2015

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

Pacharee Kulwattanaporn
University of Pennsylvania

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

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. 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 expression and protein levels were studied by immunohistochemistry, quantitative real time PCR, and ELISA. Results showed localization of Wnt5a and sFRP5 in periodontal tissue and higher level of Wnt5a expression in periodontitis group than in healthy group and vice versa for sFRP5 expression. LPS from *P. gingivalis* or *E. coli* induced Wnt5a expression and reduced sFRP5 expression. Moreover, sFRP5 mediated anti-inflammatory effects by inhibiting IL-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 be an important target for intervention in periodontitis and sFRP5 is likely a promising therapeutic compound.

Degree Type
Thesis

Degree Name
MSOB (Master of Science in Oral Biology)

Primary Advisor
George Hajishengallis

Subject Categories
Amino Acids, Peptides, and Proteins | Bacterial Infections and Mycoses | Immunology and Infectious Disease | Medical Genetics | Oral Biology and Oral Pathology | Periodontics and Periodontology

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

Pacharee Kulwattanaporn, DDS

University of Pennsylvania
School of Dental Medicine
Department of Endodontics

Presented to the Faculty of the University of Pennsylvania School of Dental Medicine in Partial Fulfillment of the Requirements for the Degree of Master of Master of Science in Oral Biology

Research project advisors

Dr. George Hajishengallis

Dr. Jonathan Korostoff

ACKNOWLEDGEMENT

I would like to express my appreciation to my advisor, Dr. George Hajishengallis, for the honorable opportunity to work and experience professional working environment in this laboratory. His initiation and creativity greatly contributes to my work. I also would like to thank Dr. Jonathan Korostoff for his guidance and help throughout the masters of oral biology program.

I want to express my deepest gratitude to Dr. Tomoki Maekawa who helped me tremendously from day one. Without his supervision and constant help, this dissertation would not have been possible. Thank you for his dedication, patience and engagement that contributes greatly to my progress.

In addition, I would like to thank Dr. Kavita Hosur and all of my postdoctoral fellows for their support both technically and mentally. They helped me thrive and enjoy the quality time in the laboratory. Finally, I would like to thank Dr. Bekir Karabucak for his understanding and support throughout this program.

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. 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 expression and protein levels were studied by immunohistochemistry, quantitative real time PCR, and ELISA. Results showed localization of Wnt5a and sFRP5 in periodontal tissue and higher level of Wnt5a expression in periodontitis group than in healthy group and vice versa for sFRP5 expression. LPS from *P. gingivalis* or *E. coli* induced Wnt5a expression and reduced sFRP5 expression. Moreover, sFRP5 mediated anti-inflammatory effects by inhibiting IL-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 be an important target for intervention in periodontitis and sFRP5 is likely a promising therapeutic compound.

LITERATURE REVIEW

Introduction

It is documented that more than 47% of the adult U.S. population suffers from periodontitis and even up to 64% of adults aged 65 years and older had either moderate or severe periodontitis [1]. Considering this high prevalence, it is important to investigate the pathogenesis of periodontitis and develop effective treatments.

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 [2]. 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 [3, 4]. Later, the important role of host immune responses contributing to disease progression has been investigated and confirmed [5, 6]. Hereditary factors, systemic conditions and environmental factors were also shown to be involved [7].

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* [8]. 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 [2]. 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 [9].

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 fed 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 [10].

Bacterial antigens, LPS, and toxins from biofilm stimulate host immune response. Once stimulated, immune cells such as PMN, 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[7].

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[11]. 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 Fz5 was present[12]. 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[13].

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-kB (NF-kB) signaling and shown to be involved in antibacterial defense against *Listeria monocytogenes* in a septic fly model[14]. Later on, Blumenthal's study in humans also demonstrated that Toll/NF-kB is required for the upregulation of Wnt5a in response to microbial challenge [15]. Furthermore, this study also demonstrated that Wnt5a upregulates the microbially-induced IL-12 response of antigen-presenting cells and IFN-γ 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[16]. These findings indicate that wnt5a may be involved in inflammatory diseases and thus might be an attractive candidate for therapeutic intervention.

Frizzled protein is a surface receptor for Wnt proteins. From 10 Fzs, Wnt5a signaling can be mediated through at least Fz3, Fz4, Fz5 and Fz8[17]. It is suggested that Wnt5a regulates cellular functions by binding to several Fzs. The soluble frizzled related protein is a family of soluble proteins that are structurally related to Frizzled (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[18]. The soluble frizzled related protein (sFRP)-5 is a known inhibitor of wnt5a signaling[19].

