

ORIGINAL ARTICLE

NAMPT enzyme activity regulates catabolic gene expression in gingival fibroblasts during periodontitis

Ka Hyon Park^{1,2}, Duck-Kyu Kim^{1,2}, Yun Hyun Huh³, Gyuseok Lee³, Su-Hyeon Lee¹, Yunkyung Hong^{1,2}, Sun-Hun Kim², Min-Suk Kook², Jeong-Tae Koh^{1,2}, Jang-Soo Chun³, Shee Eun Lee¹ and Je-Hwang Ryu^{1,2}

Periodontal disease is one of the most prevalent chronic disorders worldwide. It is accompanied by inflammation of the gingiva and destruction of periodontal tissues, leading to alveolar bone loss. Here, we focused on the role of adipokines, which are locally expressed by periodontal tissues, in the regulation of catabolic gene expression leading to periodontal inflammation. The expression of the nicotinamide phosphoribosyltransferase (NAMPT) adipokine was dramatically increased in inflamed human and mouse gingival tissues. NAMPT expression was also increased in lipopolysaccharide- and proinflammatory cytokine-stimulated primary cultured human gingival fibroblasts (GF). Adenovirus-mediated NAMPT (*Ad-Nampt*) overexpression upregulated the expression and activity of COX-2, MMP1 and MMP3 in human GF. The upregulation of IL-1 β - or *Ad-Nampt*-induced catabolic factors was significantly abrogated by the intracellular NAMPT (iNAMPT) inhibitor, FK866 or by the sirtuin (SIRT) inhibitor, nicotinamide (NIC). Recombinant NAMPT protein or extracellular NAMPT (eNAMPT) inhibition using a blocking antibody did not alter NAMPT target gene expression levels. Moreover, intragingival *Ad-Nampt* injection mediated periodontitis-like phenotypes including alveolar bone loss in mice. SIRT2, a part of the SIRT family, was positively associated with NAMPT actions in human GF. Furthermore, *in vivo* inhibition of the NAMPT-NAD⁺-SIRT axis by NIC injection in mice ameliorated the periodontal inflammation and alveolar bone erosion caused by intragingival injection of *Ad-Nampt*. Our findings indicate that NAMPT is highly upregulated in human GF, while its enzymatic activity acts as a crucial mediator of periodontal inflammation and alveolar bone destruction via regulation of COX-2, MMP1, and MMP3 levels.

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INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by irreversible destruction of the periodontium and involves bacterial infection, periodontal inflammation, degradation of gum tissues and alveolar bone resorption. Periodontitis is mainly caused by pathogenic microorganisms, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.¹ Periodontal pathogens may induce tissue damage by releasing endotoxins, such as lipopolysaccharides (LPS), which have profound effects on many cell types, including fibroblasts, osteoblasts, osteoclasts, and immune cells.^{2,3} Periodontopathogens elicit inflammatory responses in the periodontium characterized by the local production of proinflammatory factors. These factors include tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, IL-8, cyclooxygenase (COX)-2 (also known as prostaglandin G/H synthase (PTGS) 2) and matrix-degrading proteases, including matrix metalloproteinases (MMPs), all of which

infiltrate into resident cells of the periodontium.^{4–6} Gingival connective tissue is predominantly composed of stromal cells, such as fibroblasts, and a collagen-rich extracellular matrix. Gingival fibroblasts (GF) are the most common cell type in gingival tissue and are exposed to periodontopathogens at an early stage of periodontal infection.⁷ Therefore, GF may have an important role in the transition from health to disease.

Recently, obesity, metabolic syndrome and diabetes mellitus have been significantly associated with periodontitis, suggesting that adipokines have a pathomechanistic role in these associations.^{8–11} Serum levels of proinflammatory adipokines, such as nicotinamide phosphoribosyltransferase (NAMPT) (also called visfatin) and adiponectin, are increased in obesity and obesity-related diseases, and it has been speculated that such adipokines could enhance periodontal inflammation.^{12–16} Originally, adipokines were known to be secreted by adipocytes, but recently, they have also been shown to be locally produced

¹Department of Pharmacology and Dental Therapeutics, School of Dentistry, Chonnam National University, Gwangju, Republic of Korea; ²Research Center for Biomineralization Disorders, School of Dentistry, Chonnam National University, Gwangju, Republic of Korea and ³School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

Correspondence: Professor J-H Ryu, Department of Pharmacology and Dental Therapeutics, School of Dentistry, Chonnam National University, Yongbong-ro, Buk-gu, Gwangju 500-757, Republic of Korea.

E-mail: jesryu@jnu.ac.kr

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by other cell types under pathophysiological conditions.¹⁷ Recent reports have demonstrated upregulated NAMPT expression in inflamed gingival crevicular fluid¹⁸ and gingival biopsy of periodontally diseased patients.¹⁹ Furthermore, NAMPT expression is upregulated in periodontal ligament (PDL) cells following infection of periodontopathogens,^{20,21} and treatment with recombinant NAMPT stimulates increased expression of MMP1 and chemokine (C-C motif) ligand (CCL)2 in these cells.²⁰ These observations demonstrate that gingival expression of NAMPT is increased at sites of periodontitis. However, the *in vitro* and *in vivo* functions of local NAMPT expression in the periodontium, including GF, as well as the mechanisms of NAMPT function in periodontitis need to be further clarified.

NAMPT functions in both intracellular (iNAMPT) and extracellular (eNAMPT) forms. iNAMPT exhibits enzymatic activity responsible for the salvaging pathways of nicotinamide adenine dinucleotide (NAD⁺) synthesis, while eNAMPT acts as an adipokine associated with inflammatory diseases.^{22,23} A recent study, which focused on the function of eNAMPT as an adipokine, has suggested a role for adipokines in periodontal infection.¹⁷ Although this observation suggests a possible role of eNAMPT in periodontitis, the contribution of iNAMPT remains to be established. NAMPT enzymatic activity is an essential cofactor of sirtuin (SIRT) protein deacetylases. SIRT regulates gene expression by modulating chromatin function via direct deacetylation of histones and transcription factors.^{14,22} In this study, we demonstrate that local gingival expression of NAMPT regulates the pathogenesis of periodontitis by modulating the expression of catabolic factors via SIRT activation in GF.

MATERIALS AND METHODS

Human gingival tissues

Human gingival tissues containing both epithelial and connective tissues were obtained from 16 patients (20–73 years; 40.80 ± 18.80) during tooth extraction; eight healthy patients for non-inflamed gingiva and eight chronic periodontitis patients for inflamed gingiva. The Institutional Review Board at the Chonnam National University Dental Hospital (Gwangju, Republic of Korea) approved this study. Written informed consent was obtained from each study subject after all procedures had been fully explained. Gingival tissues were promptly maintained in liquid nitrogen and stored at -80 °C until further use.

