Regulation of Inflammatory Genes Involved in Periodontal Diseases by DNA Methylation

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Abstract

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(Under the direction of Steven Offenbacher)

Both bacterial infection and inflammatory immune responses by the host contribute to the pathogenesis of periodontal diseases, which include gingivitis in an early stage and periodontitis in a more advanced stage. DNA methylation, the most stable epigenetic modification, modulates gene expression without changing DNA sequences. The persistence of biofilm and the inflammation induced by periodontal pathogens may cause epigenetic modifications within the promoter region of genes in local tissues at the biofilm-gingival interface. We therefore hypothesize that DNA methylation is a regulatory mechanism for transcription of genes involved in both innate and adaptive immune responses in periodontal diseases.

Using clinical gingival biopsies, we identified an overall increased methylation level within the prostaglandin-endoperodie synthase-2 (*PTGS2*) promoter region in tissues with chronic periodontitis. The methylation level at one locus, which is -458bp in the *PTGS2* promoter region, is inversely related to the transcription of this gene. We also identified methylation changes of the tumor necrosis factor alpha (*TNFA*) promoter region specific to different stages of periodontal diseases in clinical biopsies. Transcription of *TNFA* is also inversely related to the methylation level at one locus, which is -163bp within the *TNFA* promoter. In addition, a hypomethylation profile within the interferon gamma (*IFNG*)

promoter region is only present in samples exhibiting chronic periodontitis but not in induced gingivitis samples.

In order to study whether *Campylobacter rectus* (*C. rectus*), a periodontal pathogen, is involved in the epigenetic regulation of inflammatory moleculs, we cultured THP.1 cells with *C. recuts*. An overall hypomethylation of CpG sites within the *TNFA* promoter and a progressive loss of methylation at -72bp locus are present in the THP-1 cells challenged by live *C. rectus*. In addition, the identified hypomethylation pattern is related to an increase of the transcriptional of *TNFA*. Using 5-aza-2deoxycytidine, a DNMT inhibitor, and a promoter-specific methylation luciferase reporter assay we confirmed that the methylation level of *TNFA* promoter negatively regulates the transcription of *TNFA*.

The data from this study provide evidence to support that altered DNA methylation profile identified in the promoter regions of several inflammatory genes contributes to the transcriptional regulation of those genes in periodontal diseases.

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5-Aza-2dC	5-aza-2deoxycytidine
Ap-1	activating enhancer binding protein-1
APC	adenomatous polyposis coli
Akt	v-akt murine thymoma viral oncogene homolog
B[α]PDE	benzo[α]pyrene diol epoxide
BDNF	brain-derived neurotrophic factor
BOP	bleeding upon probing
CAL	clinical attachment level
CBP/p300	CREB-binding protein
CD	cluster of differentiation
CDH1	cadherin 1 or E-cadherin
Cdkn3	cyclin-dependent kinase inhibitor
CFP1	CXXC finger protein 1
COX-2	cyclooxygenase-2 or PTGS2
CpG	cytosine-guanine dinucleotide
CREB	cAMP response element binding protein
C. rectus	*campylobacter rectus*
CTCF	CCCTC-binding factor (zinc finger protein)
DMD	differently methylated region
DNMT	DNA methyltransferases
DMSO	Dimethyl sulfoxide
eNOS	endothelial NO synthase
FGF2	fibroblast growth factor 2
FUSSEL18	functional Smad suppressor element on chromosome 18
GCF	gingival crevicular fluid

GI	experimentally induced gingivitis
GLMM	generalized linear mixed model
GSK-*3*	synthase kinase-3
GR (1)	glucocorticoid receptor
GR (2)	gingivitis resolved
H3K9	histone 3 lysine 9
HERV	human endogenous retroviruses
HDAC	histone deacetylases
HP-1	heterochromatin-binding protein-1
HPA	hypothalamic-pituitary-adrenal
H. pylori	*helicobacter pylori*
HTRA3	HtrA serine peptidase 3
ICAM	intercellular adhesion molecules
IECs	intestinal epithelial cells
IGF	insulin growth factor
LFA-1	lymphocyte function-associated antigen-1
IFN-γ	interferon gamma
IL	interleukin
IQR	interquartile range
MAOA	monoamine oxidase A
MAPK	mitogen- activated protein kinase
MBP	methyl-binding proteins
MBD	methyl-CpG-binding domain
MEcP2	methyl CpG binding protein 2
MGMT	O-6-methylguanine-DNA methyltransferase
miRNA	microRNA

Myc	V-myc myelocytomatosis viral oncogene homolog
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*)
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
LINE-1	long interspersed elements -1
LOH	loss of heterozygosity
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NO	nitric oxide
NOS2A	inducible nitric oxide synthase gene
NR3C1	neuron-specific glucocorticoid receptor
NSAID	Non-steroidal anti-inflammatory drug
PD	pocket depth
PGE_2	prostaglandin E_2
P. gingivalis	*Porphyromonas gingivalis*
PI3K	phosphatidylinositol 3-kinase
PTGS2	Prostaglandin-endoperoxidde synthase-2
RAM	regions of altered methylation
RT-PCR	real-time PCR
SAH (AdoHcy)	S-adenosylhomocysteine
SAM (AdoMet)	S-adenosylmethionine
SEPT9	Septin 9 (MLL septin-like fusion)
SINE	short interspersed elements
S-layer	surface-layer
SLE	systemic lupus erythematosus

SNP	single nucleotide polymorphism
Sp1	Specificity Protein 1
SSBP2	single-stranded DNA binding protein 2
SWI/SNF	SWItch/Sucrose NonFermentable
TLR	toll-like receptors
TNF-α	tumor necrosis factor-alpha
USF1 or 2	upstream transcription factor 1 or 2

Chapter 1

Introduction

DNA methylation overview

Genes determine all the proteins and functional RNAs on which the living biological species rely. Although all human cells share the same genetic material, cells behave differently depending on cell types, which are defined by their gene expression profiles (1). A regulatory mechanism "beyond the genome" must be present in the cells that dictates those expression profiles and can be also kept through cell division. This heritable mechanism that can control gene expression without altering genetic sequences is now described as an epigenetic regulatory approach (2)(3).

In eukaryotic cells, DNA is packed into chromatin, a highly organized structure that orchestrates DNA, histones and non-histone proteins (4). Epigenetic regulation alters the accessibility of trapped DNA sequence to trans-acting factors by modifying DNA and histones or repositioning nucleosomes, which are basic units of chromatin (5). Each nucleosome includes a stretch of DNA (about 146bp) and associated histone octamer core that consists of 2 identical copies of histone H2A, H2B, H3 and H4 polypeptides (6). DNA methylation, an enzymatic modification on certain cytosines in mammalian cells, was proposed to be the mechanism that can switch "on" or "off" genes during development (7)(8). It has been well recognized recently that such a methyl-transferring modification and post-translational modifications of N-terminal tails of histone polypeptides are key epigenetic components. The current scope of "epigenetics" is further enriched by infusing the non-coding RNA (miRNA) associated regulation, a mechanism that modulates post-transcriptional gene expression and itself can be controlled by DNA methylation (9)(10).

DNA methylation, as the most important enzymatic modification at the DNA level, involves covalent transfer of a methyl group to cytosines mainly within 5'-CpG-3' dinucleotides context. This biochemical reaction is catalyzed by DNA methyltransferases (DNMTs) utilizing S-adenosyl methionine (SAM) as the methyl donor (10)(1). The functional DNMT family includes several members such as DNMT1, DNMT3a and DNMT3b. While DNMT1 acts as a maintenance methyltransferase and is most abundant in mammals, DNMT3a and DNMT3b seem to play a more important role in *de novo* methylation at unmethylated cytosines in somatic cells (11)(6). In non-

embryonic cells, CpG rich regions, which are termed CpG islands and usually embedded within a large number of known gene promoters, usually remain unmethylated, However, about 80% of globally dispersed CpG dinucleotides, most of which reside in non-coding regions of genome, are heavily methylated. Such a hypermethylation state is associated with transcriptional repression state and essential for genomic stability by inactivating repetitive sequences, transposable and integrated retroviral elements (12).

DNA methylation is also critically involved in several biological events, such as mammalian development, imprinting and X-chromosome inactivation. A wave of active demethylation first occurs to the male genome several hours after fertilization, followed by a passive demethylation of the genome in females during subsequence cleavage division (13)(14). After implantation, the extent of remethylation in embryo genome is high due to *de novo* methylation, but the methylation decreases in specific tissues upon differentiation (15). DNA methylation also participates in establishing the X-chromosome inactivation and genetic imprinting. DNMT1, which preferentially methylates hemi-methylated CpG sites, is the key enzyme participating in genomic imprinting and X-chromosome inactivation. However, DNA methylation is responsible for maintaining and ensuring the silencing state of genes on the X-chromosome because X-chromosome inactivation is initiated by the replication of *Xist*, a non-coding RNA binding the future inactive X-chromosome, and this process precedes *de novo* methylation in embryonic development (16)(14).

Epigenetic regulatory mechanism interprets various environmental stimuli, such as toxins, stressors, carcinogens, infectious agents, cytokines, *etc*., by altering chromatin structures. Paralleling this decoding process are the cell signaling pathways through which the effects of those stimuli are transformed as the activation and/or aggregation of transcriptional factors in the nucleus. These two sets of signals work reciprocally around the genetic material: the structure of chromatin assumes a conformation due to epigenetic modification that either favors or inhibits the binding of those

transcriptional factors mobilized through signaling pathways. Therefore, the chromatin platform launched by epigenetic modifications is no less important than cell signaling pathways.

DNA methylation and bacterial infection

Epigenetic alterations induced by bacteria are possibly most studied in the gastric pathologies associated with the infection of *helicobacter pylori* (*H. pylori*), the microorganism that can cause chronic gastritis, gastric and duodenal ulcers, and is related to the pathogenesis of gastric cancer. The relationship between *H. pylori* and DNA methylation alteration has been manifested in human gastric diseases, animal models and *in vitro* co-culturing system.

The association between *H. pylori* infection and aberrant DNA methylation has been demonstrated in various gastric diseases from clinical studies. In a population-based study, the frequency of increased methylation level of *P16*, a tumor suppressor gene in regulating cell cycles, in gastric biopsies is significantly higher in *H. pylori* positive patients than *H. pylori* negative ones in each category of precancerous gastric lesions. Moreover, the presence of *P16* hypermethylation is significantly elevated with the severity of *H. pylori* infection (17). In agreement with the putative role played by *H. pylori* in the induction of abnormal DNA methylation, methylation level of promoters of several genes, such as *MGMT, CDH1, p16, COX2 and APC*, which were related to *H. pylori* infection in the gastritis or dyspeptic patients, were found to be decreased after the eradication of this infectious agent (18, 19). In addition to the co-existence of *H. pylori* and hypermethylated tumor suppressor genes in various precancerous lesions, the relationship between hypermethylated gene promoters was also widely present in the gastric malignancy (20)(21)(22). Due to this close association of *H. pylori* infection with frequent hypermethylation of tumor suppressor genes, *H. pylori* infection may contribute to the pathogenesis of gastric cancer through an epigenetic mechanism.

A plausible causal relationship between *H. pylori* infection and hypermethylation of several CpG island containing genes has been demonstrated in a gerbil model by inoculating *H. pylori* through gavage (23). In that study, the increased methylation profiles paralleled the infection process,

while the elimination of *H. pylori* resulted in a marked decrease of methylation of the same CpG islands. *usf1* and *usf2* are pleiotropic transcriptional factors regulating the expression of genes involved in immune responses, cell cycles and cell proliferation. In a murine infection model, an aberrant hypermethylation in the promoter regions of *usf1* and *usf2* in the tissues exhibiting *H. pylori* induced chronic gastritis is related to the downregulation of those genes (24).

By co-culturing gastric epithelial cells with *H. pylori*, researchers of different groups have demonstrated *in vitro* that *H. pylori* can cause DNA methylation alterations in the promoter regions of several inflammatory and/or antitumor genes. Yao et al. (25) first reported that a low level methylation within *hMLH1* promoter can be induced by incubating *H. pylori* with a gastric cancer cell line, AGS, and such a mild hypermethylation is at least partially responsible for the inhibited expression of this gene, which is critically involved in the mismatch repair process after DNA replication. Rapid cyclic DNA methylation/demethylation can be observed at several CpG sites present in a CpG island encompassing *COX-2* (or *PTGS2*) promoter region in MKN28, a gastric epithelial cell line, shortly after co-cultured with *H. pylori* (26). Most recently, a hypermethylation profile in the CpG islands within *USF1* and *USF2* promoters, both of which play important roles in tumor suppression, cell cycle and immune response, was also shown to be induced by *H. pylori* co-cultured with AGS (24).

While it seems that *H. pylori* infection is responsible for the observed aberrant epigenetic changes, the mechanistic link between infection and abnormal DNA methylation requirs the inflammatory process. Qian et al. (27) demonstrated that the hypermethylation of *E-cadherin* promoter region can be induced in several gastric cancer cell lines, such as TMK-1, MKN74, and MKN7, co-cultured with *H. pylori*. In that study, the *H. pylori* induced hypermethylation of E-cadherin seems to be largely caused by IL-1β signaling because pretreatment of cells with IL-1 receptor antagonist can reverse such a hypermethylated pattern induced by *H. pylori*. Interestingly, an induced hypermethylation by *H. pylori* within the promoter of another cancer suppressor gene, *runx3*, is mediated by nitric oxide (NO) as indicated by the fact that the addition of NO inhibitor blocked

such a hypermethylation alteration in the gastric cancer cell line co-cultured with macrophage and *H. pylori* (22). In the gerbil infection model mentioned above, the application of cyclosporine A, which suppressed the inflammation but did not affect *H. pylori* colonization in the gastric tissue, successfully blocked the induction of aberrant DNA methylation (23). Thus, the methylation alteration of some genes apprears to be dependent on the inflammation induced by infection.

In addition to *H. pylori*, other bacterial infections were also closely related to the altered methylation level in gene promoters. Bobetsis and colleagues were the first to identify the alteration of DNA methylation pattern within the *Igf2* P0 promoter region in murine placental tissues from the mothers systemically infected with a periodontopathogenic bacterium, *Campylobacter rectus* (*C. rectus*) (28). Because *C. rectus* is phylogenetically similar to *H. pylori* and both express GroEL that can stimulate IL-6 production (29), the infection of this periodontal pathogen might be causally related to the modification of various promoters of host genes and involved in pathogenesis of various diseases.

DNA methylation and inflammation

The production of inflammatory mediators including various cytokines, chemokines and growth factors, is a host defense mechanism. However, the specific combinations of innate, adaptive and regulatory cytokine profiles and cytokine networks define various inflammatory conditions. Epigenetic modifications, which carry the effect of environmental stimuli into the chromatin structure, may play a role in the pathogenesis of those inflammatory diseases. Although detailed mechanisms of host inflammation on the modification of epigenetic patterns largely remain unknown, it has been well discovered that the alteration of DNA methylation is profoundly involved in inflammatory diseases. Table 1 summarizes the studies of a wide spectrum of human chronic inflammatory diseases (gastric inflammatory diseases are not listed and mentioned in the “DNA methylation and bacterial infection” section) or chronic diseases that are related to a high inflammatory state, in which aberrant DNA methylation changes of genes have been identified.

Epigenetic regulatory approach of several important inflammatory mediators has recently been appreciated. Production of nitric oxide (NO) is involved in the inflammatory process since NO contributes to leukocyte adhesion and proliferation of vascular smooth muscle cells (30). Although the necessary transcriptional machinery for the endothelial NO synthase (*eNOS*) is apparently present in other cell types, the expression of this enzyme is strictly localized to the vascular endothelium. The molecular mechanism for this exclusive expression pattern is at least due to the regulation of promoter methylation. In endothelial cells, the promoter region of *eNOS* is hypomethylated or unmethylated as compared to other cell types that do not readily express *eNOS* (31)(1).In sharp contrast to the hypomethylated *eNOS* promoter, the methylation level of inducible nitric oxide synthase gene (*NOS2A*) in human vascular endothelial cells is very high. This hypermethylated pattern of *NOS2A* contributes to the unresponsiveness of endothelial cells upon stimulation with cytokines (1)(32).

The signaling pathway mediated by toll-like receptors (TLRs) is critically involved in the production of several key inflammatory cytokines, such as interleukin-1 beta, cyclooxygenase-2, *etc*. While expression of *TLR4* is required by myeloid lineage to function normally in innate immune response, decreased expression of *TLR4* on the surface of intestinal epithelial cells (IECs) helps to maintain intestinal homeostasis because elevated expression as seen in patients with inflammatory bowel disease incurs excessive response to commensal bacteria. This repression of *TLR4* in IECs is partially attributable to an epigenetic mechanism in which hypermethylation and histone deacetylation in the promoter region of *TLR4* are involved (33). Additionally, DNA demethylation is also involved in elevated expression of *TLR2* found in cystic fibrosis epithelial cells (34, 35).

The close association of DNA methylation with inflammation is also well documented in animal models. Adoptive transfer of $CD4^+$ T-cells pre-treated with 5-azacytidine (5-Aza-C), a known DNA methylation inhibitor, in mice induces systemic lupus erythematosus (SLE)-like phenotype (36) (37). Increased expression of lymphocyte function-associated antigen (LFA-1) by those pre-treated T-cells may be responsible for this lupus like disease (38). When injected with 5-azacytidine, arthritis

that was induced by adjuvant in rats was clearly reduced, indicating that DNA methylation is involved in the pathogenesis of adjuvant arthritis (39). Not only do those animal experiments support an epigenetic cause of certain autoimmune diseases, but they also suggest a therapeutic potential of epigenetic modifiers for those diseases.

Both increased and decreased promoter methylation profiles can be identified in the same inflammatory disease depending on individual genes in question, duration or stage of diseases (40)(41). Since DNA methylation controls gene transcription, this binary methylation pattern present in the promoter regions of different genes may help to explain expression profiles characterized by different disease conditions. Another interesting finding is that altered DNA methylation may occur either to specific genes or on a genomic scale (42, 43), changes also frequently observed in cancers (44). The continuity and agreement of those DNA methylation changes recognized in the inflammatory, precancerous and malignant lesions support the role of DNA methylation in linking inflammation and cancer (45)(46)(47).

DNA methylation and early childhood stress

It has been reported that maternal nursing behavior can alter the stress reactivity of the offspring in their adulthood. For example, the adult rats nursed from a more intimate mother-pup interaction in the first week of their childhood are less fearful and experience more moderate hypothalamic-pituitary-adrenal (HPA) responses to stress than rats raised in a less intimate relationship with the dams (48)(49). The underlying mechanism seems to be independent of genetic predisposition because in cross-fostering studies, in which rats from a less caring mother are raised by a different dam showing more intimate relationship with the fostered rats, the responses to stress of those fostered offspring are similar to those who are from and nursed by a caring dam. However, if the rats who are from a normally behaved but fostered by a less caring mother, their reaction to stress just resembles the offspring who are from and nursed by a less caring dam (49). The explanation may exist in epigenetic modifications of several stress response genes. Weaver *et al*. demonstrated that maternal behavior can affect the methylation pattern in the promoter region of glucocorticoid receptor

(*GR*) and such an effect occurred in the first week of life and can be reversed by cross-fostering (50). They also proved that the binding of an important transcription factor to the promoter region of *GR* and its expression as well as HPA responses to stress can be causally affected by DNA methylation alteration and histone acetylation. Interestingly, a human study investigating the neuron-specific glucocorticoid receptor (*NR3C1*) in the hippocampus obtained from suicidal victims who had a childhood abuse history revealed a higher DNA methylation in the promoter region and decreased mRNA level as compared to control samples obtained either from suicidal victims who had no childhood abuse history or from controls who died of unrelated causes (51). In contrast to the reversed DNA methylation pattern of *GR* promoter as mentioned above, Roth *et al*. reported that the methylation change of brain-derived neurotrophic factor (*BDNF*) promoter can be at least partially inherited to the next generation because cross-fostering pups exhibiting hypermethylation of *BDNF* from a dam experienced neonatal maltreatment to a mother who was normally nursed can not totally rescue the observed hypermethylated phenotype (52). Those studies support that epigenetic state that controls gene expression can be established by behavioral programming and such an influence induced by epigenetic modifications in the early childhood can be perpetuated through generations (52).

The effect of nutritional deficiency on DNA methylation

Chronic deficiency of group B vitamins including B6, B12, and folate acid (B9) as well as choline and methionine may disrupt the normal one-carbon metabolism network in which DNA methyltransferase reactions are integrated (53). Chronic dissipation of those group B vitamins can cause elevated homocysteine in plasma, a known independent risk factor for cardiovascular disease, in which abnormal DNA methylation is mechanistically related to its pathogenesis (54).

The only precursor of homocysteine *in vivo* is S-adenosylhomocysteine (AdoHcy, or SAH), derived from the common methyl-donor, S-adenosylmethionine (AdoMet, or SAM), after transferring the methyl group in various methyltransferase enzymatic reactions including DNA methylation (55). Although AdoHcy is physiologically hydrolyzed to homocysteine, which is further metabolized into

cysteine, a B6-dependent reaction pathway, or remethylated to methionine through a series of reactions necessitating folate acid and B12 as key coenzymes, the thermodynamics actually favors the reverse reaction to synthesize AdoHcy (53)(55)(56). Thus, metabolic perturbations, such as depletion of B6, B12 and folate acid, will lead to elevated AdoHcy level through interfering the normal removal of homocysteine. Figure 1 summarizes the methionine metabolism pathways related to methylation in cells.

As a potent inhibitor of methyltransferase involved in DNA methylation, elevated AdoHcy due to the abnormal accumulation of homocysteine caused by nutritional deficiency such as B12, which is commonly seen in elder population and an unbalanced vegetarian diet, and folate acid, widely present in alcoholism, is in close association with DNA hypomethylation (54). Yi *et al*. (53) reported that moderate elevation of plasma homocysteine level in healthy females paralleled the increases in plasma AdoHcy and global DNA hypomethylation in lymphocytes. In Castro's study (57), patients with vascular disease, as compared to controls, had significantly higher concentrations of plasma homocysteine that was correlated with AdoHcy level and a lower genomic DNA methylation. However, several groups presented contradictory data indicating elevated homocysteine level is not always related to a hypomethylation profile. By using a hyperhomocysteinemia mouse model, Bromberg et al, did not observe an altered global DNA methylation level (58). Bonsch *et al*. even found an increased genomic DNA methylation level in alcoholic patients that was significantly correlated with their increased homocysteine concentrations (59). The discrepancy might be explained by the stage of disease in question, conditions related to the secondary disease-induced alteration, such as inflammation, duration of hyperhomocysteinemia in an animal model, the ratio of AdoMet/AdoHcy (SAM/SAH) rather than the level of AdoHcy alone, and confounding nutritional factors other than group B vitamins discussed above (55).

In addition to perturbed DNA methylation on a genomic scale, an altered DNA methylation pattern also can be observed at the single gene level. As a well recognized risk factor for cardiovascular disease, hyperhomocysteinemia can arrest endothelial cell growth. Part of the

mechanism related to hyperhomocysteinemia involves the suppression of cyclin A transcription via a demethylation in a repressor site within the promoter region of this gene (60). A hypermethylation pattern in a CpG dense region within the fibroblast growth factor 2 (*FGF2*) promoter region is involved in an epigenetic mechanism in which inhibition of endothelial cell growth induced by high homocysteine concentration was achieved by transcriptional depression of *FGF2* (61). Using a hyperhomocysteinemia mouse model, Devlin *et al* (62) demonstrated that while elevated plasma level of homocysteine was related to a decreased *H19* differently methylated region (DMD), which is located to the 5' end of *H19* and 3' end of *Igf2*, the methylation level of the same *H19* DMD region in brain and aorta was significantly increased. Interestingly, such an increased methylation level paralleled an elevated transcriptional level of *H19*. These results reflect that hyperhomocysteinemia may cause either hyper- or hypo- methylation pattern in a tissue-specific manner in certain genes.

Besides the deficiency of folate acid, B12 and B9, insufficient intake of choline also disrupts the normal DNA methylation network (figure 1). Choline is another important intermediate that is involved in the conversion of homocysteine to methionine (63). Dietary choline deficiency results in decreased AdoMet concentration and hypomethylation of DNA (64). The methylation level of *cdkn3* promoter of fetal rodents whose mothers were fed with a choline deficient diet has shown to be decreased in the brain. Such a hypomethylation pattern of *cdkn3* was related to an overexpression of this gene, which inhibited cell proliferation (65).

DNA methylation and smoking

Cigarette smoke is a well known risk factor for a wide range of malignancies including oral, esophageal, pharyngeal, laryngeal and other cancers (66). Cigarette smoke contains various carcinogenic compounds of which polycyclic aromatic hydrocarbons and N-nitrosamines are the most important ones (67). Because epigenetic alteration is among the mechanisms of carcinogenesis, frequent DNA methylation changes as seen in cancers may be etiologically related to the exposure to those compounds in cigarette smoke. By exposing human bronchial epithelial cells to cigarette smoke condensate, Liu *et al*. (68) demonstrated both a hypomethylation profile in genomic scale and a local

promoter hypermethylation of several tumor suppressor genes in cells. In a large case-control study, the methylation level of *MTHFR1* in lung cancer increased with the exposure to tobacco smoke (69). The researchers also discovered a decreased global methylation pattern as represented by the methylation level of *LINE-1* repetitive sequences in the same lung cancer as compared to corresponding blood or non-cancerous lung tissue. This is probably due to the inhibited expression of *MTHFR1*, whose product plays an important role in maintaining the pool of methionine, caused by the promoter hypermethylation of *MTHFR1* (69, 70). Methylation alteration of other genes, such as *SSBP2* (single-stranded DNA binding proteins2) involved in transcription regulation) (71), *HTRA3* (HtrA serine peptidase3, involved in cell growth regulation) (72), *MAOA* (monoamine oxidase A, involved in cellular biogenic amine metabolic process) (73), *MGMT* (O-6-methylguanine-DNA methyltransferase, involved in DNA repair) , *P16* (involved in cell cycle regulation) (74), *FUSSEL18*, *SEPT9* (Septin 9, also known as MLL septin-like fusion, involved in the regulation of cell cycle and division) (75), most of which are tumor suppressor genes, was also reported to be significantly correlated with the exposure of cigarette smoke. Therefore, data from both *in vitro* and *in vivo* studies support the hypothesis that cigarette smoke is closely related with an epigenetic alteration characteristic of cancers: both a global hypomethylation and a local promoter hypermethylation.

Altered methylation patterns in the genomic scale related to cigarette smoke were, however, not always unanimously presented by different groups. Exposing SENCAR mice to cigarette smoke at different doses at different duration, Philips *et al*. discovered that non-selected genomic regions of altered methylation (RAMs) in the mouse lung tissues increased in both a dose- and time- dependent manner (76). Those RAMs include hyper-, hypo- and newly occurred methylation as compared with sham treated animals. However, in another study, the global DNA methylation pattern of normal human fibroblast exposed to benzo[α]pyrene diol epoxide (B[α]PDE), a prototype of cigarette carcinogen, was not significantly different from the cells treated with DMSO (77). It should be mentioned that as compared to animal models or other *in vivo* data, the “simplified” laboratory cell culture in this study, in which one type of cell was limitedly exposed to a single carcinogen isolated

from cigarette smoke, may not necessarily reflect the potential alteration of methylation regulatory network present in the biological individuals affected by the prolonged exposure to all the chemical compounds contained in the cigarette smoke.

DNA methylation, aging and tumorigenesis

While aging refers to a physiological process and phenotypic changes over time that are common to all species (78), tumorigenesis is characterized by a progression of pathological changes that allow cells to undergo uncontrolled growth. Although two different events, aging and tumorigenesis are related: cancer is usually a late-onsite disease and people over age 70 have the highest rate of cancer (78). Accumulation of adverse effects of carcinogens and weakening of immune system, which are etiologically involved in tumorigenesis, parallel the aging process. In addition, emerging evidence also supports that similar epigenetic changes are shared by both aging and tumorigenesis process: a hypermethylated profiles of many specific gene promoters along with a seemingly contradictory hypomethylation on the genomic scale (79).

Hypermethylation of tumor suppressor genes leads to silencing of those genes and contributes to tumorigenesis (80). Interestingly, several silenced genes caused by DNA methylation in cancers are also epigenetically altered in the normal aging tissues. For examples, promoter hypermethylation of E-cadherin gene, which is present in several epithelium derived cancers including bladder cancer, was also found in normal bladder tissues from individuals older than 70 (81). Promoter hypermethylation of estrogen receptor (ER) is present in both histologically normal colorectal mucosa from aged individuals and colorectal tumors (82). The observed hypermethylation within promoter regions not only epigenetically suppresses gene expression but promotes point mutation, such as transition from cytosine to thymine (C→T) by deamination, a genetic change that also results in inactivation of tumor suppressor genes (83).Thus, epigenetic modifications of DNA is subsequently linked to genetic changes.

In aging tissues, the content of 5-methylcytosine of cells noticeably decreased (84). Global DNA hypomethylation is a signature epigenetic change among aging population. It has been reported

that the methylation level of Alu, the most abundant short interspersed elements (SINEs) and HERV-K, a known member of human endogenous retroviruses (HERVs), was inversely related to age. Such a negative association is close to significance when the methylation level of long interspersed elements (LINEs) is considered. SINEs, LINes and HERVs are the major components of bulky genomic region, and the methylation level of those elements represents the global DNA methylation state (85)(86). Decreased methylation level in those elements seen in cancers promotes mitotic recombination, resulting in loss of heterozygosity (LOH) (14) (87). In transgenic mice with a 10% reduction of *dnmt1* as compared with wild type counterparts, a genome-wide hypomethylation was induced and an aggressive T cell lymphoma characterized by chromosome 15 trisomy was developed at the age of 4 to 8 months (88). Other studies also support the hypothesis that genomic hypomethylation is causally related to the chromosome instability, a common molecular mechanism for tumorogenesis. Because mutations of genetic sequences increase with age, DNA methylation may also contribut to those genetic changes in aging process such as through deamination as mentioned above.

