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Treponema denticola increases MMP-2 expression and activation in the periodontium via reversible DNA and histone modifications

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Abstract

Host-derived matrix metalloproteinases (MMPs) and bacterial proteases mediate destruction of extracellular matrices and supporting alveolar bone in periodontitis. The *Treponema denticola* dentilisin protease induces MMP-2 expression and activation in periodontal ligament (PDL) cells, and dentilisin-mediated activation of pro-MMP-2 is required for cellular fibronectin degradation. Here we report that *T. denticola* regulates MMP-2 expression through epigenetic modifications in the periodontium. PDL cells were treated with epigenetic enzyme inhibitors before or after *T. denticola* challenge. Fibronectin fragmentation, MMP-2 expression and activation were assessed by immunoblot, zymography and qRT-PCR, respectively. Chromatin modification enzyme expression in *T. denticola*-challenged PDL cells and periodontal tissues were evaluated using gene arrays. Several classes of epigenetic enzymes showed significant alterations in transcription in diseased tissue and *T. denticola*-challenged PDL cells. *T. denticola*-mediated MMP-2 expression and activation were significantly reduced in PDL cells treated with inhibitors of aurora kinases and histone deacetylases. In contrast, DNA methyltransferase inhibitors had little effect, and inhibitors of histone acetyltransferases, methyltransferases and demethylases exacerbated *T. denticola*-mediated MMP-2 expression and activation. Chronic epigenetic changes in periodontal tissues mediated by *T. denticola* or other oral microbes may contribute to the limited success of conventional treatment of chronic periodontitis and may be amenable to therapeutic reversal.

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Introduction

Periodontitis, a bacterially-mediated chronic inflammatory disease of the tissues supporting the tooth is one of the most common inflammatory diseases in humans and it can adversely affect systemic health (Armitage, 2004, Armitage, 2008). National surveys show that the majority of adults suffer from mild-to-moderate periodontitis, with up to 15% of the population being affected by severe forms at some stage in their lives (Pihlstrom *et al.*, 2005). Periodontitis causes largely irreversible destruction of the periodontal tissues, and in advanced stages, tooth loss, speech and masticatory problems, and an overall reduced quality of life. Moreover, the systemic burden of periodontitis on different organs has been investigated. It has been reported that periodontal infections can adversely predispose to coronary heart diseases (Stewart *et al.*, 2016), ischemic strokes (Leira *et al.*, 2016), poor glycemic control (Garcia *et al.*, 2015), preterm labor, low-birth-weight delivery (Sitholimela *et al.*, 2013), and pulmonary diseases (Prasanna, 2011). These systemic effects have been attributed to either direct bacterial invasion or modulation of specific host inflammatory and tissue destructive mediators (Williams *et al.*, 2008).

Destruction of the periodontal extracellular matrices (ECM) and detrimental changes in the cellular elements of the periodontal ligament occur as a result of disruption of normal tissue homeostatic processes. One of these disrupted processes is regulation of host-derived matrix metalloproteinases (MMP) that are both directly and indirectly involved in periodontal tissue breakdown. Destruction of the ECM in periodontitis results in the release of fibronectin (FN) fragments, which are considered markers of periodontal disease status (Huynh *et al.*, 2002). Evidence from *in vitro* studies further indicate that these FN fragments, induce several detrimental effects, including induction of apoptosis and suppression of osteoblast differentiation of periodontal ligament cells (Kapila *et al.*, 1996, Kapila *et al.*, 1998, Kapila *et al.*, 1999, Jee *et al.*, 2004, Dai *et al.*, 2005, Ghosh *et al.*, 2008, Joseph *et al.*, 2010), thereby further potentiating disease progression.

MMPs are synthesized in latent form, then activated through proteolytic cleavage to expose the catalytic site of the MMP enzyme. MMP activation is primarily extracellular, though intracellular activation is reported in certain cases (Nagase, 1997, Murphy *et al.*, 1999). MMPs are synthesized at low basal levels for maintenance of homeostatic processes; however their levels and activation are typically increased during disease (Mittal *et al.*, 2016). In addition to their role in remodeling the ECM and basement membrane during various physiologic processes, MMPs are implicated in a wide range of pathologic processes, including cardiovascular (Azevedo *et al.*, 2014), pulmonary (Navratilova *et al.*, 2016, Pardo *et al.*, 2016), renal (Aresu *et al.*, 2011, Charitaki *et al.*, 2016), and gastrointestinal diseases (Medina *et al.*, 2004), cancer (Endres *et al.*, 2016, Ligi *et al.*, 2016, Liu *et al.*, 2016, Lukaszewicz-Zajac *et al.*, 2016, Pietruszewska *et al.*, 2016) and inflammatory diseases, such as periodontitis (Shinkarenko *et al.*, 2013, Nissinen *et al.*, 2014).