Experimental animal model

Six-week-old female C57BL/6 mice were used as a periodontitis mouse model.²⁴ Before bacterial inoculation, mice were administered antibiotics (2 mg ml⁻¹ of sulfamethoxazole and 0.4 mg ml⁻¹ of trimethoprim) in drinking water for three days, followed by 3 days without antibiotics. Mice were further inoculated with 1 × 10¹⁰ colony-forming units of *P. gingivalis* (ATCC, Manassas, VA, USA) in 1 ml phosphate-buffered saline with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA). Inoculations were performed once a day for 3 days on the maxillary molar using phosphate-buffered saline as a vehicle. Mice were fostered for 40 days after the final inoculation of bacteria. Eight-week-old male C57BL/6 mice were intragingivally (IG) injected with adenovirus expressing *Nampt* (Ad-*Nampt*; 1 × 10⁹ plaque-forming units in a total volume of

10 µl). Inoculations were performed once a day for 6 days at the region of the maxillary alveolar mucosa between the first and second molars using a Hamilton syringe (32 GA, 9.25 mm, 20°; Hamilton, Reno, NV, USA). IG injection of empty adenovirus (Ad-C) was used as a control. Mice were killed 18 days after the first IG injection for histological analyses. Where it was indicated, mice were co-injected intraperitoneally (IP) with 100 µl of nicotinamide (NIC) (300 mg kg⁻¹ body weight; Sigma-Aldrich) once every 3 days. Mice were housed in specific pathogen-free barrier facilities and used in accordance with protocols approved by the Animal Care and Ethics Committees of Chonnam National University.

Cell culture and stimulation

Human GF were isolated from gingival papillary explants obtained from clinically healthy donors with no systemic and/or periodontal disease who were informed of the purpose of this study. Briefly, after dissection of gingival biopsies using dispase (Gibco BRL, Grand Island, NY, USA), the epithelial cell layer was microscopically dissected from the underlying connective tissue, and GF was extracted from the subepithelial tissue as previously described.²⁵ Isolated primary human GF were treated with the indicated amounts of LPS derived from *P. gingivalis* (Invivogen, San Diego, CA, USA) in addition to recombinant human NAMPT protein (AdipoGen, San Diego, CA, USA), human IL-1β (GeneScript, Piscataway, NJ, USA) and human TNF-α (Merck-Millipore, Billerica, MA, USA). Primary cultured human GF were infected with empty (Ad-C), *Nampt*-expressing adenovirus (Ad-*Nampt*), or *SIRT2*-expressing adenovirus (Ad-*SIRT2*) for 2 h at the indicated multiplicity of infection and incubated for an additional 24 h. The inhibitors FK866 (Cayman, Ann Arbor, MI, USA), an iNAMPT inhibitor; anti-NAMPT antibody (Bio Vision, Milpitas, CA, USA), an eNAMPT inhibitor; NIC (Sigma-Aldrich), a total SIRT inhibitor; and EX-527 (Sigma-Aldrich), a SIRT1 inhibitor, were used at the indicated concentrations in the presence of IL-1β or Ad-*Nampt*. Dimethyl sulfoxide or phosphate-buffered saline was used as a vehicle.

Immunohistochemistry and immunofluorescence microscopy

Mouse maxillas were fixed in paraformaldehyde, decalcified, embedded in paraffin, sectioned (5 µm thickness) and processed for hematoxylin and eosin staining or immunohistochemistry. Sections were briefly incubated with the following primary antibodies: goat anti-COX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NAMPT (AdipoGen) and rabbit anti-MMP3 (Abcam, Cambridge, UK). To detect osteoclasts, a leukocyte acid phosphatase (tartrate-resistant acid phosphatase; TRAP) kit (Sigma-Aldrich) was used. For double immunofluorescence labeling of human GF, cells were cultured on glass coverslips and permeabilized with 0.1% Triton X-100. Cells were incubated for 1 h with primary antibodies followed by 1 h with an Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Images were acquired using a fluorescence microscope (Carl Zeiss, Cambridge, Jena, Germany) and were analyzed by counting positively stained cells using ImageJ software.

RNA isolation, PCR with reverse transcription (RT-PCR), quantitative real-time PCR (qRT-PCR) and western blotting

Total RNA was isolated from human gingival tissues and primary culture human GF using TRIzol reagent (Ambion, Carlsbad, CA, USA). Non-inflamed or inflamed human gingival tissues were homogenized in TRIzol reagent using a glass tissue grinder. RNA was reverse-

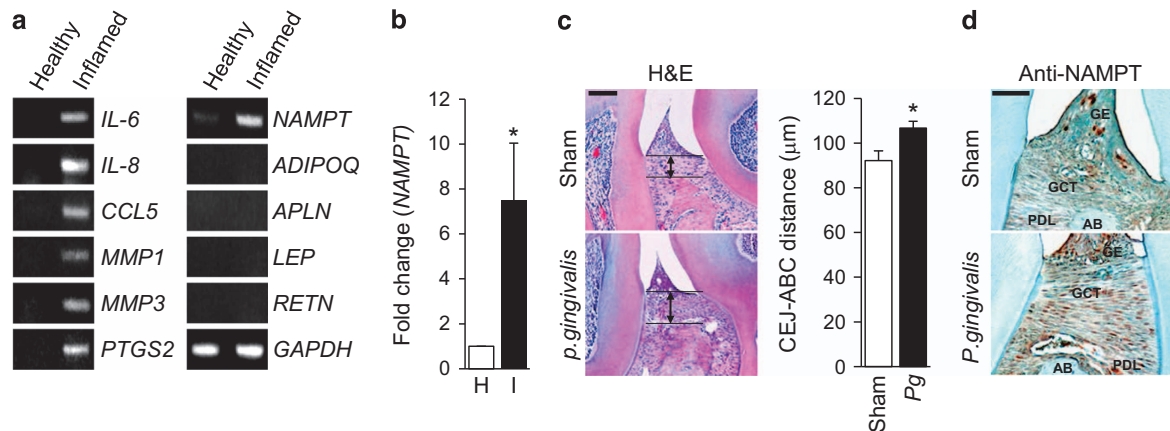


Figure 1 Increased expression of NAMPT in human and mouse inflamed gingiva. **(a and b)** Total RNA was extracted from whole gingival tissues of healthy (non-inflamed) or chronic periodontitis (inflamed) patients. mRNA levels of indicated adipokines were determined by RT-PCR **(a)**. *NAMPT* expression was quantified by qRT-PCR ($n=8$) **(b)**. **(c and d)** *P. gingivalis* was injected into the gingival margin of mouse maxillary molars for 3 days, and injection of empty adenovirus (Ad-C) was used as a control. H&E stained sections of intact periodontal tissues of control or *P. gingivalis*-infected mice and measurement of alveolar bone levels by comparing the distance from CEJ to ABC in μm ($n=4$) **(c)**. Immunostaining of gingival tissue for NAMPT in control or *P. gingivalis* infected mice **(d)**. GE, gingival epithelium; GCT, gingival connective tissue; PDL, periodontal ligament; AB, alveolar bone. Values are presented as the mean \pm s.e.m. ($*P<0.05$). Scale bar: 50 μm . ABC, alveolar bone crest; CEJ, cementoamel junction; H&E, hematoxylin and eosin.