Due to the similarity of aberrant DNA methylation changes in cancer and aging, it is possible to speculate that aging is epigenetically predisposed to cancer by altering the DNA methylation profile in cells, and cancer development perpetuates those disregulated methylation state. However, substantial studies need to be performed in order to confirm such a mechanistic link.

Mechanisms of transcriptional regulation by DNA methylation

It has been observed for a long time that DNA methylation, especially methylation in the promoter region of genes, is negatively related to transcriptional activation, though exceptions were also reported. Two models by which DNA methylation controls gene transcription are proposed and generally accepted by researchers in the epigenetic field. While one model suggests the direct exclusion of trans-acting factors through steric hindrance from the promoter region by methyl groups present in the 5-cytosine position, another model favors a mechanism in which methylated cytosines

function as a platform to which methyl-binding proteins (MBPs), histone deacetylases (HDACs), and co-repressors are recruited (12).

The best example that elucidates the "direct inhibitory" mechanism by DNA methylation comes from the study of CTCF protein. CTCF (CCCTC-binding factor, which is a zinc finger protein) is best known for its role in imprinting at the *H19/IGF2* locus. CTCF-binding element within *H19* and *IGF2* loci serves as an insulator for *IGF2* expression. In the maternally inherited allele, the binding of CTCF protein to the unmethylated element disrupts the communication between the *IGF2* promoter and enhancer that exists downstream of *H19*, resulting in silencing of *IGF2*. However, in the paternal allele, methylation in the CTCF site excludes the binding of CTCF protein and, therefore, allows enhancing the expression of *IGF2* (89). Similarly, this mechanism is also discovered in the c-Myc binding site, in which the methylated cytosines prevent c-Myc from binding to its consensus element in the DNA sequence (90).

In addition to this direct inhibition of gene transcription by methyl-groups, ample evidence suggests that methyl-group present in the gene promoters can recruit MBPs to which other transcription inhibitory factors, such as (HDACs), are further recruited. Those MBPs that contain methyl-CpG-binding domain (MBDs) include Kaiso, MeCP2, and members of the MBD family such as MBD1, MBD2, MBD3 and MBD4 (91)(92)(93). Recruited HDACs remove acetyl-group from histone tails and, thus, modify otherwise open chromatin structure to a closed conformation due to an increased association of histone molecules with DNA. Such a closed chromatin structure does not favor transcription by preventing the binding of transcription factors (94) (95).

DNA methylation may also affect nucleosome architecture within the promoter region of genes by MBPs. Nucleosome occupancy has been found to inhibit the binding of transcription factors and RNA polymerase II to the region close to the transcription start site (96). The capability of DNA methylation to affect nucleosome occupancy is possibly related to the binding of MeCP2 to the methylated cytosine. MeCP2 then can further recruit chromatin remodeling complex SWI/SNF,

which results in changes of nucelosome occupancy (12)(97). However, the detailed mechanism by which DNA methylation regulates nucleosome architecture still remains to be elucidated.

Signals and regulatory pathways that regulate DNA methylation

Most CpG dinucleotides globally interspersed in the genome of healthy cells are methylated, while most CpG islands, especially those within gene promoter regions, remain unmethylated. Therefore, there must be mechanisms that contribute to this observed methylation pattern.

One pathway by which DNA methyltransferases are regulated is through phosphatidylinositol 3-kinase (PI3K) singling via glycogen synthase kinase-3 (*gsk-3*). Popkie *et al*. (98) found that the methylation level at several imprinted loci was decreased in *gsk*-3 double knockout mouse embryonic stem cells, and such a hypomethylation profile at imprinted loci is directly related to the reduced expression of *dnmt3a2*. By utilizing a constitutively active subunit of PI3K, the same group also demonstrated that the reduced methylation at those loci was caused by activation of PI3K pathway through *akt* (also called protein kinase B, PKB), whose activation results in phosphorylation and inactivation of *gsk-3*. In their model, insulin signaling leads to activation of PI3K-AKT pathway, which inhibits the activity of *gsk-3*. The inactivation of *gsk-3* will in turn decrease *dnmt3a2* expression, which is responsible for the observed hypomethylation at those imprinted loci. They further suggested that transcriptional factor *N-myc* is a key regulator of *dnmt3a2* transcription under the control by *gsk*-3. In agreement with this regulatory role of *N-myc*, DNMT3a can be specifically recruited by MYC to the promoter of p21clip1 and contributes to the repression of its expression (99). Another study conducted by Lin *et al*. (100) also supports the role of *AKT* and *GSK*-3 in the regulation of methyltransferases. In that study, decreased phosphorylation of *DNMT1* due to inhibited activity of *GSK*-3, which is resulted by activation of *AKT,* contributes to the accumulation of *DNMT1* in the nucleus by attenuating its degradation.

Not only can DNA methylation suppress gene transcription by changing the local chromatin structure within promoter region of genes, but altered chromatin structure initiated by histone modifications can also direct DNA methyltransferases to specific loci. It has been shown that

heterochromatin-binding protein 1s (HP1s), which are recruited to the methylated histone 3 lysine 9 (H3K9), can serve as a platform with which DNMT1 associates. The enrichment of DNMT1 is responsible for the increased methylation of the analyzed genes (101).Similarly, Gazzar *et al*. (17) (102) proposed a mechanism of TNF-α unresponsiveness during endotoxin tolerance, in which silent heterochromatin is assumed due to the enrichment of DNMT1 within *TNFA* promoter region, which is recruited by HP1. This promoter-specific recruitment of HP1 is caused by dimethylated H3K9 that was established by G9a, a H3K9 dimethyltransferase. In those examples, DNMTs are recruited to the specific genomic loci as a component of chromatin-remodeling complex, and the formation of the complex will further alter chromatin structure resulting in inhibition of gene transcription.

Although certain chromatin structures favor the recruitment of DNA methyltransferases and induce methylation, specific genetic sequences can also influence the state of DNA methylation. Because most human CpG islands present in the promoter regions remain unmethylated in contrast to globally distributed and heavily methylated CpG sites, there must be mechanism(s) that prevent(s) the accumulation of DNA methyltransferases in those CpG islands. Thomson *et al*. (103) reported in their study that the specific binding of CXXC finger protein 1(CFP1) to the non-methylated CpG islands is part of this "preventive" mechanism. Using high throughput sequencing approach and chromatin immunoprecipitation, they identified unmethylated CpG sites and trimethylated H3K4 as specific binding partners of CFP1. It is noticeable that trimethylated H3K4 is frequently present within hypomethylated promoter region and considered as an active signal for gene transcription (104). Therefore, a high local concentration of CpG sites can avoid methylation by interacting with certain chromatin marks that signal active transcription state.

Small interfering RNA (siRNA) is also mechanistically involved in DNA methylation and contributes to silencing of gene transcription. Data have demonstrated that siRNA targeted EF1A promoter, a molecule essential for transcription, induces methylation *in vivo* and leads to silencing of EF1A at transcriptional level (105). Since in plants *de novo* methylation can be induced by small RNAs (106)(107)(108), siRNA mediated methylation may represent a conserved mechanism across

species and play a role in repressing retroviruses and repeated transposable elements within mammalian cells.

Therapeutic potential of DNA methylation modifiers

As compared to treatment of genetic defects, therapies targeting aberrant epigenetic alterations exhibit promising signs because epigenetic modifications can be reversible (109). Synthetic as well as natural compounds extracted from plants have already been developed to intervene and correct epigenetic defects. 5-aza-2′deoxycytidine (also known as decitabine), an analogue of 5-azacytidine that is an irreversible DNA methylation inhibitor, has already been used in clinical trials and shown promises to treat myeloid malignancies (110) (111). Combining epigenetic modifiers targeting DNA methylation and chromatin structure, such as decitabine and trichostatin (TSA), a potent HDAC inhibitor, have achieved effects in anti-cancer treatment (112).

Several natural compounds in botanical extract have been studied as potential epigenetic therapies targeting key components of epigenetic pathways, such as DNMTs. PMI-5011, a subfraction extracted from *A dracunculus* L, which was first screened as a promising dietary supplement for diabetes, showed an inhibitory effect on downregulating *DNMT1* and *DNMT3b* in cell culture (109). The similar inhibitory effect on *DNMT1* and *DNMT3b* can also been found in the extract of *A tuberosum* L (109). A wide spectrum of bioactive phytochemicals contained in the regular diet, such as epigallocatechin found in green tea, genistein in soy, resveratrol in grapes and red wine, curcumin in turmeric (*Curcuma longa L*), *etc*., are discovered to possess the activity to modify methylation profiles of genes by interacting with DNMTs. Most of those substances have an inhibitory rather than activating effect on DNMTs. The disease models in which the roles of those phytochemicals were explored include cancer and various types of inflammatory diseases (113).

Well characterized drugs in the treatment of inflammatory diseases have recently been discovered for their epigenetic modulating activity. Non-steroidal anti-inflammatory drug (NSAID) exhibits protective effects against gastric cancer. Further study has indicated a link between chronic use of NSAID and decreased promoter methylation level of several tumor suppressor genes. By

performing methylation-specific PCR on gastric mucosa samples from non-cancerous subjects, Tahara *et al*. (114) reported that the promoter methylation level of *P16*, *P14* and *E-cadherin* was significantly lower in the samples collected from NSAID users as compared to non-NSAID users. They suggested that the protective role of NSAID is related to its "hypermethylation inhibiting" activity on those tumor suppressor genes because inactivation of those genes due to hypermethylation is a critical mechanism involved in the pathogenesis of gastric cancers. Methotrexate (MTX), widely used as an anti-rheumatic drug, inhibits DNA methylation by intervening with folic acid metabolism that is involved in DNA methyltransferase pathway (115). Although we haven't known to what extent the efficacy of those drugs is due to their epigenetic modulating activity, the novel strategy of tackling dysfunctional yet reversible epigenetic marks by synthetic or natural compounds initiates new research avenues in epigenetic pharmacology. Another concern of applying epigenetic modulators is the specificity to which genes or gene promoters are targeted. However, the discovery of several zinc finger proteins in the nucleoplasm of cells, which are transcriptional factors and have a strong affinity for methyl-group or certain chromatin structures, may provide new clues for the specificity of epigenetic therapeutic approach (116).

Periodontal diseases and epigenetic mechanisms

Periodontal diseases include gingivitis, an early and reversible stage of gingival inflammation, and periodontitis, an advanced stage leading to the loss of tooth supporting tissue. Periodontal diseases are etiologically initiated by periodontal bacteria accumulated in the "bacterial biofilm", an organized structure in the crevice of tooth. Although the virulent factors possessed by those periodontal pathogens can directly cause destruction to periodontal tissues, the inflammatory and immune responses stimulated by pathogenic bacteria also results in the loss of connective tissue and alveolar bone (117, 118).

Several Gram negative and anaerobic bacteria have been identified as the pathogens of periodontal diseases. For example, *Porphyromonas gingivalis* (*P. gingivalis*), frequently detected in the subgingival microflora of patients with periodontal disease, is one of the most important

periodontal pathogens. The cell structure and virulence factors of *P. gingivalis* can directly cause damage to the periodontal tissue. The fimbriae is necessary for *P. gingivalis* to attach to and invade epithelial cells; a number of proteases synthesized by *P. gingivalis* can influence tissue integrity; short fatty acid from *P. gingivalis* can cause apoptosis in various cell types including T-cells, B-cells, keratinocytes, and fibroblasts (119). In addition, *P. gingivalis* infection can induce secretion of inflammatory cytokines in hosts, such as TNF-α, IL-1B, IL-6, IL-12, *etc* (119), which contribute to the periodontal destruction. *Campylobacter rectus* (*C. rectus*), a Gram negative, anaerobic and motile bacterium, is another periodontal pathogen that possesses several virulence factors, such as the surface-layer (S-layer), heat shock proteins (GroEL-like proteins), and flagella (120)(29) (121)(122). These virulence factors help *C. rectus* evade the phagocytic uptake and bactericidal activity of serum complement and mediate adhesion with host cells (123)(124). *C. rectus* can also upregulate both mRNA and protein levels of several cytokines in host cells, such as IL-6 and TNF-α (125).

The presence of infectious agents in the biofilm and the inflammatory responses induced by those periodontal pathogens may possibly alter gene expression in an epigenetic approach in the gingival tissue exhibiting periodontal diseases. Recently, a group reported that higher frequency of hypomethylation of *IL8* promoter region was identified in oral epithelial cells from chronic periodontitis patients (126). Yin *et al*. found that in gingival epithelial cells the expression of DNMT1 and histone deacetylase decreased upon challenge by *P. gingivalis* and *Fusobacterium nucleatum*, which is a non-oral pathogen (127). Therefore, we propose that DNA methylation contributes to the regulation of inflammatory genes involved in periodontal diseases. Therefore, in this present study, we investigated the potential methylation alterations in the promoter regions of several genes (*PTGS2*, *TNFA*, and *IFNG*) involved in inflammatory immune responses in biological samples collected from the sites in periodontal patients where periodontal diseases are evident. We also proceeded to study the epigenetic regulation of *TNFA* in a human monocytic cell line (THP-1) by a periodontal pathogen, *C. rectus*. At last, we also studied the mechanism of how DNA methylation in the *TNFA* promoter region regulates the transcription of this gene.

Table 1.1 Inflammatory diseases and aberrant DNA methylation

Diseases involved	Alteration ofmethylation	Global or specific genes	reference
inflammatory bowel disease (CD and UC)	both hyper- and hypo-methylated	*TNFRSF1A, STAT5A, SERPINA5, BGN, NOTCH4, TJP2, FMR1, etc*	(40)
inflammatory bowel disease	hypomethylated and hypermethylated*	*IFNG*	(128) (129)
ulcerative colitis	increased frequency of methylation, Methylation level is correlated to the severity of UC	*MDR1* *PAR2*	(130) (131)
inflammatory bowel disease	no significant change	*IRF5*	(132)
asthma	hypermethylated	*FOXP3*	(133)
aggressive periodontitis	hypomethylated	*IFNG*	(134)
systemic lupus erythematosus (SLE)	decreased methylated	*global*	(135) (136)
chronic pancreatitis	increased frequency of methylation	*BRCA1, CDKN1C, CCND2, PGR,HMLH1, SYK,VHL*	(137)
cardiovascular disease (131)(131)(121)	hypermethylated	*global*	(42)
psoriasis	decreased frequency of methylation, hypomethylated	*P15, P21* *P16*	(138)(139)
Inflammatory (rheumatoid or psoriatic) arthritis	hypomethylated	*global*	(43)
rheumatoid arthritis	hypomethylated	*IL6*	(140)
inflammatory bowel disease	hypermethylated	*APC2, SERP1,SFRP4, SFRP5, DKK1,WIF1*	(141)
rheumatoid arthritis	hypomethylated	*EPHRINB1*	(142)
atherosclerosis	hypomethylated, hypermethylated	genes involved in transcription and signalling specific genes far less than hypomethylated genes	(41)
Barrett's metaplasia	hypomethylated	*CDX1*	(143)
prostrate proliferative inflammatory atrophy	hypermethylated	*GSTP1*	(45)
chronic kidney disease	Hypermethylation is related to the mortality of CKD	*global*	(47)

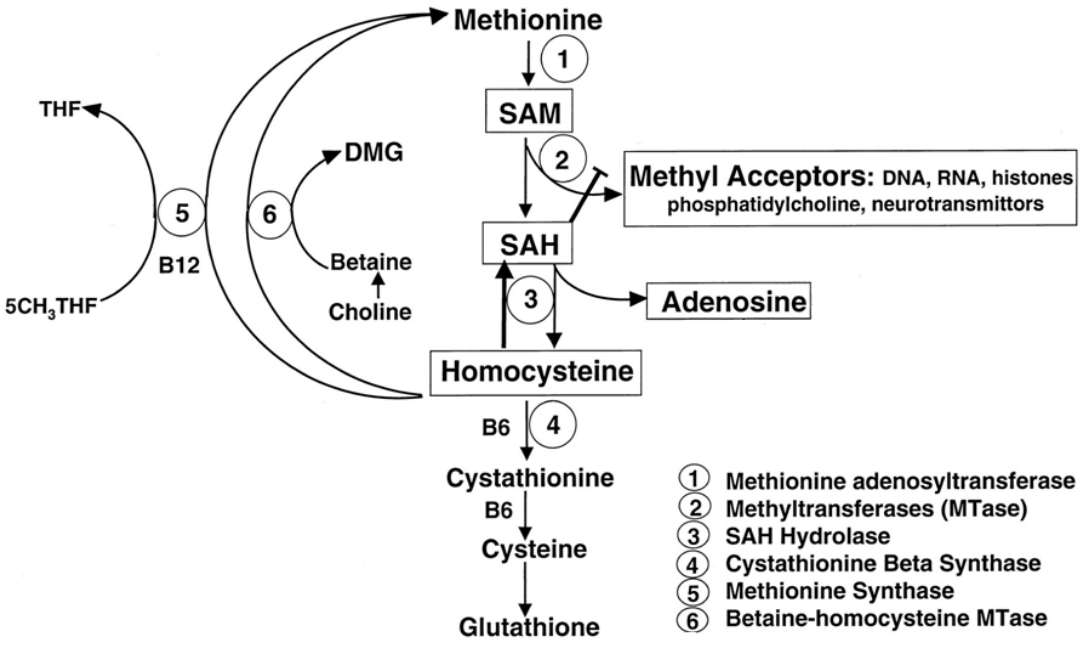

Figure 1.1: one-carbon metabolism involved in methylation reactions. S-adenosylmethionine (SAM) is generated through ATP-dependent transfer of adenosine to methionine under the enzymatic activity of methionine adenosyltransferase. SAM is the methyl-donor for most methyltransferase reactions including DNA methylation. After donating a methyl group, SAM is converted to S-adenosylhomocysteine (SAH). The removal of SAH is efficiently achieved by SAH hydrolase. SAH is then hydrolyzed to adenosine and homocysteine via SAH hydrolase. It should be emphasized that this hydrolysis reaction is actually thermodynamically favorable for the reverse reaction to synthesize SAH. Any metabolic perturbation that reduces homocysteine removal will cause SAH accumulation, which is a potent inhibitor for methyltransferases. Homocyteine can be remethylated to generate mthionine by folate /B12 dependent methionine synthase reaction. Homocysteine can also be remethylated to methionine through betaine-homocysteine methyltransferase by using betaine, the derivative of choline, as the methyl donor. However, homocysteine can be irreversibly removed from methionine cycle through vitamine B6-dependent transsulfuration pathway by cystathionine beta synthase. THF: tetrahydrofolate; DMG: dimethylglycine. The figure is adopted from the figure 1 by Yi P *et al* (53).

Chapter 2

Alteration of *PTGS2* Promoter Methylation in Chronic Periodontitis

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Abstract

Levels of prostaglandin E_2 and the prostaglandin-endoperoxide synthase-2 (PTGS2, or COX-2) increase in actively progressing periodontal lesions, but decrease in chronic disease. We hypothesized that chronic inflammation is associated with altered DNA methylation levels within the *PTGS2* promoter, with effects on COX-2 mRNA expression. *PTGS2* promoter methylation levels from periodontally inflamed gingival biopsies showed a 5.06-fold increase as compared with non-inflamed samples (p=0.03), and the odds of methylation in a CpG site in the inflamed gingival group is 4.46 times higher than in the same site in the non-inflamed group (p=0.016). The level of methylation at -458bp was inversely associated with transcriptional levels of *PTGS2* (RT-PCR) (p=0.01). Analysis of the data suggests that, in chronically inflamed tissues, there is a hypermethylation pattern of the *PTGS2* promoter in association with a lower level of *PTGS2* transcription, consistent with a dampening of COX-2 expression in chronic periodontitis. These findings suggest that the chronic persistence of the biofilm and inflammation may be associated with epigenetic changes in local tissues at the biofilm-gingival interface.

Introduction

The production of prostaglandin E_2 (PGE_2) has been associated with periodontal inflammation (144, 145). In periodontitis, increased levels of gingival crevicular fluid (GCF) PGE_2 have been predictive of longitudinal periodontal attachment loss (146), and associated with the clinical signs of bleeding on probing (147), both of which would suggest that increased PGE_2 expression is associated with progressive lesions (148). The biosynthesis of PGE_2 and other prostanoids is tightly coupled to the inducible expression of COX-2, and the transcriptional control of COX-2 levels appears to be the key regulatory gate for modulating tissue PGE_2 levels (149). Although levels of PGE_2 increase during certain stages of disease progression, little is known regarding the regulation of local PGE_2 synthesis, in which some down-regulation must be needed to prevent a continued and ever-expansive loss of connective tissue. This homeostasis in chronic inflammation in the omnipresence of a microbial burden is probably due to the establishment of what has been referred as a "metastable" equilibrium (150). This metastable equilibrium arises as the presence of a chronic inflammatory stimulus creates a new "set-point", in which higher levels of inflammatory mediators are tolerated or down-regulated by some compensatory molecular mechanism(s) that prevent the unrestricted tissue destruction and serve to dampen the uncontrolled inflammatory response.

Analysis of data in the literature provides some evidence of this down-regulatory mechanism. In a community study (147), it was reported that the level of GCF PGE_2 is negatively associated with attachment loss (as a marker of total history of disease activity), indicating that the greater the cumulative historical tissue damage, the lower the GCF-PGE_2 level. Presumably, this is a result of a site-specific history of an episode of progression with increased attachment loss and elevations of local GCF PGE_2 levels that eventually become dormant with lowered levels of PGE_2.

Recently, we have found that certain periodontal bacteria can induce epigenetic alterations in host tissues, such as gene-specific methylation of CpG sequences (151). In eukaryotes, DNA methylation occurs almost exclusively at the 5' end of cytosine within the CpG dinucleotide context (152). It has been generally accepted that an increase of methylation in the gene promoter region is related to the decrease of gene expression, though exceptions have been identified (153).

In this study, we sought to investigate the potential alteration in the DNA methylation pattern of the *PTGS2* (*COX2*) gene promoter and its effect on the transcriptional control of COX-2. We also sought to identify potential feedback mechanisms that might lead to suppression of PGE_2 synthesis following periods of disease activity.

Materials & Methods

Participants and tissue specimens

A total of 16 participants, aged between 18-65 years, provided written informed consent and were enrolled into this study, which was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Exclusion criteria included: 1) the use of either antibiotics or non-steroidal anti-inflammatory drugs within one month prior to scheduled surgery; and 2) medical treatment for other diseases 3 months prior to recruitment. Measurements included probing depths, clinical attachment level, and bleeding on probing at six sites *per* tooth. One interproximal gingival site was biopsied from each participant. Ten gingival biopsies were removed during routine periodontal flap surgeries from participants clinically diagnosed with chronic adult periodontitis. Those biopsied tissues were from sites with probing depths of 5mm or more, bleeding on probing and radiographic evidence of localized bone loss. These tissues are referred to as "inflamed" in the data presentation. Non-inflamed tissues were collected from participants who were periodontally healthy or had localized mild gingivitis at non-study sites. Six non-inflamed gingival biopsies were removed from participants who were undergoing crown extension surgery at sites with probing depth measurements of 4mm or less at all four interproximal probing sites and no bleeding on probing. Upon removal, gingival tissues were incubated with RNA*later*® (Applied Biosystems/Ambion, Austin, TX) overnight at 4°C, and then transferred to -80°C freezer for storage.

DNA Preparation and Sodium Bisulfite Modification

Genomic DNA was isolated from gingival biopsies by the use of a DNeasy Tissue Kit (Qiagen Inc, Valencia, CA). A 2ug quantity of genomic DNA from each sample was treated with sodium bisulfite according to the method recommended (154).

Bisulfite Specific PCR, Cloning, and Sequencing

The promoter sequence of *PTGS2* (155) was analyzed by using MethPrimer software (156). Two CpG islands encompassing -541bp and -216bp were identified. Sodium bisulfite treated genomic DNA was amplified using primers that are specific to the CpG islands within the *PTGS2* promoter. Primer sequences and PCR condition are provided in Table 2.1 and Table 2.2, respectively. A 334bp PCR product including those two CpG islands was purified through electrophoresis, and the gel-purified PCR product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). Colonies showing positive PCR fragment insertion were selected, and the insert was amplified with standard Sp6 and T7 primers (Promega) listed in Table 2.1 and 2.2. 4 to 7 clones for each individual clinic gingival sample were sequenced by Sp6 primer by UNC-CH genomic analysis facility.

RNA Isolation and Real-time PCR

Total RNA was isolated from RNA*later*® treated gingival tissues with the use of a RNeasy Mini Kit (Qiagen). cDNA was then synthesized from 1 µg of total RNA using the Omniscript Kit (Qiagen) by random decamer primers (Applied Biosystems/Ambion). Real-time PCR was performed with 1 µL synthesized cDNA, TaqMan Universal PCR mix, and 20X on-demand primers (Applied Biosystems, Foster City, CA) specific for *PTGS2* gene, in a 7000 Sequence Detection System apparatus (Applied Biosystems). Amplification of 18s rRNA from each gingival sample was included as internal control. The relative quantity of PTGS2 mRNA was calculated against 18S rRNA values (Livak and Schmittgen, 2001). Two samples, one from inflamed and one from non-inflamed gingival groups, did not provide enough RNA for analyses, and therefore, were excluded from the quantitative RT-PCR study.

Statistical Analysis

Two-sample independent *t* tests were applied for the statistical analysis of clinical data and mRNA expression levels. We used the Mann-Whitney/Wilcoxon two sample test (SAS v9.2) to

compare overall percent methylation of each gingival sample between the two gingival groups. We used a generalized linear mixed model (GLMM) to estimate the odds ratio describing the relationship between methylation and inflamed versus non-inflamed groups, conditional on CpG site and gingival tissue sample. Specifically, a three-level logistic regression model (157), with fixed effect for group and random effects for samples and sites within samples was used. A 95% confidence interval for the odds ratio was constructed such that the observed difference between groups was assessed with respect to the variation between gingival tissue samples. We applied linear regression analysis to test for the significance of slope to evaluate the association between the percentage of methylation at a specific CpG dinucleotide (-458bp) within the *PTGS2* promoter region and the *PTGS2* transcriptional level. We applied Chi-square approximation to test gender difference between participants in two groups. Alpha levels less than 0.05 were considered statistically significant.

Results

Participants

There are no significant differences in age and gender comparing the participants in the two groups (Table 2.3). As expected, there were differences in mean probing depth, clinical attachment loss and the presence of bone loss at inflamed sites as compared to non-inflamed controls.

Methylation Status of CpG-rich *PTGS2* Promoter Region

The overall methylation of the studied *PTGS2* promoter region (-541bp~-216bp) in chronically inflamed gingival tissues was 5.06 fold higher than the methylation level exhibited in non-inflamed gingival tissues when the methylation level of individual gingival tissue in both groups were compared [4.3% (1.8%, 8.5%) vs. 0.85% (0.7%, 1.3%), respectively, shown as median and interquartile range (IQR), p=0.03] (Figure 2.1). There are 23 CpG dinucleotides (Figure 2.2A) present in two CpG islands (shaded area, Figure 2.2B) within the upstream sequences of *PTGS2* (-831bp to +69bp, Fig. 2B). Several transcription factor binding sequences (cis-elements), such as NF-κB, AP-2 and Sp-1 (Figure 2.2A), were also identified within these CpG rich regions. Therefore, we analyzed the methylation state of all 23 CpG sites within those regions to compare methylation levels between the two gingival sample groups. The individual clonal bisulfite sequencing map for the 16 participants appears in figure 2.4. It can be seen that the increased methylation shown in inflamed tissues extends over almost all the 23 potential methylation sites analyzed (Figure 2.2C). However, this diffuse methylation pattern was absent from the non-inflamed samples, in which methylation could be detected at only 3 CpG sites. In addition, the odds of methylation at a CpG site from a sample in the inflamed gingival group was 4.46 times higher (95% CI: 1.38,14.35) than the odds of methylation at the same CpG site from a sample in the non-inflamed gingival group (p=0.016), as estimated by the GLMM.

Interestingly, the most heavily methylated site in both gingival tissue groups occurred at what we are designating as “site 8” (-458bp, Figure 2.2C), a CpG dinucleotide that is physically close to a NF-κB binding site (Figure 2.2A). The methylation level of that particular CpG site was 23.5% in inflamed and 20.6% in non-inflamed gingival tissues, and higher than the methylation levels of other CpG dinucleotides in both groups (Figure 2.2C).

CpG Methylation Status as Related to PTGS2 mRNA Expression Level

The mRNA level of *PTGS2* in inflamed gingival samples, as determined by real-time PCR, was lower than the level of non-inflamed group, although the difference was not significant (p=0.36, Figure 3.3A). We then plotted the percentage methylation level of “site 8” against mRNA expression level of each individual sample. Samples from both gingival tissues groups are shown on this plot to demonstrate the association between methylation status and mRNA expression. The overall regression analysis pooling all clinical samples shows a statistically significant negative association between percentage of methylation of CpG site at -458bp and *PTGS2* mRNA expression (p=0.01, Figure 3.3B).