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 [20]. 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[21]. 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 PD, CAL 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.

Immunohistochemistry

Gingival biopsy specimens were fixed in 4% paraformaldehyde and embedded in OCT compound. Cryostat sections were cut at 8 µm and mounted on glass slides. The sections were fixed in PFA for 10 minutes, washed with mixed solution of Phosphate buffered saline (PBS) and 0.1% Tween 20 followed by solution of PBS and 0.1% TritonX-100. Final rinse with PBS 15 minutes before incubation. Sections were stained using mouse monoclonal antibody against human Wnt5a (clone6F2, Lsbio), rabbit polyclonal antibody against human sFRP (Novus), followed by secondary reagents (Alexa Fluor 488–conjugated goat anti-rabbit

IgG or Alexa Fluor 594–conjugated goat anti-mouse IgG,; Life Technologies). The specificity of staining was confirmed by using appropriate isotype controls or nonimmune rabbit IgG followed by Alexa Fluor 488– or Alexa Fluor 594–conjugated anti-IgG. Images were captured using a Nikon Eclipse NiE automated upright fluorescent microscope.

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 (ΔΔCt) 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 ELISA kits (eBioScience) according to the manufacturer's instructions.

Statistical Analysis

Results were expressed as the mean ± 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

Localization of Wnt5a and sFRP5 in periodontal tissue

To determine the localization of Wnt5a and sFRP5 protein expression in periodontal tissue, we performed immunohistochemistry of periodontal biopsy specimens. Expression of sFRP5 was much stronger in healthy tissue compared to diseased periodontal tissue (Fig. 1). In contrast, Wnt5a expression was comparable in diseased and healthy tissue. Both Wnt5a and sFRP5 were expressed in the epithelial layer but not in the connective tissue.

Gene expressions of Wnt5a and sFRP5 in human periodontal tissue

We next investigated the gene expressions of Wnt5a and sFRP5 in human periodontal tissue. We found that sFRP5 expression was upregulated in healthy tissue relative to its expression in diseased tissue (Fig. 2), consistent with our immunohistochemical observations. On the other hand, Wnt5a expression was increased in periodontitis tissue as compared with healthy tissue (Fig. 2). Although Wnt5a expressed comparably in both health and diseased periodontal tissues in IHC, its mRNA expression was significantly upregulated in diseased tissue.

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 sFRP5 expression (Fig. 3).

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. 4).

Discussion

Wnt5a is strongly associated with inflammatory responses as shown by several studies. Wnt5a was found to be a proinflammatory molecule that stimulates inflammatory function of antigen-presenting cells, such as macrophages[15, 21-23].

Interestingly, our immunofluorescent staining demonstrated that Wnt5a and sFRP5 were expressed in epithelial layer of gingival tissues. Furthermore, Wnt5a and sFRP5 were co-localized. More pronounced expression of sFRP5 in healthy group than in periodontitis group was observed; however, the expression level of Wnt5a was found to be similar in both groups. Difference was noted in merged Wnt5a and sFRP5 images of periodontitis and healthy group in a way that Wnt5a appeared to predominate over sFRP5 expression in periodontitis group and vice versa in the healthy group. Periodontitis tissues in this experiment were obtained from periodontal surgeries. These tissues were periodontally treated previously, thus it is likely that inflammation was not as pronounced as in cases of untreated periodontitis. This might explain why we did not detect an obvious difference in the levels of Wnt5a expression between periodontitis and healthy group. Results from the immunofluorescent study showed clearly that Wnt5a and Sfrp5 were expressed in the epithelial layer.

This led to the next step of quantitative analysis of Wnt5a and sFRP5 mRNA expression using qPCR in both groups. 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 [21], which showed significantly higher Wnt5a mRNA expression in periodontitis tissues, although they did not examine the expression of Wnt5a protein. Moreover, we are the first group that investigated the expression of sFRP5 mRNA and protein 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. Results clearly demonstrated 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[24]. IL-8 is a chemokine which mainly attracts neutrophils to sites of inflammation. It is up-regulated by pro-inflammatory cytokines such as TNF-α and IL-1, bacterial or viral products. Increased expressions of IL-8 and neutrophil accumulation have been shown to be involved in the pathology of periodontitis[25]. Another experiment was set up to study the level of IL-8 mRNA expression using qPCR. Human gingival epithelial cells 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[26]. Moreover, Wnt/ROR2 pathway also can inhibit the canonical pathway[27]. 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[28, 29]. 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[19]. 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.