MMP-2, one of several MMPs involved in tissue homeostasis and remodeling, is constitutively expressed by periodontal ligament cells as pro-MMP-2 (Kapila *et al.*, 1996). Thus, control of the process or rate of activation of MMP- 2 has been proposed as a key

regulatory step in periodontal tissue homeostasis (Madsen *et al.*, 2013, Mosig *et al.*, 2013, Borkham-Kamphorst *et al.*, 2015). Activation of pro-MMP-2 is promoted by proteolytic cleavage by the membrane type-1-MMP (MT1-MMP/MMP-14) (Nagase, 1997, Murphy *et al.*, 1999, Zucker *et al.*, 2003). Extracellular MMP activities are controlled by the blockage of autolytic MMP activation or by endogenous proteinase inhibitors, such as tissue inhibitors of MMPs (TIMPs) (Nagase *et al.*, 2006, Brew *et al.*, 2010). Regulation of the proteolytic activity of MMP-2 is dependent on the balance between MT1-MMP/TIMP-2 (Shofuda *et al.*, 1998, Hernandez-Barrantes *et al.*, 2001, Oyarzun *et al.*, 2010). This mechanism of MMP-2 activation by an MT1-MMP–TIMP-2 complex has been well recognized in other systems (Strongin *et al.*, 1995, Butler *et al.*, 1998, Kinoshita *et al.*, 1998). Disturbances in that balance may result in excessive tissue degradation associated with inflammatory diseases (Ejeil *et al.*, 2003).

T. denticola along with *Porphyromonas gingivalis* and *Tannerella forsythia* become prevalent in late stages of subgingival biofilm formation and comprise the bacterial “red complex” that is considered pathogenic in the etiology of periodontal disease (Socransky *et al.*, 1998). While our understanding of the periodontal disease microbiome has greatly increased, the “red complex” bacteria continue to be recognized as important pathogens in the disease process. Oral spirochetes including *T. denticola* often predominate in periodontal disease, though they are typically below detectable levels in healthy gingival plaque (Choi *et al.*, 1994, Ellen *et al.*, 2005). The levels of *T. denticola* increase with the severity of periodontitis, underscoring its major role in the disease (Simonson *et al.*, 1988, Yoshida *et al.*, 2004).

Recognized virulence factors of *T. denticola* include the acylated serine protease complex (dentilisin; PrtP complex; CTLP/chymotrypsin-like protease) that degrades gelatin, laminin and various serum components and bioactive peptides (Uitto *et al.*, 1988, Grenier *et al.*, 1990, Makinen *et al.*, 1995). The dentilisin complex contributes to *T. denticola* adherence and cytotoxic effects on epithelial cells and fibroblasts (Ellen *et al.*, 1994, Mathers *et al.*, 1996, Fenno *et al.*, 1998), penetration of epithelial tissue (Chi *et al.*, 2003), and it may play a role in complement-mediated bactericidal activity (McDowell *et al.*, 2009) and complement evasion (McDowell *et al.*, 2011). Of particular relevance to the current study, we previously demonstrated that dentilisin proteolytic activity induces activation of pro-MMP-2 in cultured PDL cells, and that activated MMP-2 is required for cleavage of cellular FN into fragments similar to those observed in gingival crevicular fluid from periodontal lesions (Miao *et al.*, 2011). Furthermore, transcription and expression of MT1/MMP and TIMP-2 increased in response to *T. denticola* challenge (Miao *et al.*, 2014). Taken together, these properties suggest important links between the *T. denticola* protease activity and regulation of the cellular and tissue processes that result in periodontal tissue destruction.

Epigenetics is defined as heritable and potentially reversible changes in gene expression without alterations in the DNA sequence (Goldberg *et al.*, 2007, Waddington, 2012). Such modifications are not only associated with diseases but are also essential for the incorporation and integration of endogenous and environmental signals in cells. Epigenetic status can be affected by environmental factors, such as, nutrients, toxins, infections, and hypoxia with subsequent up- or down-regulation of specific gene expression patterns (Barros

et al., 2009, Safronova *et al.*, 2010, Bayarsaihan, 2011, Yin *et al.*, 2011). Emerging evidence suggests that epigenetic modifications play a major role in inflammatory diseases, including periodontal disease (Barros *et al.*, 2014). Several factors that mediate periodontal disease pathogenesis, including bacteria and their byproducts, smoking, and diabetes, induce marked epigenetic changes in tissue components (Offenbacher *et al.*, 2008, Khansari *et al.*, 2009, Medzhitov *et al.*, 2009, Yin *et al.*, 2011, Razzouk *et al.*, 2013, Martinez *et al.*, 2014, Pasquier *et al.*, 2015). For example, chronically inflamed periodontal tissues demonstrated an increased methylation of CpG-rich regions of the PTGS2/COX2 promoter compared to healthy periodontal tissues (Zhang *et al.*, 2010). Also, there is marked hypomethylation of the IL8 promoter in oral epithelial cells of subjects with Generalized Aggressive Periodontitis compared to control subjects (Andia *et al.*, 2010). Emerging studies suggest that microbial pathogens, including oral species such as *Porphyromonas gingivalis,* induce epigenetic modifications in host cells (reviewed in (Niller *et al.*, 2017)). We recently identified potential epigenetic links between *T. denticola* and genes in PDL cells involved in activation of MMP-2 (Miao *et al.*, 2014). Thus, *T. denticola* may mediate epigenetic modifications that regulate MMP-2 activation and subsequent ECM degradation in the periodontium.

Epigenetic modifications are potentially reversible, and, therefore, a thorough understanding of these changes may identify new therapeutic targets for disease management. The aim of this study was to investigate *T. denticola's* ability to chronically activate MMP expression through epigenetic modifications in periodontal ligament cells/tissues, and to examine potential therapeutic approaches for reversal/modification of these changes.