Discussion

Although aging and environmental exposures can affect global genome methylation, new evidence indicates that some promoter methylation sites can be targeted by specific toxins, nutrient deficiencies and infectious stimuli to modify methylation levels (158)(159). In this investigation we report that chronically inflamed periodontal tissues exhibit an increased (5.06 fold) generalized methylation of the CpG rich region of the *PTGS2* promoter, as compared to non-inflamed periodontal tissues. In a previous study using a mouse model of *Campylobacter rectus* infection, we identified a hypermethylated *Igf2* P0 promoter region suggesting, for the first time, that infection with an oral pathogen can lead to epigenetic modifications (151). The association between infection and alteration of DNA methylation is also supported by another study in an *in vitro* infection model (160), which reported that the promoter region of *hMLH1* from a gastric cell line was hypermethylated by persistent *H. pylori* infection. Therefore, our findings are consistent with the concept that infections at mucosal surfaces can modify the epigenetic status of the tissues in a gene-specific manner. The role of inflammation in modulating methylation status either by altering the density of cells at local sites of inflammatory infiltration, or by modifying the methylation status of existing cells, cannot be determined from these experiments. Furthermore, the intra-differences of methylation status within the same gingival tissue sample (e.g. Figure 4, participant 8 had 10 methylated sites in one clone but none in 4 other clones) may reflect the epigenetic impact imposed by infection/inflammation on different cell types present in the biopsy samples.

The increase in methylation in chronic disease was associated with a metastable steady state level of *PTGS2* mRNA expression that was lower than that seen in non-inflamed participants with shallow sites. This finding is consistent with the report (147) that shows lower PGE_2 levels in deeper sites. It has been suggested (161) that most of the periodontal disease progression that occurs within participants likely involves shallow sites rather than deep sites. In this context, the chronic state of

deep pockets reflects a historical episode of disease activity and a re-instatement of a new steady state equilibrium, resulting in a metastable shift in COX-2 expression.

The site-specific methylation of CpG dinucleotide at site -458bp ("site 8"), which is located 12bp upstream of a NF-κB binding site, was higher in both gingival groups. Since NF-κB activation enhances *PTGS2* expression (162), the observed increase in methylation at this specific site may impair NF-κB activation. It has been reported (153) that transcription can be critically affected by the methylation status of specific or "key" CpG sites within a regulatory region. In the present study, the methylated cytosine of this CpG dinucleotide just upstream the NF-κB binding site may possibly either directly exclude the binding by this transcriptional activator or condense local chromatin structure by recruiting methyl CpG binding proteins (152)(158).

One limitation of this report is the relatively small number of participants studied. Relationships between methylation status and clinical status are tenuous and should be confirmed in larger studies. However, this study provides the first evidence and proof-of-principle that epigenetic modifications of local tissues may occur in periodontal disease. Additional studies will be needed to understand whether epigenetic changes also occur in gingivitis or in other mucosal pathologies. Although we demonstrate that promoter methylation is linked to decreased mRNA expression, we do not have direct evidence that this is associated with altered levels of COX-2 protein expression or levels of PGE_2 within the tissue. However, once the PTGS2 mRNA is translated into protein, the enzyme is unstable, having a short biological half-life (163). COX-2 does not exist in a zymogen form nor as a pre-existing mRNA pool. Thus, the transcription of PTGS2 mRNA directly results in the synthesis of inducible COX-2 and appears to be the key regulatory gate for modulating tissue COX-2 activity.

Our working model of how epigenetic modification may impact periodontal status is based upon: 1) changes in DNA methylation patterns alter gene expression profiles; 2) epigenetic changes

are not readily reversible and are retained following cell division creating a sustained change in gene expression and tissue phenotype that would persist, even following the reduction in the inflammatory infiltrate; 3) epigenetic alterations may induce tissue tolerance to the chronic stress imposed by the biofilm 4) epigenetic alterations may influence wound healing and the dynamics of biofilm emergence. This suggests that epigenetic modifications may result in long-lived alterations in the metastable state of the local periodontal tissues.

Table 2.1. Primer Sequences

Technique	Primers	Primer Sequences
Bisulfite specific PCR for *PTGS2* promoter region	Forward	5′-AAATATGTTAGTTTTTTTTAATTTTATT-3′
	Reverse	5′- ATAATCCCCACTCTCCTATCTAATC-3′
Colony-PCR (Clone amplification)	T7	5′-TAATACGACTCACTATA-3′
	Sp6	5′-TATTTTAGGTGACACTATAG-3′

Table 2.2. PCR Conditions

	35 Cycles				
Technique	Denaturation °C, Time	Denaturation °C, Time	Annealing °C , Time	Elongation °C , Time	Elongation °C , Time
Bisulfite specifc PCR *PTGS2* Promoter region	94, 2min	94, 1min	52, 1min	72, 2min	72, 10min
Colony-PCR (Clone amplification)	94, 4min	94, 30sec	55, 30sec	72, 30sec	72, 10min

Table 2.3. Clinical Parameters of Study Participants

Clinical Measurements	Inflamed (n=10)	Non-inflamed (n=6)
Mean age (years)	45.8± 7.4	44.2±15.6
Gender (Male/Female)	5/5	1/5
Mean Probing depth (mm)[a]	6.2±0.6	2.5±0.8
Mean Clinical Attachment loss (mm)[b]	3.8±1.1	1.6±1.1
Alveolar bone loss	Yes	No

a. statistically significant difference by t-test (P=0.00001). Value reflects mean interproximal Probing Depth aggregated over four interproximal sites at the biopsy region.

b. indicates statistically significant difference by t-test (p=0.01). Value reflects mean interproximal clinical attachment loss aggregated over four interproximal sites at the

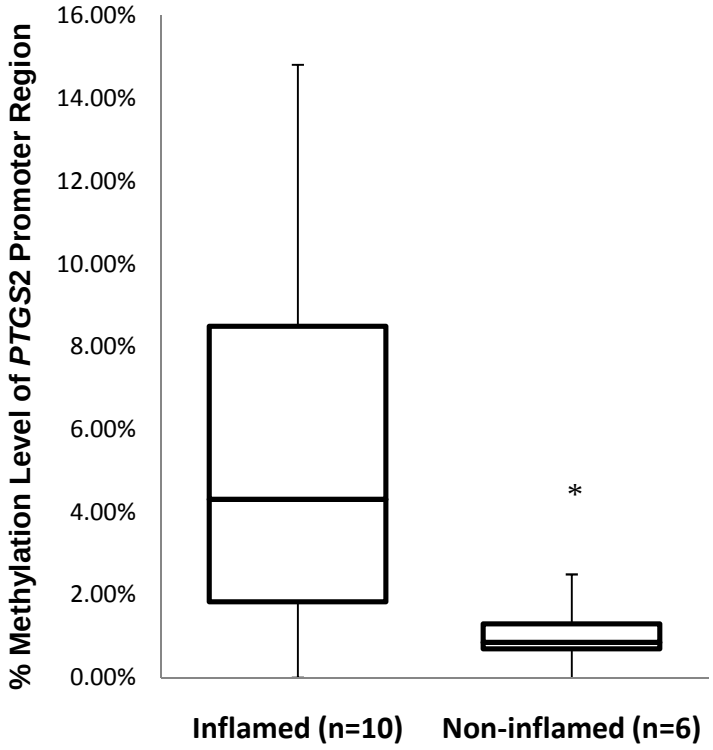


Figure2.1. *PTGS2* promoter region (-541bp~-216bp) DNA methylation levels for both groups of gingival samples. The methylation level in chronic inflamed periodontitis samples (4.3% (1.8%, 8.5%)) was significantly higher than non-inflamed samples (0.85% (0.7%, 1.3%), *p=0.03) as determined by Mann-Whitney/Wilcoxon two-sample test. Box plot shows the median (center line in box), 25% quartile (bottom line in box), 75% quartile (top line in box), maximum (plus error bar) and minimum (minus error bar).

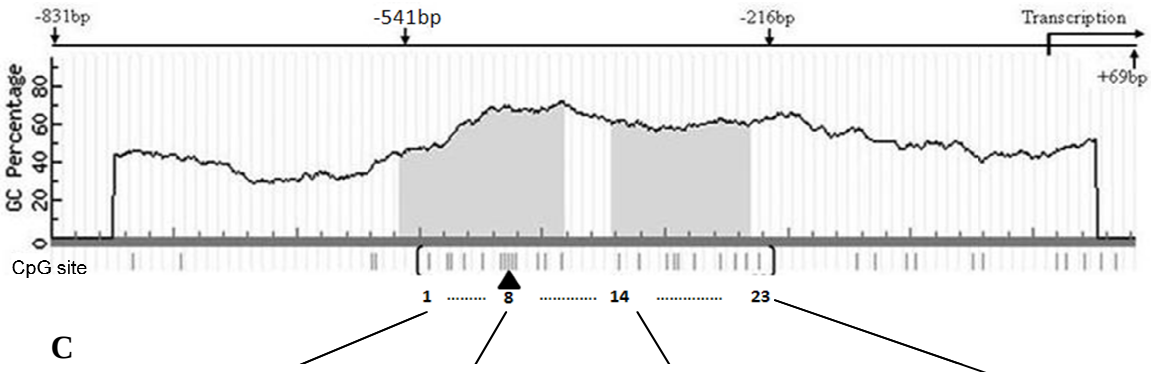
ctctaaa

g*ccccc**g***

ccc**cg***gta

agactg**cg***

tgggagga



C

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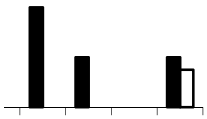

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31bp to +69bp is
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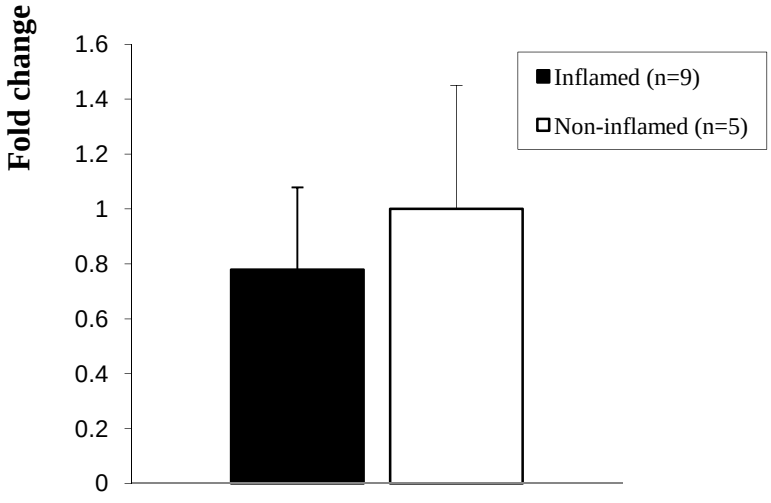
A



B

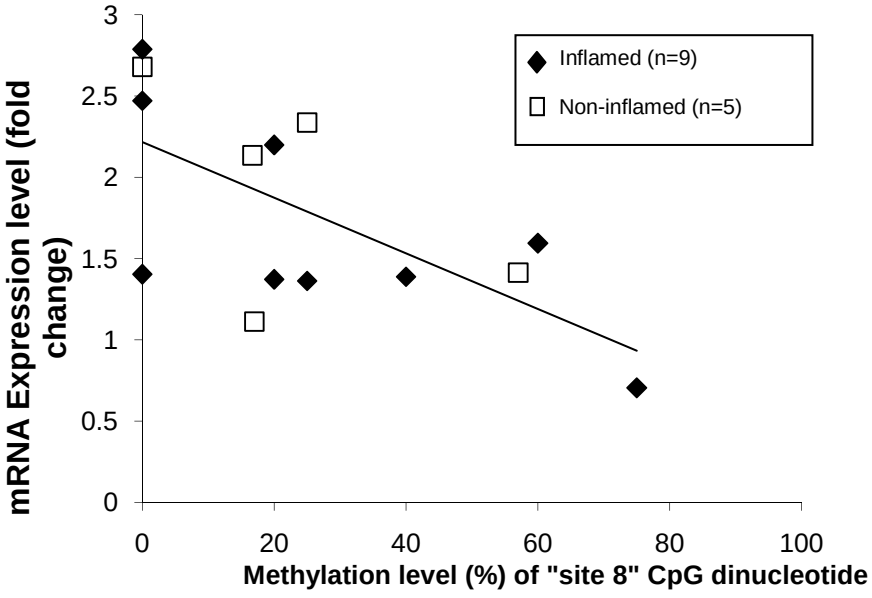


Figure 2.3. Transcriptional level of *PTGS2*, determined by quantitative RT-PCR, from the inflamed and non-inflamed gingival samples. (A) mRNA expression of PTGS2 in the inflamed gingival group showed lower yet non-statistically different (P=0.36) level as compared with non-inflamed gingival tissues . (B) PTGS2 mRNA expression level of individual sample from both groups is inversely related to its methylation level of "site 8" CpG dinucleotides. Regression analysis indicates statistical significance (p=0.01).

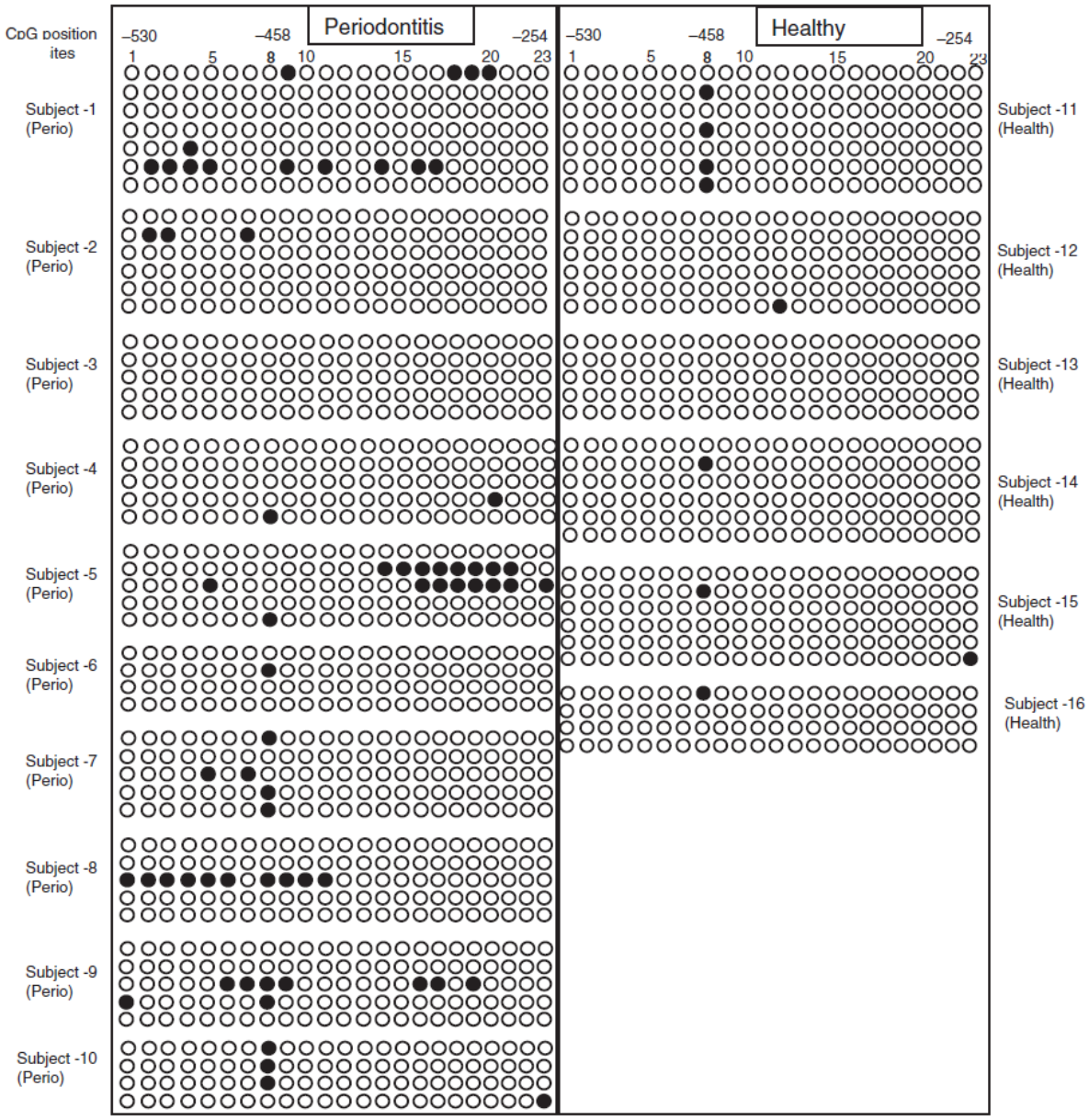


Figure2.4. Methylation sequencing analysis of *PTGS2* promoter region. In total, 23 CpG potential methylation sites from the PTGS2 promoter region located from -523through -254 bp were examined. “Site 8”, the CpG site (-458bp), is indicated in bold. Each circle represents a CpG site, with solid circles showing methylation. Each block of circles represents a single subject biopsy, and each row of circles represents a somg;e c;pme/

Chapter 3

Interferon-Gamma Promoter Hypomethylation and Increased Expression in Chronic Periodontitis

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Abstract

The goal of this investigation was to determine whether epigenetic modifications in the *IFNG* promoter are associated with elevation of *IFNG* transcription in different stages of periodontal diseases. DNA was extracted from gingival biopsy samples collected from total 47 sites from different subjects: 23 periodontally healthy sites, 12 experimentally induced gingivitis sites and 12 chronic periodontitis sites. Levels of DNA methylation within the *IFNG* promoter containing 6 CpG dinucleotides were determined using Pyrosequencing technology. IFN-γ mRNA expression was analyzed by quantitative polymerase chain reactions using isolated RNA from part of the biological samples mentioned above. The methylation level of all 6 analyzed CpG sites within the *IFNG* promoter region in the periodontitis biopsies {52% [interquartile range, IQR (43.8%, 63%)]} was significantly lower than periodontally healthy samples { 62%[IQR(51.3%,74%)], p=0.007} and gingivitis biopsies {63%[IQR (55%, 74%)], p=0.02}.The transcriptional level of *IFNG* in periodontitis biopsies was 1.96 fold and significantly higher than tissues with periodontal health (p=0.04). Although the mRNA level from experimental gingivitis samples exhibited an 8.5 fold increase as compared to periodontally healthy samples, no significant methylation difference can be identified. A hypomethylation profile within *IFNG* promoter region is related to an increase of *IFNG* transcription present in the chronic periodontitis biopsies, while such an elevation of *IFNG* exhibited in experimentally induced gingivitis seems independent of promoter methylation alteration.

Introduction

The fundamental mechanisms that lead to the development of periodontal diseases are closely related to the dynamics of the host immune and inflammatory responses to periodontal pathogens present in the dental biofilm (164). Although much is known regarding the innate immune response in periodontal disease, the specific role of T cells in modulating local Th1 and Th2 responses is not fully characterized. Cell-mediated immunity modulated by a Th1 response, which involves interferon gamma (IFN-γ) production and IL-2, and the humoral immune response, which is favored by a Th2 response and driven by the secretion of IL-4, IL-6 and IL-10, are well established and these responses have been described in periodontal diseases (165) (166) (167)(164)(168).

The expression of IFN-γ is noteworthy, not only because of its elevated transcriptional and translational expression in inflamed gingival tissues and gingival crevicular fluid (GCF), but its association with advanced periodontal disease and disease progression (164)(169)(170). In a recent molecular epidemiologic study with 6,768 community-based subjects, Offenbacher *et al*. (171) reported a significant increase in the GCF concentration of IFN-γ in those subjects with deep periodontal pockets and severe gingival bleeding as compared to subjects with probing depth of ≤3mm. It was previously demonstrated that a high level of Th1 cytokines was found in the gingival crevicular fluid (GCF) of patients with extremely severe periodontits (terminal dentition stage), including a 10-fold increase in the concentration of IFN-γ when compared to the Th2 mediators IL-4 and IL6 (169). The presence of high IFN-γ level is shown to enhance phagocytic activity of monocytes and neutrophils, which helps containment of infection (164) as well as upregulates monocytic response to LPS, which results in elevated monocytic secretion of proinflammatory molecules, such as $PGE_{2,}$ IL-1β, and TNF-α, all of which play important roles in bone loss and the disintegration of soft tissue in the periodontium (169)(172). Literatures demonstrate that IFN-γ can be secreted by type-1 CD4+, CD8+ T lymphocytes, NK cells, mononuclear cells and dendritic cells found in periodontal tissues (170) (173). However, the molecular signaling pathways that result in a

chronically elevated level of IFN- γ expression in periodontal diseases are still the subject of investigation.

The control of inflammatory responsiveness by the host to the recurrent and omnipresent challenges imposed by the oral biofilm is complex, involving genetically determined traits, regulation by cytokine networks and changes in epigenetic patterns. For example, genetic studies suggest that specific single nucleotide polymorphism (SNP) haplotypes of *IL1B* in the population are associated with the level of IL-1□ within the GCF (174). IL-12 has been shown to potently enhance *IFNG* expression (175). Recently, alterations in epigenetic patterns have been discovered as another important mechanism for the regulation of gene expression at mucosal surfaces (176). In eukaryotes, DNA methylation occurs almost exclusively at the 5' end of cytosine nucleotide within the CpG dinucleotide context (177). The change of methylation status in CpG islands, which are regions of genome that contain high percentage of CpG dinucleotides, are profoundly associated with diseases such as developmental abnormalities, cancer, and chronic inflammatory states (178)(179). It has been generally accepted that increased methylation (hypermethylation) in the gene promoter region is associated with a decrease of gene expression, while a hypomethylation pattern is closely associated with transcriptional upregulation (180)(181). Recently, we have described an increased methylation of CpG islands within the *PTGS2* promoter region in human gingival biopsies associated with a suppression of PGE_2 mRNA expression (182).

The aim of this study is to understand whether IFN- γ expression in the gingival tissues from subjects with different stages of periodontal diseases, including experimentally induced gingivitis and chronic periodontitis, is associated with an altered methylation status of the promoter region of *IFNG*, as evaluated in the context of *IFNG* SNPs known to regulate expression levels.

Materials & Methods

Participants, experimental gingivitis, and tissue specimens

A total of forty seven participants, aged between 19-63 years, provided informed consent and were enrolled into this study that was approved by the Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill. Exclusion criteria for recruiting participants exhibiting chronic periodontitis and periodontal health included: 1) the use of either antibiotics or non-steroidal anti-inflammatory drugs within one month prior to scheduled surgery; and 2) medical treatment for other diseases 3 months prior to recruitment. Measurements included probing depths (PD), clinical attachment levels (CAL), and bleeding on probing (BOP) at six sites per tooth. One interproximal gingival site was biopsied from each participant. Twelve gingival biopsies were removed during routine periodontal flap surgeries from participants clinically diagnosed with chronic periodontitis. Scaling and root planning as initial therapy were performed on those patients 4-6 weeks prior to periodontal surgeries. Those biopsied tissues were from sites exhibiting probing depths of 5mm or more, bleeding on probing and radiographic evidence of localized bone loss. Twenty three gingival tissues were collected from different participants who were periodontally healthy or had localized mild gingivitis at non-study sites. Those gingival biopsies were removed from either periodontally healthy volunteers or participants who were undergoing crown lengthening procedures at sites with probing depth measurements of 4mm or less at all six interproximal probing sites, no bleeding on probing and no evidence of radiographic bone loss.

Another twelve biopsied samples were collected from different participants exhibiting experimental gingivitis. The gingivitis was induced following a 3 week (21 days) stent-induced biofilm overgrowth protocol as described previously (183). The protocol for this experimental gingivitis was approved by the IRB of UNC-CH. In addition to the exclusion criteria mentioned above, all participants had at least 8 teeth in functional dentition and with at least 4 teeth in each

posterior sextant. In this protocol gingivitis participants with BOP scores of ≥10% (184) and pocket depth probing <5mm were brought to periodontal health by initial dental prophylaxis and supragingival scaling. After one week following this treatment, baseline GI scores were collected and the subjects were instructed to wear 2 stents during routine toothbrushing and not to floss the stent-covered teeth. This stent covered the tooth surfaces and promoted biofilm overgrowth during a 3-week induction period. At day 21, biopsies were collected from one interproximal site of gingiva in one of the stent sextants.

Upon removal, all biopsied gingival tissues were divided into two comparable samples. One half used for DNA methylation analysis, was placed and kept in -80°C freezer immediately, while the other piece for real-time polymerase chain reaction (RT-PCR) was incubated with RNAlater (Applied Biosystems/Ambion, Austin, TX) overnight at 4°C, and then transferred to -80°C.

DNA isolation and sodium bisulfite conversion

Genomic DNA was isolated from collected gingival tissue samples using a DNeasy Mini Kit (Qiagen, Valencia,CA) according to the manufacturer's instructions. Genomic DNA was bisulfite treated using published procedures (154). Briefly, 1~2 µg of genomic DNA in 45 µl of nuclease-free water was denatured at 42°C for 20 minutes with 5 µl of freshly prepared 3 M sodium hydroxide. Denatured DNA was incubated with freshly prepared sodium bisulfite (saturated) and hydroquinone solution in the water bath at 55°C for 16 hours. The bisulfite-converted DNA was purified using a Wizard DNA Clean-up Column (Promega, Madison, WI) and then desulfonated by incubation with 5.5 µl of a 3 M NaOH solution at 37°C for 20 minutes. The bisulfate treated DNA was finally precipitated with ethanol and then, resolved in 25 µl of 1mM Tris-Cl pH 8.

Bisulfite specific PCR and pyrosequencing

The detailed information of primers used in the bisulfite-specific polymerase chain reactions (PCR) can be found in Table 3.1. For methylation analysis, five amplicons, which were amplified by using a HotStar Taq kit (QIAgen, Valencia, CA), include a total of 6 CpG sites within the proximal promoter region of *IFNG*. PCR condition for each individual amplicon, in which specific CpG sites were included, can be found in Table 3.2. Direct quantification of the ratio of methylated to unmethylated cytosine nucleotide for each analyzed CpG site present in the amplicons was determined by pyrosequencing with the PSQ HS 96 Pyrosequencing System (Biotage, Charlottesville, VA) and Pyro Gold CDT Reagents (AIQgen, Valencia, CA) as previously described (185). We have also checked a polymorphism at site -179bp in the same amplicon containing the CpG site of -186bp by pyrosequencer. In each pyrosequencing assay, one amplicon was used for sequencing and the corresponding sequencer can be found in table 1. Internal controls for bisulfite conversion efficiency were included in each pyrosequencing assay. A genomic sequence that is artificially methylated on all its CpG dinucleotides (Millipore, Billerica, MA) was also used in the bisulfite conversion, PCR and pyrosequencing with the primers and sequencers mentioned above as a technical control.

Quantitative real time PCR

Total RNA was isolated from gingival tissues with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA from 500ng of total RNA was synthesized using an Omniscript Kit (Qiagen, Valencia, CA) and random decamer primers. Real-time PCR was performed with 1 µl of synthesized cDNA, 12.5 µl TaqMan Universal PCR mix, and 1.25 µl 20X Assay on demand gene expression assay mix (Mm00445273_m1, Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA). Each sample was performed in duplicates. The ribosomal 18s, which was also from Applied Biosystems, was used as an endogenous control for data normalization. The relative quantity of *IFNG* mRNA was calculated against 18S rRNA values according to the method recommended by Livak and Schmittgen (186).

Immunofluorescence

After sectioned, the frozen gingival tissues from healthy gingival and chronic periodontitis tissues were fixed with 70% ethanol for 15 seconds and followed by acetone for another 5 minutes. Then, the frozen tissue slides were blocked for one hour at room temperature, and then incubated overnight at -4°C with monoclonal antibodies specific for CD4 (Cat#14-0049, eBioscience, San Diego, CA) , CD56 (Cat# 14-0567, eBioscience). All the antibodies expect for anti-CD4 were 1:50 diluted in a blocking buffer containing 8% bovine serum albumin, 1% goat serum and 1% Trion-X. Anti-CD4 antibody was 1:20 diluted with the same blocking buffer. After vigorous washing with 1X PBS contains 0.1% Triton-X, the slides were incubated for 1 hour at room temperature with a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Invitrogen, Carlsbad, CA). Sections were then washed in 0.1% Triton/PBS, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and coverslipped. Sections were analyzed using confocal microscopy (Carl Zeiss LSM 710 Confocal Microscope, Thornwood, NY).

Statistical analysis

Analysis of Variance (ANOVA) was applied for the statistical analysis of clinical measurement. Fisher's exact test was used to test gender difference among participants in different groups. Mann-Whitney/Wilcoxon two sample test was used to compare methylation level of each CpG site and overall percent methylation of gingival samples among different biopsy groups. Linear regression analysis was applied for testing for significance of the slope to analyze the *IFNG* messenger level of different sample groups. The threshold for all statistical significance was set at a p-value less than 0.05.

Results

Selected characteristics between the three subsets of individuals are summarized in Table 3.3 There were no age differences between periodontally diseased subjects (either periodontitis or experimental gingivitis) and participants with periodontal health. As expected, in the periodontitis tissue group, both probing depth and clinical attachment level were greater than the periodontal health group ($p<0.001$ for both). Although there are more male participants in chronic periodontitis group as compared to periodontal health group ($p=0.01$) , there is no evidence so far, that gender has differential effect on methylation status of *IFNG* promoter in periodontal diseases.