FIGURE LEGENDS

Figure. 1. Expression of Wnt5a and sFRP5 in sections of periodontal specimens. Periodontal biopsy specimens from healthy and periodontitis patients were processed for immunofluorescence and hematoxylin and eosin staining. Overlays of differential interference contrast (DIC) and fluorescent images stained for wnt5a (red) and sFRP5 (green) are shown. Scale bar, 50 µm.

Figure. 2. Gene expression of Wnt5a and sFRP5 in periodontal tissue. The mRNA expression levels were normalized against GAPDH mRNA. Data are means ± SD (N=9 healthy, N=8 periodontitis) . *$p < 0.01$.

Figure. 3. LPS increases Wnt5a expression and inhibites sFRP5 expression. HGECs were stimulated for 12 h with *P. gingivalis* lipopolysaccharide (Pg LPS; 100ng/ml or 1 µg/mL), *E. coli* LPS (Ec LPS; 100ng/ml or 1 µg/mL). Data are means ± SD (n = 5 sets of cells). *$p < 0.01$ compared with no pretreatment.

Figure. 4. sFRP5 inhibit 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 ± SD (n = 5 sets of cells). *$p < 0.01$.

References

1. Eke, P.I., et al., *Prevalence of periodontitis in adults in the United States: 2009 and 2010.* J Dent Res, 2012. **91**(10): p. 914-20.

2. Hajishengallis, G., *Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response.* Trends Immunol, 2014. **35**(1): p. 3-11.

3. Lindhe, J., S. Hamp, and H. Loe, *Experimental periodontitis in the beagle dog.* J Periodontal Res, 1973. **8**(1): p. 1-10.

4. Loe, H., E. Theilade, and S.B. Jensen, *Experimental Gingivitis in Man.* J Periodontol, 1965. **36**: p. 177-87.

5. Seymour, G.J., *Possible mechanisms involved in the immunoregulation of chronic inflammatory periodontal disease.* J Dent Res, 1987. **66**(1): p. 2-9.

6. Ranney, R.R., *Immunologic mechanisms of pathogenesis in periodontal diseases: an assessment.* J Periodontal Res, 1991. **26**(3 Pt 2): p. 243-54.

7. Page, R.C. and K.S. Kornman, *The pathogenesis of human periodontitis: an introduction.* Periodontol 2000, 1997. **14**: p. 9-11.

8. Holt, S.C. and J.L. Ebersole, *Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis.* Periodontol 2000, 2005. **38**: p. 72-122.

9. Hajishengallis, G. and R.J. Lamont, *Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.* Mol Oral Microbiol, 2012. **27**(6): p. 409-19.

10. Hajishengallis, G., *The inflammophilic character of the periodontitis-associated microbiota.* Mol Oral Microbiol, 2014.

11. Kikuchi, A., H. Yamamoto, and A. Sato, *Selective activation mechanisms of Wnt signaling pathways.* Trends Cell Biol, 2009. **19**(3): p. 119-29.

12. He, X., et al., *A member of the Frizzled protein family mediating axis induction by Wnt-5A.* Science, 1997. **275**(5306): p. 1652-4.

13. Kikuchi, A., et al., *Wnt5a: its signalling, functions and implication in diseases.* Acta Physiol (Oxf), 2012. **204**(1): p. 17-33.

14. Gordon, M.D., et al., *WntD is a feedback inhibitor of Dorsal/NF-kappaB in Drosophila development and immunity.* Nature, 2005. **437**(7059): p. 746-9.

15. Blumenthal, A., et al., *The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation.* Blood, 2006. **108**(3): p. 965-73.

16. Divaris, K., et al., *Exploring the genetic basis of chronic periodontitis: a genome-wide association study.* Hum Mol Genet, 2013. **22**(11): p. 2312-24.

17. Takada, R., et al., *Analysis of combinatorial effects of Wnts and Frizzleds on beta-catenin/armadillo stabilization and Dishevelled phosphorylation.* Genes Cells, 2005. **10**(9): p. 919-28.

18. Bovolenta, P., et al., *Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease.* J Cell Sci, 2008. **121**(Pt 6): p. 737-46.