Results

T. denticola chronically upregulates expression of MMP-2, MT1-MMP and TIMP-2, with concomitant fibronectin fragmentation

To determine the long-term effects of a brief exposure to *T. denticola* on MMP-2 expression in host cells, PDL cells were briefly challenged with *T. denticola,* then MMP-2 expression and MMP-2 activation in long-term cultures with daily medium changes were assessed by gelatin zymography and qRT-PCR. As shown in Fig. 1A, PDL cells constitutively expressed basal levels of pro-MMP-2 with minimal activation for maintenance of homeostatic functions. However, challenge with *T. denticola* triggered both chronic increased MMP-2 expression (pro-MMP2) and activation (active MMP-2) in PDL cells. Following a 2h exposure to *T. denticola*, dentilisin activity (visible as a ~100kDa band on the zymogram, Fig. 1A) persisted within these cultures throughout the experiment, though it was detected at greatly reduced levels at days 9 and 12. Expression and activation levels of MMP-2 were chronically sustained for up to 12 days, with minor reductions in the levels of activated MMP-2 on days 9 and 12 (Fig. 1A). *T. denticola*-mediated increases in MMP-2 expression and activation were mirrored by concomitant fibronectin fragmentation throughout the experiment (Fig. 1B).

The chronic effects of *T. denticola* on MMP-2 expression in PDL cells were regulated at the transcriptional level. MMP-2 mRNA levels were upregulated for up to 12 days as assessed by qRT-PCR (Fig. 1C). Given that the MT1-MMP/TIMP-2 complex is a well-known

regulator of MMP-2 activation, MT1-MMP and TIMP-2 expression were examined in *T. denticola* challenged periodontal ligament cells in long-term cultures. Expression of the MT1-MMP/TIMP-2 complex was also chronically upregulated by the *T. denticola* challenge, mirroring the changes induced in MMP-2 transcriptional expression (Fig. 1C).

T. denticola levels and MMP-2 transcription are elevated in periodontal disease

Examination of human tissues from periodontally diseased and healthy sites confirmed the association between *T. denticola* and elevated MMP-2 expression in diseased tissues. Human tissue specimens from periodontally diseased sites exhibited elevated levels of *T. denticola* concomitant with elevated levels of MMP-2 mRNA expression compared with healthy sites (Fig. 2). Tissue specimens from healthy sites exhibited negligible levels of *T. denticola* and low levels of MMP-2 expression. Low levels of MMP-2 expression in healthy tissues are consistent with the low basal levels of MMP-2 expression necessary for homeostatic functions within the periodontium, while increased MMP-2 expression is consistent with dysbiotic alterations of periodontal homeostasis in disease.

Expression of genes encoding chromatin modification enzymes are significantly altered in periodontal disease

To further examine the role of epigenetics in periodontal disease, the major chromatin modification enzymes that might be involved in the disease process (Table S1) were assessed via a focused gene array. Applying a “2-fold change” as a threshold value, we found that several chromatin modification enzymes were significantly altered in diseased periodontal tissue specimens compared to healthy control tissues (Fig. 3A, B and Table S2). The most significantly altered enzymes were the histone methyltransferases (HMTs) and histone deacetylases (HDACs), including PRMT8 and HDAC11, which exhibited a significant down-regulation. Other enzymes, especially those related to SET Domain proteins, histone acetyltransferases (HATs), and histone phosphorylation kinases showed significant up-regulation, such as SETD4, ESCO1, ESCO2, and AURKB. Other significantly altered enzymes included HATs, HDACs, histone ubiquitinases, DNA demethylases (DDMs) and histone demethylases (HDMs), and DNA methyltransferases (DNMTs).

T. denticola significantly alters expression of genes encoding chromatin modification enzymes in PDL cells

To examine the potential role of epigenetics in the *T. denticola*-mediated upregulation of MMP-2 expression, a focused gene array was employed to study all major epigenetic chromatin modification enzymes that might be involved in this process (Table S1). Applying a “2-fold change” as a threshold value, we found that *T. denticola*-challenged PDL cells exhibited significantly decreased levels of all major chromatin modification enzymes (and Fig. 3C, D and Table S3). No chromatin modifying enzymes assayed showed increased expression (data not shown). The most significantly down-regulated enzymes included aurora kinases, which mediate histone phosphorylation, and HMTs. Specifically, within these classes of enzymes, the most downregulated enzymes included Aurora Kinase B and EHMT2. Other significantly downregulated enzymes included HATs, histone deacetylases (HDAC), histone ubiquitinases, DNA (DDM) and histone demethylases (HDM), and DNMTs.

Inhibitors of histone kinase/aurora kinase, DNMT and HDAC block *T. denticola*-mediated increases in MMP-2 activity and expression in PDL cells

Given the broad landscape of epigenetic changes mediated by *T. denticola* on PDL cells and the changes exhibited in diseased tissues, targeted approaches were employed to examine the role of representative members of each major class of epigenetic enzymes (Table 1) in the process of MMP-2 modulation and FN fragmentation in *T. denticola*-challenged PDL cells.

Pretreatment of PDL cells with the histone kinase/phosphorylation inhibitor, PF-03814735, inhibited the potential increase in MMP-2 activation and expression mediated by *T. denticola* (Fig. 4A to C). Pretreatment with PF-03814735 also prevented the transcriptional increase in the MMP-2 activator complex, MT1-MMP/TIMP-2, and FN fragmentation mediated by the *T. denticola* challenge. This aurora kinase inhibitor exhibited a dose-response effect in terms of preventing the changes in MMP-2 expression and activation. At the highest dose tested, PF-03814735 also suppressed MMP-2 expression and activation in control cells. All enzyme inhibitor concentrations were selected in ranges that did not alter proliferation or induce cytotoxicity in PDL cells (data not shown).

