used cell-cell communication

pathway involving Wnt signaling. Because of their homology with the Wnt-binding domain on the Fz receptors, sFRPs sequester Wnt proteins in the extracellular space and prevent Wnt from binding to their receptors. They have therefore been characterized as antagonists that bind to Wnt proteins to prevent signal activation<sup>17)</sup>. The soluble frizzled related protein (sFRP)-5 is a known inhibitor of wnt5a signaling<sup>18)</sup>.

Dominik and colleagues measured wnt5a and sFRP5 serum concentrations in lean control subjects and patients with obesity. They found that pro-inflammatory Wnt5a was not measurable in any serum sample of lean control subjects. In patients with obesity, however, Wnt5a was significantly upregulated consistent with low-grade inflammation in such subjects. sFRP5 was detected in both groups with no significant difference. Interestingly, after caloric restriction, there was a significant increase in serum concentrations of sFRP5. Furthermore, they found that adipose tissue macrophages of obese and type 2 diabetic human subjects express wnt5a secreted by macrophage and inhibits differentiation of pre-adipocytes.

These recent findings suggest that Wnt5a might act as an important pro-inflammatory molecule in low-grade inflammation of adipose tissue in obese humans, whereas sFRP5 may play an anti-inflammatory role<sup>19)</sup>. Since both obesity and periodontitis are inflammatory diseases, these studies prompted us to study the expression of Wnt5a and sFRP5 in periodontitis tissue and healthy tissue in order to dissect the possible roles of Wnt5a and sFRP5 in the periodontal tissue in health and disease. It should be noted that knowledge about the expression and modulation of Wnt homologs in inflammatory settings, such as periodontitis, is still limited. A study demonstrated that Wnt5a mRNA expression was up-regulated in chronic periodontitis tissue as compared to healthy control tissue using RT-PCR<sup>[20]</sup>. However, no study thus far has investigated how the expression of Wnt5a is correlated with sFRP5 expression in periodontitis and healthy tissue. We expect that our approach will advance the knowledge of Wnt5a and sFRP5 in the progression and prevention of periodontitis and serve as a platform for the development of new therapies.

## Materials and methods

### Human samples

Human gingival tissues were obtained during routine periodontal surgeries and crown-lengthening surgeries conducted on 69 physically healthy adults in the University of Pennsylvania School of Dental Medicine Graduate Periodontics Clinic. Samples of gingival tissues were obtained from 29 individuals without periodontal disease (control group) during crown-lengthening surgery and 40 patients with generalized and localized moderate to severe chronic periodontitis. The probing depth and clinical attachment level of the sampling sites was evaluated using a periodontal probe (Hu-Friedy, Leimen, Germany). Research was performed under an Institutional Review Board-approved protocol and all donors provided informed consent.

### Quantitative real-time PCR (qPCR)

Total RNA was extracted from excised gingival tissue or cultured cells using the PerfectPure RNA cell kit (5 Prime, Fisher) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Life Technologies) and qPCR with cDNA was performed using the Applied Biosystems 7500 Fast Real-Time PCR System according to the manufacturer's protocol (Life Technologies). Data were analyzed using the comparative ( $\Delta\Delta C_t$ ) method. TaqMan probes, sense primers, and antisense primers for detection and quantification of genes investigated in this paper were purchased from Life Technologies.

### Cell preparation and culture

Human gingival epithelial cells (HGECs) were prepared from clinically normal gingival tissue. The tissues were treated overnight with Dulbecco's modified minimal essential medium (DMEM) containing 0.025% trypsin and 0.01% EDTA. After washing with PBS and subsequent chopping into small pieces, the tissues were suspended in Epilife, containing supplement S7 and penicillin-streptomycin- amphotericin B solution (all from Invitrogen). The tissues were removed when the cells started to grow and were maintained until the cells reached confluence. For the stimulation experiments, HGECs were seeded into a 12-well culture plate. After 24 h of incubation, the attached cells were washed

extensively with Epilife and co-cultured with 100ng/mL of *P. gingivalis* LPS (Invivogen), 100ng/mL of *E. coli* LPS (Invivogen), Wnt5a 300ng/ml (R&D) or sFRP5 500ng/ml (R&D) in Epilife containing Supplement S7 for 12 h (gene expression assay) or 24 h (Chemokine assay).

### Chemokine assay

The level of IL-8 in the supernatants of HGECs culture were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioScience) according to the manufacturer's instructions.