19. Ouchi, N., et al., *Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity.* Science, 2010. **329**(5990): p. 454-7.

20. Schulte, D.M., et al., *Pro-inflammatory wnt5a and anti-inflammatory sFRP5 are differentially regulated by nutritional factors in obese human subjects.* PLoS One, 2012. **7**(2): p. e32437.

21. Nanbara, H., et al., *Modulation of Wnt5a expression by periodontopathic bacteria.* PLoS One, 2012. **7**(4): p. e34434.

22. Pereira, C., et al., *Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the antiinflammatory action of activated protein C and interleukin-10.* Arterioscler Thromb Vasc Biol, 2008. **28**(3): p. 504-10.

23. Sen, M., et al., *Expression and function of wingless and frizzled homologs in rheumatoid arthritis.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2791-6.

24. Kim, J., et al., *Wnt5a induces endothelial inflammation via beta-catenin-independent signaling.* J Immunol, 2010. **185**(2): p. 1274-82.

25. Gamonal, J., et al., *Levels of interleukin-1 beta, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment.* J Periodontol, 2000. **71**(10): p. 1535-45.

26. Bhatt, P.M. and R. Malgor, *Wnt5a: A player in the pathogenesis of atherosclerosis and other inflammatory disorders.* Atherosclerosis, 2014. **237**(1): p. 155-162.

27. Angers, S. and R.T. Moon, *Proximal events in Wnt signal transduction.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 468-77.

28. Glass, D.A., 2nd, et al., *Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation.* Dev Cell, 2005. **8**(5): p. 751-64.

29. Glass, D.A., 2nd and G. Karsenty, *Canonical Wnt signaling in osteoblasts is required for osteoclast differentiation.* Ann N Y Acad Sci, 2006. **1068**: p. 117-30.

Fig. 1

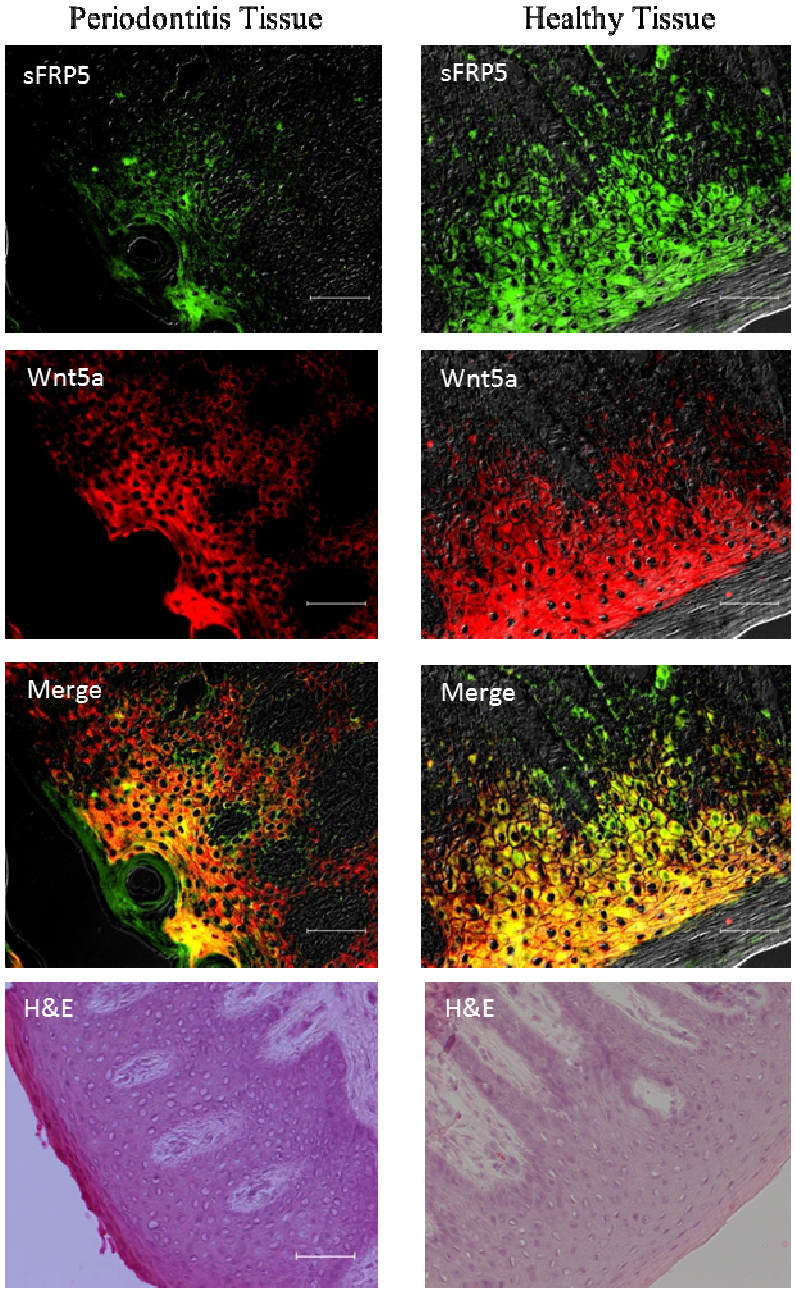
Periodontitis Tissue
Healthy Tissue
sFRP5
sFRP5
Wnt5a
Wnt5a
Merge
Merge
H&E
H&E