Prevention of the potential increase in MMP-2 activation and expression mediated by *T. denticola* was also achieved by pretreatment of PDL cells with Azacytidine (AZA), a DNMT inhibitor (Fig. 5A to C). AZA had a dose-response effect, inhibiting increases in MMP-2 expression and activation, MT1-MMP/TIMP-2 expression, and fibronectin fragmentation mediated by the *T. denticola* challenge. In contrast, epigallocatechin gallate (EGCG), a different DNMT inhibitor with a broad spectrum of activity that includes inhibition of HATs, had considerably less effect than AZA. At the highest concentration tested, EGCG had only minor effects on *T. denticola*-mediated MMP-2 activation and TIMP-2 expression, but this did not result in modulation of FN fragmentation (Fig. 6A to C).

Examination of HDAC inhibitors Apicidin and Trichostatin revealed that pretreatment with these two inhibitors at the highest doses tested (1 μM and 0.1 μM, respectively) also inhibited the *T. denticola*-mediated increase in MMP-2 expression and activation as well as that of its activator complex, MT1-MMP/TIMP-2. However, as with EGCG, neither of these inhibitors affected FN fragmentation (data not shown).

HMT, HDM and HAT inhibitors exacerbate *T. denticola*-mediated increases in MMP-2 expression and activity in PDL cells

Inhibitors of histone demethylases (HDM), histone methyltransferases (HMT), and histone acetyltransferases (HAT) were not effective in preventing the *T. denticola*-mediated increases in pro-MMP-2 expression and activation. Treatment with Curcumin, a HAT inhibitor, resulted in moderately increased expression of pro-MMP-2, but significantly decreased its MMP-2 activation in a dose-dependent manner (Fig. 7A). In contrast, the HDM inhibitor, Tranylcypromine, HCl (TCP) and the HMT inhibitor, BIX 01295/ trihydrochloride hydrate further augmented the *T. denticola*-mediated increases in MMP-2 expression and activation in PDL cells (Fig. 7B, C).

Inhibitors of histone phosphorylation and histone deacetylase reverse *T. denticola*-mediated increases in MMP-2 activity and expression in PDL cells

An important remaining question was whether existing epigenetic modifications on periodontal ligament cells mediated by *T. denticola* could be reversed in a post-treatment scenario. To this end, periodontal ligament cells were first challenged with *T. denticola* then the same enzyme inhibitors tested above were employed to address this question. In agreement with the pretreatment results, post-treatment of *T. denticola* challenged PDL cells with three different epigenetic enzyme inhibitors reversed the effects on MMP-2 expression and activation: the histone kinase inhibitor, PF-03814735 (Fig. 8A) and the HDAC inhibitors apicidin (Fig. 8B) and trichostatin (Fig. 8C).

It is important to note that the results of the pre- and post-challenge inhibition experiments should not be compared in parallel because of different sample collection times used. In the pre-treatment experiments, samples were collected after 3 days of a *T. denticola* (2-hour exposure) challenge, whereas in the post-challenge treatment experiments, the samples were collected 2 hours to 4 days after a *T. denticola* (2 hour exposure) challenge, depending on the inhibitor used. Similarly, in contrast to the pre-challenge enzyme inhibition experiments, no decrease in FN fragmentation was observed in post-challenge inhibition experiments (data not shown), presumably because the MMP-2-mediated fragmentation process had begun before addition of enzyme inhibitors.

Discussion

ECM destruction, a key event in the pathogenesis of periodontitis, is mediated by host-derived enzymes such as MMP-2 that are involved in ECM homeostasis and remodeling. Strong evidence has accumulated over the past two decades that bacterial components, including proteases and lipopolysaccharides, are key factors contributing to dysregulation of ECM homeostasis. It is of particular interest that activation of pro-MMP-2, which is constitutively expressed in PDL cells, is required for the fragmentation of cellular FN that is typically seen in periodontal disease (Miao *et al.*, 2011). Thus, identifying factors that control MMP-2 expression or activation can help us better understand and modulate periodontal disease pathogenesis.

T. denticola is a member of a very complex microbiota involved in the pathogenesis of periodontal diseases. MMP-2 production and activation is a major event in the pathogenesis of these diseases via its role in extracellular matrix destruction. The *T. denticola* dentilisin protease plays an important role in the up-regulation and activation of MMP-2 in PDL cells (Miao *et al.*, 2011, Miao *et al.*, 2014), thereby promoting further ECM destruction and release of fibronectin fragments, which have deleterious effects on the periodontal environment (Kapila *et al.*, 1996, Kapila *et al.*, 1999, Kapila *et al.*, 2002, Jee *et al.*, 2004, Dai *et al.*, 2005, Tafolla *et al.*, 2005, Ghosh *et al.*, 2008, Joo *et al.*, 2008, Joseph *et al.*, 2010, Miao *et al.*, 2011, Miao *et al.*, 2014, Pereira *et al.*, 2014). The oral spirochete *T. lecithinolyticum* is also reported to activate MMP-2, as are *P. gingivalis* and *A. actinomycetemcomitans* (Choi *et al.*, 2001, Chang *et al.*, 2002, Song *et al.*, 2003). The molecular mechanism(s) involved remain unstudied or unresolved for these species.