### Statistical Analysis

Results were expressed as the mean  $\pm$  standard error of the mean. When two groups were compared, unpaired *t* test was used. Multiple comparisons were performed by ANOVA-Williams test was used for post hoc comparisons. A probability value  $<0.05$  was considered statistically significant.

## Results

### *Gene expressions of Wnt5a and sFRP5 in human periodontal tissue*

We investigated the gene expressions of Wnt5a and sFRP5 in human periodontal tissue. We found that sFRP5 expression was downregulated in diseased tissue relative to its expression in healthy tissue (Fig. 1). On the other hand, Wnt5a expression was increased in periodontal tissue as compared with healthy tissue (Fig. 1).

### *Effects of LPS on Wnt5a and sFRP5 expression in HGECs*

Although *P. gingivalis* LPS can induce inflammatory cytokine, it is not clear whether these affect Wnt5a and sFRP5 expression. First, we isolated HGECs from healthy patient. Both stimuli used, LPS from *P. gingivalis* or *E. coli*, induced Wnt5a expression and reduced sFRP5a expression (Fig. 2).

### *sFRP5 inhibited chemokine production in HGECs*

To determine whether sFRP5 mediates anti-inflammatory effects, HGECs were treated with both Wnt5a and LPS in the presence or absence of sFRP5. Importantly, the production of IL-8 in HGECs was significantly inhibited in the presence of sFRP5 (Fig. 3).

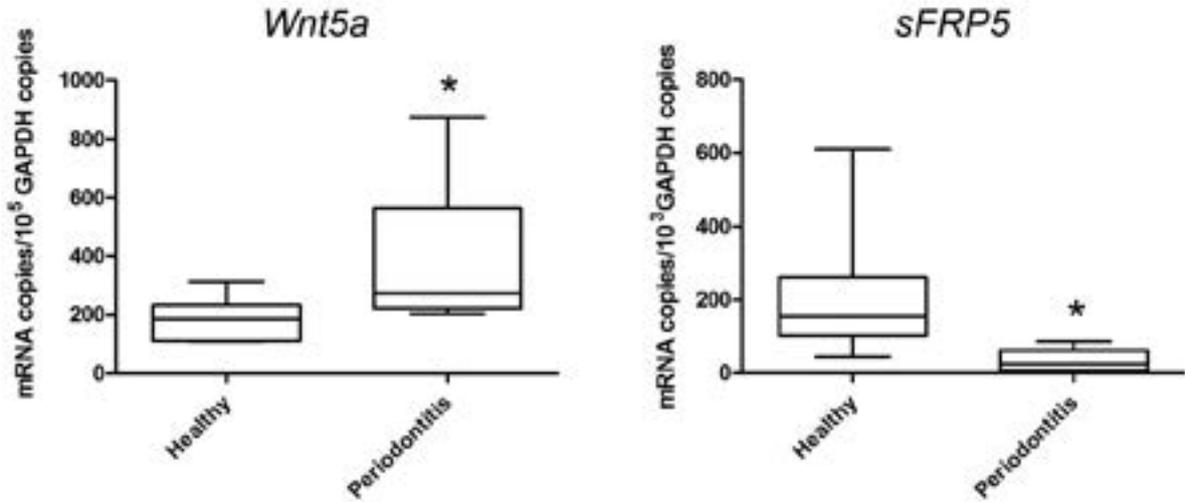


Figure 1. Gene expression of Wnt5a and sFRP5 in periodontal tissue. The mRNA expression levels were normalized against GAPDH mRNA. Data are means  $\pm$  SD (N=9 healthy, N=8 periodontitis). \* $p < 0.01$ .