transcribed, and the complementary DNA was amplified by PCR using *Taq* polymerase (GeneAll, Songpa, Seoul, Republic of Korea). Quantitative real-time PCR (qRT-PCR) was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and the SYBR premix Ex *Taq* (Takara Bio, Kyoto, Japan). All qRT-PCR was performed in duplicate, and the target gene amplification signal was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the same reaction. The relative levels of SIRT family (*SIRT1–7*) gene expression were analyzed using the comparative C_t (cycle threshold) method. The average C_t was calculated for *SIRT1* to *SIRT7* and *GAPDH*, as previously described.²⁶ Primers and experimental conditions are shown in Supplementary Table 1. Western blot analysis was performed to detect the cellular and secreted levels of NAMPT, COX-2 and MMP3 in total cell lysates or in conditioned culture media using standard techniques. The following primary antibodies were used for western blotting: NAMPT (AdipoGen), MMP3 (Abcam), COX-2 (Cayman) and TUBULIN (Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA)

Primary cultured human GF were cultured under each experimental condition. For MMP and COX-2 activity assays, conditioned medium was harvested and centrifuged for 3 min at 842 g. The total MMP activity in collected supernatants was evaluated by using a SensoLyte 520 generic MMP assay kit (Anaspec, Fremont, CA, USA), and COX-2 activity was assayed using a PGE₂ assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Nuclear proteins were isolated for the SIRT deacetylase activity assay using the NE-PER Nuclear kit (Thermo Scientific, Rockford, IL, USA). Total SIRT deacetylase activity was measured using a colorimetric assay kit (Abcam) according to the manufacturer's instructions. Absorbance was detected using an Epoch microplate reader (BioTek, Winooski, VT, USA) at 450 nm or FLx800 Fluorescence Reader (BioTek) at 520 nm.

Statistical analysis

The non-parametric Mann–Whitney *U*-test was used to analyze the ordinal grades (for example, cementoamel junction to alveolar bone

crest distance and bone surface to bone volume ratio). The data obtained with qRT-PCR and enzymatic activity assays were initially tested for conformation to normal distribution using the Shapiro–Wilk test and subsequently analyzed with Student's *t*-test (pair-wise comparisons) or analysis of variance with *post hoc* tests (multi-comparison) where appropriate. The threshold for significance was set at the 0.05 level of probability ($P<0.05$).

RESULTS

NAMPT is upregulated in inflamed human and mouse gingiva

To investigate gingival expression of adipokines at sites of periodontitis, we examined the transcript levels of adipokines with previously detected expression in serum.⁸ We assessed the expression of adiponectin (*ADIPOQ*), apelin (*APLN*), leptin (*LEP*), resistin (*RETN*) and *NAMPT*. Of the examined adipokines, we observed that only *NAMPT* expression was highly upregulated in chronically inflamed gingival tissues obtained from human periodontitis patients compared with non-inflamed gingiva (Figures 1a and b). Upregulation of *IL-6*, *IL-8*, *CCL5*, *PTGS2*, *MMP1* and *MMP3* represents the severity of inflammation during periodontal disease (Figure 1a). To confirm the upregulation of *NAMPT* expression during periodontitis, we generated a *P. gingivalis*-infected periodontitis mouse model. Histomorphometric measurements of hematoxylin and eosin-stained tissue sections showed that the distance from the cementoamel junction to alveolar bone crest was significantly greater in *P. gingivalis*-infected mice than in control mice (Figure 1c). Consistent with previous studies,^{19,20} immunohistochemistry using an anti-NAMPT antibody showed NAMPT expression in gingival epithelial cells, GF, and PDL cells (Figure 1d). These data suggest that local gingival expression of NAMPT has a potential role in periodontal inflammation and subsequent alveolar bone erosion.