Fig. 2

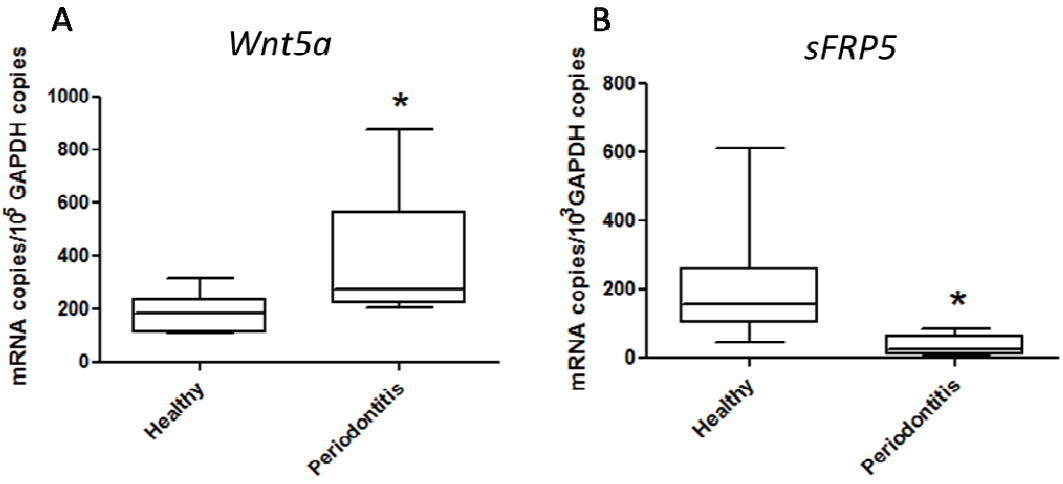

Fig. 3

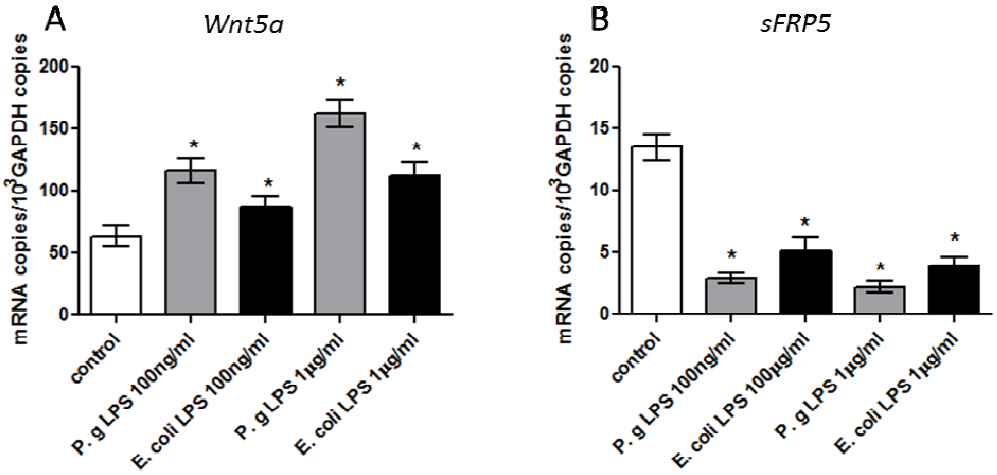

Fig. 4