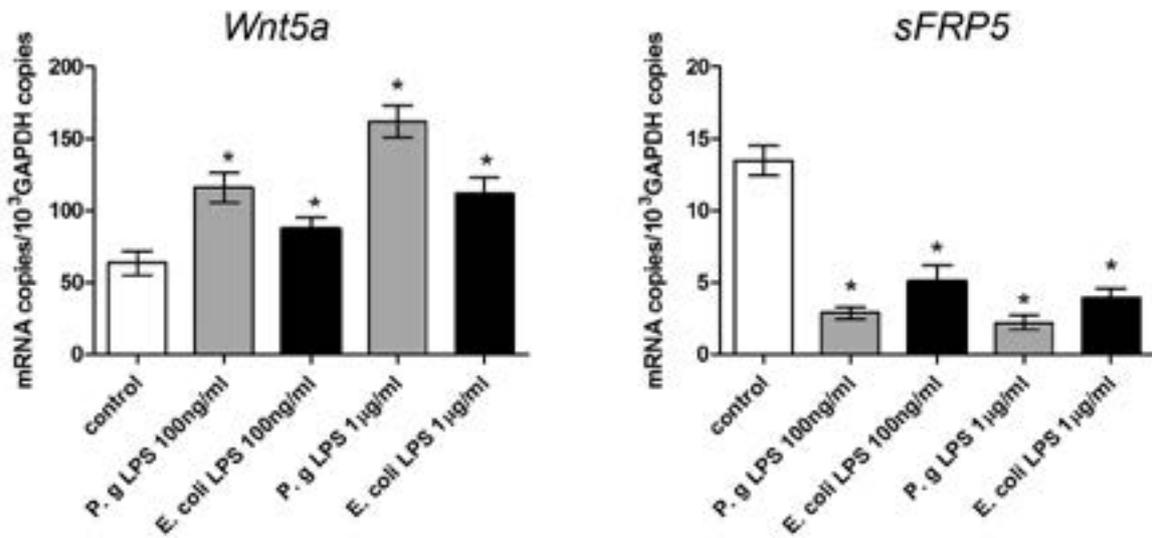


Figure 2. LPS increases Wnt5a expression and inhibites sFRP5 expression. HGECs were stimulated for 12 h with *P. gingivalis* lipopolysaccharide (Pg LPS; 100ng/ml or 1  $\mu$ g/ml), *E. coli* LPS (Ec LPS; 100ng/ml or 1  $\mu$ g/ml). Data are means  $\pm$  SD (n = 5 sets of cells). \* $p < 0.01$  compared with no pretreatment.

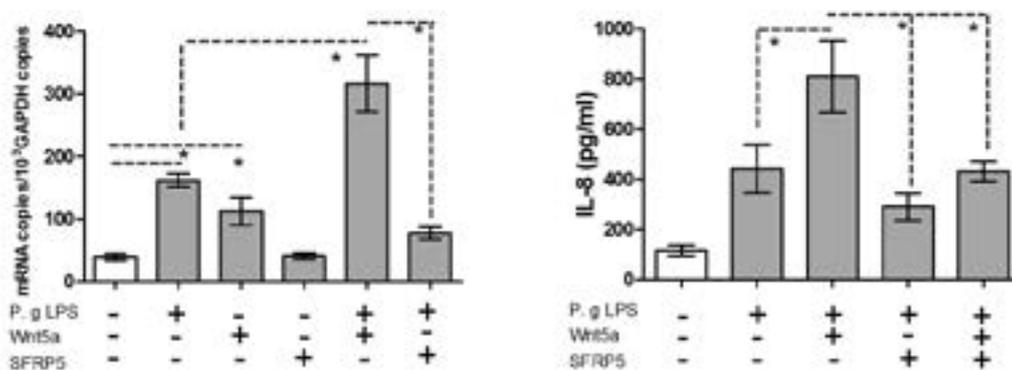


Figure 3. sFRP5 inhibits production of chemokine IL-8 by Wnt5a and Pg LPS stimulation. HGECs were pretreated for 1 h with sFRP5 (500ng/ml), followed by 12 h (mRNA) or 24 h (chemokine assay) stimulation with medium only or with Pg LPS; 100ng/ml or Wnt5a (300ng/ml). Subsequently, mRNA was collected for gene expression assay and culture supernatants were collected for measuring IL-8 by ELISA. Data are means  $\pm$  SD (n = 5 sets of cells). \* $p < 0.01$ .

## Discussion

Wnt5a is strongly associated with inflammatory responses as shown by several studies. Wnt5a was found to be a proinflammatory molecule that stimulate inflammatory function of antigen-presenting cells, such as macrophages<sup>14, 20-22</sup>.

Wnt5a mRNA was shown to be significantly higher in periodontitis group when compared to healthy group. sFRP5 mRNA expression, on the contrary, was shown to be significantly higher in healthy group. These results confirm our hypothesis that the expression of the proinflammatory Wnt5a is more pronounced in inflamed tissues and expression of the anti-inflammatory sFRP5 is more pronounced in healthy tissues. Our findings agree with Nanbara's study<sup>20</sup>, which showed significantly higher Wnt5a mRNA expression in periodontitis tissues. Moreover, we are the first group that investigated the expression of sFRP5 mRNA in periodontitis and healthy gingival tissues.