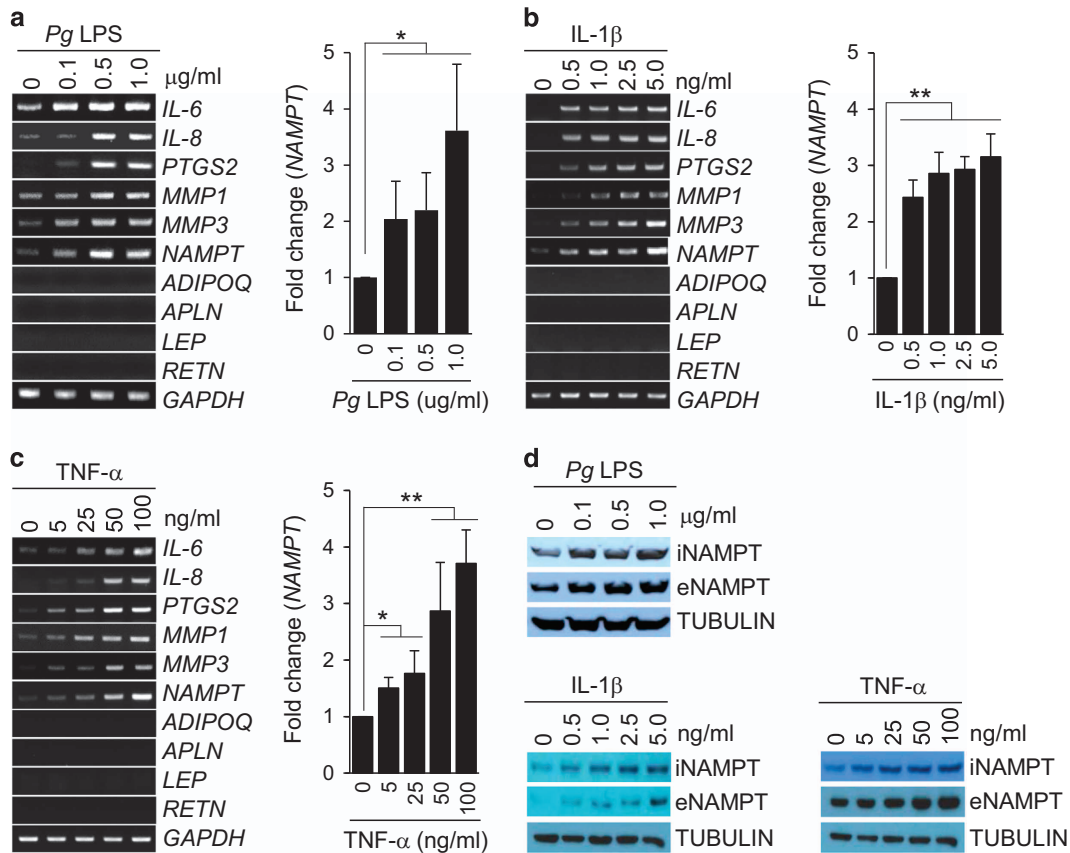


Figure 2 NAMPT is upregulated in human GF during periodontal inflammation. (a) Primary cultures of human GF were left untreated or treated for 24 h with the indicated concentrations of LPS obtained from *P. gingivalis*. The mRNA levels of the indicated genes were detected by RT-PCR analysis. Relative *NAMPT* mRNA levels (compared to those of untreated cells) were quantified by qRT-PCR ($n=5$). (b and c) Human GF were treated with the indicated doses of cytokines IL-1β (b) and TNF-α (c) for 24 h. Transcript levels of the indicated genes were determined by RT-PCR and quantified by qRT-PCR analysis ($n\geq 5$). (d) Western blot analysis of the extracellular (eNAMPT) and intracellular (iNAMPT) forms of NAMPT in human GF treated with the indicated concentrations of LPS, IL-1β and TNF-α. Values are presented as the mean \pm s.e.m. (* $P<0.05$, ** $P<0.01$).

NAMPT expression is upregulated in pathogenic human GF
 GF are the most abundant structural cells in periodontal tissue; they are exposed to pathogens at an early stage of periodontal infection and are critical in sustaining inflammation in periodontal disease.⁷ Therefore, we focused on NAMPT upregulation in human GF in inflamed gingiva. First, we examined expression patterns of adipokines following treatment with *P. gingivalis*-derived LPS (*Pg* LPS). We observed that *Pg* LPS induced increased expression of well-known periodontitis inflammatory factors, such as *IL-6*, *IL-8*, *PTGS2*, *MMP1* and *MMP3*, that are correlated with the upregulation of *NAMPT* expression in a dose-dependent manner. The expression levels of other adipokines, including *ADIPOQ*, *APLN*, *LEP* and *RETN*, were very low or not detected (Figure 2a). These data are consistent with the results obtained using gingival tissues of periodontally diseased patients (Figure 1a). Moreover, proinflammatory cytokines IL-1β (Figure 2b) and TNF-α (Figure 2c), which are involved in periodontitis pathogenesis, dramatically increased *NAMPT* expression in a dose-dependent manner but did not increase the expression of other examined adipokines. The two different forms of NAMPT have distinct

functions. iNAMPT acts as a regulator of cell metabolism and eNAMPT has a role in inflammation.^{22,23} Following treatment with *Pg* LPS, IL-1β or TNF-α, pathogenic human GF produced both iNAMPT and eNAMPT (Figure 2d). In addition, IL-1β-induced *NAMPT* upregulation was abolished by inhibition of p38 kinase and NF-κB using SB203580 and SC514, respectively (Supplementary Figure 1).

NAMPT regulates expression and activities of MMP1, MMP3 and COX2 in human GF

To explore the function of local NAMPT expression in gingiva during periodontitis pathogenesis, we overexpressed NAMPT. We examined the effects of NAMPT overexpression on the expression of catabolic factors involved in periodontal inflammation, including cytokines, chemokines, inflammatory mediators and matrix-degrading enzymes. Overexpression of NAMPT by Ad-*Nampt* infection in human GF led to a marked increase in the mRNA levels of *PTGS2*, *MMP1* and *MMP3* (Figure 3a). Additionally, increased protein levels of COX-2 and MMP3 were observed with immunoblotting (Figure 3b), and their increased enzymatic activities were determined by the

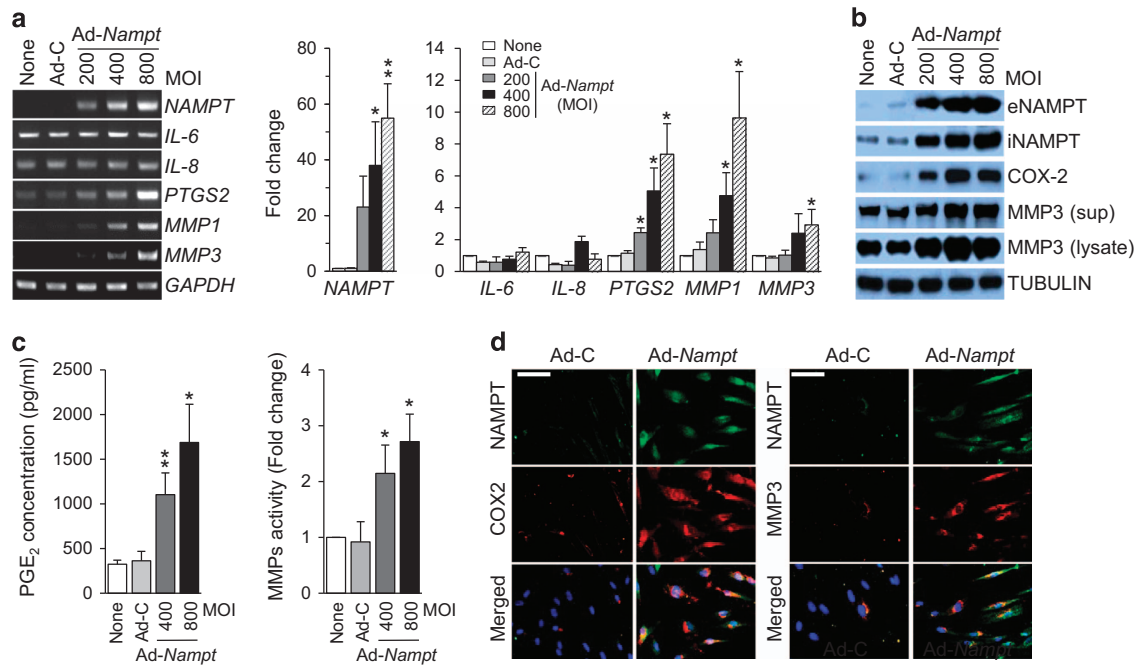


Figure 3 Ectopic expression of NAMPT regulates the expression of COX-2, MMP1 and MMP3 in human GF. Primary cultured human GF were left untreated (None) or were infected with 800 MOI of empty virus (Ad-C) or the indicated MOI of *Nampt* adenovirus (Ad-Nampt) for 24 h. (a) mRNA levels of the indicated genes were detected by RT-PCR (left) and quantified by qRT-PCR ($n \geq 5$) (right). (b) Western blots of cellular NAMPT (iNAMPT), COX-2, MMP3 (Lysate), secreted NAMPT (eNAMPT) and MMP3 (Sup) in human GF infected with the indicated MOI of Ad-Nampt. (c) MMP activity and PGE₂ release in human GF infected with Ad-C (800 MOI) or the indicated MOI of Ad-Nampt ($n = 5$). (d) Immunofluorescence microscopy of NAMPT, COX-2 and MMP3 in human GF infected at an MOI of 800 with Ad-C or Ad-Nampt. DAPI was used for nuclear staining. Values are presented as the mean \pm s.e.m. (* $P < 0.05$, ** $P < 0.01$). Scale bar, 50 μ m. MOI, multiplicity of infection.

overexpression of NAMPT (Figure 3c). Ad-Nampt-infected human GF expressed both eNAMPT and iNAMPT (Figure 3b), and cells with a high iNAMPT expression were strongly positive for target genes, such as COX-2 and MMP3, as shown in double-staining images (Figure 3d).