T. denticola dentilisin activity was present for several days in PDL cell conditioned medium, long after the brief (2 hour) challenge, washing and multiple changes of growth medium. This is consistent with our prior reports (Miao *et al.*, 2011, Miao *et al.*, 2014) and is likely due to persistent adherence of *T. denticola* to PDL cells, though we cannot yet rule out potential downstream effects of previously documented uptake of *T. denticola* by PDL cells (Miao *et al.*, 2014) and gingival epithelial cells (Shin *et al.*, 2012, Jo *et al.*, 2014, Inagaki *et al.*, 2016). Importantly, while dentilisin activity levels decreased steadily over time, MMP-2 and its activating complex, MT1-MMP and TIMP-2, were clearly expressed and activated through the 12th day of culture. These data confirm our previous results that dentilisin is an important factor in the activation of pro-MMP-2 (Miao *et al.*, 2011). Additionally, in agreement with our earlier findings (Miao *et al.*, 2014), the current data show that *T. denticola* chronically activates MMP-2, in concert with MT1-MMP and TIMP-2 expression up to 12 days. Importantly, degradation of cellular FN in PDL cell cultures is dependent on MMP-2 activation, which is the result of *T. denticola* dentilisin activity (Miao *et al.*, 2011). The persistent fibronectin fragmentation in these samples may help explain the chronicity of tissue destruction mediated by bacterial proteases during periodontal disease pathogenesis.

Previous studies showed that *T. denticola* has the ability to adhere to and be internalized by several different host cells, including gingival epithelial cells, PDL cells, and polymorphonulear leukocytes (Ding *et al.*, 1997, Konermann *et al.*, 2012, Shin *et al.*, 2012, Miao *et al.*, 2014). It should be noted that, unlike other oral pathogens, such as *Porphyromonas gingivalis* which exhibits a cellular-invasive phenotype (Lamont *et al.*, 1995), there is no reported evidence that *T. denticola* survives in the intracellular environment. Taken in aggregate, these results and the findings from this study, support the concept that the alteration of the PDL cells towards a destructive phenotype is a consequence of exposure to *T. denticola*. These data are consistent with evidence from other inflammatory diseases pointing toward an epigenetic role in the pathogenic process. We hypothesized that epigenetic mechanisms may imprint the periodontium and set in motion the process of chronic periodontal tissue destruction.

Mechanisms of epigenetic modifications include, histone acetylation, histone methylation, DNA methylation, positioning of histone variants, and gene regulation by non-coding micro RNAs (miRNAs) (Bayarsaihan, 2016, Herceg, 2016, Perkins *et al.*, 2016). Several enzymes are involved in these mechanisms including; histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone phosphorylases, and DNA methyltransferases (DNMTs).

Perturbing the balance between acetylation/deacetylation or methylation/demethylation is profoundly associated with numerous diseases including developmental abnormalities, cancer and chronic inflammatory conditions (Bird, 2002, Barros *et al.*, 2009). Epigenetic modifications are potentially reversible; therefore, a thorough understanding of these changes may identify new therapeutic targets for disease management. Using gene arrays that target chromatin modification enzymes, our data provides the first evidence that *T. denticola* mediates downregulation of many of these enzymes in PDL cells. In similar studies, *P. gingivalis* lipopolysaccharides were shown to downregulate many chromatin modification enzymes in cultured oral keratinocytes (de Camargo Pereira *et al.*, 2013) and

change the expression of multiple miRNAs in PDL cells (Du *et al.*, 2016). However, since *T. denticola*-dependent MMP-2 activation requires dentilisin proteolytic activity (Miao *et al.*, 2011), it is likely that the molecular mechanisms responsible for this downregulation differ from those driven by *P. gingivalis* lipopolysaccharides. Specific mechanisms by which *T. denticola* or its dentilisin protease may regulate epigenetic enzyme expression are under study in our laboratories.

The association between *T. denticola* and periodontitis has been reported in numerous studies (Simonson *et al.*, 1988, Sakamoto *et al.*, 2001, Asai *et al.*, 2002, Yoshida *et al.*, 2004). Consistent with the existing literature, we found that *T. denticola* levels were significantly elevated in human tissue specimens from periodontally diseased sites compared with healthy sites, as measured by qPCR normalized to human GAPDH (data not shown). To date, studies of the role of epigenetic modifications in periodontal disease have focused on DNA methylation patterns in genes involved in inflammatory responses (Barros *et al.*, 2014). The present study used gene array approaches to examine healthy and diseased periodontal tissues, revealing for the first time significant alterations in the expression of several chromatin modification enzymes in diseased tissues. Although expression levels of some enzymes (such as Aurora Kinase B) differed in the gene array of the PDL cells compared to the tissues, this likely reflects the fact that tissues exhibit the net expression of diverse cell types that comprise the periodontium, as well as the net effects of the diverse oral microbiome on the periodontium, and net effects of other epigenetic effectors, including smoking and medications.

Different inhibitors show promise in reversing epigenetic changes in the context of our study and in other reports. Chronic *T. denticola*-mediated MMP-2 expression and activation were decreased in *T. denticola* challenged PDL cells either pre- or post-treated with inhibitors of histone phosphorylases, histone deacetylases (HDACs), and DNA methyltransferases (DNMTs). This indicates that *T. denticola* induces epigenetic changes mediated by histone phosphorylation, histone deacetylation, or DNA methylation pathways. Specifically, pre- or post-treatment inhibition of histone phosphorylation in PDL cells using PF-03814735 significantly prevented or reversed the *T. denticola*-mediated increase in MMP-2 and the MT1-MMP/TIMP-2 complex. This study is the first to show the effect of inhibition of histone phosphorylation on MMP-2 expression in PDL cells. In a broader context, PF has been used in phase I clinical trials for the treatment of advanced solid tumors (Jani *et al.*, 2010, Schoffski *et al.*, 2011).