The positions of all six analyzed CpG sites within the promoter region of the *IFNG* gene are depicted in figure 3.1. Since the methylation pattern of the CpG sites shown in this region has been demonstrated to be mechanistically related to the control of IFN-γ expression in various studies (187-189), we performed DNA methylation analysis on those CpG sites in our biopsied gingival tissues.

Figure 3.2 demonstrates two representative sequencing pyrograms of the methylation level of the *IFNG* promoter region taken from a periodontally healthy and a chronic periodontitis sample. The pyrogram in figure 3.2A shows that, at the CpG dinucleotide at position -295bp, 71% of the amplification products from one healthy gingival tissue sample contained a methylated cytosine nucleotide. The pyrogram in figure 3.2B demonstrates that at position -295bp, 49% of the amplification products from one periodontitis tissue sample contained a methylated cytosine residue.

A lower level of methylation as determined by pyrosequencing was found at each individual CpG site within the *IFNG* promoter region in the DNA samples from the chronic periodontitis tissues as compared to tissues with periodontal health (figure 3.3A). Of all the analyzed CpG dinucleotides, the methylation levels at site -295bp, -54 bp and +171bp from the chronic periodontitis DNA samples were significantly lower than the healthy gingival samples (44.5% [IQR(37%, 50.5%)] vs. 60% [IQR(51.5%, 64.5%)], $p=0.002$ for site -295bp, 58.5% [IQR(55.3%, 62%)] vs. 65%[IQR(58.5%,71%)], $p=0.04$ for -54bp, and 49% [IQR(47.5%, 50.3%)] vs. 55% [IQR(52.5%,

59%)] ,p=0.0007 for site +171bp). In contrast, there is no significant difference of DNA methylation percentage in each analyzed CpG sites in experimentally induced gingivitis group as compared to periodontal health samples. One of the designed amplicons encompassing one methylation site at -186bp also contained a reported SNP at position -179bp. The G/T SNP (rs2069709) reported at site -179bp is reported to be associated with an elevated level of *IFNG* gene expression (190)(191). We examined this polymorphism for all the samples along with DNA methylation analysis. No minor allele (-179T) was detected in any of the analyzed samples (data not shown).

The overall methylation percentage of all the 6CpG sites within *IFNG* promoter was 52% [(IQR) 43.8%-63%)] in the chronic periodontitis biopsies and significantly lower than the methylation percentage in samples with periodontal health and experimental gingivitis biopsies, which were 62% (IQR, 51.3%-74%), (p=0.007) and 63%(IQR, 55%-74%), (p=0.02), respectively (Figure 3.3B). No significant difference can be found in the *IFNG* promoter methylation levels comparing samples with periodontal health to samples with experimental gingivitis.

Since DNA methylation level is a critical regulatory mechanism for gene transcription, we also measured the messenger (mRNA) level of *IFNG* in biopsied tissues. The transcriptional level of *IFNG* was 1.96 fold increase and significantly higher in the periodontitis biopsy samples as compared with the healthy gingival samples as determined by quantitative real-time PCR (p=0.04, figure 3.4). In contrast, there was a 8.5 fold induction of *IFNG* transcription in experimentally induced gingivitis samples as compared to samples with periodontal health, and such an increase of messenger level is statistically significant (p=0.01).

We also performed immunofluorescence experiment to identify the IFN-γ secreting cell populations and compare them between chronic periodontitis samples and tissues with periodontal health. In chronic periodontitis tissues more CD4+ T cells were present in the epithelial and lamina propia as compared to the tissues with periodontal health (figure 3.5A and 3.5B). In addition, we also found more CD56+ (figure 3.5C and 3.5D) positive cells, which are markers for NK and dendritic

cells, respectively, infiltrated in the chronically diseased biopsies than samples in health gingival tissues.

Discussion

Although B-cell mediated adaptive immune responses dominate in chronic periodontitis, differential distribution of Th1 or Th2 cytokine secretion profiles have been reported to be related to the severity and/or progression of periodontal diseases (164)(192). Among the Th1 cytokines, IFN-γ plays a central role in the containment of infection and represents one of the most efficient cytokines for triggering antimicrobial activity in both macrophages and neutrophils (193)(194)(195). In addition to priming the antimicrobial activities of phagocytes, over expression of IFN-γ levels may also lead to direct and indirect host tissue destruction through the activation of these phagocytes (196).

In our study, we noted a significant 1.96 fold increase in IFN-γ messenger level in chronically inflamed periodontal biopsies as compared to periodontally healthy samples, consistent with several earlier reports (171)(167). Studies have shown an increased IFN-γ level within the GCF and higher IFN-γ expression in gingival tissues from progressive periodontal lesions (167). By contrast, other studies have suggested that elevated IFN-γ is a characteristic of the stable periodontal lesion and that active lesions favor a Th2 cell response(197)(198)(199). We also found that the transcriptional level of *IFNG* was 8.5 fold higher in the experimentally induced gingivitis samples as compared to tissues with periodontal health. Such a more pronounced transcriptional expression seems independent of *IFNG* methylation in the analyzed promoter region. Although the inflammatory lesion from experimental gingivitis is histologically similar to the chronic periodontitis in that inflammatory infiltrates dominated by lymphocytes and antigen presenting cells such as macrophage and dendritic cells are noticeably present (200)(201), the mechanisms for IFN-γ production may possibly different in the different stages of periodontal diseases. For example, cytokine-dominating mechanism such as high IL-12 secretion from antigen presenting cells and IL-18 may be possibly responsible for the observed high level production of IFN-γ in Th1 committed cells in experimental gingivitis lesions (202, 203). In addition, p38 and JNK signaling pathways have also been shown to be critically involved in IFN-γ production (204). However, this study supports that a decreased

methylation profile within *IFNG* promoter region may contribute to a higher gene transcriptional level, as found in chronically diseased gingival tissues. We hypothesized here that the promoter region of *IFNG* in infiltrating Th1 cells may be already epigenetically modified prior to migration into the lesion after prolonged and systemic exposure to periodontal pathogens present in the oral biofilm in chronic periodontitis patients.

It has been extensively reported that genetic polymorphisms can regulate cytokine expression levels. Bream *et al* (191) reported a guanidine to thymidine transition at site -179 (G/T) within the *IFNG* proximal promoter, those authors reported that, as compared to -179G allele, the -179T allele exhibited a six to thirteen fold increase in expression of *IFNG* in a promoter assay. The absence of SNP at position -179bp within IFNG promoter in all the gingival biopsies analyzed by pyrosequencing supports the argument that the differences in *IFNG* expression in the chronic periodontitis biopsies were less likely attributable to *IFNG* polymorphisms, but more likely due to epigenetic influences conferred by prolonged environmental exposures.

In this study, the general demethylation pattern across all 6 CpG sites within the *IFNG* promoter region in the periodontal lesions could be due to chronic inflammation or the direct action and/or invasion of periodontal pathogens. Hypomethylation in chronically diseased gingival tissues could reflect a dilution of the tissue DNA pool by an influx of non-methylated DNA-bearing cells or the loss of methylation from the resident cells. We have found that more CD4+ T cells and CD56+ NK infiltrated in the periodontitis gingival samples than tissues in healthy gingival samples. Therefore, the observed lower methylation pattern in *IFNG* promoter region in periodontitis may be associated with altered methylation patterns on those cells capable of IFN-γ production. Nares et al (205) showed a marked infiltration by various inflammatory cell types in periodontally inflamed tissues in comparison to healthy gingival tissues, and also demonstrated that IFN-γ immunostaining was related to the presence of monocytes, macrophages and lymphocytes in periodontally diseased tissues. This evidence is consistent with our hypothesis that in periodontally diseased gingival tissues, hypomethylation status of *IFNG* promoter in those inflammatory cell types contributes to the

observed higher IFN-γ expression in comparison with healthy gingival tissues. This association has also been reported and discussed by others; Gonsky and collaborators (129)examining patients with Inflammatory Bowel Disease collected intestinal specimens from patients undergoing surgical resection of the colon, and showed that the infiltrated T cells isolated from the lamina propria in the mucosal tissues exhibited lower level of IFNG methylation in comparison to T cells isolated from peripheral blood from the same patients, suggesting that the epigenetic methylation status of IFNG plays a mechanistic role in the modulation of IFNG secretion in the mucosa. In another recent study on the methylation levels of *IFNG* promoter in human dental pulp tissues, the authors found an elevated level of hypomethylation in symptomatic pulpitis as compared to control pulp samples from impacted teeth, and also associated the IFNG hypomethylation levels with an increased number of infiltrating mononuclear cells in the inflamed tissues (206).

A hypomethylation pattern of the *IFNG* promoter region has also be reported by several groups as the hallmark of T cell commitment to a Th1 phenotype (187)(188)(207). In this study, although the methylation level of *IFNG* from periodontal lesions is significantly lower than control sites (52% (IQR, 43.8%-63%) vs. 62% (IQR, 51.3%-74%) p=0.007), the magnitude of the difference (~ 10%) is not large, probably due to the fact that the modulation of methylation patterns of IFN-γ competent cell populations, which are infiltrated inflammatory cells, constitute a relatively small percentage of the total cell numbers within the periodontal lesion. Therefore, the magnitude of methylation difference between chronic periodontitis biopsies and gingival tissue with periodontal health would be expected to be relatively small, diluted by the overwhelming presence of non-IFN-γ competent cell populations in the tissue. However, this small decrease in methylation can account for a greater induction of messenger level of *IFNG* since it may be targeted to the IFN-γ producing cells. Gonsky et al (128)reported that a 5% lower methylation level observed in the *IFNG* promoter region of lamina propria T cells from patients with inflammatory bowel disease as compared to controls was associated with an almost 3-fold induction of *IFNG* transcription. Thus, the difference of the *IFNG* methylation level that we observed in the oral mucosa is in close agreement with the levels observed

in inflammatory bowel disease. However, the causal relationship between a small change of methylation in the promoter region of *IFNG* and increase of its transcription level requires further mechanistic evidence supported by experiments such as chromatin immunoprecipitation, electrophoretic mobility shift assay, etc.

The findings from this study indicate the potential role of local epigenetic effect that result in regional modifications of the tissue DNA methylation status in the pathogenesis of chronic inflammatory periodontal disease. The observed hypomethylation of the *IFNG* promoter is a characteristic of a chronic inflammatory state. The study of methylation in inflamed tissues under chronic infection is still at an early stage and altered methylation patterns likely effect many genes in disease. Additional research is needed to elucidate the potential diagnostic utility of epigenetic markers as a determinant of disease progression, as well as response to treatment. Since the DNA methylation status can be modified by certain drugs, the possibility of reversing epigenetic modifications may have profound effects on periodontal treatment responses.

Table 3.1 Oligonucleotides used for bisulfite specific PCR and pyrosequencing

CpG site (*)	Details	Sequences
-295	Forward	5'-[Biotin] TTTGTAAAGGTTTGAGAGGTTTTAGAAT-3'
	Reverse	5'-CAAACCCATTATACCCACCTATACCA-3'
	Sequencer	5'-TTTTATACCTCCCCACTT-3'
-186	Forward	5'-TTAGAATGGTATAGGTGGGTATAATGG-3'
	Reverse	5'-[Biotin] TATTATAATTAAAATTTCCTTTAAACTCCT-3'
	Sequencer	5'-GGGTATAATGGGTTTGTT-3'
-54	Forward	5'-GGGTTTGTTTTATAGTTAAAGGATTTAAGG-3'
	Reverse	5'-[Biotin] AATCAAAACAATATACTACACCTCCTCTAA-3'
	Sequencer	5'-TATTTTATTTTAAAAAATTTGTG-3'
+122 ~ +128	Forward	5'-[Biotin] TTTTGGATTTGATTAGTTTGATATAAGAA-3'
	Reverse	5'-AAAACCCAAAACCATACAAAACTAAAA-3'
	Sequencer	5'-CTAAAAAACCAAAATATAACTTAT-3'
+171	Forward	5'- [Biotin] TTTTGGATTTGATTAGTTTGATATAAGAA-3'
	Reverse	5'- CATTTTCAACCACAAACAAATACTATTAA-3'
	Sequencer	5'- ACAACCAAAAAAACCC-3'

*CpG sites indicate nucleotides position in relation to transcription start. "-" or "+"indicates upstream or downstream of transcription start, respectively.

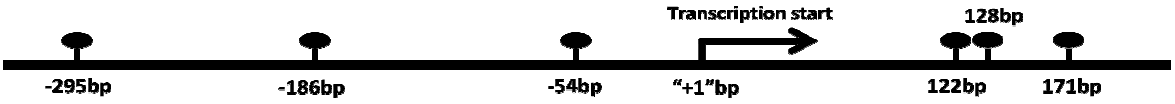
Table 3.2 Bisulfite specific PCR conditions for CpG containing amplicons

Amplicons (CpG site(s) included)	Initial Denaturing T(°C),T(min)	Cycles (X45)			Final Elongation T (°C),T (min)
		Denaturing T(°C),T(sec)	Annealing T(°C),T(sec)	Elongation T(°C),T(sec)	
-295	94, 15	94, 30	58, 30	72, 30	72, 10
-186	94, 15	94, 30	54, 30	72, 30	72, 10
-54	94, 15	94, 30	60, 30	72, 30	72, 10
+122~+128	94, 15	94, 30	55, 30	72, 30	72, 10
+171	94, 15	94, 30	55, 30	72, 30	72, 10

Table 3.3 Demographic information of the participants and clinical parameters in the biopsied gingival sites

Demographic/ Clinical parameters	Periodontal Health (n=23)	Experimental Gingivitis (n=12)	Periodontitis (n=12)
Mean age (years)	40.8± 11.6	35.8±11.2	47.2± 7.4
Gender Males/Females	6/17	5/7	9/3*
Probing Depth (Mean±SD, mm)	2.2±0.6	2.24±0.63	6.3±0.8**
Clinic Attachment Level (Mean± SD, mm)	1.2±0.6	1.3±0.45	4.7+1.5**

"*" indicates $p<0.05$ as compared with periodontal health' "**" indicates $p<0.001$ as compared with periodontal health



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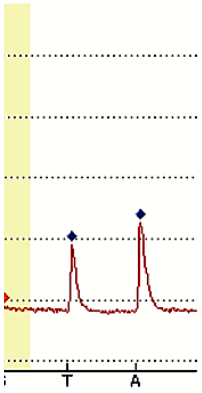

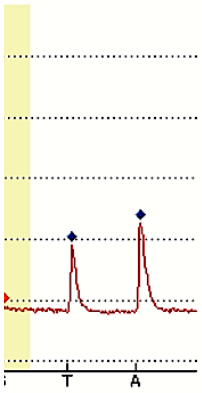

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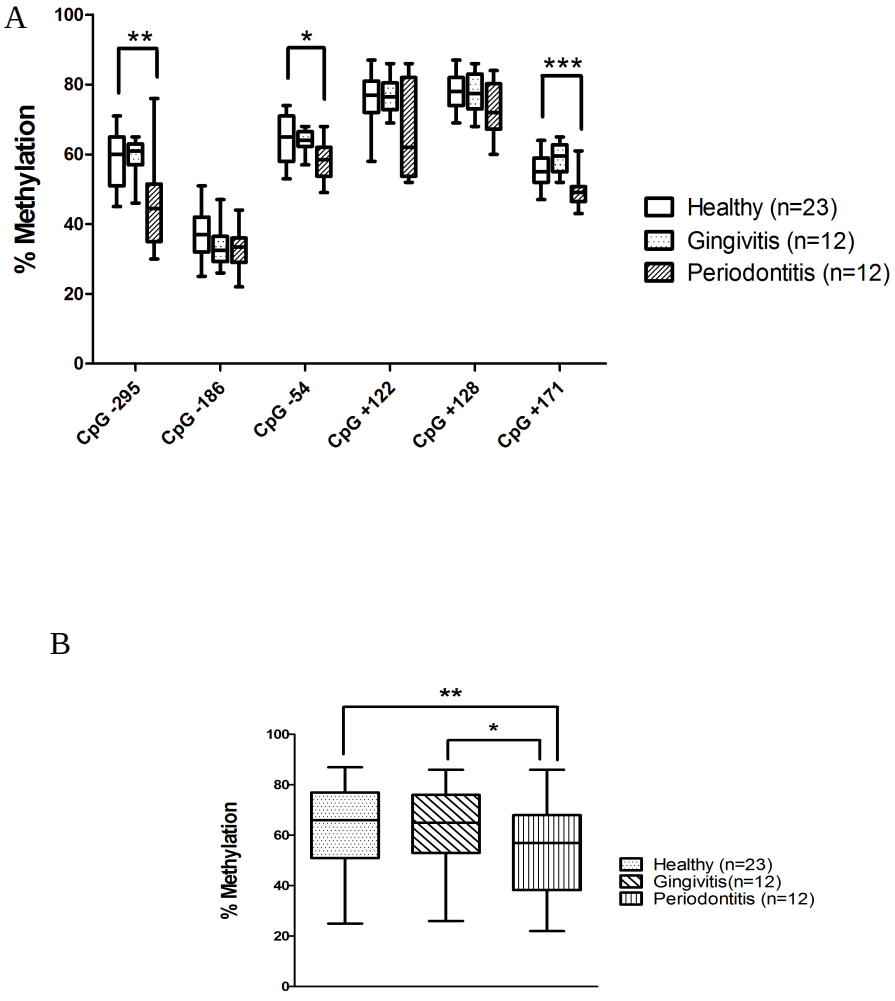


Figure 3.3 The percentage of methylation from healthy gingival tissues, experimentallygingivitis and chronic periodontitis biopsies. 3.3A: The percentage of methylation in each individual CpG site from healthy gingival tissues, experimentally gingivitis and chronic periodontitis biopsies. "*", "**", and "***" indicate significantly lower methylation level in chronic periodontitis biopsies than in healthy samples at CpG site -54 (p=0.04), CpG site -295 (p=0.002), and CpG site +171 (p= 0.0007), respectively. Plus error bars and minor error bars demonstrate the maximal and minimal methylation percentages, respectively, while the top line, middle line and the bottom lines of boxes illustrate 75% percentile, median, and 25% percentile of the methylation levels in each group analyzed, respectively. 3.3B*:* Percentage of overall methylation level combinging all 6 analyzed CpG sites within IFNG promoter region in biopsied tissues exhibiting periodontal health, experimental gingivitis and chronic periodontitis. "**" and "*" indicate that the overall methylation level of *IFNG* promoter region in chronic periodontitis gingival tissuesis was statistically lower than the percentage of methylation in healthy tissues (p=0.007) and experimental gingivitits biopsies (p=0.02), respectively. The top, middle and bottom lines of the box plot indicate the 75% percentile, median and 25% percentile methylation levels, respectively. Plus error bars and minor error bars demonstrate the maximal and minimal methylation percentages, respectively.

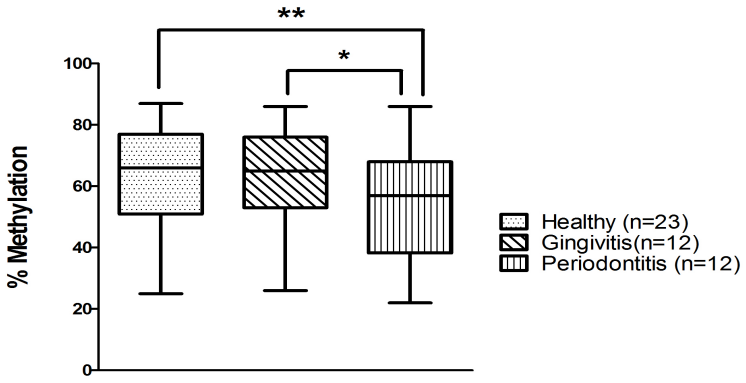


Figure 3.4. mRNA expression level of *IFNG* in the healthy gingival biopsies, experimental gingivitis and chronic periodontitis samples. Fold induction is shown using 18s as a internal housekeeping gene. As compared to gingival biopsies with periodontal health, a 1.96 fold increase in the transcriptional level of *IFNG* is significantly higher (p=0.04) in the chronic periodontitis biopsies, which was indicated by "*". In contrast, the transcriptional level of *IFNG* in experimental gingivitis showed an 8.5 fold increase and was significantly higher (p=0.01) than tissues with peridontal health, which was indicated by "**".

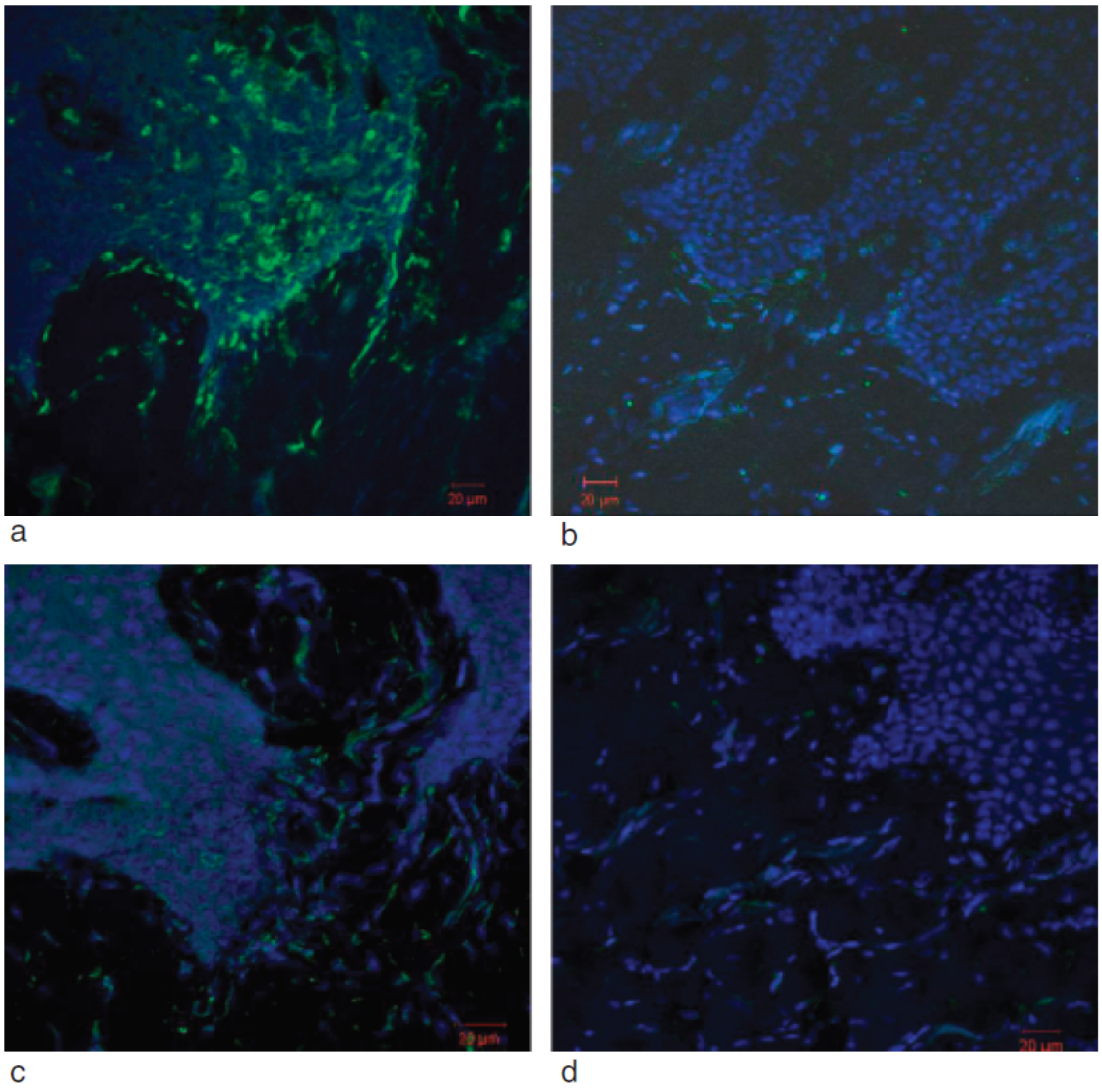


Figure 3.5. Immunofuorescence staining of CD4+ and CD56+ cells in 1 representative chronic periodontitis biopsy and 1 biopsy with periodontal helath. 3.5A and 3.55B: biopsy section from either a site with chronic periodontits (3.5A) or a site with periodontal health (3.5B) showing CD4+ (green) T cells within the gingival epithelium and lamina propria. 3.5C and 3.5D: A chronic periodontitis (3.5C) and a healthy gingival tissue (3.5D) were stained for CD56+ (green) NK cells. Nuclei were stained with DAPI (blue).

Chapter 4

Regulation of *TNFA* Expression in Periodontal Diseases by DNA Methylation

Abstract

The knowledge of epigenetic control of tumor necrosis factor-alpha (TNF-α) in the pathogenesis of periodontal diseases is scarce. In this study, we investigated the DNA methylation alteration within *TNFA* promoter in gingival biopsies from different stages of periodontal diseases and a monocytic cell line exposed to a periodontal pathogen, *Cambylobacter rectus* 314. The regulatory mechanism by promoter methylation on *TNFA* transcription is also explored. A decreased methylation level at -244bp in the promoter region of *TNFA* in the experimentally induced gingivitis biopsies as compared to the resolution phase of gingivitis (46.1±10.3% vs. 53.6±8.0%, p=0.01). Such a hypomethylation status at -244bp also persisted to the chronic periodontitis, a more advanced stage of periodontal diseases. The methylation level at that specific site was 46.7±6.7% in gingival tissues with chronic periodontitis, and was significantly lower than biopsies with periodontal health (52.4±6.1%, p=0.01). Paradosically, an increased methylation at CpG site -163bp within the *TNFA* promoter observed in chronic periodontitis tissues was significantly higher than in periodontal health (16.1±5.1% vs.11.0±4.6%, p=0.0016). In addition, the methylation level at -163bp was also invserly associated with its transcriptional level among periodontitis and periodontal health tissues (r=0.16, p=0.018). Exposure of THP-1 cells to live *C. rectus* 314 significantly decreased the overall methylation across the studied CpG sites within the *TNFA* promoter as compared to mock challenged cells 14% (10.1%, 25.5%) vs. 21.8% (17.8%, 27.4%), shown as median and interquartile range, p=0.013]. Interestingly, a progressive loss of methylation at site -72bp was also found in the co-cultured THP-1 cells. By treating THP-1 cells with a DNA methylation inhibitor as well as employing a promoter-specific methylation luciferase reporter, we further confirmed that the methylation status of *TNFA* negatively controls its transcription.

Introduction

Periodontal diseases, though etiologically initiated by periodontal pathogens present within microbial flora of dental biofilm, are pathogenically shaped by host inflammatory and immune responses to those organisms (164). Various cytokines produced by the periodontal tissues and inflammatory infiltrates are actively involved in the pathogenesis of periodontal diseases (208, 209).Of those cytokines, unconstrained secretion of tumor necrosis factor-α (TNF-α) has been demonstrated to contribute at different levels to periodontum destruction, such as cellular apoptosis, activation of bone resorption, upregulation of intercellular adhesion molecules (ICAMs), *etc* (210).Abnormal level of TNF-α has been also associated with periodontal diseases of different stages. Gorska *et al*. reported that the concentration of TNF-α was significantly elevated in both serum samples and gingival tissues biopsied from one active site in patients with severe chronic periodontitis as compared to periodontally healthy control subjects (211). At the terminal dentition stage of both adult periodontitis and early on site periodontitis patients, the TNF-α level in gingival crevicular fluid (GCF) is among the second-high tier of inflammatory mediators (169). As a primary mediator produced largely by macrophages/monocytes in response to the challenge of periodontal pathogens, TNF-α can sustain or amplify inflammation by inducing secondary inflammatory mediators such as cyclooxygenase-2, which is the rate-limiting enzyme in the production of prostaglandins, and matrix metalloproteinases (MMPs), which is responsible for extracellular matrix degradation (212). Although it is typically associated with innate immune response, TNF-α is also involved in the regulation of adaptive immunity. For example, in an acute graft-vs-host disease mouse model, Via et al. found that TNF-α plays a crucial role in the induction of cytotoxic lymphocytes and blockage of TNF-α clearly favors a humoral-mediated response (213).

Due to its global effects and potent biological activities, fine-tuned control of TNF-α is necessary in maintaining periodontal health. The regulation of TNF-α by signaling network is complex and depends on cell types and stimuli in question: transcriptional activation of TNF-α by

lipopolysaccharide (LPS) in macrophages or monocytes can be mediated by NF-kB (214), or mitogen- activated protein kinase (MAPK) family such as P38 or extracellular regulated kinase (215)(216); activated T cells by TCR engagement or virus induced TNF-α production require the coactivator proteins of cAMP response element binding protein (CREB)-binding protein (CBP)/p300 family(217); P38 MAPK, NF-kB and phosphatidylinositol 3-kinase (PI3K) pathways are involved in TNF-α by mature adipose cells in response to LPS stimulation(218). Regulatory approaches also exist at post-transcriptional, translation, or post-translational levels (219)(220)(221).In addition, genetic variants also contribute to the control of TNF-α and are associated with diseases. For example, in the promoter region of TNF-α, -308 A carrier is associated with increased risk of chronic obstructive pneumonia disease (COPD) in Asian population, while the same single nucleotide polymorphism (SNP) is found to be associated with Grave's disease (222)(223)(182). Certain allelic variations are also functionally relevant. -1031 C carrier is associated with increased expression of TNF-α in COPD patients, and a SNP within the first intron (+123A) increased the activity of TNF-α reporter compared to G allele (224)(225).