In order to confirm our speculation that gingival epithelial cells express Wnt5a and sFRP5, another qPCR experiment was set up. Previous studies showed that Wnt5a and sFRP5 mRNA were expressed by human gingival epithelial cells. Furthermore, modulation of Wnt5a and sFRP5 expression by periodontal bacteria was also investigated. Wnt5a mRNA was highly upregulated in the presence of *P. gingivalis* when compared to unstimulated group and *E. coli* challenged group. The significance of *P. gingivalis* in the pathogenesis of periodontitis is well documented. *P. gingivalis* is a keystone pathogen in periodontitis which subverts the host response and tips the balance from homeostasis to dysbiosis.

As shown by this study that Wnt5a mRNA was highly up-regulated in the presence of *P. gingivalis* when compared to unstimulated group. In contrast, sFRP5 mRNA was suppressed in the presence of *P. gingivalis* and *E. coli*.

As stated previously, Wnt5a was found to be involved in a large number of inflammatory diseases. Kim's group demonstrated up-regulation of inflammatory cytokines, for example, IL-8, IL-6 etc. by Wnt5a in endothelial cells through the Wnt-Ca2+ signaling pathway<sup>23</sup>. IL-8 is a chemokine which mainly attracts neutrophils to sites of inflammation. It is up-

regulated by pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  and IL-1, bacterial or viral products. Increased expression of IL-8 and neutrophil accumulation have been shown to be involved in the pathology of periodontitis<sup>24</sup>. Another experiment was set up to study the level of IL-8 mRNA expression using qPCR. HGECs challenged with Wnt5a or *P. gingivalis* LPS showed more expression of IL-8 while *P. gingivalis* LPS combined with Wnt5a triggered the most pronounced IL-8 mRNA expression. This demonstrates synergistic effect of *P. gingivalis* LPS and proinflammatory Wnt5a in eliciting inflammation. In contrast, *P. gingivalis* LPS combined with sFRP5 decreased expression of IL-8 mRNA compared to stimulation with *P. gingivalis* LPS alone. This demonstrates antagonistic effect of *P. gingivalis* LPS and anti-inflammatory sFRP5. ELISA experiment confirmed these results at the cytokine protein level.

Overall, it's shown from our study that Wnt5a and sFRP5 have important roles in inflammatory process of periodontitis. Wnt5a usually stimulates non-canonical Wnt signaling which consists of at least three different B-catenin-independent pathways, the Wnt/PCP, Wnt/Ca2+, and Wnt/ROR2. Interestingly, CAMKII and PKC which are products from Wnt/Ca2+ pathway have been shown to block the Wnt canonical pathway<sup>25</sup>. Moreover, Wnt/ROR2 pathway also can inhibit the canonical pathway<sup>26</sup>. In periodontitis, canonical pathway is known to regulate bone homeostasis. Activation of canonical pathway leads to more bone formation; on the contrary, inhibition of this pathway leads to bone destruction<sup>27, 28</sup>. On the basis of the above-discussed findings, we can speculate that apart from the ability of Wnt5a to enhance inflammation through non-canonical pathways, mediators induced via non-canonical pathways can also contribute to blockage of canonical pathway and thus lead to further bone destruction.

Ouchi's group conducted experiment in sFRP5 knockout mice, which were put on high-calorie diet. They observed systemic metabolic dysfunction and severe adipose tissue inflammation. However, administration of sFRP5 improved metabolic function and down-regulated adipose tissue inflammation<sup>18</sup>. A similar approach could be applied to the treatment of periodontitis. Our study found higher level of sFRP5 in healthy gingival tissues when compared to periodontitis tissues. sFRP5 also interferes with

inflammatory chemokine expression induced by Wnt5a or *P. gingivalis* LPS. Our results therefore suggest a role of sFRP5 as a potential anti-inflammatory molecule in periodontitis.

### Conclusion

We have shown for the first time an inverse correlation between Wnt5a and sFRP5 in periodontal disease vs. health. We have moreover shown for the first time that both molecules can be expressed by human gingival epithelial cells. Wnt5a appears to be an important target for intervention in periodontitis and in this context, sFRP5 is likely a promising therapeutic compound. This notion could be tested in future studies in preclinical models, hopefully paving the way for clinical trials.

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