NAMPT enzymatic activity (iNAMPT) is necessary for NAMPT action

To further investigate the regulatory mechanism of gingival expression of NAMPT during periodontitis pathogenesis, we determined the inflammatory actions of iNAMPT and eNAMPT in human GF. Addition of human recombinant NAMPT protein (rNAMPT) to the culture media of GF, which essentially mimics eNAMPT action, did not induce the expression of *PTGS2*, *MMP1* or *MMP3* (Figure 4a). Treatment with a neutralizing antibody against NAMPT did not alter the IL-1 β - (Figure 4b) or Ad-Nampt-induced upregulation of NAMPT target genes (Figure 4c). Treatment with FK866, a highly specific noncompetitive inhibitor of iNAMPT, in human GF significantly blocked the IL-1 β - or Ad-Nampt-induced upregulation of *PTGS2*, *MMP1* and *MMP3* (Figures 4d and e). Moreover, FK866-mediated inhibition of iNAMPT enzymatic activity suppressed the actions of IL-1 β (Figure 4f) and NAMPT overexpression by Ad-Nampt infection (Figure 4g) on the activation of MMPs and COX-2.

NAMPT enzymatic activity is known to stimulate the synthesis of NAD⁺, an essential cofactor of SIRT protein deacetylases.²² Therefore, we next examined whether the effects of iNAMPT in human GF were due to the NAD⁺-SIRT axis. IL-1 β treatment or Ad-Nampt infection increased total SIRT deacetylase activity in GF (Figures 5a and b). These increases were efficiently blocked by the inhibition of NAMPT using FK866 (Supplementary Figure 2), or by the inhibition of SIRT using NIC, a broad SIRT inhibitor (Figures 5a and b).²⁷ As expected, inhibition of SIRT activity with NIC significantly blocked IL-1 β -induced or Ad-Nampt-induced upregulation of *PTGS2*, *MMP1* and *MMP3* (Figures 5c and d) in addition to the activities of these target genes (Figures 5e and f). EX527, a SIRT1-specific inhibitor,²⁸ had no effect on the expression of catabolic factors upregulated by IL-1 β or NAMPT overexpression (Supplementary Figure 3). These results suggest that members of the SIRT family other than SIRT1 are associated with NAMPT-induced upregulation of COX-2, MMP1 and MMP3 in periodontal inflammation. Thus, we next determined which SIRT isoforms are involved in NAMPT regulation in GF. Although NAMPT overexpression increased SIRT activity, the mRNA levels of SIRT family members were not changed by overexpression of NAMPT (Figure 5g). Among the SIRT family members (SIRT1–7) examined, *SIRT2* was found to be the most abundantly expressed in human GF (Figure 5h). Overexpression of *SIRT2* by Ad-SIRT2 infection significantly