Although much is known about the control of TNF-α at genetic level by signaling pathways, the knowledge of the epigenetic regulation related to periodontal diseases and periodontal pathogens is scarce. This regulatory mechanism, which is independent of DNA sequence, includes DNA methylation, histone post-translational modifications, and small non-coding RNA modulation. Exposure to various environmental stimuli such as diet, live style, toxins, inflammation, etc., have profound effects on gene expression, and such influence can be modulated by those epigenetic mechanisms (10)(226). In eukaryotes, DNA methylation, as the heritable and least reversible epigenetic modifications, occurs almost exclusively at the 5' end of cytosine nucleotide within the CpG dinucleotide context (227). It has been generally accepted that the degree of DNA methylation within gene promoter regions is usually inversely related to the transcriptional level of those genes, though exceptions are also reported (228)(181)(229). Recently, we have described an increased methylation (hypermethylation) of CpG rich region within the *PTGS2* promoter region in human

gingival biopsies, which is associated with a subdued *PGE*2 mRNA expression (182). Bobetsis *et al.* using a mouse model demonstrated that maternal infection by *Campylobacter rectus* (*C. rectus*), a periodontal pathogen, can induce a hypermethylation profile within promoter region of *IGF2* P0 in fetal placenta (28). These data suggest that inflammation and/or pathogenic oral bacteria can induce epigenetic changes in gene promoters.

Studies have shown that TNF-α can be transcriptionally regulated by epigenetic mechanisms. The high-glucose condition induced *TNFA* transcription is associated with the increased histone acetylation at the *TNFA* promoter region (230). Both histone and DNA methylation as well as chromatin remodeling factors are involved in the silence of *TNFA* expression during *E. coli* LPS induced tolerance (231). However, whether or how cytokines are epigenetic modulated by periodontal pathogens is not understood. The chronic exposure to the omnipresent oral microorganism flora present in the bioflim and the inflammatory state of periodontal diseases may alter the gene expression file of the local gingival tissue of hosts by inducing epigenetic changes. Therefore, we hypothesize that methylation profile of *TNFA* promoter region was altered in periodontal diseases and the expression of this gene is modulated by promoter methylation. We are also seeking to study how the methylation pattern of *TNFA* in cells, modulated by the presence of periodontal pathogens exampled by *C. rectus*.

Materials & Methods

Participants and tissue specimens

A total of thirty five participants, aged between 19 and 63 years, provided informed consent and were recruited into this study wherein all components were approved by the Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill (UNC-CH). All the participants reported no use of either antibiotics or non-steroidal anti-inflammatory drugs (NSADs) within 1 month before enrollment. Nor had the participants have medical treatment for other diseases 3 months before the biopsy or periodontal surgery. Clinical measurements, such as probing depth (PD), clinical attachment loss (CAL), and bleeding on probing (BOP) at six sites per tooth were obtained. Tissue from one interproximal gingival site was harvested from each participant. Seventeen gingival tissues were collected during routine periodontal flap surgeries from participants clinically diagnosed with chronic periodontitis (CP), on whom initial therapies including scaling and root planning were performed four to six weeks before the scheduled periodontal surgery. Tissues from CP biopsies exhibited PDs at least 5mm and radiographic evidence of localized bone loss. Another 18 gingival biopsies were collected from different control participants (C) who were periodontally healthy or had mild gingivitis at non-study sites. Those biopsy samples, which were harvest from either volunteers or participants undergone crown lengthening procedures, exhibited PD measurement of 4mm or less, no BOP and no evidence of radiographic bone loss.

Another 11 pairs of biopsied gingival samples (total 22) were collected from 11 diffeent participants when gingivitis was experimentally induced (GI) and at the stage of gingivitis resolved (GR). The gingivitis induction was followed a 21-day stent-induced biofilm overgrowth protocol as described previously (183). No contradiction with the medical and medicine history mentioned above, all the recruited participants had at least four teeth in each posterior sextant. In this protocol, gingivitis participants with BOP scores of 10% (184) and pocket depth probing <5 mm were brought

to periodontal health by initial dental prophylaxis and supragingival scaling. After 1 week following this treatment, baseline gingival index scores were collected and the subjects were instructed to wear two stents during routine toothbrushing and not to floss the stent-covered teeth. This stent covered the tooth surfaces and promoted biofilm overgrowth during a 3-week induction period. At day 21, biopsies were collected from one interproximal site of gingiva exhibiting GI in one of the stent sextants. The participants were instructed to resume dental hygiene measures on the stent covered teeth. After a 4-week resorption phase, at day 49, another piece of biopsy with GR from the same participants was collected at a different interproximal site from the last biopsied one but covered by the same stent.

Upon removal, all biopsied gingival tissues were divided into two comparable samples. One piece, used for DNA methylation analysis, was kept in the −80°C freezer immediately, while the other half, used for real-time polymerase chain reaction (RT-PCR), was incubated with RNA later (Applied Biosystems/Ambion, Austin, TX, USA) overnight at 4°C, and then transferred to −80°C for storage.

Cell cultures and co-culture with *C. rectus*:

THP-1 cells, a human monocytic cell line, originally purchased from American Type Culture Collection (ATCC# TIB-202, Manassas, VA, USA) were obtained from Tissue Culture Facility at UNC-CH. The cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), $5X10^{-5}$M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin (Invitrogen). RAW264.7 cells, a murine monocytic cell line, were a generous gift from Dr. Patrick Flood at the School of Dentistry, UNC-CH. Cells were maintained in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, and 1% penicillin/streptomycin, 2mM L-glutamine (Cat.# 17-605E, Lonza). All cells were grown in a humidified incubator with 5% CO_2 at 37°C.

C. rectus strain 314 was grown under an anaerobic condition at 37 °C on Enriched Tryptic soy agar (Cat.# AS-546, Anaerobe systems, Morgan Hill, CA, USA) in an anaerobic chamber.

After washed with PBS, $1X10^6$ THP-1 cells resuspended with the antibiotic-free culture medium as mentioned above were plated into each well of a 6-well plate prior to bacterial stimulation. *C. rectus* were collected at the late logarithmic phase and resuspended in the same antibiotic-free culture medium to an optical density of 1.00 (at 600 nm). Bacteria were added into THP-1 cells to obtain a multiplicity of infection (MOIs) of 100 at different time points as indicated. Fresh antibiotic-free cell culture medium was added 48 hours after *C. rectus* challenge to maintain the cell density no more than $1X10^6$/ml.

DNA isolation and sodium bisulfite conversion:

Genomic DNA was extracted from collected gingival biopsies and THP-1 cells using a DNeasy Mini kit (QIAgen, Valencia, CA, USA) according to manufacturer's manual. Genomic DNA was treated with sodium bisulfite by published procedures (232). Briefly, 1-2ug genomic DNA dissolved in 45µl of water was denatured at 42°C for 20 minutes with 5µl of freshly prepared 3M sodium hydroxide solution. After denatured, DNA was incubated with saturated sodium bisulfite (Cat.# 243937, Sigma) solution containing 10mM of hydroquinone (Cat.# H9003, Sigma), with a final pH at 5.0, at 55°C overnight. Converted DNA was then purified using a Wizard DNA Clean-up Column (Promega, Madison, WI, USA) and desulfonated by incubation with the 3M sodium hydroxide solution at 37°C for 20 minutes. DNA was finally precipitated with ethanol, and then resolved in 25ul of 1mM Tris-Cl (pH 8).

Bisulfite-specific PCR and pyrosequencing

The information of primers used in the bisulfite-specific polymerase chain reactions (PCR) can be found in Table 1. Four PCR amplicons, which were amplified by a HotStar Taq kit (QIAgen,

Valencia, CA), include a total of 10 CpG sites within the proximal promoter region of *TNFA*. The annealing temperature for each PCR can be found in Table 1. Direct quantification of the percentage of methylated cytosine nucleotide for each CpG site present in the amplicons was determined by pyrosequencing. In brief, 6-8µl of PCR product was mixed with 2µl of streptavidin-coated sepharose beads and 40µl of PyroMark binding buffer (QIAgen, Valencia, CA). After captured by the PyroMark vacuum prep filter tips (QIAgen), the beads to which biotin-labeled single strand PCR product attached were washed sequentially with 70% ethanol, denaturing buffer and washing buffer and released into a PyroMark Q96 HS plate (QIAgen) onto which 12µl of PyroMark annealing buffer mixed with 3.6pmol of sequencing primer specific for each amplicon was already preloaded. After heated for 2 minutes at 86°C, the plate was loaded onto a PSQ HS 96 pyrosequencing system (QIAgen). The sequencing was determined by Pyro Gold CDT reagents (QIAgen) as previously described (185). For each pyrosequencing assay, one amplicon was used for sequencing and the corresponding sequencer can be also found in Table 4.1. A genomic sequence that is artificially methylated on all its CpG dinucleotides (Cat.#S7821, Millipore, Billerica, MA, USA) was used in the bisulfite conversion, PCR and pyrosequencing with the primers and sequencers mentioned above as a technical control.

Quantitative realtime PCR:

Total RNA was isolated from gingival tissues and cells with an RNeasy Mini Kit (Qiagen). cDNA from 500ng of isolated RNA was synthesized by an Omniscript Kit (Qiagen) and random decamer primers (Cat. # 5722E, Ambion, Austin, TX, USA). In a volume of 25µl, real-time PCR for transcriptional expression of *TNFA* was performed with 1µl of such synthesized cDNA, 12.5µl TaqMan Universal PCR mix (Applied Biosystems, Foster City, CA, USA) , 1.25 µl 20X assay on demand gene expression assay mix (Cat. # hs_99999043_m1, Applied Biosystems), and nuclease-free water in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems). The ribosomal 18S (18S rRNA) Control Reagents (part# 4308329, Applied Biosystems), which was also purchased from

Applied Biosystems, was included in the real-time PCA assay as an endogenous control. The relative quantity of *TNFA* mRNA was calculated against 18S rRNA values according to the $\Delta\Delta C_t$ method according to Livak & Schmittgen (233).

5-azacytidin treatment of cells:

Dissolved 5-Aza-2′-deoxycytidine (5-Aza-2dC, Cat.# A3656, Sigma), an inhibitor of DNA methylation, was added to THP-1 cells to achieve a final concentration of 5μM. After two days of treatment, cells were washed with PBS and treated again with freshly prepared 5-Aza-2dC of the same concentration for another two days. THP-1 cells that were treated with diluents in which 5-Aza-2dC was dissolved (mock treatment) served as control. DNA and RNA samples were extracted from those treated cells or treated cells post challenged with *C. rectus*.

Cloning of *TNFA* promoter, transfection and luciferase reporter assay:

A promoter fragment ranging from -291bp and +44bp relative to the transcription start site of the *TNFA*, which includes 11 CpG dinucleotides, was generated by PCR using the following primers: 5'-TCCGGTACCCCTCCAGGGTCCTACACACA-3 for forward; 5'- TCCAAGCTT TAGCTGGTCCTCTGCTGTCC-3' for reverse. Two restrictive endonuclease recognition sites KpnI and HindIII were underlined, respectively. Digested PCR product was directly ligated using a T4 DNA ligase (Cat.#M1801, Promega) to a pGL-3 luciferase reporter vector (Cat.#E1751, Promega) that was digested with the same endonucleases mentioned above. After transformation, the construct pGL3-PTNFA291 was extracted from *E. Coli* DH5α using a QIAprep Spin Miniprep kit (QIAgen) and sequenced to confirm the correct insert.

For transfection, $1.8X10^5$ RAW 294.7 cells per well were seeded on a 48-well plate one day before transfection and grown without antibiotics at 37°C. Cells were transfected with either 120ng pGL3-PTNFA291 or 220ng modified pGL3-PTNFA291 (methylated or mock-methylated) with a

Lipofectamin™ 2000 reagent (Invitrogen). The reporter constructs were always co-transfected with a *Renilla* luciferase vector (pRL-TK vector, Cat.# E2241, Promega). 18 hours after transfection, the cells were challenged with *E. coli* LPS (500ng/µl) for another 21 hours.

Cells were harvested and the luciferase activities were measured by the Dual Luciferase Reporter Assay System (Cat.# 1910, Promega) following the manufacturer's instruction using a Lumat LB9507 luminometer (Berthold, Oak Ridge, TN, USA). The luciferase activities of the constructs were normalized with *Renilla* luciferase reporter activity to account for transfection efficiency.

***In vitro* methylation:**

In vitro methylation of cloned TNFA promoter fragment was performed according to the protocol recommended by Dell *et al* (234). In brief, 80µg of pGL3-PTNFA291 isolated by a QIAgen plasmid Maxi kit (Cat.#12163, QIAgen) were digested by HindIII and KpnI, and the insert and vector backbone were then purified by a QIAquick gel extraction kit (Cat.#28704, QIAgen). For *in vitro* methylation, the insert was incubated with 24 units of *M. SssI*, a CpG methyltransferase from New England Biolabs (Cat.# M0226S, Ipswich, MA, USA), in the presence of S-adenosylmethionine (SAM) with a final concentration of 160µM, at 37°C overnight. In parallel, a mock methylation reaction as described above but without the supplement of SAM was also performed. The methylation efficiency was estimated by BstUI (Cat.#R0518S, New England Biolabs) digestion, as the presence of methylated CpG dinucleotides will block its endonuclease activity. After purification, the methylated or mock methylated insert was directly religated with the digested pGL3 vector by the T4 DNA ligase mentioned above to achieve either pGL3-PTNFA291(methylated) or pGL3-PTNFA291(mock). After purification with a QIAquick PCR purification kit (Cat.#28104,QIAgen), the religated luciferase reporter was transfected to RAW294.7 cells.

Statistics:

Analysis of variance (ANOVA) was applied for the statistical analysis of clinical measurement. Fisher's exact test was used to test gender difference among participants in different groups. Mann-Whitney/Wilcoxon two-sample test was applied to comparing the percentage methylation of the overall percentage methylation of gingival samples among different biopsy groups. Student *t*-test was used to determine the significant difference of percentage methylation of each CpG site, real-time PCR and luciferase report assay data. Linear regression analysis was applied to test for the significance of slope to evaluate the association between the percentage of methylation of specific CpG site and *TNFA* mRNA expression from the gingival biopsies.

Results

Participants

The demographic information of those study participants is listed in Table 4.2. No significant difference in gender and age was found among participants with chronic periodontitis and with periodontal health. As expected, sites with chronic periodontitis exhibited deeper pocket depth and more attachment loss than sites with periodontal health.

Promoter methylation level and transcription of *TNFA* in clinical samples:

The human *TNFA* promoter region does not contain classic CpG islands. Because DNA methylation affects the architecture of chromatin structure and initiation of transcription machinery around the transcription start site (TSS), we analyzed the methylation status of 10 CpG dinucleotides that are present just upstream of *TNFA* TSS (figure 4.1). Several transcription factor binding sequences, such as NF-κB, AP1, Sp1 and CRE, are also identified within this region.

Although the overall methylation levels from samples with chronic periodontitis did not exhibit a significant overall difference from samples with periodontal health, both lower methylation (hypomethylation) and higher methylation (hypermethylation) were identified at individual sites in the chronical periodontits samples as compared to periodontal health (figure 4.2A). At site -244bp, the methylation level of periodontitis samples was significantly lower than found in periodontal health samples (46.1$\pm$10.3% vs. 53.6$\pm$8.0%, shown as mean$\pm$ standard deviation, p=0.01), while the methylation level from the periodontitis samples was significantly higher at CpG site -163 and -161bp than the same sites from the periodontal health gingival biopsies (16.1$\pm$5.1% vs. 11.0$\pm$4.6%, p=0.0016, 19.75$\pm$4.1% vs. 15.4$\pm$3.6%p<0.001, respectively). The transcriptional levels of *TNFA* between those two biopsy groups did not show significant difference (p=0.08, figure 4.2B). Because the methylation levels at 3 CpG sites are different between periodontal health and chronic

periodontitis samples, we plotted the *TNFA* messenger level of each individual gingival biopsy against its percentage methylation at those 3 CpG sites. Samples from both gingival tissue groups are shown on this plot (figure 4.2C) to demonstrate a significant but inverse association between promoter methylation level at -163bp and mRNA expression of *TNFA,* and such a significant association is only present for this site (r=0.16,p=0.018, figure 4.2C).

For those self-controlled induced gingivitis participants, though no significant difference of overall methylation combing all studied CpG dinucleotides can be detected within the *TNFA* promoter region between samples collected from different phases (data not shown), methylation level at -244bp is significantly lower in the induced phase than in the resolved phase (46.7$\pm$6.7% vs. 52.4$\pm$6.1%, p=0.01, figure 4.3A). This reduced methylation level reminds us a similar hypomethylation pattern at the same CpG site identified in the chronically inflamed gingival samples as shown in figure 4.2A. A trend of increased *TNFA* messenger level, though not significantly different, is observed in induced phase of experimental gingivitis compared to the resolved phase (p=0.06, figure 4.3B).

Promoter methylation and *TNFA* transcription change of THP-1 challenged by *C. rectus* 314:

After the alteration of *TNFA* promoter methylation was detected in the clinical gingival samples, we further want to identify whether the methylation change is related to the presence of periodontal pathogens using a co-culture cell model. In this model, THP-1 cells, which is a monocytic cell line that can readily produce TNF-α upon challenge LPS, were stimulated by *C. rectus* 314, a periodontal pathogen that was shown to be involved in the epigenetic regulation of *IGF2* in the placenta tissue from a systemic infection mouse model (28). With a MOI 100, we could not find any significant overall methylation change at early time points (data not shown). However, after 96 hours a significant hypomethylation profile in the *TNFA* promoter was identified in the live *C. rectus 314* challenged THP-1 cells [14% (10.1%, 25.5%), shown as median and interquartile range (IQR)] compared to mock challenged cells [21.8% (17.8%, 27.4%), p=0.013, figure 4.4A]. Although the

TNFA promoter methylation level in THP-1 cells challenged with heat-killed *C. rectus 314* decreased [16.0% (13%, 27.5)] at the same time point, it was not significantly different from either mock or live *C. rectus* challenged cells (figure 4.4A).

Upon challenge by *C. rectus*, the methylation level at one specific CpG site, -72bp, within *TNFA* promoter region exhibited a progressive demethylation at different time points after one hour post challenge (figure 4.4B). Therefore, besides overall decreased methylation change, continuous methylation loss at specific CpG site within *TNFA* promoter region is also present in THP-1 cells upon oral pathogen challenge.

We next examined the messenger level of *TNFA* as DNA methylation is a regulatory mechanism for gene transcription. Although TNF-α is among the early responsive inflammatory mediators challenged by LPS, its mRNA level of THP-1 cells exhibited a 4.1-fold induction 96 hours post *C. rectus* challenge in comparison to mock challenged cells (figure 4.4C).

DNA methylation alteration within *TNFA* promoter region upon 5-azacytidine treatment:

Although we found a hypomethylation either at specific site or in overall promoter region and a higher transcriptional level of *TNFA*, it does not necessarily indicate that promoter hypomethylation of *TNFA* is contributable to the induction of *TNFA* transcription. In order to identify the effect of hypomethylation on *TNFA* gene expression, we used a DNA methylation inhibitor, 5-azacytidine, to treat THP-1 cells and studied its transcriptional change. 5-azacytidine treatment increased baseline *TNFA* expression in a time dependent manner. After 24 hours treatment, messenger level of *TNFA* was induced 1.6-fold as compared to mock treated cells, while the induction level is increased to 3-fold after 96 hours treatment (figure 4.5A). The treatment of this non-specific DNA inhibitor resulted in a general reduction of DNA methylation level at almost all the 10 CpG dinucleotides, with 5 of them showing significant decrease (figure 4.5B). To test whether demethylated promoter region can increase the responsiveness to periodontal pathogens by elevating

the transcriptional expression of *TNFA*, we challenged pre-treated THP-1 cells with *C. rectus* 314 for half an hour, a duration not long enough to cause DNA methylation changes by this periodontal pathogen. A 1.6-fold induction is identified in the pre-treated cells as compared to cells treated with diluents. Therefore, a hypomethylation pattern of *TNFA* promoter region increased the responsiveness of THP-1 cells to *C. rectus*.

The effects of methylation status of *TNFA* promoter on gene transcription:

To investigate the direct effect of the *TNFA* promoter methylation on its transcription, we measured the luciferase activities in THP-1 cells transfected with reporter construct containing either mock methylated or *in vitro* methylated *TNFA* promoter region. We first cloned the *TNFA* promoter region ranging from -291bp to +44bp that includes all the CpG sites that were analyzed with only one extra CpG site being downstream the TSS. After transfection into a mouse monocytic cell line, the luciferase activity of the reporter construct was induced by LPS. The pGL3-PTNFA291 showed sufficient promoter activity upon induction (figure 4.6A), which proved the promoter activity of this cloned fragment. We then either *in vitro* methylated or mock methylated this cloned *TNFA* promoter and confirmed the methylation modification by digesting the fragment with methylation sensitive restrictive endonuclease. After transfection of the reporter construct containing either mock or *in vitro* methylated *TNFA* promoter fragment and induction by LPS, we observed that the promoter activity of *in vitro* methylated construct was only 35% of the mock modified construct ($p=0.03$, figure 4.6C).

Discussion

Cell signaling networks are involved in the transcriptional control of *TNFA* in inflammatory and infectious diseases. Although transcription of *TNFA* can be transiently induced upon challenge by bacterial structures such as LPS, the persistence of microbe flora present in the dental biofilm may regulate its expression by altering the local chromatin structure of inflammatory genes including *TNFA*. As the most stable epigenetic modification, the regulation by DNA methylation is highly dynamic and connects with histone modifications to modulate the binding between transcriptional factors mobilized through signaling pathways and their cognate recognition sites in the genomic DNA(235). From chronic periodontitis gingival biopsies, both hypo- and hyper- methylation pattern exist in the *TNFA* promoter region (figure 4.2A). However, only the methylation level at site -163bp is correlated with the transcription of *TNFA* in both periodontally healthy and periodontitis samples. Interestingly, CpG site at -244bp, whose methylation level is significantly decreased in the chronic periodontitis samples, was also hypomethylated and the only site exhibiting differential methylation level across the promoter region in the GI samples as compared to samples from GR. It seems that the methylation pattern of *TNFA* promoter is affected by disease stages. If the pathogenic effects are eliminated soon enough before the dieases progresses into an advanced lesion, the methylation change is slight and reversible; otherwise, not only are the epigenetic marks resulted from gingivitis maintained but new modifications unique to the chronic state of periodontal disease presented as chronic periodontitis will occur. This evolution of methylation pattern at key CpG sites within *TNFA* promoter region may reflect the transition from a mild and reversible periodontal inflammation to a more advanced and relatively irreversible stage. The methylation pattern at key promoter CpG sites that are involved in other physiopahologies has been also reported by different groups. For example, Camion *et al*., found that the methylation levels at -169bp and -119bp within *TNFA* promoter region were associated with successful weight loss in the obese males (236). The methylation status of one key CpG site within the second CpG island in the promoter region of *15-LO-1* is counterintuitively

related to its transcriptional activation in several prostate cell lines (229) . Murayama *et al*. proved that the demethylation at CpG site 1 within the promoter region of human *IL4* is required for IL-4 expression in CD 4+ cells (270). The difference of identified key CpG sites of methylation between their studies and the current one may be due to the different diseases in question.

The identification of key CpG sites within promoter region whose methylation levels are significantly different among clinical samples representing different stage of periodontal diseases renders us to ask whether methylation alteration is related to the presence of periodontal pathogens. To achieve this end, we utilized an *in vitro* co-culture system to test whether *TNFA* promoter region in a monocytic cell line can be modulated by a pathogenic oral bacterial strain, *C. rectus* 314. Although TNF-α is among the early response gene in monocytes upon challenge by bacterial product, such as LPS, the increase of *TNFA* transcription in THP-1 cells is maintained as long as 96 hours after co-cultured with live *C. rectus* 314. Interestingly, an overall hypomethylation pattern in the promoter region was evident at 96 hours only after the cells were challenged with live bacteria, indicating the delayed involvement of DNA methylation in the regulation of *TNFA* transcription upon *C. rectus* 314 stimulation. It is also noted that a non-significant decrease of the overall methylation was found in cells challenged by heat-killed *C. rectus 314*. It is possibly because the preserved bacterial structures in heat-killed *C. rectus*, such as LPS or fimbrae, account for this change, while a more significant change may necessitate the presence of live bacteria. The epigenetic regulation of mammalian gene promoter regions by bacteria has been studied by several groups. Yao et al. reported that a hypermethylation of *hMLH1* promoter region in a gastric cell line was induced by a persistent *Helicobacter pylori (H. pylori)* stimulation as early as 4 days(237). Another study also identified a hypermethylated *E-cadherin* promoter region in several gastric cancer cell lines upon long term (2-4 days) challenge by *H. pylori*(238). Similarly, the methylation status of promoter regions of *USF1* and *USF2*, which are pleiotropic transcriptional regulators of immune responses, was also modulated by the presence of *H. pylori* in a gastric epithelial cell line(239). Because *H. pylori* infection has been

shown to be closely associated with aberrant DNA methylation in the gastric mucosa and it is phylogenetically similar to *C. rectus*, it is not surprising that this periodontal pathogen possess the capacity of modulating expression of inflammatory cytokines through epigenetic mechanisms (240)(241).

In our *in vitro* study, the overall methylation decrease within analyzed *TNFA* promoter region and a site-specific progressive demethylation paralleling cell cycle, was supported by a recent paper reporting that the expression of human β-defensin 2 (*BD2*) and CC chemokine ligand 20 (*CCL20*) in oral epithelial cells can be epigenetically regulated by another periodontal pathogen, *Porphyromonas gingivalis* (*P. gingivilis*), and a nonpathogen *Fusobacterium nucleatum* (*F. nucleatum*) in a co-culture system(127). In that study, the authors found that the transcriptional of *DNMT1* and histone deacetylases (*HDAC*), was inhibited in gingival epithelial cells with the presence of those bacteria. Because *DNMT1* is the major form of DNA methyltransferase, which is mainly responsible for maintaining newly synthesized DNA strand, and works coordinately with *HDACs* to enforce a close chromatin structure around gene promoter, the decreased expression of those enzymes may relieve the gene promoters from an inhibitory chromatin structure and, thus, activate transcription process. We also noticed that the site at -72bp exhibiting continuous methylation loss is different from the key CpG sites found in clinical samples (-244bp or -163bp). The mixed cell types and the presence of a dynamic biofilm-gingival interface interaction may explain the difference of this site-specific DNA methylation pattern within *TNFA* promoter region.

Several transcriptional factors binding sites, such as NF-κB, Sp1, Ap1, Ap2, etc., are located within the analyzed promoter region just above TSS (figure 4.1). The presence of those binding sites indicates the importance of this promoter region in transcription initiation. The luciferase activity reporter construct containing the unmodified upstream of *TNFA* proved the promoter activity of the cloned sequence. The methylation of CpG sites within or around those sites may affect the binding by those transcriptional factors and, thus, decrease the transcriptional activity. For example, the methylated cytosine at of -72bp, -49bp and -38bp that are either located within or close to the cognate

binding sites of Ap1, Ap2, and Sp1 significantly inhibited the binding to those transcriptional factors(242). In order to further understand how methylation status of *TNFA* promoter region affects its transcription, we first treated cells with DNA methylation inhibitor. A general decrease of methylation within *TNFA* promoter in THP-1 cells treated by 5-azacytidine results in a higher transcription level in a time-dependent manner. This suggests that, in addition to other mechanisms that activates *TNFA* transcription in the presence of live periodontal pathogens, hypomethylation alone within *TNFA* promoter region can result in an elevated transcriptional level. Someone may argue that a global demethylation due to the continuous treatment by a DNA methylation inhibitor may not be specifically linked to *TNFA* transcriptional activation since higher transcription can be a secondary effect of 5-azacytidine treatment. In addressing this issue, we compared luciferase activities from reporter constructs containing either methylated or mock methylated promoter region of *TNFA*. This promoter-specific methylation luciferase assay further proved that the transcription of *TNFA* can be negatively regulated by promoter DNA methylation level.

In this study, we analyzed the regulation by DNA methylation in *TNFA* promoter region either in gingival biopsies with periodontal diseases and cell-perioodntal pathogens interactions. In conclusion, although a decreased methylation level at site -244bp cytosine within *TNFA* promoter region is related to a trend of increased transcription of this inflammatory gene in experimentally induced gingivitis biopsies, a hypermethylated CpG dinucleotides at site -163bp identified in the chronic periodontitis biopsied tissues, which also retained the decreased methylation change at -244bp possibly resulted from early stage of periodontal disease, is inversely associated with its transcription in both periodontitis and periodontal health samples. Exposure of Monocytic cells to a periodontal pathogen caused an overall decreased methylation pattern in *TNFA* promoter region and a unique progressive demethylation at site -72bp that are mechanistically related to higher *TNFA* transcription.

Table 4.1. Oligonucleotides used for bisulfite specific PCR and pyrosequencing

CpG site (*)	Details	Sequences	Annealing T (°C)
	Forward	5'-TAGGTTTTGAGGGGTATGGG -3'	
-244, -238	Reverse	5'-[Biotin]TCAAAAATACCCCTCACACTCC -3'	62
	Sequencer	5'-GTTAGTGGTTTAGAAGATTT-3'	
-169, -163,	Forward	5'-[Biotin]GAGTGTGAGGGGTATTTTTGATG -3'	
-161	Reverse	5'-GCAACCATAATAAACCCTACACCTTC-3'	63
	Sequencer	5'-AAACCCTACACCTTCTATCT -3'	
	Forward	5'-GAGGGGTATTTTTGATGTTTGTGT -3'	61
-146, -119	Reverse	5'-[Biotin] CAACCAACCAAAAACTTCCTTAAT-3	
	Sequencer	5'-TTTAGAGATGGAGAAGAAA-3'	
	Forward	5'-GAGGGGTATTTTTGATGTTTGTGT-3'	63
-72, -49, -38	Reverse	5'-[Biotin]CCAACAACTACCTTTATATATCCC -3	
	Sequencer	5'-TTATGGGTTTTTTTATTAAG-3'	

*CpG sites indicate nucleotide position in relation to transcription start.
"−" or "+", indicates upstream or downstream of transcription start, respectively.

