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Bone biology-related gingival transcriptome in aging and periodontitis in non-human primates

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Abstract

Aim—Cellular and molecular immunoinflammatory changes in gingival tissues drive alveolar bone loss in periodontitis. Since aging is a risk factor for periodontitis, we sought to identify age-related gingival transcriptome changes associated with bone metabolism in both healthy and in naturally-occurring periodontitis.

Materials and Methods—Adult (12–16 years) and aged (18–23 years) non-human primates (*M. mulatta*) (n=24) were grouped into healthy and periodontitis. Gingival tissue samples were obtained and subjected to microarray analysis using the Gene Chip Macaque Genome Array. Gene expression profiles involved in osteoclast/osteoblast proliferation, adhesion, and function were evaluated and compared across and between the age groups. QPCR was also performed on selected genes to validate microarray data.

Results—Healthy aged tissues showed a gene profile expression that suggest enhancement of osteoclastic adhesion, proliferation/survival and function (SPP1, TLR4, MMP8, and TFEC) and impaired osteoblastic activity (SMEK3P and SMAD5). The gingival transcriptome in both adult and aged animals with naturally-occurring periodontitis (FOS, IL6, TLR4, MMP9, MMP10 and SPP1 genes) was consistent with a local inflammatory response driving towards bone/connective tissue destruction.

Conclusion—A pro-osteoclastogenic gingival transcriptome is associated with periodontitis irrespective of age; however, a greater bone-destructive molecular environment is associated with aging in healthy tissues.

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Conflict of Interest:

Authors report no conflicts of interest related to this study.

Keywords

non-human primates; periodontitis; aging; osteoclast; gene expression

1. Introduction

A hallmark of periodontal disease is alveolar bone loss as a consequence of persistent inflammation triggered by a dysbiotic biofilm (Graves et al. 2011). An understanding of how an inflammatory response drives bone loss during periodontitis, and similar diseases like rheumatoid arthritis, has been the focus of interest to investigators in the field of osteoimmunology (Arron and Choi, 2000). In general, *in vitro* and *in vivo* evidence indicates that several types of immune cells infiltrating the gingival tissues can be active modulators of osteoclastogenesis during periodontal disease. T and B cells have both been shown to secrete RANKL in the diseased periodontium (Kawai et al., 2006), whereas dendritic cells and macrophages appear to be potential osteoclast precursors (Maitra et al., 2010). Select pro-inflammatory cytokines (e.g. TNF α , IL-1 β , and IL-6) also appear to play critical role in enhancing osteoclastogenesis (Boyle et al., 2003), whereas other molecules (e.g. IFN β , IL-4 and IL-10) could provide a molecular feedback dampening the bone loss processes (Araujo-Pires et al., 2015). In addition to osteoclast-dependent bone loss induced by the inflammatory mediators, chronic inflammation may interfere with bone formation altering the physiological coupling process (i.e. episodes of bone resorption followed by bone formation), which could lead to bone loss due to predominant osteoclastic activity (Liu et al., 2006). The regulatory mechanisms of bone formation and resorption have been extensively studied (Zaidi, 2007). These events involve a growing number of molecules that control: cell proliferation, differentiation into osteoblasts and osteoclasts and expression of matrix proteins. Bone metabolism can also be modulated by multiple factors such as, diet, drugs, hormones and aging (Feng and McDonald, 2011). Aging, in particular affects maintenance of normal bone remodeling and metabolism, the development of an inflammatory environment leading to inherent changes in bone architecture, and less resistance to fracture and increased resorption (Demontiero et al., 2012, Li et al., 2014). Accordingly, it has also been shown that the prevalence and severity of periodontitis increases with aging (Eke et al., 2012, Kassebaum et al., 2014, Gonzalez et al., 2016). Growing evidence indicates that cellular and molecular variations in gingival tissues, particularly associated with the immunoinflammatory response during periodontal disease, play an important role in alveolar bone loss. Beyond these genes, there are other crucial molecules involved in bone metabolism whose expression has not been previously evaluated in gingival tissues during periodontal disease. In this study we sought to determine the transcriptional differences associated with aging and periodontitis of around 200 genes in gingival tissues representing different steps in bone metabolism, using the translational nonhuman primate model.