Inhibition of HDACs using Apicidin and/or Trichostatin was also effective in preventing or reversing the *T. denticola*-mediated effects on the expression of MMP-2 in PDL cells. Similar data were obtained by other studies investigating the effect of Trichostatin on MMP-2 expression in murine fibroblasts (Ailenberg *et al.*, 2002). Trichostatin was further shown to decrease MMP-2 and MMP-9 expression in murine heart cells (Mani *et al.*, 2015) and in human esophageal squamous cell carcinoma cells (Wang *et al.*, 2013). Trichostatin, in combination with SAHA, was reported to inhibit the respiratory syncytial virus (RSV)-mediated increase in HDAC2 expression with resultant decrease in airway inflammation and oxidative stress *in vivo* (Feng *et al.*, 2016). Apicidin was also reported to inhibit the expression of MMP-2 in different cancer cells (Kim *et al.*, 2000, Park *et al.*, 2011, Ahn *et al.*,

2012). On the other hand, inhibition of DNMTs using AZA showed a reduction in the expression of MMP-2 and its activating complex, while the use EGCG was not effective in this process.

Our results are consistent with those in other studies reporting that AZA causes a down-regulation of MMP-2 and MMP-9 expression in cultured esophageal squamous cell carcinoma cells (Liu *et al.*, 2014) and breast cancer cells (Chang *et al.*, 2014b) respectively. AZA was approved by the U. S. Food and Drug Administration (FDA) for treatment of myelodysplastic syndromes (Nebbioso *et al.*, 2012). It was also investigated in many clinical trials for treatment of several disorders, including hematological and neoplastic disorders (Liu *et al.*, 2005, Mirza *et al.*, 2010, Chen *et al.*, 2012). Regarding the effect of EGCG on MMP-2 expression in our study, the data are inconsistent with other studies, which reported the ability of EGCG to inhibit MMP-2 in different cancer cells (Chang *et al.*, 2014a, Nowakowska *et al.*, 2016). This inconsistency may be due to the use of different cell types, different concentrations of EGCG, or both. Concentrations higher than 10 μM were toxic to PDL cells as examined by cell proliferation and cytotoxicity assays (data not shown). Due to the weak effect of EGCG on the expression of MMP-2, we did not test its effects in a post-challenge scenario.

Negative consequences of using epigenetic enzyme inhibitors are possible, as seen in the current study. Specifically, some inhibitors, namely those inhibiting HDM with TCP and those inhibiting HMT with BIX 01295, further increased MMP-2 expression and activation. Although TCP and BIX are thought to mediate opposite actions, namely BIX inhibits histone methylation and TCP promotes histone methylation, both increased MMP-2 expression and activation. Thus, due to these undesirable effects, BIX and TCP were not evaluated further in post-challenge scenarios. There are no previous studies investigating their effect on expression of MMPs. However, *Pereira et al.* showed that increases in MMP-2 expression were stimulated by decreases in histone methylation in the context of the MMP-2 promoter (Pereira *et al.*, 2014). Additionally, in another study, the MMP-1, 3, 9, and 13 genes were shown to be actively transcribed in rheumatoid arthritis-derived synovial fibroblasts, and this transcription correlated with an elevation in H3K4me3 and suppression of H3K27me3 in the MMP promoter genes (Araki *et al.*, 2016). These studies, which both showed increases in MMP-2 expression despite different degrees of histone methylation, can be explained by the fact that activation or repression of gene expression by histone modifications/methylations depends on the type of lysine being modified and the degree of its methylation. For example, H3K4me, H3K36me, or H3K79me are associated with transcriptional activation (Jenuwein *et al.*, 2001, Zhang *et al.*, 2001, Barski *et al.*, 2007, Guenther *et al.*, 2007, Koch *et al.*, 2007, Kouzarides, 2007), whereas H3K9me, H3K27me, or H4K20me are implicated in gene repression (Jenuwein *et al.*, 2001, Nakayama *et al.*, 2001, Talbert *et al.*, 2006, Barski *et al.*, 2007, Kouzarides, 2007).

The HAT inhibitor curcumin also increased the expression of MMP-2 but significantly inhibited its activation. The effect of curcumin in decreasing MMP-2 and MMP-9 expression levels was reported in cancer cells, such as squamous cell carcinoma and osteoclastoma (Cao *et al.*, 2015, Lee *et al.*, 2015). Additionally, *in vivo* studies in humans and animals demonstrated curcumin's effectiveness in decreasing the severity of periodontal diseases

(Elburki *et al.*, 2014, Nagasri *et al.*, 2015, Bakir *et al.*, 2016, Elburki *et al.*, 2016). Curcumin has been investigated in many clinical trials for treatment of several disorders, such as ulcerative colitis (Baliga *et al.*, 2012), breast cancer (Nagaraju *et al.*, 2012), pancreatic cancer (Veeraraghavan *et al.*, 2011), and diabetes (Abdel Aziz *et al.*, 2012). Additional investigation into the role of curcumin in MMP activation and regulation in periodontal diseases is further warranted, given these reports and the current study findings.