Table 4.2 Demographic information of the participants and clinical parameters in the biopsied gingival sites

Demographic/ Clinical parameters	Periodontal Health (n=17)	Periodontitis (n=18)	Gingivitis Induced (n=11)	Gingivitis Resolved
Mean age (years)	40.9± 13.5	48.7±8.7	36.8±9.7	
Gender Males/Females	5/12	11/7	5/6	
Probing Depth (Mean±SD, mm)	1.9±0.9	5.7±1.1**	2.4±0.3	2.1±0.2
Clinic Attachment Level (Mean± SD, mm)	0.9±0.6	4.1+1.0**	1.1±0.7	1.3±0.6
Alveolar bone loss	No	Yes	No	

"**" indicates $p<0.001$ as compared with periodontal health

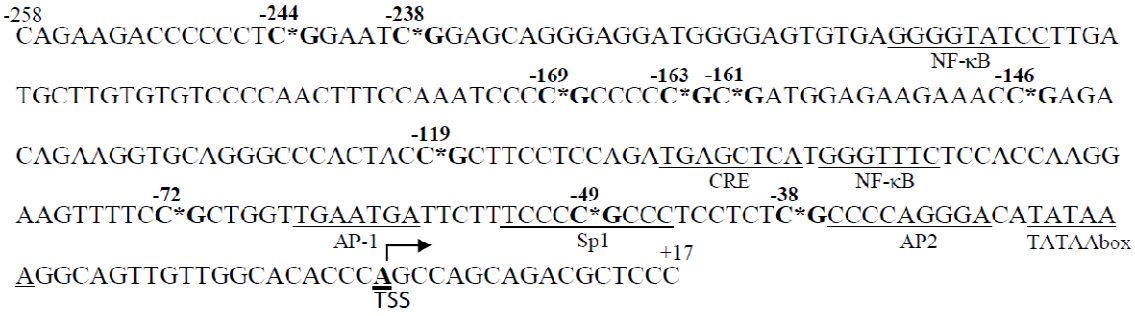
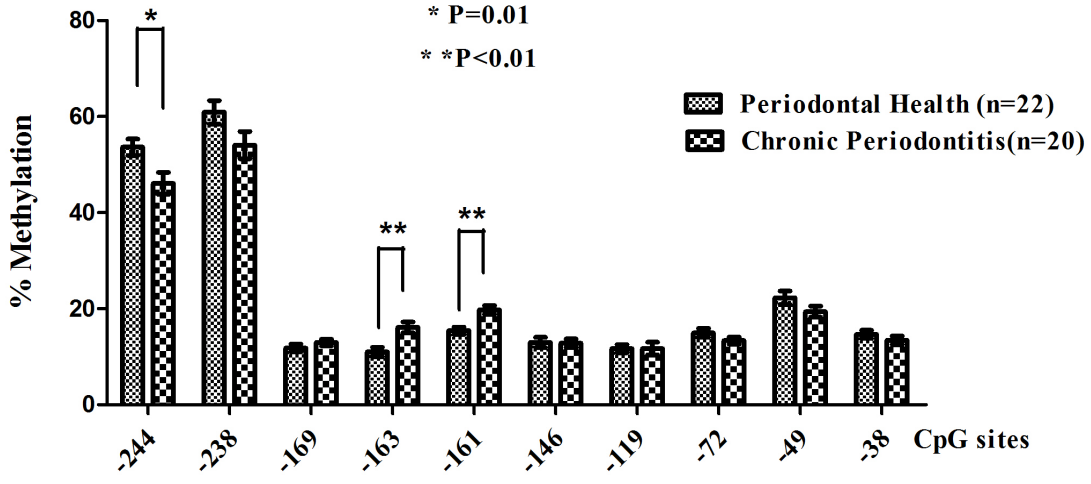
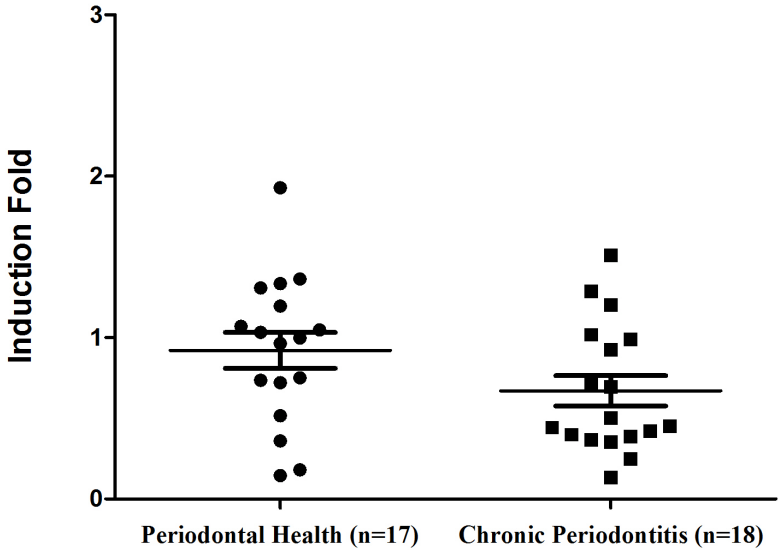


Figure 4.1. Genomic sequence of *TNFA* promoter fragment. Sequence ranging from -258bp to +17bp relative to transcription start site (TSS) before bisulfite modification is presented. This promoter fragment just upstream of TSS contains ten CpG dinucleotides that are marked with "*" and in bold. Their position in relation to TSS is also indicated above. Sites for potential transcriptional factor binding and TATA box are underlined.

A


* P=0.01
* *P<0.01
Periodontal Health (n=22)
Chronic Periodontitis(n=20)
% Methylation
80
60
40
20
0
*
**
**
-244
-238
-169
-163
-161
-146
-119
-72
-49
-38
CpG sites

B


Induction Fold
3
2
1
0
Periodontal Health (n=17)
Chronic Periodontitis (n=18)

C

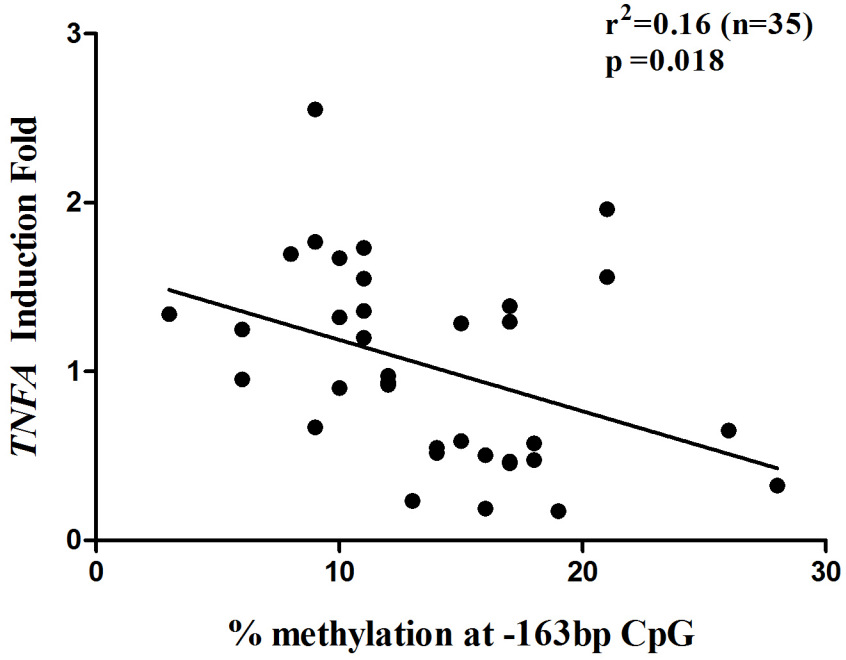


Figure 4.2. Methylation level of *TNF*A promoter region as well as transcriptional level of *TNFA* in clinical gingival biopsies collected from sites either with chronic periodontitis or periodontal health. (Figure 4.2A) The percentage methylation of each individual CpG dinucleotide from chronic periodontitis tissues is compared with gingival tissues with periodontal health. "*" indicates significantly lower methylation level at site -244bp in chronic periodontitis samples compared to samples with periodontal health (p=0.01); "**" indicates significantly higher methylation level at sites -163bp and -161bp in chronic periodontitis samples compared to periodontal health (p<0.01 for both sites). (Figure 4.2B) Individual *TNFA* transcriptional expression from periodontitis biopsies (shown as solid circle) is compared with gingival biopsies with periodontal health. The transcription of *TNFA* is lower in periodontitis sample but not significantly different from the samples with periodontal health (p=0.08). (Figure 4.3C) The messenger level of *TNFA* of individual sample from both periodontitis group and periodontal health group is plotted against its methylation level at site -163bp. Regression analysis indicates that the transcriptional level of *TNFA* is significantly and inversely related to the methylation level at -163bp (r=0.16, p=0.018).

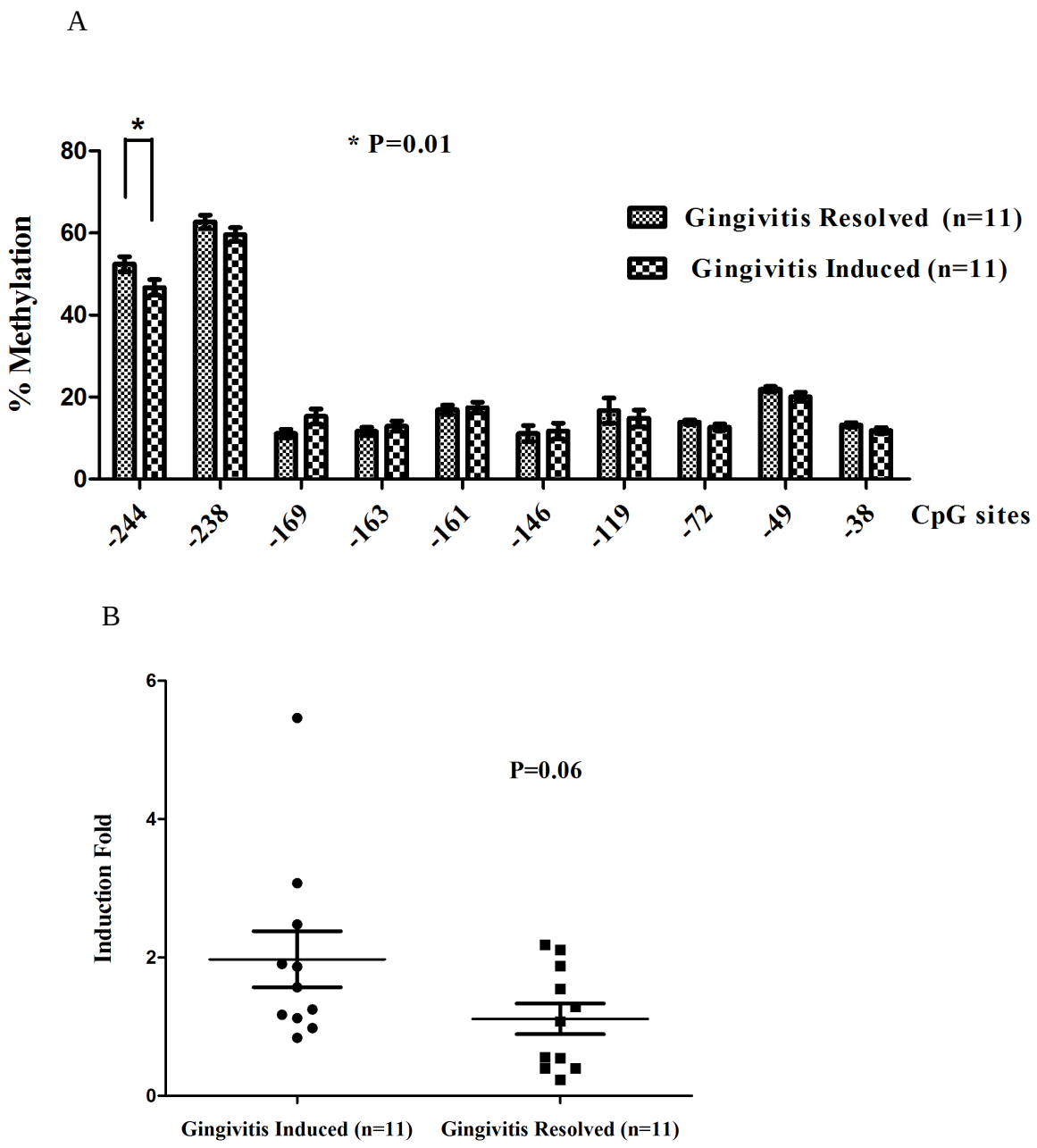
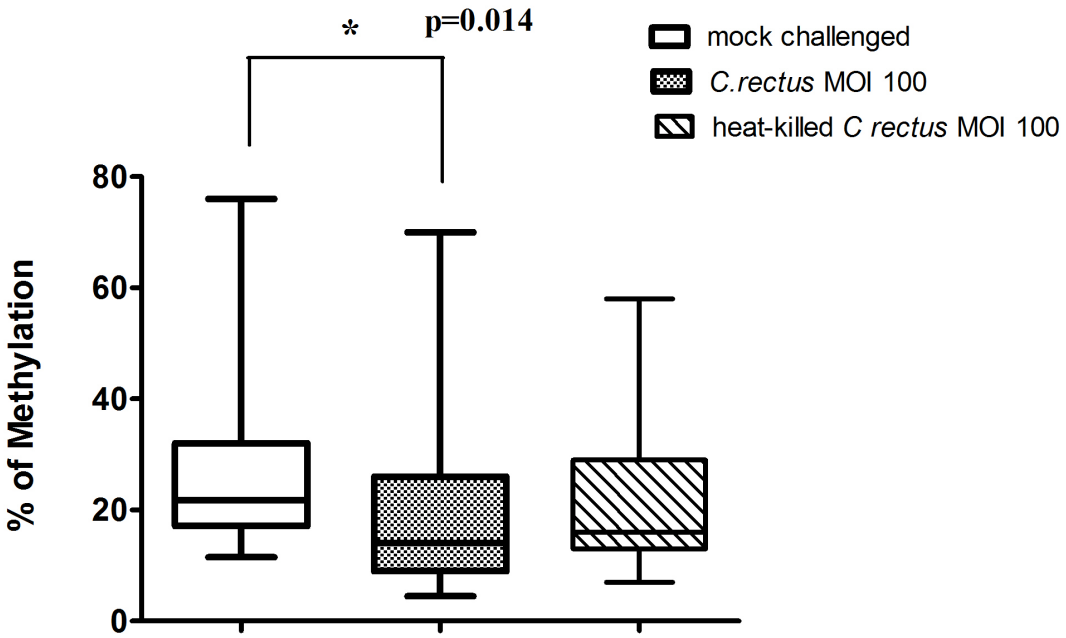
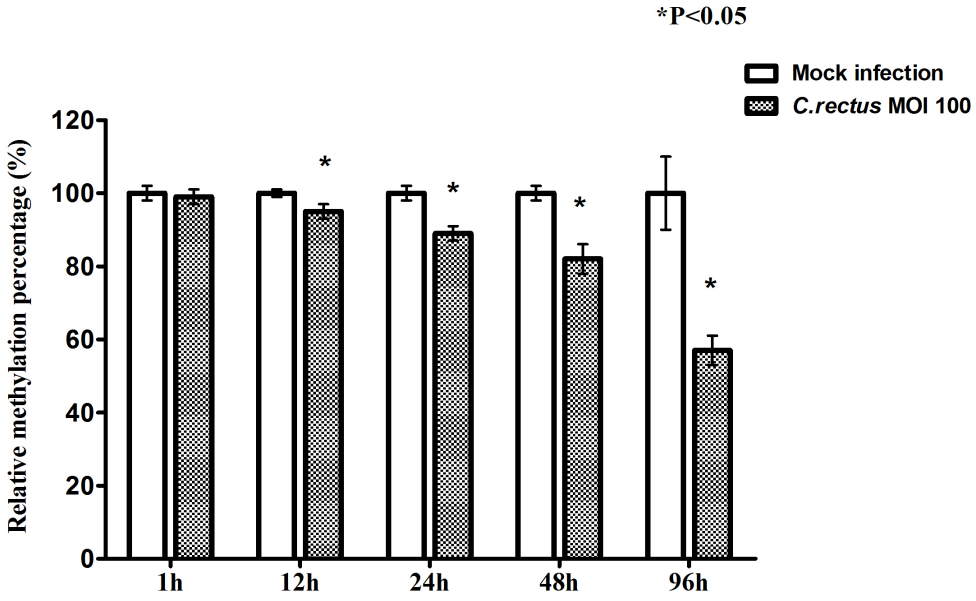


Figure 4.3. Methylation level of *TNF*A promoter region and transcriptional level of *TNFA* in biopsies collected from sites exhibiting experimentally induced gingivitis and gingivitis resolved. (Figure 4.3A) The percentage methylation of each individual CpG dinucleotide from experimentally induced gingivitis is compared with gingival tissues with gingivitis resorption. "*" indicates significantly lower methylation level at site -244bp in gingivitis samples compared to samples collected from resolved phase (p=0.01). (Figure 4.3B) Individual *TNFA* transcriptional expression from the induced phase of gingivitis biopsies (solid circles) is higher but not significantly different from the self-controlled resolved phase of gingivitis (solid squares) (p=0.06).

A


*
p=0.014
mock challenged
C.rectus MOI 100
heat-killed C rectus MOI 100
% of Methylation
80
60
40
20
0

B


*P<0.05
Mock infection
C.rectus MOI 100
Relative methylation percentage (%)
120
100
80
60
40
20
0
*
*
*
*
1h
12h
24h
48h
96h

C

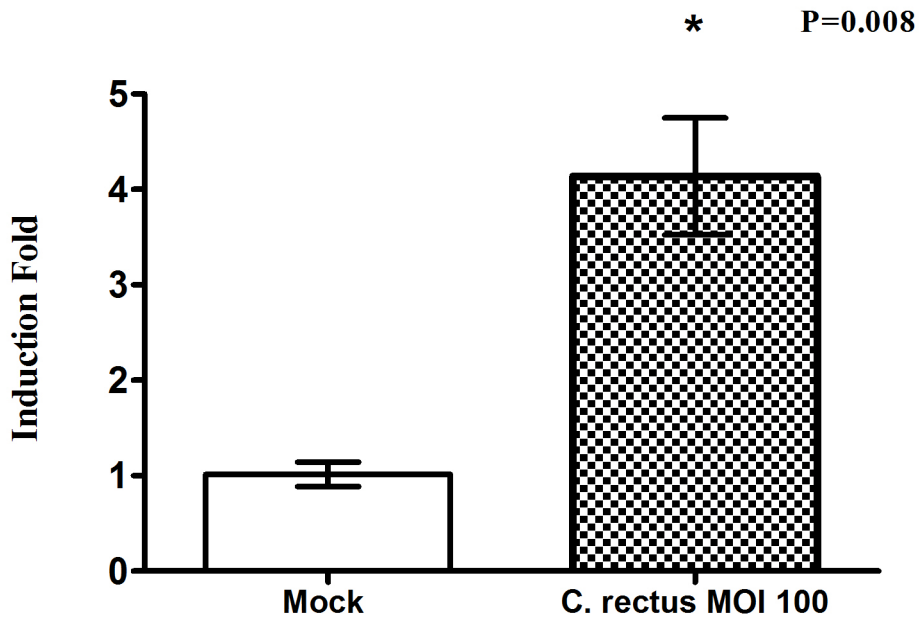
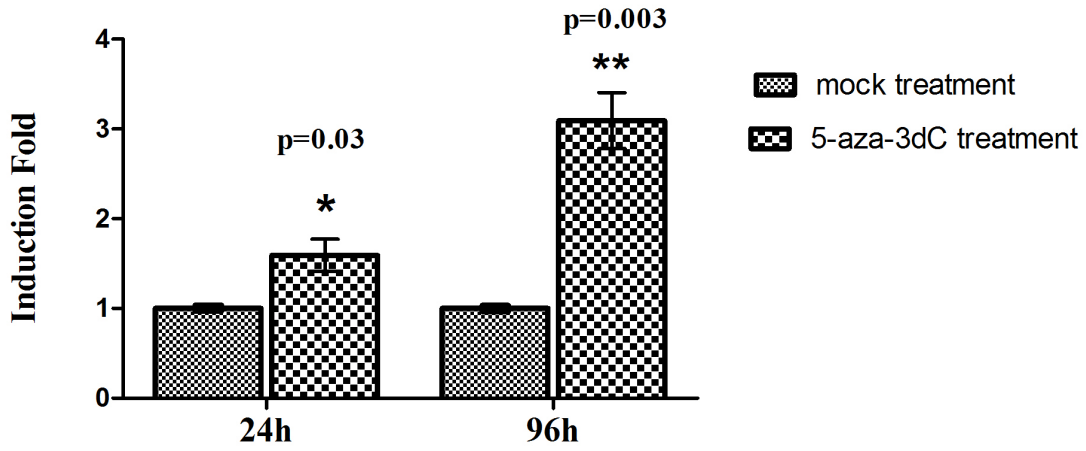
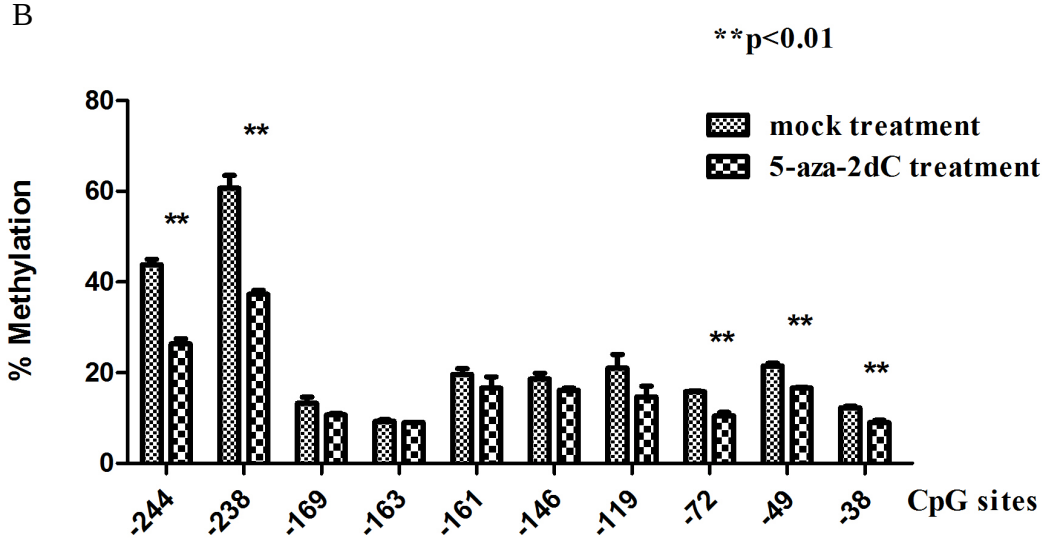


Figure 4.4. Methylation alteration of *TNFA* promoter and transcriptional expression of *TNFA* in THP-1 cells co-cultured with *C. rectus 314.* (Figure 4.4A) After 96 hours post challenge, the overall methylation level of the ten CpG sites within *TNFA* promote in THP-1 cells challenged by live *C. rectus 314* showed a significant decrease as compared to mock challenged cells (p=0.014, indicated by "*"). Although *TNFA* promoter methylation level also decreased in heat-killed *C. rectus* treated cells in comparison to mock challenged cells, there is no statistical difference between those treatment (p=0.12). (Figure 4.4B) Methylation level at -72bp in live C. rectus 314 challenged cells, which was normalized to the level of mock-challenged cells, is significantly lower than the mock-challenged cells at all the time points except 1 hour. "*" indicates statistical difference ($p<0.05$). (Figure 4.4C) The transcriptional level of *TNFA* in live *C. rectus* challenged THP-1 cells is higher than the mock challenged cells at 96 hours (p=0.008, as indicated by "*").

A


p=0.003
**
p=0.03
*
Induction Fold
4
3
2
1
0
24h
96h
mock treatment
5-aza-3dC treatment

B


**p<0.01
mock treatment
5-aza-2dC treatment
% Methylation
80
60
40
20
0
**
**
**
**
**
-244
-238
-169
-163
-161
-146
-119
-72
-49
-38
CpG sites

C

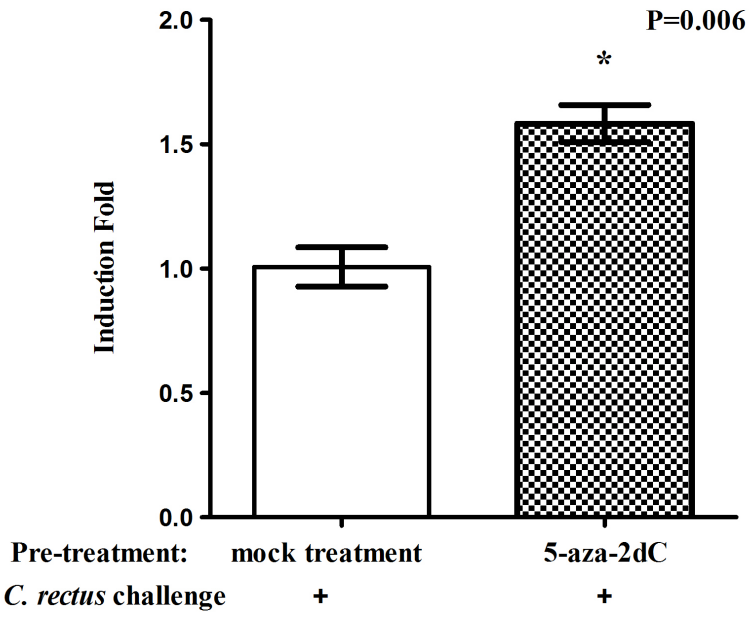


Figure 4.5. Methylation of *TNFA* promoter and its transcription in THP-1 cells treated with 5-aza-2dC. (Figure 4.5A) Cells treated with 5-aza-2dC exhibited an increase of TNFA transcription in a time-dependent manner in comparison to mock treated cells ("*" indicates p=0.03 and "**" indicates p=0.003). (Figure 4.5B) A general reduction of methylation level at CpG sites of *TNFA* promoter region is present in cells treated with 5-aza-2dC, with 5 CpG sites at -244bp, -238bp, -72bp, -49 and -38bp showing significant decrease, as compared to mock treated cells ("**" indicates $p<0.01$). (Figure 4.5C) THP-1 cells pretreated with 5-aza-2dC are more responsive to C. rectus challenge by increasing *TNFA* transcription (p=0.03, as indicated by "*").

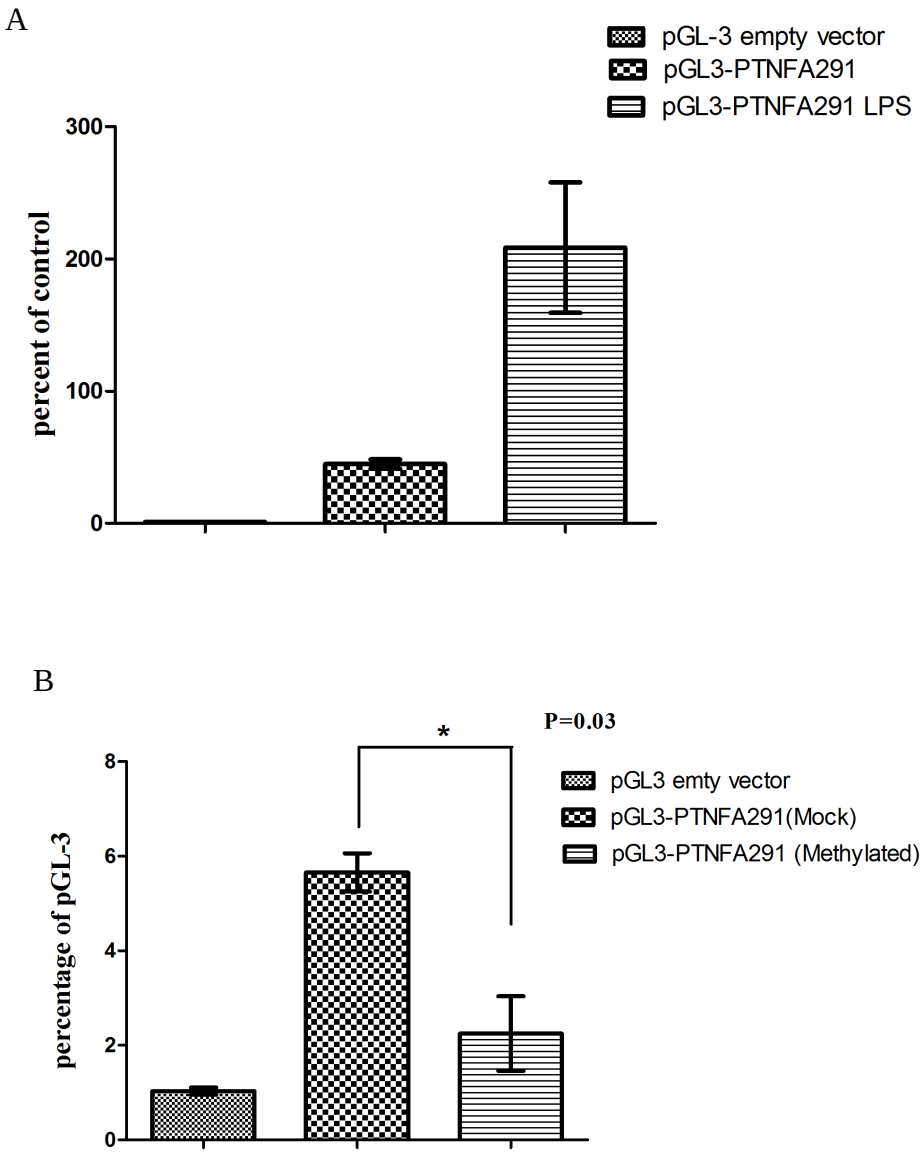


Figure 4.6. Activity of luciferase reporter construct containing either unmodified or modified *TNFA* promoter fragment ranging from -291bp to +44bp relative to TSS. (Figure 4.6A) Cells transfected with pGL-3PTNFA291 construct showed increased luciferase activity upon induction by LPS as compared to uninduced construct. Lucfirase activity is relative to the empty vector. (Figure 4.6B) Cells transfected with in vitro methylated pGL-3PTNFA291showed a significantly decreased luciferase activity as compared to cells transfected with a mock methylated pGL-3PTNFA291 upon LPS induction (*p=0.03).