2. Materials and Methods

2.1 Animals and diet

Rhesus monkeys (*Macaca mulatta*) (n=24; 14 females and 10 males) were housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, PR. The sample consisted of two age groups (adult: 12–16 years and aged: 18–23 years) representing healthy (adult: n=7; aged: n=6) and diseased animals with naturally-occurring periodontitis (adult: n=5; aged: n=6). The clinical examination for periodontal health status and sample collection was done under anesthesia by a single investigator. Probing pocket depth (PPD) and bleeding on probing (BOP) measures (scale 0–5) on two interproximal sites per tooth were obtained, excluding canines and 3rd molars (Ebersole et al. 2008). Healthy sites were defined as PPD ≤3mm and mean BOP ≤1, and periodontitis sites were defined as PPD>3mm and mean BOP ≥2. Animals were fed commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). They also had access to fruits, vegetables and water *ad libitum*, in an enclosed corral setting. All protocols and procedures are in compliance with ARRIVE guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico and the University of Kentucky.

2.2 Tissue sampling and gene expression microarray analysis

Using a standard gingivectomy technique (a crevicular incision followed by an interdental incision at the base of the papillae using a #15 surgical blade), a buccal gingival tissue specimen per animal from healthy or periodontitis affected sites from the same premolar/molar regions in the maxilla or mandible was obtained. The tissue specimen was stored in RNAlater solution (Invitrogen, IN) until further processing. Total RNA from each gingival tissue was isolated using a manufacturer's protocol and was further cleaned using RNeasy Mini kit (Qiagen, CA) and submitted to the University of Kentucky Microarray Core Facility to assess RNA quality and transcriptome analysis using the GeneChip® Rhesus Macaque Genome Array (Affymetrix, CA) (Gonzalez et al., 2011).

2.3 QPCR analysis

Based on the microarray outcomes we selected four genes and performed a QPCR analysis using a standard technique employing a Roche 480 Light-Cycler (Roche, IN). Four to seven specimens for each group were pooled and a total of 1µg was used for cDNA synthesis and further amplification using the following primers FOS (F- GTCTTCCTTCGTCTTCACCTAC; R-AGTCAGAGGAGGG CTCATT), SPP1 (F- TCCGATGAACTGGTTACTGATTT; R- CTCAGTC CATAAGCCACACTATC), MMP9 (F- CGTCTTCCAGTACCAAGAGAAA; R-GGATGTCATAGGTCACGTAGC), PIK3CG (F- GGCAACCTTTGTTCTTGGAATAG; R- AGAATGTGCCCCGAAGTCAATA) and GAPDH (F-GGTGTGAACCATGAGAAGTATGA; R- GAGTCCTTCCACGATACCAAAG), purchased from IDT-DNA (IA, USA). Concentration ratios of target genes were normalized to the GAPDH gene and the gene expression levels were compared across the samples prepared from each of the healthy, adult and aging samples and their respective periodontitis groups.

2.4 Histology and immunohistochemistry

Gingival tissue specimen (3/group) were fixed in 4% neutral buffered formalin for 24 hours (at RT) and changed to 70% ethanol. Tissues were processed by graded dehydration in alcohols and paraffin embedded. Deparaffinized 5µm serial sections were obtained using microtome (Leica RM2255) and were stained with hematoxylin-eosin. For immunohistochemistry, sections were subjected to protease antigen retrieval (0.1% trypsin in 0.1% CaCl₂, 20 mM Tris-HCl, pH 8.0) and peroxidase quenched. Gingival sections were incubated for 1hr in 10% normal horse serum and 0.01% Tween-20 in phosphate buffer to block nonspecific binding following incubation overnight with the polyclonal goat IgG anti-Osteopontin antibody (R&D systems, MN) at 4°C. Sections were then applied with donkey anti-goat HRP secondary antibody (Santacruz, TX) for 1hr followed by DAB staining (Invitrogen, CA), counterstained with hematoxylin and images were captured using Olympus microscope with QImaging software (QImaging Inc., Canada). DAB staining intensity measurements were done on acquired images using ImageJ.