In summary, *T. denticola* plays a key role in the transcriptional regulation of MMP-2 and its activating complex MT1-MMP/TIMP-2 in PDL cells. *T. denticola* also mediates alterations of chromatin modification enzyme expresssion in PDL cells, and an array of epigenetic modifications are associated with periodontally diseased tissues. Furthermore, inhibition of enzymes that mediate epigenetic modifications can prevent *T. denticola*-mediated increases in MMP-2, MT1-MMP, and TIMP-2 in PDL cells. These inhibitors can also reverse the *T. denticola*-mediated effects on MMP-2 and its activating complex in PDL cells. These data indicate that *T. denticola* mediates its effects on MMP-2 activation through epigenetic modifications in these cells. This knowledge can be useful as a first step toward the development of novel targeted therapeutics for the treatment of periodontal diseases.

Materials and Methods

Periodontal ligament (PDL) cell culture

As described previously, the primary culture of PDL cells was obtained via the direct cell outgrowth method by isolating cells from the PDL tissue around the middle third of extracted healthy human teeth (Scanlon *et al.*, 2011, Tanaka *et al.*, 2011). Cells were maintained in minimal essential medium-α (MEM-α) augmented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 1% amphotericin B (Gibco, Grand Island, NY, USA) in a humid atmosphere with 95% air and 5% CO_2 at 37°C. Cell outgrowths were passaged when they reached confluency. Cells passaged three to six times were used for experimentation. The cell counting kit-8 (Dojindo, Rockville, MD, USA) was used for assaying PDL cell proliferation and cytotoxicity at different time intervals in response to different concentrations of the inhibitors used in the study (data not shown). Protocols involving the collection and use of human teeth and PDL cells/tissue were approved by the Health Sciences Institutional Review Board of the University of Michigan.

Human periodontal tissues

Periodontal tissues were obtained by collecting the tissues around the coronal third of the roots of extracted periodontally-involved teeth and healthy teeth, both from the posterior mandibular region. Periodontal status was diagnosed clinically by testing for bleeding on probing of the gingival sulcus and periodontal pocket depth measurements. The samples were collected from six different periodontitis patients and six healthy subjects. The collection and use of human teeth and PDL tissues was approved by the Health Sciences Institutional Review Board of the University of Michigan.

Culture of *Treponema denticola*

Treponema denticola ATCC 35405 was grown as described previously under anaerobic conditions at 37°C in New Oral Spirochete (NOS) broth medium (Haapasalo *et al.*, 1991, Fenno, 2005). Purity of spirochete cultures was confirmed by darkfield microscopy prior to use in experiments.

Challenge of PDL cells with *Treponema denticola*

PDL cells were prepared in MEM-α free of serum and antibiotics. The bacteria in broth culture were collected by centrifugation, then re-suspended in serum- and antibiotic-free MEM-α to an optical density of 0.1 at 600 nm, such that the cellular density was approximately 2.4×10^8 cells/ml.

T. denticola in serum-and antibiotic-free MEM-α was added to the test group of PDL cells (*T. denticola* group) at a multiplicity of infection (MOI) = 100, whereas only MEM-α was added to the control group. Both groups were then incubated for two hours at 37°C in 5% CO2-containing air. After the two-hour challenge, PDL cells were washed three times with PBS and incubated for the planned time periods in serum-and antibiotic-free MEM-α with daily medium changes as described previously (Miao *et al.*, 2011). Subsequently, the culture conditioned medium and cell lysates were collected, RNA was extracted from the cell lysates, and all samples were stored at −80°C for further investigations.

Pre-challenge inhibition of epigenetic chromatin modification enzymes in PDL cells

Different inhibitors of epigenetic chromatin modification enzymes (listed in Table 1) were obtained and prepared according to the manufacturer's instructions, then brought to the desired concentrations via dilution in serum- and antibiotic-free MEM-α. With the exception of tranylcypromine (EMD Millipore, Temecula, CA, USA), all enzyme inhibitors were purchased from Sigma Aldrich (St. Louis, MO, USA).

PDL cells were treated with the indicated concentrations of the enzyme inhibitors either as single agents or in combinations, and incubated for the indicated times with daily culture medium replacement. The cells were then challenged with *T. denticola* at an MOI of 1:100 for 2 hours, washed, and incubated for three days in serum- and antibiotic-free MEM-α with daily culture medium refreshment. At the end of this incubation period, conditioned culture medium and cell lysates were harvested and stored at −80°C for further investigations.

Post-challenge inhibition of epigenetic chromatin modification enzymes in PDL cells

For the post-challenge experiments, the PDL cells were first challenged with *T. denticola* as described before, then treated with the indicated concentrations of the inhibitors either as single agents or in combinations for certain periods. At the end of the treatment period, the conditioned culture medium and cell lysates were harvested and stored at −80°C for further investigations. All experiments were repeated at least three times and each experiment was performed in triplicate.