Chapter 5

Discussion

Discussion

Periodontal diseases include gingivitis, an early and less severe form of gingival inflammation characterized by red and/or swollen gingiva and bleeding upon probing, and periodontitis, a more advanced and destructive inflammation causing loss of tooth-supporting structures such as alveolar bone (243). Although periodontal diseases are infectious diseases initiated by periodontal pathogens present in the dental biofilm and plaques, the destruction of soft and mineralized periodontal tissue is actually more contributable to the inflammatory immune responses by the host mobilized to fight against those pathogens (244) (243) (245). In the pathogenesis of periodontal diseases, both innate and adaptive immune responses are critically involved, and the roles played by those immune responses depend on the stage of disease progression. For examples, infiltrates of innate-immunity competent cells such as polymorphonuclear neutrophils and monocytes/macrophages are typically seen in the initial or early stage of periodontal diseases, while T or B lymphocytes are the dominating cell populations present in an established or advanced lesion (246). However, recent studies have clearly indicated that immune responses that are involved in tissue destruction are also critical to controlling periodontal infection (247)(248). Therefore, balanced production of cytokines, chemokines, growth factors, enzymes, vasoactive small molecules, *etc*., controlled by a fine-tuned regulation network is necessary to keep homeostasis of periodontum irrespective of disease status.

Once secretion of proinlfammatory cytokines surpasses “a critical level”, a protective response will transform into a pathological one (249). Although the level of TNF-α either in GCF or in gingival tissues from the periodontitis lesion has been reported to be increased, the transcription of this proinflammatory molecule in the current study from the periodontitis sites did not show such an elevation in comparison to gingival biopsies with periodontal health. Actually, a trend of lower transcriptional expression is evident in those periodontitis tissues (figure 4.2B). Interestingly, such a non-significant reduction of *PTGS2* transcription is also seen in those periodontitis biopsy samples as compared to control periodontal health (figure 2.3A). Such a concordant trend for both *TNFA* and

PTGS2 transcription is supported by studies demonstrating that TNF-α is a potent inducer for COX-2 expression in macrophages and fibroblasts from gingivae and temporomandibular joint (250)(251)(252). It is generally believed that primary inflammatory cytokines, such as TNF-α or IL-1B, stimulate the production of secondary response cytokines such as PGE_2 by upregulating COX-2, which is the rate-limiting enzyme for prostaglandins (119)(249). This failed induction of *TNFA* and *PTGS2* in those chronic periodontitis gingival tissues may be due to the shift of a metastable stead-state of cytokine production. All those periodontitis tissues were collected from the chronically inflamed gingival sites with deep pocket, which reflects a historical episode of disease activity. After initial periodontal therapy such as root planning and scaling, the inflammation in those biopsied tissues is most likely in a quiescent, rather than an advanced progressive state. During the prolong battle between periodontal pathogens and host defense, the body may develop a "protective" mechanism that resets the equilibrium of those molecule production to avoid an uninhibited insults by cytokines or enzymes.

DNA methylation may be part of this mechanism that is involved in the control of *TNFA* and *PTGS2* transcription in periodontally diseased gingival tissues. Epigenetic regulatory mechanisms translate various environmental stimuli into different forms of modification in either DNA or key residues of histone molecules. Those modifications build the suitable platform by altering chromatin structure for the bindings of transcriptional factors to their cognate sites in DNA. In both *TNFA* and *PTGS2* promoter region, we identified the altered methylation patterns in periodontally diseased samples compared to non-diseased tissues. However, the observed methylation alterations are different for both promoter regions possibly due to their different promoter structures. The promoter region of *PTGS2* contains a CpG island, in which a general but low level of methylation is present in periodontitis samples but not in control samples (figure 2.2C), while the *TNFA* promoter region does not possess such a CpG island and showed both hypo- and hyper- methylation patterns at different CpG sites (figure 4.2A). A general lack of methylated cytosines within *PTGS2* CpG island identified in non-diseased periodontal tissues (figure 2.2B) suggests a low nucleosome occupancy that favors

chromatin remodeling to an activated state (253)(254). In this sense, a general increase of methylation in this CpG island may lead to an inhibitory chromatin remodeling that does not favor transcription activity. In spite of the absence of a CpG island in *TNFA* promoter, the methylation level of CpG sites ranging from -169bp to -38bp is much lower in both periodontitis samples or samples with periodontal health (< 20%) than the methylation level at -244bp and -238bp (around 60%). This observed methylation pattern from this study is also seen in peripheral blood mononuclear cells (255). It is also reported by another group that such a low methylation level extends into the first exon at least around CpG site +310bp in TNF-α competent cells (256). Therefore, it is reasonable to assume that nucleosome occupancy is also relatively low around this region and an increase of methylation at CpG sites within this region may negatively affect the *TNFA* transcription efficiency. Methylation changes at key sites within those low-nucleosome occupancy promoter regions, such as CpG site at -458bp within *PTGS2* promoter or site -163bp within *TNFA* promoter in clinical samples, may have a greater effect on the binding by transcriptional factors, and thus, are negatively related to its transcription (figure 2.3B, figure 4.2C).

Another explanation for this failed induction of those inflammatory molecules, especially *TNFA*, in chronic periodontitis gingival tissues may be attributable to a tolerance mechanism. Using RT-PCR and immunostaining, Muthukuru *et al*. found that the transcriptional level of TLR2 and TLR4 decreased 30-fold and nine-fold, respectively, in chronic periodontitis gingival samples compared to periodontal health, though more TLR2 and TLR4 positive cells were present in the inflamed gingival samples (257). Rechallenging LPS pre-treated peripheral blood monocytes with LPS again, the same group observed a 10-fold reduction of *TNFA* transcription as well as decreased production of TLR2, TLR4, IL-1B, and other proinflammatory cytokines. The abundance of highly tolerable macrophages present in the infiltrate of a stable and chronically inflamed gingival lesion may thus contribute to the absence of *TNFA* induction in our periodontitis tissues.

This tolerant mechanism, however, has also been closely related to epigenetic modifications. Gazzar *et al*. proposed a tolerance model for *TNFA* transcription in which in LPS tolerated cells

DNMT3a/3b was recruited into the promoter region through HP1, an adaptor protein that binds to the di-methylated lysine 9 H3 catalyzed by G9a, a mono- or di- lysine methyltransferase (258). The recruited DNMT3a/3b further reinforces this inhibitory chromatin structure by adding methyl- group into cytosines within the *TNFA* promoter region. This group also found that the enrichment of inhibitory epigenetic marks in the tolerated *TNFA* promoter region excluded the binding by NF-κB P65 to *TNFA* promoter region but increased the binding of RelB, which is a NF-κB repressor (259). Therefore, this mechanism links epigenetic remodeling in the local chromatin structure of *TNFA* promoter region with an important signaling pathways mediated by NF-κB.

Similar studies aiming at untangling the relationship between periodontal diseases and methylation alteration of inflammatory cytokine genes came out just after our data were published. For example, in a most recent paper, an increased DNA methylation pattern in *PTGS2* promoter in periodontitis patients compared to non-periodontitis control subjects from a larger subject pool also confirmed our findings (260). They even found the hypermethylation occurs more frequent in periodontitis samples than in breast cancer biopsies. A higher frequency of hypomethylation of *IL8* promoter region was found in oral epithelial cells from chronic periodontitis patients independent of smoking habit (126). The same group later found that patients with generalized aggressive periodontitis have a higher frequency of hypomethylation of *IL8* promoter than in periodontally healthy controls (261).

In the *in vitro* co-culture model, we observed an overall hypomethylation in cells challenged by live *C. rectus* after 96 hours (Figure 4.4A). A progressive methylation loss at -72bp was also identified in THP-1 cells co-cultured with *C. rectus* (figure 4.4 B). This overall hypomethylation and site-specific loss of methylation is related to an elevation of *TNFA* transcription. Although TNF-α production is among the primary response of inflammation, this observed hypomethylation pattern may contribute to the sustaining of increased transcription in a late time course. The identified pattern of methylation change in co-culture model is different from the change observed in clinical samples. This is possibly because the epigenetic alteration in the *TNFA* promoter region induced by one

periodontal pathogen in a single cell type does not necessarily reflect the overall effect of bacteria flora present in the biofilm on mixed cell populations seen in the gingival tissue. However, we confirmed that *C. rectus 314* is capable of altering epigenome in host genes. Previously, using a mouse model, we found that the *ifg2* P0 promoter region was hypermethylated at several sites in placenta from the maternally infected mice by *C. rectus 314* compared to the mock infected mothers (262). In this co-culture model, we found that such an epigenetic modulating activity requires live *C. rectus* upon challenge. We also found that the overall methylation pattern also decreased in the heat-killed *C. rectus*, though not significantly different. Because bacterial structural components, such as LPS and fregellae, are still preserved in the heat-killed bacteria, the observed decrease of methylation may be possibly related to those preserved components. Therefore, the capacity of *C. rectus 314* to modulate promoter methylation is related to its metabolism or virulence factors. It is important to point out that *C. rectus* is phylogenetically similar to another anaerobic gram negative bacterium, *H. pylori*, a known gastric pathogen that can modify DNA methylation pattern of mammalian gene promoters(263)(264). For example, they both share a 64-kDa antigen, GroEL protein (263). In addition to *H. pylori*, other bacteria are also found to regulate host gene expression through an epigenetic approach. For example, infection of *Anaplasma phagocytophilum*, an intracellular bacterium, can lead to silencing of dense genes by increasing HDAC1 expression, which results in H3 deacetylation and a more compact chromatin structure (265). The research on the mechanisms of how microorganisms affect host's epigenome is still in its infancy stage, and the results will greatly benefit our understanding towards the pathogenesis of infectious diseases.

Not only are innate immune responses involved in periodontal diseases, but adaptive immunity is another critical component that contributes to the pathogenesis of periodontal diseases. Lymphocytes and plasma cells are prevalently present in the infiltrate of periodontal lesions as early as the on-site of gingivitis, the reversible and early stage of periodontal diseases (246). Those identified cell types and the abundance of immunoglobulin in the periodontal lesion indicate the active participation of both T-cell and B-cell mediated adaptive immune responses (246)(266). By

immunofluorescence, we also identified more CD4+, CD56+ and CD11c+ cells in the infiltrate of periodontal lesions compared to gingival biopsies with periodontal health (figure 3.5). In agreement with this pathological finding, the transcriptional level of *IFNG* in the periodontally diseased samples was also elevated (figure 3.4). Both Th1 and Th2 responses are involved in the pathogenesis of periodontal diseases and related to the disease progression (266). The increase of *IFNG* in the periodontitis biopsies is not unexpected because those samples were collected from the gingivae undergone initial therapies and representing stable lesions. However, the methylation change within *INFG* promoter region in chronic periodontitis samples is different from experimentally induced gingivitis compared to gingival tissues with periodontal health (figure 3.3A and 3.3B). This difference of methylation pattern may indicate a late involvement of epigenetic mechanism in the regulation of *IFNG* transcriptional expression, while the elevation of *IFNG* messenger level in gingivitis samples is independent of DNA methylation. However, a recently published report with the similar aim of investigating the methylation alteration in chronically inflamed periodontitis samples couldn't find a significantly different level of methylation at two sites: -54bp and -186bp, though unmethylated samples are only present in the periodontitis group (267). In the current study, we did identify a significantly lower methylation level at site -54bp and a non-significant decrease of methylation at site -186bp in chronic periodontitis samples (figure 3.3A). The difference between their study and ours is probably because methylation-specific PCR, the method they used for methylation analysis, is semi-quantitative and less sensitive to discovering difference of relatively small magnitude compared to pyrosequencing. However, both studies agree that a demethylation pattern within *IFNG* promoter region may exist in samples with chronic periodontitis.

It is also noteworthy that a Th1 response as exhibited by a higher transcriptional level of *IFNG* in the chronic periodontitis samples echoes a reduction of messenger level of *PTGS2* identified among the chronic periodontitis samples in the current study. Studies have strongly supported an inhibitory effect of IFN-γ on *PTGS2* transcription in several cell types including human umbilical vascular endothelial cells (HUVEC), human foreskin fibrolasts, and U937, a monocytic cell line (268).

Reciprocally, the inhibition of Cox-2 or suppression of PGE_2 production also leads to the upregulation of INF-γ in NK cells (269). This mutual exclusive relationship between Cox-2, an enzyme playing import roles in alveolar bone destruction, and IFN-γ, a classic Th1 cytokine in adaptive immunity, may reflect a protective mechanism developed by the host aiming at resetting the homeostasis by favoring a cell-mediated immune responses in chronic inflamed but relatively stable periodontal lesions.

Although we identified alterations of DNA methylation within several gene promoters critically involved in either innate or adaptive immune responses in gingival biopsies with different stages of periodontal diseases, a direct mechanism linking promoter DNA methylation and transcriptional expression needs to be proved. We studied the epigenetic regulation of *TNFA* transcription in THP-1 cells to address this important mechanism. The general demethylation across the promoter region of *TNFA* and an increase of the messenger level of *TNFA* after 5-aza-2dC treatment provide the evidence to indicate that the methylation level of *TNFA* is inversely associated with its transcription (figure 4.5A and 4.5B). In addition, the decreased methylation level of *TNFA* promoter also increased the responsiveness of THP-1 cells to the challenge by periodontal pathogen. Such an increased messenger level of *TNFA* in treated cells compared to mock treated cells indicates that a permissive chromatin structure in the promoter region further facilitates the transcription of inflammatory genes. However, a globally decreased methylation level resulted from a non-specific inhibition of DNMT1 by 5-aza-2dC can also justify other explanations to the increased transcription of inflammatory genes. For example, this observed transcriptional increase can be due to a higher expression of molecules in the regulatory network of *TNFA*, which was actually caused by a hypomethylation upon 5-aza-2dC treatment in their promoters. In order to investigate the primary effect of methyl groups in the *TNFA* promoter on its transcription, we methylated the promoter fragment of *TNFA in vitro* and compared its luciferase activity with the mock methylated insert. A significant reduction of luciferase activity, which measures the constructed promoter activity, in

methylated *TNFA* promoter fragment showed direct evidence that promoter methylation of *TNFA* exerts a negative regulatory influence on its transcription.

In conclusion, in the current study we investigated the alteration of promoter methylation of inflammatory and immune response genes involved in the pathogenesis of periodontal diseases and their transcriptional expression using clinical samples. We have found that an increased methylation level of *PTGS2* promoter was present in chronically inflamed periodontitis samples as compared to non-inflamed gingival tissues with periodontal health. The methylation level at a specific CpG site (-458bp) within *PTGS2* promoter, which was higher than other CpG site within the same CpG island in both sample groups, was inversely associated with the transcriptional level of *PTGS2*. Those inflammation associated epigenetic changes in the local tissue were also evident within *TNFA* promoter in clinical samples with different stages of periodontal diseases. A decreased methylation level at site -244bp was found in gingival biopsies exhibiting experimentally induced gingivitis, which represents an early and a reversible stage of periodontal disease, compared to the tissues with gingivitis resolution. The maintenance of this hypomethylation pattern at the same site and the newly occurred increased methylation level at site -161bp and -163bp were characteristic epigenetic changes present in the samples with chronic periodontitis, an established and advanced stage of periodontal disease, in comparison to periodontal health. In addition, the hypermethylated CpG site at -163bp was also inversely related to the transcription of *TNFA* in both groups. Not only do epigenetic changes exist in promoter regions of genes mediating innate immune responses in the pathogenesis of periodontal diseases, the altered methylation pattern modulated by inflammation and/or microbial flora in the biofilm is also present in the genes involved in adaptive immunity. Although no significant difference of DNA methylation level was found within the promoter region of *IFNG* in the experimentally induced gingivitis biopsy samples from tissues with gingival health, an overall and a site-specific hypomethylation pattern were identified in the chronically inflamed periodontitis gingival samples. Such a hypomethylation pattern that was related to an increased transcriptional expression of *IFNG* in the periodontitis tissues may possibly reflect a dilution of the tissue DNA pool

by an influx of IFN-γ competent cells, such as CD4+ T cells, CD56+ NK cells and CD11C+ dendritic cells as indicated by immunofluorescence, with minimally methylated *IFNG* promoter region. The more pronounced DNA methylation changes as identified in the promoter regions of *TNFA* and *IFNG* at a more advanced rather than an early stage of periodontal diseases may possibly suggest a role of epigenetic regulation of inflammatory genes triggered by the accumulative effect of chronic exposure to the periodontal pathogenic stimuli. One limitation of the in vivo studies using gingival biopsies is the absence of profiling methylation changes in different cell types. The methylation levels of those inflammatory genes from those biopsy samples reflect the overall alterations of a mixed cell population contained in the biopsies. The larger alterations of methylation in certain cell types may be overwhelmed by the presence of other cell types that experience little epigenetic modifications within the promoter region of genes in question. Laser capture microdissection experiment should be employed to address this important question in the future study. In order to further investigate whether periodontal pathogens are related to the modulation of promoter methylation, we proceeded to analyze the promoter methylation changes in a monocytic cell line co-cultured with *C. rectus*. In contrast to the heat-killed pathogen, live *C. rectus* 314 challenged THP-1 cells exhibited a general hypomethylation within the promoter region of *TNFA* at a late time course and a progressive loss of methylation at the CpG site -72bp. The hypomethylation pattern is related to an increased transcription of *TNFA*. This finding suggests that live periodontal pathogen is capable of modulating host inflammatory genes in an epigenetic approach. At last, we studied the effect of DNA methylation on the transcriptional control of inflammatory genes. Both DNMT inhibitor treatment and promoter-specific methylation luciferase reporter assay confirmed that the methylation level of *TNFA* promoter negatively regulates its transcription.

References

1. Wierda RJ, Geutskens SB, Jukema JW, Quax PH & van den Elsen PJ (2010) Epigenetics in atherosclerosis and inflammation. J Cell Mol Med 14: 1225-1240.

2. Fraga MF, et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A 102: 10604-10609.

3. Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. Nature 447: 433-440.

4. Luger K, Mader AW, Richmond RK, Sargent DF & Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389: 251-260.

5. Jenuwein T & Allis CD (2001) Translating the histone code. Science 293: 1074-1080.

6. Kim JK, Samaranayake M & Pradhan S (2009) Epigenetic mechanisms in mammals. Cell Mol Life Sci 66: 596-612.

7. Riggs AD (1975) X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet 14: 9-25.

8. Holliday R & Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 187: 226-232.

9. Bayarsaihan D (2011) Epigenetic mechanisms in inflammation. J Dent Res 90: 9-17.

10. Barros SP & Offenbacher S (2009) Epigenetics: Connecting environment and genotype to phenotype and disease. J Dent Res 88: 400-408.

11. Jeltsch A (2006) Molecular enzymology of mammalian DNA methyltransferases. Curr Top Microbiol Immunol 301: 203-225.

12. Miranda TB & Jones PA (2007) DNA methylation: The nuts and bolts of repression. J Cell Physiol 213: 384-390.

13. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3: 662-673.

14. Jaenisch R & Bird A (2003) Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl: 245-254.

15. Ehrlich M, et al (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. Nucleic Acids Res 10: 2709-2721.

16. Csankovszki G, Nagy A & Jaenisch R (2001) Synergism of xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol 153: 773-784.

17. Dong CX, et al (2009) Promoter methylation of p16 associated with helicobacter pylori infection in precancerous gastric lesions: A population-based study. Int J Cancer 124: 434-439.

18. Perri F, et al (2007) Aberrant DNA methylation in non-neoplastic gastric mucosa of H. pylori infected patients and effect of eradication. Am J Gastroenterol 102: 1361-1371.

19. Sepulveda AR, et al (2010) CpG methylation and reduced expression of O6-methylguanine DNA methyltransferase is associated with helicobacter pylori infection. Gastroenterology 138: 1836-1844.

20. Peterson AJ, et al (2010) Helicobacter pylori infection methylates and silences trefoil factor 2 leading to gastric tumor development in mice and humans. Gastroenterology

21. Kondo T, et al (2009) Accumulation of aberrant CpG hypermethylation by helicobacter pylori infection promotes development and progression of gastric MALT lymphoma. Int J Oncol 35: 547-557.

22. Katayama Y, Takahashi M & Kuwayama H (2009) Helicobacter pylori causes runx3 gene methylation and its loss of expression in gastric epithelial cells, which is mediated by nitric oxide produced by macrophages. Biochem Biophys Res Commun 388: 496-500.

23. Niwa T, et al (2010) Inflammatory processes triggered by helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 70: 1430-1440.

24. Bussiere FI, et al (2010) H. pylori-induced promoter hypermethylation downregulates USF1 and USF2 transcription factor gene expression. Cell Microbiol 12: 1124-1133.

25. Yao Y, Tao H, Park DI, Sepulveda JL & Sepulveda AR (2006) Demonstration and characterization of mutations induced by helicobacter pylori organisms in gastric epithelial cells. Helicobacter 11: 272-286.

26. Pero R, et al (2010) Chromatin and DNA methylation dynamics of helicobacter pylori-induced COX-2 activation. Int J Med Microbiol

27. Qian X, et al (2008) E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. Cancer Lett 263: 107-113.

28. Bobetsis YA, et al (2007) Bacterial infection promotes DNA hypermethylation. J Dent Res 86: 169-174.

29. Tanabe S, et al (2003) Helicobacter pylori and campylobacter rectus share a common antigen. Oral Microbiol Immunol 18: 79-87.

30. Fish JE & Marsden PA (2006) Endothelial nitric oxide synthase: Insight into cell-specific gene regulation in the vascular endothelium. Cell Mol Life Sci 63: 144-162.

31. Chan Y, et al (2004) The cell-specific expression of endothelial nitric-oxide synthase: A role for DNA methylation. J Biol Chem 279: 35087-35100.

32. Chan GC, et al (2005) Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. J Immunol 175: 3846-3861.

33. Takahashi K, Sugi Y, Hosono A & Kaminogawa S (2009) Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 183: 6522-6529.

34. Shuto T, et al (2006) Promoter hypomethylation of toll-like receptor-2 gene is associated with increased proinflammatory response toward bacterial peptidoglycan in cystic fibrosis bronchial epithelial cells. FASEB J 20: 782-784.

35. Furuta T, et al (2008) DNA demethylation-dependent enhancement of toll-like receptor-2 gene expression in cystic fibrosis epithelial cells involves SP1-activated transcription. BMC Mol Biol 9: 39.

36. Quddus J, et al (1993) Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice. J Clin Invest 92: 38-53.

37. Backdahl L, Bushell A & Beck S (2009) Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. Int J Biochem Cell Biol 41: 176-184.

38. Yung R, et al (1996) Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupuslike disease in syngeneic mice. J Clin Invest 97: 2866-2871.

39. Kroger H, Dietrich A, Gratz R, Wild A & Ehrlich W (1999) The effect of tryptophan plus methionine, 5-azacytidine, and methotrexate on adjuvant arthritis of rat. Gen Pharmacol 33: 195-201.

40. Lin Z, et al (2010) Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. Clin Genet

41. Castillo-Diaz SA, Garay-Sevilla ME, Hernandez-Gonzalez MA, Solis-Martinez MO & Zaina S (2010) Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries. Int J Mol Med 26: 691-700.

42. Sharma P, et al (2008) Detection of altered global DNA methylation in coronary artery disease patients. DNA Cell Biol 27: 357-365.

43. Kim YI, Logan JW, Mason JB & Roubenoff R (1996) DNA hypomethylation in inflammatory arthritis: Reversal with methotrexate. J Lab Clin Med 128: 165-172.

44. Breivik J & Gaudernack G (1999) Genomic instability, DNA methylation, and natural selection in colorectal carcinogenesis. Semin Cancer Biol 9: 245-254.

45. Nakayama M, et al (2003) Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: A detailed study using laser-capture microdissection. Am J Pathol 163: 923-933.

46. Kanai Y (2010) Genome-wide DNA methylation profiles in precancerous conditions and cancers. Cancer Sci 101: 36-45.

47. Peng DF, et al (2006) DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. Carcinogenesis 27: 1160-1168.

48. Caldji C, et al (1998) Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc Natl Acad Sci U S A 95: 5335-5340.

49. Francis D, Diorio J, Liu D & Meaney MJ (1999) Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286: 1155-1158.

50. Weaver IC, et al (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7: 847-854.

51. McGowan PO, et al (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 12: 342-348.

52. Roth TL, Lubin FD, Funk AJ & Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 65: 760-769.

53. Yi P, et al (2000) Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 275: 29318-29323.

54. Varela-Moreiras G, Murphy MM & Scott JM (2009) Cobalamin, folic acid, and homocysteine. Nutr Rev 67 Suppl 1: S69-72.

55. Ingrosso D & Perna AF (2009) Epigenetics in hyperhomocysteinemic states. A special focus on uremia. Biochim Biophys Acta 1790: 892-899.

56. Jamaluddin MS, Yang X & Wang H (2007) Hyperhomocysteinemia, DNA methylation and vascular disease. Clin Chem Lab Med 45: 1660-1666.

57. Castro R, et al (2003) Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin Chem 49: 1292-1296.

58. Bromberg A, Levine J, Belmaker RH & Agam G (2010) Hyperhomocysteinemia does not affect global DNA methylation and nicotinamide N-methyltransferase expression in mice. J Psychopharmacol

59. Bonsch D, Lenz B, Reulbach U, Kornhuber J & Bleich S (2004) Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. J Neural Transm 111: 1611-1616.

60. Wang H, et al (2002) Cyclin A transcriptional suppression is the major mechanism mediating homocysteine-induced endothelial cell growth inhibition. Blood 99: 939-945.

61. Chang PY, et al (2008) Homocysteine inhibits arterial endothelial cell growth through transcriptional downregulation of fibroblast growth factor-2 involving G protein and DNA methylation. Circ Res 102: 933-941.

62. Devlin AM, Bottiglieri T, Domann FE & Lentz SR (2005) Tissue-specific changes in H19 methylation and expression in mice with hyperhomocysteinemia. J Biol Chem 280: 25506-25511.

63. Zeisel SH (2007) Gene response elements, genetic polymorphisms and epigenetics influence the human dietary requirement for choline. IUBMB Life 59: 380-387.

64. Tsujiuchi T, Tsutsumi M, Sasaki Y, Takahama M & Konishi Y (1999) Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. Jpn J Cancer Res 90: 909-913.

65. Niculescu MD, Craciunescu CN & Zeisel SH (2005) Gene expression profiling of choline-deprived neural precursor cells isolated from mouse brain. Brain Res Mol Brain Res 134: 309-322.

66. Hecht SS (2006) Cigarette smoking: Cancer risks, carcinogens, and mechanisms. Langenbecks Arch Surg 391: 603-613.

67. Toh Y, et al (2010) Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: Molecular mechanisms of carcinogenesis. Int J Clin Oncol 15: 135-144.

68. Liu F, et al (2010) Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. Oncogene 29: 3650-3664.

69. Vaissiere T, et al (2009) Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. Cancer Res 69: 243-252.

70. Chen Z, et al (2001) Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet 10: 433-443.

71. Huang Y, et al (2010) Cigarette smoke induced promoter methylation of single-strand DNA-binding protein 2 in human esophageal squamous cell carcinoma. Int J Cancer

72. Beleford D, et al (2010) Methylation induced gene silencing of HtrA3 in smoking-related lung cancer. Clin Cancer Res 16: 398-409.

73. Philibert RA, et al (2010) The effect of smoking on MAOA promoter methylation in DNA prepared from lymphoblasts and whole blood. Am J Med Genet B Neuropsychiatr Genet 153B: 619-628.

74. Brait M, et al (2009) Association between lifestyle factors and CpG island methylation in a cancer-free population. Cancer Epidemiol Biomarkers Prev 18: 2984-2991.

75. Bennett KL, et al (2010) HPV status-independent association of alcohol and tobacco exposure or prior radiation therapy with promoter methylation of FUSSEL18, EBF3, IRX1, and SEPT9, but not SLC5A8, in head and neck squamous cell carcinomas. Genes Chromosomes Cancer 49: 319-326.