2.5 Data Analysis

Normalization of values across the chips was accomplished through signal intensity standardization across each chip using Affymetrix PLIER algorithm. The GeneChip® Rhesus Macaque Genome Array contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. Gene expression and immunostaining intensity by immunohistochemistry from healthy and periodontitis tissues were then compared across and between the age groups using a *t*-test. The microarray data has been uploaded to ArrayExpress database (www.ebi.ac.uk) under accession number E-MTAB-1977. JMP version 12 (SAS, Inc. NC) software was used for statistical analysis. Since our study focuses on 228 genes, we expect at most about 10 false positives by chance using a *p*-value cut off of 0.05. Of the 177 pairwise comparisons with fold changes more than 1.5, 81 had *p*-values less than 0.05, approximately nine of which may be false positives. Two way ANOVA followed by two sample *t*-tests was used for analysis of clinical measures (PPD and BOP) at each site among the groups. A *p*-value 0.05 was accepted as significant for all comparisons. Furthermore, ontology enrichment analysis is designed to be robust to random false positives in gene lists. Heat maps were generated using MeV_4_9_0 (open source genomic analysis software; www.tm4.org).

3. Results

Clinical characterization for healthy and inflamed/periodontitis sampled sites in each group were as follows: adult healthy group (mean PPD 2.4±0.2 mm, mean BOP 0.9±0.4); aged healthy group (mean PPD 2.5±0.4 mm, mean BOP 1.1±0.7); adult periodontitis group (mean PPD 3.9±0.2 mm, mean BOP 2.6±0.6); and aged periodontitis group (mean PPD 4.6±0.7 mm, mean BOP 2.7±0.4). Both PPD and BOP were significantly higher (*p* 0.0001) in periodontitis samples from both adult and aged when compared with their corresponding healthy counterpart. BOP levels associated with periodontitis were not significantly different (*p*>0.05) between adult and aged sites. PPD levels in periodontitis sites were significantly higher (*p* 0.05) in aged compared with adult diseased sites (Suppl. Figure 1).

3.1 Gene expression profiles in adult and aged healthy gingival tissues

To explore gene expression patterns in health and aging, samples isolated from gingival tissue were processed for RNA and analyzed using oligonucleotide microarrays. The amount of RNA required for microarray analysis and the yields that were obtained from gingival tissues from each animal were sufficient enough allowing samples from individual animals to be analyzed by the microarrays. The biological variability among samples is presented in the form of heat maps plotted against the means of the healthy adult group (Fig. 1a). Only known genes annotated for these microarray chips but not uncharacterized, expressed sequences were included in the analysis. A total of 228 genes related to osteoclast/osteoblast proliferation, survival, cell adhesion, differentiation and maturation, as well as genes associated with bone extracellular matrix, were analyzed and found to be differentially regulated (Suppl. Table 1). A stringent criteria was applied to determine the effects of differentially expressed genes where only those with level of expression such that $p < 0.05$ and fold change was > 1.5 vs healthy adults were pursued further. At least 20 genes (11 up-regulated and 9 down-regulated) had an expression which was significantly different and only 6 of these (SPP1, SMEK3P, MMP8, TLR4, TFEC, and BMP3) were induced at least at a 1.5-fold level in the aging group (Fig. 1b).