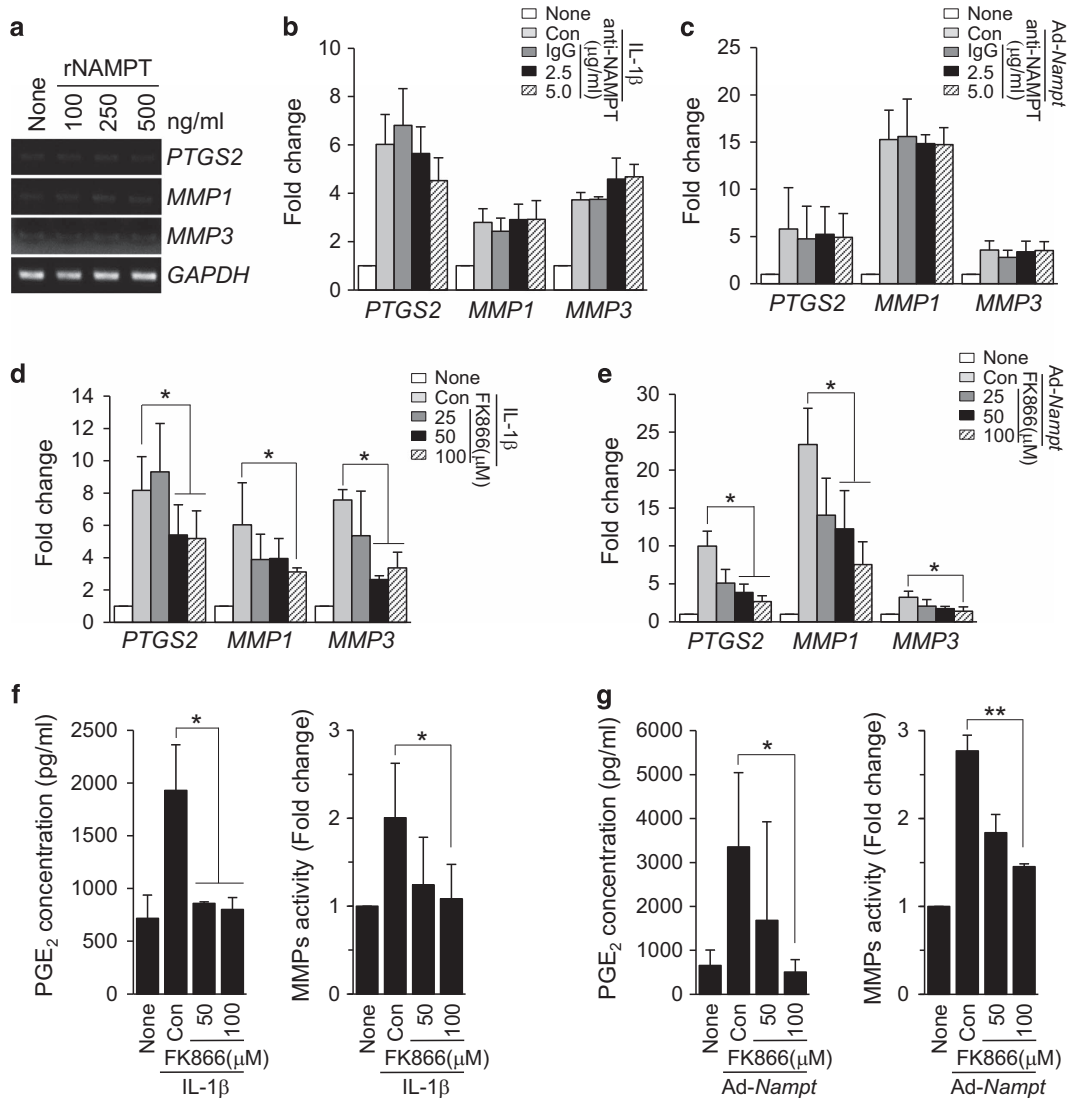


Figure 4 NAMPT enzyme activity is required for the upregulation of catabolic genes in human GF. (a) Expression levels of *PTGS2*, *MMP1*, and *MMP3* as determined by RT-PCR in human GF treated with recombinant NAMPT (rNAMPT). (b) and (c) mRNA levels of catabolic factors determined by qRT-PCR in human GF treated with IL-1 β (b), infected with Ad-Nampt (c) or exposed to the indicated concentrations of the NAMPT neutralizing antibody ($n \geq 5$). (d) and (e) Human GF treated with IL-1 β (d) or infected with Ad-Nampt (e) in the absence or presence of the indicated amounts of FK866 for 24 h. Expression levels of *PTGS2*, *MMP1* and *MMP3* were detected by qRT-PCR ($n \geq 5$). (f) and (g) MMP activity and PGE₂ release were measured in human GF treated with IL-1 β (f) or infected with Ad-Nampt (g) in the presence of FK866 ($n \geq 4$). Values are presented as the mean \pm s.e.m. (* $P < 0.05$, ** $P < 0.01$).

increased the expression of the NAMPT target genes *PTGS2*, *MMP1* and *MMP3* (Figure 5i). Taken together, these results indicated that iNAMPT/SIRT activity, rather than eNAMPT, promotes the upregulation of *PTGS2*, *MMP1* and *MMP3* in pathogenic human GF.

Local gingival NAMPT is a regulator of periodontitis

NAMPT enzyme activity is required for COX-2 and MMP expression in pathogenic GF. Therefore, we examined the effects of iNAMPT on periodontitis in mice. Intragingival injection of Ad-Nampt induced alveolar bone loss, which was determined by volumetric measurements from μ CT analysis (Figure 6a). The level of exposed root surface was found to

be significantly higher in the maxilla (Figure 6b), while TRAP-positive osteoclasts were increased in the alveolar bone of Ad-Nampt-injected mice (Figure 6c). Consistent with these findings, upregulated expression of RANKL was detected in Ad-Nampt-infected human GF (Figure 6d). Co-injection of nicotinamide significantly ameliorated Ad-Nampt-induced periodontal bone destruction as determined by volumetric measurements from μ CT analysis (Figure 6e) and histomorphometric measurements of hematoxylin and eosin-stained tissue sections displaying the cemento-enamel junction to alveolar bone crest distance (Figure 6f). In addition, MMP3 upregulation and TRAP-positive osteoclast numbers were decreased by blockade of the NAMPT-SIRT axis (Figure 6g).