76. Phillips JM & Goodman JI (2009) Inhalation of cigarette smoke induces regions of altered DNA methylation (RAMs) in SENCAR mouse lung. Toxicology 260: 7-15.

77. Tommasi S, et al (2010) Investigating the epigenetic effects of a prototype smoke-derived carcinogen in human cells. PLoS One 5: e10594.

78. Liu L, Wylie RC, Andrews LG & Tollefsbol TO (2003) Aging, cancer and nutrition: The DNA methylation connection. Mech Ageing Dev 124: 989-998.

79. Wilson AG (2008) Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. J Periodontol 79: 1514-1519.

80. Jones PA & Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415-428.

81. Bornman DM, Mathew S, Alsruhe J, Herman JG & Gabrielson E (2001) Methylation of the E-cadherin gene in bladder neoplasia and in normal urothelial epithelium from elderly individuals. Am J Pathol 159: 831-835.

82. Issa JP, et al (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 7: 536-540.

83. Gonzalgo ML & Jones PA (1997) Mutagenic and epigenetic effects of DNA methylation. Mutat Res 386: 107-118.

84. Wilson VL & Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220: 1055-1057.

85. Jintaridth P & Mutirangura A (2010) Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiol Genomics

86. Bollati V, et al (2009) Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 130: 234-239.

87. Chan MF, et al (2001) Reduced rates of gene loss, gene silencing, and gene mutation in Dnmt1-deficient embryonic stem cells. Mol Cell Biol 21: 7587-7600.

88. Gaudet F, et al (2003) Induction of tumors in mice by genomic hypomethylation. Science 300: 489-492.

89. Bell AC & Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405: 482-485.

90. Prendergast GC, Lawe D & Ziff EB (1991) Association of myn, the murine homolog of max, with c-myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell 65: 395-407.

91. Prokhortchouk A, et al (2001) The p120 catenin partner kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev 15: 1613-1618.

92. Sansom OJ, Maddison K & Clarke AR (2007) Mechanisms of disease: Methyl-binding domain proteins as potential therapeutic targets in cancer. Nat Clin Pract Oncol 4: 305-315.

93. Jones PL, et al (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19: 187-191.

94. Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389: 349-352.

95. Nan X, et al (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393: 386-389.

96. Li B, Carey M & Workman JL (2007) The role of chromatin during transcription. Cell 128: 707-719.

97. Harikrishnan KN, et al (2005) Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. Nat Genet 37: 254-264.

98. Popkie AP, et al (2010) Phosphatidylinositol 3-kinase (PI3K) signaling via glycogen synthase kinase-3 (gsk-3) regulates DNA methylation of imprinted loci. J Biol Chem 285: 41337-41347.

99. Brenner C, et al (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J 24: 336-346.

100. Lin RK, et al (2010) The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients. J Clin Invest 120: 521-532.

101. Smallwood A, Esteve PO, Pradhan S & Carey M (2007) Functional cooperation between HP1 and DNMT1 mediates gene silencing. Genes Dev 21: 1169-1178.

102. El Gazzar M, et al (2008) G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. J Biol Chem 283: 32198-32208.

103. Thomson JP, et al (2010) CpG islands influence chromatin structure via the CpG-binding protein Cfp1. Nature 464: 1082-1086.

104. Ruthenburg AJ, Allis CD & Wysocka J (2007) Methylation of lysine 4 on histone H3: Intricacy of writing and reading a single epigenetic mark. Mol Cell 25: 15-30.

105. Morris KV, Chan SW, Jacobsen SE & Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. Science 305: 1289-1292.

106. Chan SW, et al (2004) RNA silencing genes control de novo DNA methylation. Science 303: 1336.

107. Matzke MA & Birchler JA (2005) RNAi-mediated pathways in the nucleus. Nat Rev Genet 6: 24-35.

108. Cao X, et al (2003) Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. Curr Biol 13: 2212-2217.

109. Kirk H, Cefalu WT, Ribnicky D, Liu Z & Eilertsen KJ (2008) Botanicals as epigenetic modulators for mechanisms contributing to development of metabolic syndrome. Metabolism 57: S16-23.

110. Issa JP, et al (2005) Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. J Clin Oncol 23: 3948-3956.

111. Leone G, Voso MT, Teofili L & Lubbert M (2003) Inhibitors of DNA methylation in the treatment of hematological malignancies and MDS. Clin Immunol 109: 89-102.

112. Cameron EE, Bachman KE, Myohanen S, Herman JG & Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21: 103-107.

113. vel Szic KS, Ndlovu MN, Haegeman G & Vanden Berghe W (2010) Nature or nurture: Let food be your epigenetic medicine in chronic inflammatory disorders. Biochem Pharmacol 80: 1816-1832.

114. Tahara T, et al (2009) Chronic nonsteroidal anti-inflammatory drug (NSAID) use suppresses multiple CpG islands hyper methylation (CIHM) of tumor suppressor genes in the human gastric mucosa. Cancer Sci 100: 1192-1197.

115. Huber LC, Stanczyk J, Jungel A & Gay S (2007) Epigenetics in inflammatory rheumatic diseases. Arthritis Rheum 56: 3523-3531.

116. Bartke T, et al (2010) Nucleosome-interacting proteins regulated by DNA and histone methylation. Cell 143: 470-484.

117. Darveau RP, Tanner A & Page RC (1997) The microbial challenge in periodontitis. Periodontol 2000 14: 12-32.

118. Madianos PN, Bobetsis YA & Kinane DF (2005) Generation of inflammatory stimuli: How bacteria set up inflammatory responses in the gingiva. J Clin Periodontol 32 Suppl 6: 57-71.

119. Taylor JJ (2010) Cytokine regulation of immune responses to porphyromonas gingivalis. Periodontol 2000 54: 160-194.

120. Socransky SS, Haffajee AD, Cugini MA, Smith C & Kent RL,Jr (1998) Microbial complexes in subgingival plaque. J Clin Periodontol 25: 134-144.

121. Miyamoto M, et al (1998) The S-layer protein from campylobacter rectus: Sequence determination and function of the recombinant protein. FEMS Microbiol Lett 166: 275-281.

122. Hinode D, Yokoyama M, Tanabe S, Yoshioka M & Nakamura R (2002) Antigenic properties of the GroEL-like protein of campylobacter rectus. Oral Microbiol Immunol 17: 16-21.

123. Okuda K, et al (1997) Role for the S-layer of campylobacter rectus ATCC33238 in complement mediated killing and phagocytic killing by leukocytes from guinea pig and human peripheral blood. Oral Dis 3: 113-120.

124. Wang B, Kraig E & Kolodrubetz D (1998) A new member of the S-layer protein family: Characterization of the crs gene from campylobacter rectus. Infect Immun 66: 1521-1526.

125. Arce RM, et al (2010) Characterization of the invasive and inflammatory traits of oral campylobacter rectus in a murine model of fetoplacental growth restriction and in trophoblast cultures. J Reprod Immunol 84: 145-153.

126. Oliveira NF, et al (2009) DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. J Clin Periodontol 36: 719-725.

127. Yin L & Chung WO (2011) Epigenetic regulation of human beta-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria. Mucosal Immunol

128. Gonsky R, Deem RL & Targan SR (2009) Distinct methylation of IFNG in the gut. J Interferon Cytokine Res 29: 407-414.

129. Gonsky R, et al (2010) Distinct IFNG methylation in a subset of ulcerative colitis patients based on reactivity to microbial antigens. Inflamm Bowel Dis

130. Tahara T, et al (2009) Effect of MDR1 gene promoter methylation in patients with ulcerative colitis. Int J Mol Med 23: 521-527.

131. Tahara T, et al (2009) Promoter methylation of protease-activated receptor (PAR2) is associated with severe clinical phenotypes of ulcerative colitis (UC). Clin Exp Med 9: 125-130.

132. Balasa A, et al (2010) Assessment of DNA methylation at the interferon regulatory factor 5 (IRF5) promoter region in inflammatory bowel diseases. Int J Colorectal Dis 25: 553-556.

133. Nadeau K, et al (2010) Ambient air pollution impairs regulatory T-cell function in asthma. J Allergy Clin Immunol 126: 845-852.e10.

134. Andia DC, et al (2010) DNA methylation status of the IL8 gene promoter in aggressive periodontitis. J Periodontol 81: 1336-1341.

135. Wang GS, et al (2009) Ultraviolet B exposure of peripheral blood mononuclear cells of patients with systemic lupus erythematosus inhibits DNA methylation. Lupus 18: 1037-1044.

136. Richardson B, et al (1990) Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. Arthritis Rheum 33: 1665-1673.

137. Liggett T, et al (2010) Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. Cancer 116: 1674-1680.

138. Zhang K, Zhang R, Li X, Yin G & Niu X (2009) Promoter methylation status of p15 and p21 genes in HPP-CFCs of bone marrow of patients with psoriasis. Eur J Dermatol 19: 141-146.

139. Zhang K, et al (2007) The mRNA expression and promoter methylation status of the p16 gene in colony-forming cells with high proliferative potential in patients with psoriasis. Clin Exp Dermatol 32: 702-708.

140. Nile CJ, Read RC, Akil M, Duff GW & Wilson AG (2008) Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum 58: 2686-2693.

141. Dhir M, et al (2008) Epigenetic regulation of WNT signaling pathway genes in inflammatory bowel disease (IBD) associated neoplasia. J Gastrointest Surg 12: 1745-1753.

142. Kitamura T, et al (2008) Enhancement of lymphocyte migration and cytokine production by ephrinB1 system in rheumatoid arthritis. Am J Physiol Cell Physiol 294: C189-96.

143. Wong NA, et al (2005) CDX1 is an important molecular mediator of barrett's metaplasia. Proc Natl Acad Sci U S A 102: 7565-7570.

144. Goodson JM, Dewhirst FE & Brunetti A (1974) Prostaglandin E2 levels and human periodontal disease. Prostaglandins 6: 81-85.

145. Noguchi K & Ishikawa I (2007) The roles of cyclooxygenase-2 and prostaglandin E2 in periodontal disease. Periodontol 2000 43: 85-101.

146. Offenbacher S, Odle BM & Van Dyke TE (1986) The use of crevicular fluid prostaglandin E2 levels as a predictor of periodontal attachment loss. J Periodontal Res 21: 101-112.

147. Zhong Y, Slade GD, Beck JD & Offenbacher S (2007) Gingival crevicular fluid interleukin-1beta, prostaglandin E2 and periodontal status in a community population. J Clin Periodontol 34: 285-293.

148. Champagne CM, et al (2003) Potential for gingival crevice fluid measures as predictors of risk for periodontal diseases. Periodontol 2000 31: 167-180.

149. Noguchi K, Yanai M, Shitashige M, Nishihara T & Ishikawa I (2000) Cyclooxygenase-2-dependent prostaglandin production by peripheral blood monocytes stimulated with lipopolysaccharides isolated from periodontopathogenic bacteria. J Periodontol 71: 1575-1582.

150. Feinberg AP & Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4: 143-153.

151. Bobetsis YA, et al (2007) Bacterial infection promotes DNA hypermethylation. J Dent Res 86: 169-174.

152. Bird AP & Wolffe AP (1999) Methylation-induced repression--belts, braces, and chromatin. Cell 99: 451-454.

153. Kelavkar UP, et al (2007) DNA methylation paradigm shift: 15-lipoxygenase-1 upregulation in prostatic intraepithelial neoplasia and prostate cancer by atypical promoter hypermethylation. Prostaglandins Other Lipid Mediat 82: 185-197.

154. Grunau C, Clark SJ & Rosenthal A (2001) Bisulfite genomic sequencing: Systematic investigation of critical experimental parameters. Nucleic Acids Res 29: E65-5.

155. Appleby SB, Ristimaki A, Neilson K, Narko K & Hla T (1994) Structure of the human cyclo-oxygenase-2 gene. Biochem J 302 (Pt 3): 723-727.

156. Li LC & Dahiya R (2002) MethPrimer: Designing primers for methylation PCRs. Bioinformatics 18: 1427-1431.

157. Kim HY, Preisser JS, Rozier RG & Valiyaparambil JV (2006) Multilevel analysis of group-randomized trials with binary outcomes. Community Dent Oral Epidemiol 34: 241-251.

158. Vanselow J, et al (2006) DNA-remethylation around a STAT5-binding enhancer in the alphaS1-casein promoter is associated with abrupt shutdown of alphaS1-casein synthesis during acute mastitis. J Mol Endocrinol 37: 463-477.

159. Wasson GR, et al (2006) Global DNA and p53 region-specific hypomethylation in human colonic cells is induced by folate depletion and reversed by folate supplementation. J Nutr 136: 2748-2753.

160. Yao Y, Tao H, Park DI, Sepulveda JL & Sepulveda AR (2006) Demonstration and characterization of mutations induced by helicobacter pylori organisms in gastric epithelial cells. Helicobacter 11: 272-286.

161. Beck JD, Sharp T, Koch GG & Offenbacher S (1997) A study of attachment loss patterns in survivor teeth at 18 months, 36 months and 5 years in community-dwelling older adults. J Periodontal Res 32: 497-505.

162. Newton R, Kuitert LM, Bergmann M, Adcock IM & Barnes PJ (1997) Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. Biochem Biophys Res Commun 237: 28-32.

163. Perkins DJ & Kniss DA (1997) Rapid and transient induction of cyclo-oxygenase 2 by epidermal growth factor in human amnion-derived WISH cells. Biochem J 321 (Pt 3): 677-681.

164. Gemmell E & Seymour GJ (2004) Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. Periodontol 2000 35: 21-41.

165. Yamazaki K, et al (1994) IL-4- and IL-6-producing cells in human periodontal disease tissue. J Oral Pathol Med 23: 347-353.

166. Aramaki M, Nagasawa T, Koseki T & Ishikawa I (1998) Presence of activated B-1 cells in chronic inflamed gingival tissue. J Clin Immunol 18: 421-429.

167. Dutzan N, et al (2009) Levels of interferon-gamma and transcription factor T-bet in progressive periodontal lesions in patients with chronic periodontitis. J Periodontol 80: 290-296.

168. Wright HJ, Matthews JB, Chapple IL, Ling-Mountford N & Cooper PR (2008) Periodontitis associates with a type 1 IFN signature in peripheral blood neutrophils. J Immunol 181: 5775-5784.

169. Salvi GE, et al (1998) Inflammatory mediators of the terminal dentition in adult and early onset periodontitis. J Periodontal Res 33: 212-225.

170. Takeichi O, et al (2000) Cytokine profiles of T-lymphocytes from gingival tissues with pathological pocketing. J Dent Res 79: 1548-1555.

171. Offenbacher S, et al (2007) Periodontal disease at the biofilm-gingival interface. J Periodontol 78: 1911-1925.

172. Nichols FC & Garrison SW (1987) Interferon-gamma potentiation of lipopolysaccharide-induced eicosanoid release from human monocytes. J Interferon Res 7: 121-129.

173. Fujihashi K, et al (1996) Selected Th1 and Th2 cytokine mRNA expression by CD4(+) T cells isolated from inflamed human gingival tissues. Clin Exp Immunol 103: 422-428.

174. Rogus J, et al (2008) IL1B gene promoter haplotype pairs predict clinical levels of interleukin-1beta and C-reactive protein. Hum Genet 123: 387-398.

175. Watford WT, Moriguchi M, Morinobu A & O'Shea JJ (2003) The biology of IL-12: Coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14: 361-368.

176. Nakajima T, et al (2009) The presence of a methylation fingerprint of helicobacter pylori infection in human gastric mucosae. Int J Cancer 124: 905-910.

177. Bird AP & Wolffe AP (1999) Methylation-induced repression--belts, braces, and chromatin. Cell 99: 451-454.

178. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16: 6-21.

179. Barros SP & Offenbacher S (2009) Epigenetics: Connecting environment and genotype to phenotype and disease. J Dent Res 88: 400-408.

180. Jones PA & Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21: 163-167.

181. Shuto T, et al (2006) Promoter hypomethylation of toll-like receptor-2 gene is associated with increased proinflammatory response toward bacterial peptidoglycan in cystic fibrosis bronchial epithelial cells. FASEB J 20: 782-784.

182. Zhang S, et al (2010) Alteration of PTGS2 promoter methylation in chronic periodontitis. J Dent Res 89: 133-137.

183. LOE H, THEILADE E & JENSEN SB (1965) Experimental gingivitis in man. J Periodontol 36: 177-187.

184. Loe H, Anerud A, Boysen H & Morrison E (1986) Natural history of periodontal disease in man. rapid, moderate and no loss of attachment in sri lankan laborers 14 to 46 years of age. J Clin Periodontol 13: 431-445.

185. Colella S, Shen L, Baggerly KA, Issa JP & Krahe R (2003) Sensitive and quantitative universal pyrosequencing methylation analysis of CpG sites. BioTechniques 35: 146-150.

186. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25: 402-408.

187. White GP, et al (2006) CpG methylation patterns in the IFNgamma promoter in naive T cells: Variations during Th1 and Th2 differentiation and between atopics and non-atopics. Pediatr Allergy Immunol 17: 557-564.

188. White GP, Watt PM, Holt BJ & Holt PG (2002) Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. J Immunol 168: 2820-2827.

189. Gonsky R, Deem RL & Targan SR (2009) Distinct methylation of IFNG in the gut. J Interferon Cytokine Res 29: 407-414.

190. Smith AJ & Humphries SE (2009) Cytokine and cytokine receptor gene polymorphisms and their functionality. Cytokine Growth Factor Rev 20: 43-59.

191. Bream JH, Ping A, Zhang X, Winkler C & Young HA (2002) A single nucleotide polymorphism in the proximal IFN-gamma promoter alters control of gene transcription. Genes Immun 3: 165-169.

192. Garlet GP, Martins W,Jr, Ferreira BR, Milanezi CM & Silva JS (2003) Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. J Periodontal Res 38: 210-217.

193. Huang S, et al (1993) Immune response in mice that lack the interferon-gamma receptor. Science 259: 1742-1745.

194. Meda L, Gasperini S, Ceska M & Cassatella MA (1994) Modulation of proinflammatory cytokine release from human polymorphonuclear leukocytes by gamma interferon. Cell Immunol 157: 448-461.

195. Farrar MA & Schreiber RD (1993) The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 11: 571-611.

196. Boehm U, Klamp T, Groot M & Howard JC (1997) Cellular responses to interferon-gamma. Annu Rev Immunol 15: 749-795.

197. Lappin DF, MacLeod CP, Kerr A, Mitchell T & Kinane DF (2001) Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. Clin Exp Immunol 123: 294-300.

198. Gemmell E, Yamazaki K & Seymour GJ (2002) Destructive periodontitis lesions are determined by the nature of the lymphocytic response. Crit Rev Oral Biol Med 13: 17-34.

199. Reinhardt RA, McDonald TL, Bolton RW, DuBois LM & Kaldahl WB (1989) IgG subclasses in gingival crevicular fluid from active versus stable periodontal sites. J Periodontol 60: 44-50.

200. Zachrisson BU (1968) A histological study of experimental gingivitis in man. J Periodontal Res 3: 293-302.

201. Seymour GJ, Gemmell E, Walsh LJ & Powell RN (1988) Immunohistological analysis of experimental gingivitis in humans. Clin Exp Immunol 71: 132-137.

202. Berenson LS, Ota N & Murphy KM (2004) Issues in T-helper 1 development--resolved and unresolved. Immunol Rev 202: 157-174.

203. Yang J, Murphy TL, Ouyang W & Murphy KM (1999) Induction of interferon-gamma production in Th1 CD4+ T cells: Evidence for two distinct pathways for promoter activation. Eur J Immunol 29: 548-555.

204. Lu B, et al (2001) GADD45gamma mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector TH1 cells. Immunity 14: 583-590.

205. Nares S, et al (2009) Rapid myeloid cell transcriptional and proteomic responses to periodontopathogenic porphyromonas gingivalis. Am J Pathol 174: 1400-1414.

206. Cardoso FP, et al (2010) Methylation pattern of the IFN-gamma gene in human dental pulp. J Endod 36: 642-646.

207. Kwon NH, Kim JS, Lee JY, Oh MJ & Choi DC (2008) DNA methylation and the expression of IL-4 and IFN-gamma promoter genes in patients with bronchial asthma. J Clin Immunol 28: 139-146.

208. Garlet GP (2010) Destructive and protective roles of cytokines in periodontitis: A re-appraisal from host defense and tissue destruction viewpoints. J Dent Res 89: 1349-1363.

209. Graves D (2008) Cytokines that promote periodontal tissue destruction. J Periodontol 79: 1585-1591.

210. Iacopino AM (2001) Periodontitis and diabetes interrelationships: Role of inflammation. Ann Periodontol 6: 125-137.

211. Gorska R, et al (2003) Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. J Clin Periodontol 30: 1046-1052.

212. Graves DT & Cochran D (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 74: 391-401.

213. Via CS, et al (2001) In vivo neutralization of TNF-alpha promotes humoral autoimmunity by preventing the induction of CTL. J Immunol 167: 6821-6826.

214. Collart MA, Baeuerle P & Vassalli P (1990) Regulation of tumor necrosis factor alpha transcription in macrophages: Involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. Mol Cell Biol 10: 1498-1506.

215. Carter AB, Monick MM & Hunninghake GW (1999) Both erk and p38 kinases are necessary for cytokine gene transcription. Am J Respir Cell Mol Biol 20: 751-758.

216. Tudhope SJ, et al (2008) Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. J Pharmacol Exp Ther 324: 306-312.

217. Falvo JV, et al (2000) A stimulus-specific role for CREB-binding protein (CBP) in T cell receptor-activated tumor necrosis factor alpha gene expression. Proc Natl Acad Sci U S A 97: 3925-3929.

218. Hoareau L, et al (2010) Signaling pathways involved in LPS induced TNFalpha production in human adipocytes. J Inflamm (Lond) 7: 1.

219. Dumitru CD, et al (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103: 1071-1083.

220. Paludan SR, Ellermann-Eriksen S, Kruys V & Mogensen SC (2001) Expression of TNF-alpha by herpes simplex virus-infected macrophages is regulated by a dual mechanism: Transcriptional regulation by NF-kappa B and activating transcription factor 2/Jun and translational regulation through the AU-rich region of the 3' untranslated region. J Immunol 167: 2202-2208.

221. Amour A, et al (1998) TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435: 39-44.

222. Zhang S, Wang C, Xi B & Li X (2010) Association between the tumour necrosis factor-alpha -308G/A polymorphism and chronic obstructive pulmonary disease: An update. Respirology

223. Kammoun-Krichen M, et al (2008) TNF gene polymorphisms in graves' disease: TNF-308 A/G meta-analysis. Ann Hum Biol 35: 656-661.

224. Chen YC, et al (2010) Association of tumor necrosis factor-alpha-863C/A gene polymorphism with chronic obstructive pulmonary disease. Lung 188: 339-347.

225. Susa S, et al (2008) A functional polymorphism of the TNF-alpha gene that is associated with type 2 DM. Biochem Biophys Res Commun 369: 943-947.

226. Bayarsaihan D (2011) Epigenetic mechanisms in inflammation. J Dent Res 90: 9-17.

227. Bird AP & Wolffe AP (1999) Methylation-induced repression--belts, braces, and chromatin. Cell 99: 451-454.

228. Jones PA & Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21: 163-167.

229. Kelavkar UP, et al (2007) DNA methylation paradigm shift: 15-lipoxygenase-1 upregulation in prostatic intraepithelial neoplasia and prostate cancer by atypical promoter hypermethylation. Prostaglandins Other Lipid Mediat 82: 185-197.

230. Miao F, Gonzalo IG, Lanting L & Natarajan R (2004) In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. J Biol Chem 279: 18091-18097.

231. El Gazzar M, et al (2008) G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. J Biol Chem 283: 32198-32208.

232. Grunau C, Clark SJ & Rosenthal A (2001) Bisulfite genomic sequencing: Systematic investigation of critical experimental parameters. Nucleic Acids Res 29: E65-5.

233. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25: 402-408.

234. Dell G, Charalambous M & Ward A (2001) In vitro methylation of specific regions in recombinant DNA constructs by excision and religation. Methods Mol Biol 181: 251-258.

235. Cedar H & Bergman Y (2009) Linking DNA methylation and histone modification: Patterns and paradigms. Nat Rev Genet 10: 295-304.

236. Campion J, Milagro FI, Goyenechea E & Martinez JA (2009) TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. Obesity (Silver Spring) 17: 1293-1297.

237. Yao Y, Tao H, Park DI, Sepulveda JL & Sepulveda AR (2006) Demonstration and characterization of mutations induced by helicobacter pylori organisms in gastric epithelial cells. Helicobacter 11: 272-286.

238. Qian X, et al (2008) E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. Cancer Lett 263: 107-113.

239. Bussiere FI, et al (2010) H. pylori-induced promoter hypermethylation downregulates USF1 and USF2 transcription factor gene expression. Cell Microbiol 12: 1124-1133.

240. Maekita T, et al (2006) High levels of aberrant DNA methylation in helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 12: 989-995.

241. Tanabe S, et al (2003) Helicobacter pylori and campylobacter rectus share a common antigen. Oral Microbiol Immunol 18: 79-87.

242. Pieper HC, et al (2008) Different methylation of the TNF-alpha promoter in cortex and substantia nigra: Implications for selective neuronal vulnerability. Neurobiol Dis 32: 521-527.

243. Garlet GP (2010) Destructive and protective roles of cytokines in periodontitis: A re-appraisal from host defense and tissue destruction viewpoints. J Dent Res 89: 1349-1363.

244. Graves D (2008) Cytokines that promote periodontal tissue destruction. J Periodontol 79: 1585-1591.

245. Liu YC, Lerner UH & Teng YT (2010) Cytokine responses against periodontal infection: Protective and destructive roles. Periodontol 2000 52: 163-206.

246. Page RC & Schroeder HE (1976) Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab Invest 34: 235-249.

247. Garlet GP, et al (2007) The dual role of p55 tumour necrosis factor-alpha receptor in actinobacillus actinomycetemcomitans-induced experimental periodontitis: Host protection and tissue destruction. Clin Exp Immunol 147: 128-138.

248. Garlet GP, et al (2008) The essential role of IFN-gamma in the control of lethal aggregatibacter actinomycetemcomitans infection in mice. Microbes Infect 10: 489-496.

249. Graves DT & Cochran D (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 74: 391-401.

250. Zhang F, et al (2003) The overexpression of cyclo-oxygenase-2 in chronic periodontitis. J Am Dent Assoc 134: 861-867.

251. Yucel-Lindberg T, Nilsson S & Modeer T (1999) Signal transduction pathways involved in the synergistic stimulation of prostaglandin production by interleukin-1beta and tumor necrosis factor alpha in human gingival fibroblasts. J Dent Res 78: 61-68.

252. Ke J, et al (2007) Role of NF-kappaB in TNF-alpha-induced COX-2 expression in synovial fibroblasts from human TMJ. J Dent Res 86: 363-367.

253. Medzhitov R & Horng T (2009) Transcriptional control of the inflammatory response. Nat Rev Immunol 9: 692-703.

254. Ramirez-Carrozzi VR, et al (2009) A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell 138: 114-128.

255. Campion J, Milagro FI, Goyenechea E & Martinez JA (2009) TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. Obesity (Silver Spring) 17: 1293-1297.

256. Sullivan KE, et al (2007) Epigenetic regulation of tumor necrosis factor alpha. Mol Cell Biol 27: 5147-5160.

257. Muthukuru M, Jotwani R & Cutler CW (2005) Oral mucosal endotoxin tolerance induction in chronic periodontitis. Infect Immun 73: 687-694.

258. El Gazzar M, et al (2008) G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. J Biol Chem 283: 32198-32208.

259. El Gazzar M, Yoza BK, Hu JY, Cousart SL & McCall CE (2007) Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. J Biol Chem 282: 26857-26864.

260. Loo WT, Jin L, Cheung MN, Wang M & Chow LW (2010) Epigenetic change in E-cardherin and COX-2 to predict chronic periodontitis. J Transl Med 8: 110.

261. Andia DC, et al (2010) DNA methylation status of the IL8 gene promoter in aggressive periodontitis. J Periodontol 81: 1336-1341.

262. Bobetsis YA, et al (2007) Bacterial infection promotes DNA hypermethylation. J Dent Res 86: 169-174.

263. Tanabe S, et al (2003) Helicobacter pylori and campylobacter rectus share a common antigen. Oral Microbiol Immunol 18: 79-87.

264. Minarovits J (2009) Microbe-induced epigenetic alterations in host cells: The coming era of patho-epigenetics of microbial infections. A review. Acta Microbiol Immunol Hung 56: 1-19.

265. Garcia-Garcia JC, Barat NC, Trembley SJ & Dumler JS (2009) Epigenetic silencing of host cell defense genes enhances intracellular survival of the rickettsial pathogen anaplasma phagocytophilum. PLoS Pathog 5: e1000488.

266. Gemmell E & Seymour GJ (2004) Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. Periodontol 2000 35: 21-41.

267. Viana MB, et al (2011) Methylation pattern of IFN-gamma and IL-10 genes in periodontal tissues. Immunobiology

268. Deng WG, Montero AJ & Wu KK (2007) Interferon-gamma suppresses cyclooxygenase-2 promoter activity by inhibiting C-jun and C/EBPbeta binding. Arterioscler Thromb Vasc Biol 27: 1752-1759.

269. Inada T, Kubo K & Shingu K (2010) Promotion of interferon-gamma production by natural killer cells via suppression of murine peritoneal macrophage prostaglandin E production using intravenous anesthetic propofol. Int Immunopharmacol 10: 1200-1208.

270. Murayama A, et al (2006) A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. EMBO J 25: 1081-1092.