3.2 Gene expression profiles in Adult and Aged Periodontitis

Gene expression changes associated with periodontitis in adult and aging animals are shown in Fig. 1a. Six genes (DKK1, IL6, MMP9, NAIP, SPP1 and TLR4) were significantly up-regulated more than 1.5-fold with periodontitis in adult samples when compared to healthy adults (Fig. 1c). Apart from a few overlapping candidates (e.g. SPP1 and TLR4), gene expression of IL6 and MMP9 was only found significantly elevated in the adult periodontitis group. Comparison of the gene expression patterns in aged periodontitis tissues with respect to the corresponding healthy controls demonstrated that 7 genes (ALPL, BIRC3, FOS, MMP10, MMP9, PTK2, and ZHX2) were up-regulated significantly (Fig. 1a; 1d). Interestingly, when comparison was made between adult and aged periodontitis groups, 4 distinct genes (C1orf61, MMP10, PTK2B, and TGFB1) were up-regulated and only one (FCER1A) was down-regulated significantly in aged tissues when compared with the adult counterpart (Fig. 2a).

3.3 Global bone-related gene expression for periodontitis irrespective of age

The gene profile obtained after comparing periodontitis with healthy samples irrespective of age showed several genes noted previously in adult and aging diseased animals analyzed independently. Among those that were consistently up-regulated were FOS, IL6, SPP1, and MMP9 genes (Fig. 2b). In addition, COL4A4 and RAC2 were found to be up-regulated significantly, whereas the osteoclast marker CA2 and CAV1 were down-regulated.

3.4 Validation of microarray data

To further validate these results, we performed QPCR for three of the most relevant genes that demonstrated a significant change in the microarray data. These are genes known to particularly influence osteoclast proliferation/survival or function (FOS, SPP1 and MMP9). All three genes showed expression profiles via QPCR that were consistent with that of

microarray-based measurements (Fig. 3). In addition, PIK3CG expression which was not altered among the groups as evidenced by microarray was also analyzed by QPCR to validate the differential expression of the above mentioned genes.

Further validation of microarray data was obtained from immunohistochemistry analysis of gingival tissues. Osteopontin protein (OPN) encoded by SPP1 gene was found to correlate with the microarray data in both healthy and disease tissue samples (Fig. 4a). Interestingly, histological characteristics reveal moderate to intense staining in gingival tissue sections in both periodontitis and aging groups with extracellular OPN expression evident in all layers of the gingival epithelium, and underlying connective tissue (Fig. 4b). As OPN appears in the early stage of osteogenic maturation, high OPN expression in periodontitis indicates concurrent bone formation parallel to osteoclastic resorption.

4. Discussion

The emerging field of osteoimmunology has paved way to better understand the interactions between immune responses and resulting biological processes in juxtaposed bone that occur in chronic inflammation and destructive bone loss (Takayanagi, 2009). Host responses have been linked to innate and adaptive immune, and inflammatory responses to emphasize the critical nature of regulating these responses in a local microenvironment (Takayanagi, 2012). Nevertheless, it is clear that in periodontitis triggered by an altered microbial ecology interfacing with a susceptible host, these persistent responses can result in both soft tissue destruction and alveolar bone loss (Hajishengallis, 2015). However, much of the knowledge of these processes in periodontitis has been derived from systems in which individual molecules are targeted and models created to develop proof of their role in the tissue destruction (Ebersole et al., 2013). These studies do not address the systems biologic nature of the complexity of the host-bacterial interactions that occur in the subgingival sulcus, translating the microbial stimulatory signals across numerous cellular pathways that results in disease. This study used a nonhuman primate model of naturally-occurring periodontitis to explore this complexity, not only in periodontitis, but also how the local gingival tissue environment changes with aging.