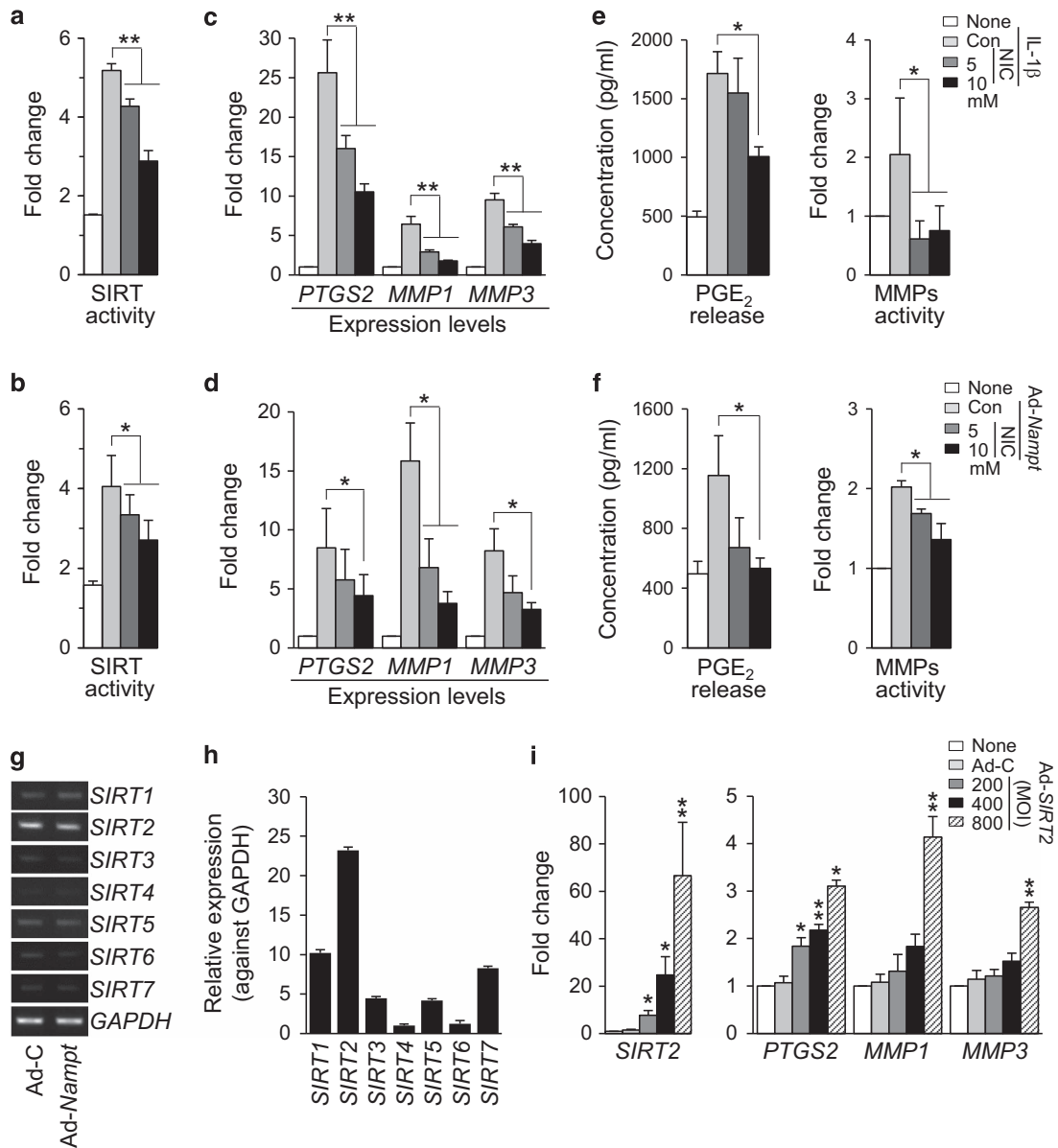


Figure 5 The NAMPT-SIRT2 pathway mediates NAMPT-induced gene expression in human GF. (a–f) Human GF were untreated (None), treated with IL-1 β (a, c and e), or infected with Ad-Nampt (b, d and f) in the absence or presence of NIC at the indicated concentrations. Total SIRT activity (a and b) and the expression levels of *PTGS2*, *MMP1* and *MMP3* (c and d) were determined ($n=5$). MMP activity and PGE₂ production were measured in human GF treated with IL-1 β or infected with Ad-Nampt in the presence of NIC ($n\geq 4$) (e and f). (g) Primary cultured human GF were infected with Ad-C or Ad-Nampt at an MOI of 800. mRNA levels were determined by RT-PCR. (h) The relative amount of *SIRT* mRNA in untreated human GF was normalized to that of *GAPDH* mRNA. (i) Human GF were left untreated (None) or were infected with Ad-C (800 MOI) or Ad-SIRT2 at the indicated MOI. mRNA levels were determined by qRT-PCR ($n\geq 5$). Values are presented as the mean \pm s.e.m. (* $P<0.05$, ** $P<0.001$). MOI, multiplicity of infection.

Furthermore, increased *RANKL* transcript levels were reduced by SIRT inhibition in the presence of Ad-Nampt in human GF (Figure 6h). Collectively, these results demonstrate the essential role of NAMPT enzyme activity in periodontal inflammation and in stages subsequently following alveolar bone resorption.

DISCUSSION

Periodontitis is caused by periodontopathogens in combination with other risk factors. Obesity and metabolic diseases enhance

the risk for periodontitis and compromised periodontal healing. Adipokines are suggested to be pathomechanistic links between periodontitis and systemic inflammatory diseases.¹⁷ The pathogenesis of periodontitis primarily occurs through periodontal inflammation and alveolar bone resorption. Here, we demonstrate that iNAMPT serves as an essential catabolic regulator of periodontitis pathogenesis. iNAMPT upregulates COX-2, MMP1 and MMP3, all of which are critical effectors of periodontal inflammation, osteoclast activation and the likelihood of bone destruction around teeth.

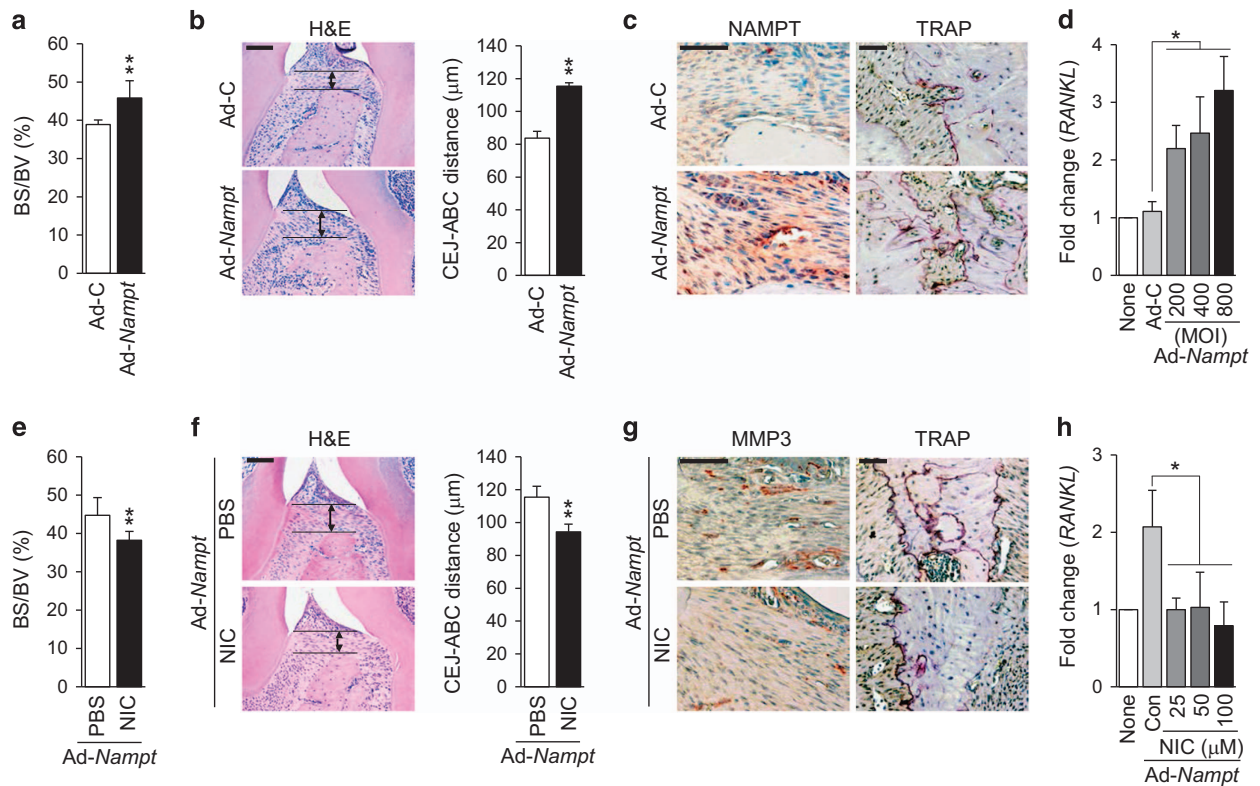


Figure 6 Blockade of SIRT activity ameliorates NAMPT-mediated periodontal inflammation and alveolar bone loss. Mice were intragingivally injected with 1×10^9 PFU of empty virus (Ad-C) or Ad-Nampt (a–d), and co-injected intraperitoneally with NIC (e–h). After 18 days, mice were killed for further analysis. Periodontal inflammation and alveolar bone erosion were detected by volumetric measurements (BS to BV ratio) via μ CT reconstruction (a and e) and histomorphometric analysis (distance from CEJ to ABC) using H&E stained sections (b and f) of the mice maxilla. NAMPT or MMP3 protein levels, and TRAP-positive osteoclasts in Ad-Nampt-infected gingival tissue with or without injection of NIC were examined by immunostaining ($n=4$) (c and g). Transcript level of RANKL was determined by qRT-PCR in GF infected with Ad-Nampt in the absence or presence of NIC ($n=5$) (d and h). Values are presented as the mean \pm s.e.m. (* $P < 0.05$, ** $P < 0.005$). Scale bar: 50 μ m. ABC, alveolar bone crest; BS, bone surface; BV, bone volume; CEJ, cementoenamel junction; H&E, hematoxylin and eosin.