The upregulated genes were those promoting osteoclast differentiation and activity which include SPP1, SMEK3P, MMP8, TLR4, TFEC, and BMP3 expression. SPP1 encodes for Osteopontin (OPN) which is major component of mineralized matrix and is expressed by a variety of cells in soft and hard tissues (Sodek et al., 2000, Lao et al., 2006) indicating multiple functions. OPN is produced by T-cells, macrophages, osteoclasts and osteoblasts, binds to mineral and signals through several integrin receptors which includes $\alpha v \beta 3$ and CD44. OPN induces chemotaxis, cytoprotection, survival in osteoclasts and regulates TH1/TH2 balance (Wang and Denhardt, 2008, Tanabe et al., 2011). It is also an important regulator of innate immune responses, enhancing neutrophil and macrophage accumulation at sites of injury (Diao et al., 2004). High Osteopontin levels have been previously noted in inflammation and in sepsis as inflammatory cytokines like TNF α and IL-1 β modulate its expression (Yu et al., 1999) and a few reports have outlined elevated OPN in periodontal diseases previously (Kido et al., 2001, Sharma and Pradeep, 2007). Recent findings suggest that OPN impairs host defenses during established septic melioidosis (van der Windt et al.,

2010). Suppression of Mek1 pseudogene homolog 3 (SMEK3P), a putative MAP kinase activator in lower multicellular organisms was also up-regulated. It is not clear at the moment, how this gene product might reflect or contribute to functional alterations of the gingival microenvironment; however, since Mek1/Erk-1 pathway is involved in negative regulation of osteoclastogenesis (Boyle et al., 2003), higher levels of SMEK3P could be contributing to enhance osteoclast differentiation in aged gingival tissues. In addition, both BMP3 and MMP8 are significantly upregulated in aging tissues. Typically, matrix metalloprotease expression is low in healthy periodontal tissues, and their secretion is regulated by various pro-inflammatory cytokines such as IL1 β and TNF α , which stimulate gingival fibroblasts to secrete MMP3, MMP8 and MMP9 thereby enhancing ECM and basement membrane breakdown. MMP8 (neutrophil collagenase) also plays a role in fracture healing, hematopoietic stem cell mobilization and immune cell recruitment (Gamer et al., 2009, Thirkettle et al., 2013, Steinl et al., 2013) contributing to osteoclast activation. BMP3 suppresses osteoblast proliferation (Daluiski et al., 2001, Kokabu et al., 2012) and is elevated in aging tissues causing imbalance in bone remodeling. TLR4 was significantly elevated which could link osteoclast proliferation and differentiation with the host response to the microbial challenge and sustained engagement of the NF κ B activity. TFEC (Transcription Factor EC) is a transcriptional activator known to collaborate with MITF in target gene activation, contributing to enhanced osteoclastic differentiation and activity (Kuiper et al., 2004) in aging tissues. Thus, the bone biology global transcriptome analysis suggested a gingival pro-osteoclastogenic environment accompanied of an impaired osteoblastic activity associated with aging in healthy tissues reflecting an increased risk for bone loss in aged gingival tissues exposed to a dysbiotic microbiota during periodontitis.

As expected, gene expression changes with periodontitis in both adult and aged animals were skewed towards creating an environment with substantial osteoclastogenic potential consistent with the bone resorption in periodontitis. Nevertheless, there were aging-periodontitis associated genes such as FOS, MMP10, ALPL, BIRC3, PTK2 and ZHX2 that were up-regulated more than 1.5-fold. Of note, most of these genes are related to osteoclast proliferation, differentiation and function (except for ALPL and ZHX2 which regulate osteoblast function).

Among the bone-related genes that are up-regulated 1.5-fold or greater with periodontitis specifically in adult gingival tissues were SPP1, IL-6, MMP9, TLR4, NAIP and DKK1. Most of these genes (except DKK1) are related to osteoclasts. Interestingly, genes differentially expressed with periodontitis in adult tissues were mostly different than those changing with disease in aged tissues. Of the 6 altered genes in adults and 7 in aged diseased gingival tissues, only MMP9 was upregulated in both age groups. MMP9 (gelatinase B) is a protease that degrades type IV and V collagen and has a role in local proteolysis of the extracellular matrix, in leukocyte migration and, in bone osteoclastic resorption (Hu et al., 2007). The level of gelatinases (MMP-2, MMP-9) in oral fluids was reported to be elevated in patients with periodontal disease, and standard mechanical periodontal treatment reduces these levels suggesting a potential role in tissue destruction in periodontitis (Rai et al., 2008). Moreover, MMP-9 knockout mice develop larger periapical lesions and greater inflammatory responses, suggesting a potential role in peri-radicular infections and bone loss (Wan et al., 2014). ZHX2 (Zinc fingers and homeoboxes protein 2) is a homodimeric

transcriptional repressor that interacts with the A subunit of nuclear factor- κ B (NF- κ B) (Ma et al., 2015). This transcription factor can stimulate an array of promoters including those for Type I collagen, albumin, beta-actin genes and bone sialoprotein (Kim and Sodek, 1999). In addition, NF- κ B interacts with serum response factor (SRF) that binds to the serum response element (SRE) in the promoter region of target genes to regulate cell cycle, apoptosis, cell growth/differentiation (Yamada et al. 1999).

Consistently, aged periodontitis tissues exhibited significantly higher levels of MMP10 and PTK2B than adult diseased tissues. MMP10, besides being a regulator of matrix modeling, plays a role in osteoblast differentiation through BMP signaling (Mao et al., 2013). PTK2B (Protein tyrosine kinase 2 beta/PYK2) is a calcium dependent tyrosine kinase that regulates reorganization of cytoskeleton, migration, adhesion and bone remodeling (Lakkakorpi et al., 2003). In particular, PTK2B has been associated to osteoclast proliferation/survival (Gil-Henn et al., 2007).

Interestingly, expression levels of the genes C1orf61, TGFB1 and FCER1A were significantly different in aged periodontitis tissues with respect to adult periodontitis tissues, even though these genes did not show significant changes with disease neither in aged nor adult tissues. C1orf61 is a transcriptional activator of the c-Fos promoter, which is known to play a role in Fos signaling during development and remodeling of neurons (Jeffrey et al., 2000). Though direct evidence is lacking in terms of osteoclast/osteoblast function, enhanced c-Fos activity in osteoclasts can be interpreted by analogy. TGFB1 is a multifunctional protein that controls proliferation, differentiation and many others cellular functions. Specifically, it plays an important role in bone remodeling as it is a potent stimulator of osteoblastic bone formation causing chemotaxis, proliferation and differentiation in committed osteoblasts (Nesti et al., 2007). FCER1A or IgE receptor alpha is normally known to mediate the release of mediators associated with allergy, and induce the secretion of important cytokines. Nevertheless, it is not clear how the change in FCER1A expression could affect bone physiology. Evidence indicates that other immunoglobulin receptors (e.g. Fc γ RIII) are negative regulators of osteoclast differentiation (Negishi-Koga et al., 2015). Thus, it will be tempting to hypothesize that a significant reduction in the levels of FCER1A in aging-periodontitis tissues (particularly in osteoclast precursors), may increase the likelihood for an impaired regulation of osteoclastic differentiation/activity. Aging-specific variation in the expression of these genes with periodontitis could involve age-associated epigenetic changes that may impact the gingival transcriptional responses normally occurring with disease, and could ultimately lead to tissue destruction as observed clinically with higher PPD values in aging periodontitis compared with adult diseased sites.

When healthy tissues were compared with periodontitis irrespective of age, genes like FOS, SPP1, IL-6 and MMP9 were consistently up-regulated in disease. However, as discussed before, only MMP9 was similarly up-regulated with disease in both adult and aged tissues, whereas increased expression of SPP1 and IL-6 were associated only with adult periodontitis, and FOS up-regulation with aged periodontitis. These genes could be potential targets for disease prevention or intervention; however, an age-dependent variation in the expression of these molecules with disease would need to be considered for such purposes. Furthermore, our findings may be valuable to guide future studies on the development of

gene expression-based predictive models and using RNA-*seq* complementary to Microarrays for reliable assessment of transcript abundance would be beneficial.