Here, we focused on the upregulated expression of GF-derived NAMPT and demonstrated its possible roles in the modulation of periodontal inflammation and stages following alveolar bone loss. Adipokines are secreted mainly by adipose tissues and regulate insulin sensitivity, energy homeostasis and inflammation.¹² A number of previous studies have suggested an association between periodontitis and systemic adipokines, such as NAMPT, along with a relationship between periodontitis, obesity and type 2 diabetes.^{29,30} In periodontitis plasma levels, leptin³¹ and resistin³² are known to be increased, while gingival crevicular fluid leptin levels are known to be reduced.³³ Recent studies have reported that increased NAMPT levels were detected in gingival crevicular fluid, serum¹⁸ and inflamed gingival biopsy¹⁹ of human periodontally diseased patients. Plasma levels of NAMPT are also elevated in patients with obesity, metabolic syndrome, diabetes mellitus, cardiovascular diseases and insulin resistance.¹² To investigate the possible involvement of systemic NAMPT during periodontal inflammation, we verified the effect of circulating NAMPT (eNAMPT) in human GF. Unexpectedly, we observed that eNAMPT was not sufficient in mediating the pathogenesis of periodontitis. Recombinant

NAMPT protein and blocking antibody against NAMPT did not alter the NAMPT overexpression-mediated gene expression. However, whether eNAMPT functions in the regulation of other unexamined catabolic factors remains to be elucidated. Here, we examined the local expression of several adipokines in inflamed gingival tissue. With the exception of NAMPT, the local expression levels of the adipokines examined in inflamed gingival tissues and pathogenic human GF were extremely low. The expression of *adiponectin*, *apelin*, *leptin* and *resistin* was barely detected by qRT-PCR. Thus, we cautiously suggest that of the examined adipokines, NAMPT might be the most effective in locally regulating periodontal inflammation.

The role of NAMPT in inflammatory diseases has been widely investigated. NAMPT has been shown to stimulate a variety of cells to produce inflammatory mediators and proteases by using different intracellular pathways.²⁹ However, NAMPT expression and regulatory mechanisms in periodontal cells have not been clearly defined yet. NAMPT expression was upregulated by *F. nucleatum*,²⁰ *P. gingivalis* and IL-1 β .²¹ Furthermore, NAMPT supplementation in PDL cells produced MMP1 and CCL2.²¹ Consistent with previous studies, our

results demonstrate that *NAMPT* levels were markedly upregulated in inflamed gingival tissues from periodontitis patients and from a *P. gingivalis*-induced periodontitis mouse model. *NAMPT* upregulation in inflamed gingiva was verified by mimicking pathogenic conditions with LPS, IL-1 β and TNF- α treatments. LPS, IL-1 β and TNF- α are well-known proinflammatory cytokines involved in periodontitis in human GF. Pathogenic human GF produce significant amounts of *NAMPT*, cytokine (*IL-6*), chemokines (*IL-8* and *CCL5*), inflammatory mediator (*PTGS2*) and matrix-degrading enzymes (*MMP1* and *MMP3*), which are known to be expressed in pathogenic periodontium. Our gain-of-function studies using *Ad-Nampt* demonstrated that *NAMPT* upregulated various catabolic factors, including COX-2, *MMP1* and *MMP3* in human GF. Other types of adipokines are also known to upregulate catabolic gene expression, while leptin and proinflammatory cytokines may synergistically drive *MMP1* and *MMP3* production in human GF.³⁴ Alveolar bone loss is a hallmark of periodontitis progression. The influx of inflammatory cells during periodontitis produces proinflammatory cytokines, such as IL-1, IL-6 and TNF- α , which have been implicated in the stimulation of osteoclastic resorption in periodontitis.³⁵ These inflammatory cells also interact with periodontal stromal cells, such as osteoblasts, PDL cells and GF, resulting in the upregulation of RANKL, a mediator of osteoclast activation. Osteoblasts and GF stimulated by LPS and IL-1 induce RANKL expression.³⁵ Here, we demonstrate that in human GF, RANKL expression is increased by *Ad-Nampt*-mediated *NAMPT* overexpression, while NIC-mediated inhibition of SIRT decreases the *NAMPT*-induced upregulation of RANKL. These results suggest that the *NAMPT*-SIRT axis plays a pivotal role in periodontal inflammation by regulating inflammatory mediators and MMPs, in addition to alveolar bone loss by regulating RANKL expression.

Our loss-of-function studies, by FK866 treatment in the presence of cytokines or *Ad-Nampt*, suggest that iNAMPT enzyme activity has an important role in the regulation of expression and activities of COX-2, *MMP1* and *MMP3*. To date, the contribution of iNAMPT to periodontitis pathogenesis has not been established *in vivo*, although *in vitro* studies have suggested possible roles of eNAMPT in periodontitis.^{18–21} Therefore, our study provides the first evidence for an *in vivo* function of iNAMPT in the pathogenesis of periodontitis. Intragingival injection of *Ad-Nampt* caused periodontitis, which was blocked by inhibition of the NAD⁺-SIRT axis. *NAMPT* enzymatic activity is known to stimulate the synthesis of NAD⁺,^{14,22} an essential cofactor of SIRT protein deacetylases. *NAMPT* overexpression or IL-1 β treatment increased NAD⁺-dependent SIRT activity in human GF, and SIRT activity was inhibited by NIC, a broad SIRT inhibitor.²⁷ The mammalian SIRT family is composed of seven members, which are found in different subcellular locations, including the nucleus (SIRT6 and SIRT7), nucleus and cytosol (SIRT1 and SIRT2), and mitochondria (SIRT3–5). Among SIRT family members, SIRT1 is the best characterized

in the periodontium. SIRT1 is thought to be involved in the differentiation of human PDL cells into osteoblast-like cells³⁶ and in the remodeling of PDL in response to mechanical stress.³⁷ However, EX527, a SIRT1-specific inhibitor,²⁸ had no effect on *NAMPT* overexpression-induced upregulation of *MMP1*, *MMP3* and *PTGS2*. We found that the endogenous expression level of SIRT2 was higher than that of other family members, although the expression of all seven SIRT proteins was not affected by *Ad-Nampt* infection. Involvement of SIRT2 in *NAMPT*-mediated *PTGS2*, *MMP1* and *MMP3* expression was verified by SIRT2 overexpression using *Ad-SIRT2*. Our results demonstrate that SIRT2 might be involved in *NAMPT*-induced gene expression in periodontal inflammation.

In summary, we demonstrated fundamental roles of the *NAMPT*-SIRT axis in human GF. *NAMPT* expression is increased in inflamed human and mouse gingival tissues during periodontitis. Furthermore, *NAMPT* has a pivotal role in the expression of catabolic factors (COX-2, *MMP1*, *MMP3* and RANKL) in human GF, resulting in periodontal inflammation and alveolar bone loss. Effective treatment of periodontitis by various stimuli remains a significant medical need that is currently unachieved. Our findings suggest that future studies are warranted to examine the potential for targeting *NAMPT* and/or the *NAMPT*-SIRT axis as a therapeutic strategy in periodontitis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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