In summary, this study provides a broad picture of local inflammatory responses that could be contributing to enhanced bone and connective tissue destruction in aged periodontitis transitioning from a protective response to typical bacterial challenge to a pathognomonic complex response with an osteoimmunological interface resulting in disease. Moreover, findings reinforce the concept that the mucosal tissue characteristics in aging are different from other age groups even in healthy gingival tissues (Gonzalez et al., 2013, Ebersole et al., 2016). Thus, tissue alterations may presage clinical changes in mucosal tissues in aging individuals resulting in an enhanced risk of deregulated responses, breakdown in homeostasis, and subsequent soft tissue and bone destruction resulting from the chronic periodontal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Relevance

Scientific rationale for the study

The cellular and molecular mechanisms involved in higher risk of periodontitis with aging remain unclear. Changes in the bone biology-related gingival transcriptome could be involved in aging- associated periodontitis.

Principal findings

Aging supports an osteoclastogenic gingival transcriptome in healthy tissues that could increase the risk for periodontitis with age.

Practical implications

Understanding the potential mechanisms involved in inflammation and activation of pro-osteoclastic genes in aging-associated periodontal disease could provide novel therapeutic targets for the elderly.

Figure 1. Expression profiles of bone biology-related genes in healthy and periodontitis gingival tissues from adult and aging nonhuman primates

(a) Gene expression profiles of healthy and periodontitis gingival tissues from adult and aged animals obtained by microarray are presented as heat maps using MeV_4_9_0 (open source genomic analysis software; www.tm4.org). A total of 228 genes related to osteoclasts/osteoblasts were identified and means of raw expression values from healthy aging, adult periodontitis and aging periodontitis groups were projected against mean of adult healthy group. Few significantly upregulated genes are marked with arrow heads. (b)

Histogram indicates genes that were significantly different from aging healthy group with at least 1.5 fold over adult healthy ($p < 0.05$ vs adult healthy group). **(c)** Histogram indicates genes that were significantly different ($p < 0.05$ vs healthy control group) with at least 1.5 fold change in periodontitis adult group compared with its corresponding healthy group. **(d)** Histogram indicates genes that were significantly different ($p < 0.05$ vs healthy control) with at least 1.5 fold change of gene expression occurring with periodontitis in aging tissues.

a)

b)

Figure 2. Gene expression profiles of gingiva from periodontitis samples from nonhuman primates

(a) Histogram shows the bone-related genes whose expression levels (fold change) was significantly different in adult periodontitis vs aged periodontitis tissues ($p < 0.05$). (b) Relative expression profiles of bone-related genes associated to periodontal disease irrespective of age factor that were significantly different ($p < 0.05$ vs healthy group). Histogram indicates the fold change of gene expression levels in periodontitis over healthy samples.

Figure 3. Validation of gene expression of selected candidate genes in gingival tissues

Samples were processed to prepare cDNA from pooled RNA obtained from tissue samples (n=5 to 7) from each group and gene expression was analyzed using triplicates. Histograms indicate the fold change of gene expression levels over adult healthy samples.

Figure 4. Validation of Osteopontin expression in healthy and periodontitis gingival tissues from adult and aged nonhuman primates

(a) (A1–D1) Hematoxylin and eosin staining of serial sections from paraffin embedded gingival tissues. (A2–D2) Negative staining (secondary antibody alone). (A3–D3 & A4–D4) Immunostaining of Osteopontin shows extracellular expression in gingival epithelium and connective tissue. Some inflammatory cells show intense nuclear Osteopontin reaction (arrow heads). (b) Sections were analyzed to quantify relative staining intensity using ImageJ from 5–7 random areas from each group (n=3). Histograms indicate the fold change

over adult healthy samples, asterisk indicate $p < 0.05$ vs adult healthy group. Gingival epithelium (GE); Connective tissue (CT). (Magnification: A1–3; B1–3; C1–3 and D1–3 at 100×, A4–D4 at 200×) Scale bar: 100µm.