Gelatin zymography

Culture supernatants were concentrated approximately 10-fold in Amicon centrifugal concentrators (10,000-molecular-weight cutoff; Millipore) and total protein concentration was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) normalized to an albumin standard. Equivalent protein concentrations from each sample were mixed with non-reducing sample buffer (0.25 M tris-base, 40% glycerol, 0.8% SDS, and 0.05% bromophenol blue stain in distilled deionized water/ddH_2O at pH 6.8) and loaded into 8% polyacrylamide gels co-polymerized with 0.4% SDS and 0.2% gelatin. Samples were electrophoretically resolved on gelatin-containing gels at 125 V for 110 minutes at 4°C. Gels were then washed twice for 15 minutes under continuous agitation using renaturation/washing buffer (2.5% v/v Triton-X100 and 0.05 M Tris-base in ddH_2O at pH 7.5) to eliminate SDS and promote the renaturation of MMP enzymes. Subsequently, gels were incubated in developing/incubation buffer (0.05 M Tris-base, 0.15 M sodium chloride, 0.01 M calcium chloride, and 0.02% sodium azide in ddH_2O at pH 7.5) for 30 minutes under agitation, then the buffer was replaced and gels incubated for 16–20 hours at 37°C. After that, gels were stained using filtered Coomassie Brilliant blue stain for one or two hours under agitation. Destaining of the gels was performed using a methanol/acetic acid destaining buffer (40% methanol and 10% acetic acid in ddH_2O) until the bands on the gel appeared clear. Zymograms were scanned and the densitometry of the gelatinolytic activity represented by the clear bands was analyzed using ImageJ software (NIH, USA). Brightness and contrast levels of zymogram images were slightly adjusted for publication only.

Immunoblotting

Equivalent protein samples consisting of 10 fold concentrates of PDL cell culture conditioned media were standardized as described above, subjected to standard SDS-PAGE (4–12% polyacrylamide gels; Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes using standard techniques. The membranes were exposed to a rabbit polyclonal anti-fibronectin IgG (Santa Cruz, Dallas, TX, USA) primary antibody diluted 1:2000 in TBST solution for 2 hours at room temperature followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz, Dallas, TX, USA) diluted 1:10,000 in TBST for 1 hour at room temperature with agitation. Western blots using anti-GAPDH antibodies were used to further confirm equal protein loading (data not shown). Blots were developed using the SuperSignal® West Pico kit (ThermoFischer Scientific, Pittsburgh, PA, USA) and scanned for digitization.

Quantitative reverse transcriptase PCR (qRT-PCR)

qRT-PCR was performed to assess the transcriptional levels of MMP-2, MT1-MMP, and TIMP-2 in PDL cells. Cell lysates were collected and the RNA was extracted and purified using the RNeasy® mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription of the RNA into cDNA was then performed using the SuperScript™ II RT kit (Invitrogen, Carlsbad, CA, USA). The cDNA was then amplified by qPCR using the TaqMan® Universal PCR Master Mix (Invitrogen, Carlsbad, CA, USA)" on a ViiA™ 7 Applied Biosystems® PCR system. The following TaqMan® human probes were used; MMP-2 (Hs01548727_m1), MT1-MMP (Hs01037003_g1), TIMP-2

(Hs00234278_m1), and GAPDH (Hs03929097_g1) (Invitrogen, Carlsbad, CA, USA). The cycle threshold (C_t) values were obtained, analyzed and the quantitative expression of target genes in challenged PDL cells was normalized to GAPDH and compared to the control cells using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001), applying a minimum 2 fold change in expression as the cut off.

Similar methodology was utilized to assess the levels of MMP-2 transcription and the levels of *T. denticola* in human periodontal tissue samples. Periodontal tissue specimens were collected by scraping the most coronal portion of the tissue around the roots of extracted healthy and periodontally-diseased teeth. Total RNA was extracted from healthy and diseased periodontal tissue specimens and cDNA was generated. Target genes were amplified using TaqMan® Universal PCR Master Mix (Invitrogen, Carlsbad, CA, USA). Tissue sample human genome content was normalized using a custom TaqMan® primer/probe set for GAPDH (Hs03929097_g1; Invitrogen, Carlsbad, CA, USA). *T. denticola* 16S rRNA was amplified in parallel using the SYBR® Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) with the following primer set: 16SrRNA-987F AGGGATATGGCAGCGTAGCA and 16SrRNA-1077R TTGCGGGACTTAACCCAACA.

Quantitative reverse transcription (qRT-PCR) gene micro-array

qRT-PCR arrays were used to assess the transcriptional levels of the main epigenetic chromatin modification enzymes in cultured PDL cells and in periodontal tissues using the RT^2 Profiler PCR Array kit (Qiagen, Valencia, CA, USA) to assay transcription of the genes listed in Table S1. Periodontal tissue specimens were collected by scrapping the most coronal portion of the tissue around the roots of extracted healthy and periodontally-diseased teeth. RNA was extracted and purified from PDL cell cultures using the RNeasy® Mini kit, while extraction and purification of RNA from periodontal tissues was achieved using the RNeasy® Protect Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reverse transcription of RNA into cDNA was performed using the RT^2 First Strand Kit (Qiagen, Valencia, CA, USA). The target genes were then amplified using the RT^2 Profiler PCR Array kit and RT^2 SYBR® Green Master mix (Qiagen, Valencia, CA, USA) on a ViiA™ 7 Applied Biosystems® PCR system. Applying a minimum 2 fold change in expression as the cut off, C_t values were analyzed and the quantitative expression of the genes of interest in *T. denticola*-challenged PDL cells was normalized to supplied housekeeping genes; β-actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin, Hypoxanthine phosphoribosyltransferase-1, and large ribosomal protein-P0 and then compared to expression levels in unchallenged PDL cells and the healthy tissues (Livak *et al.*, 2001).

Statistical analysis

The data were analyzed using the statistical software SPSS® v.22 (IBM, Armonk, NY, USA). Results were evaluated by a one-way ANOVA when comparing more than two groups, whereas the student's t-test was used when comparing two groups. $p \leq 0.05$ was considered significant, whereas $p \leq 0.001$ was considered highly significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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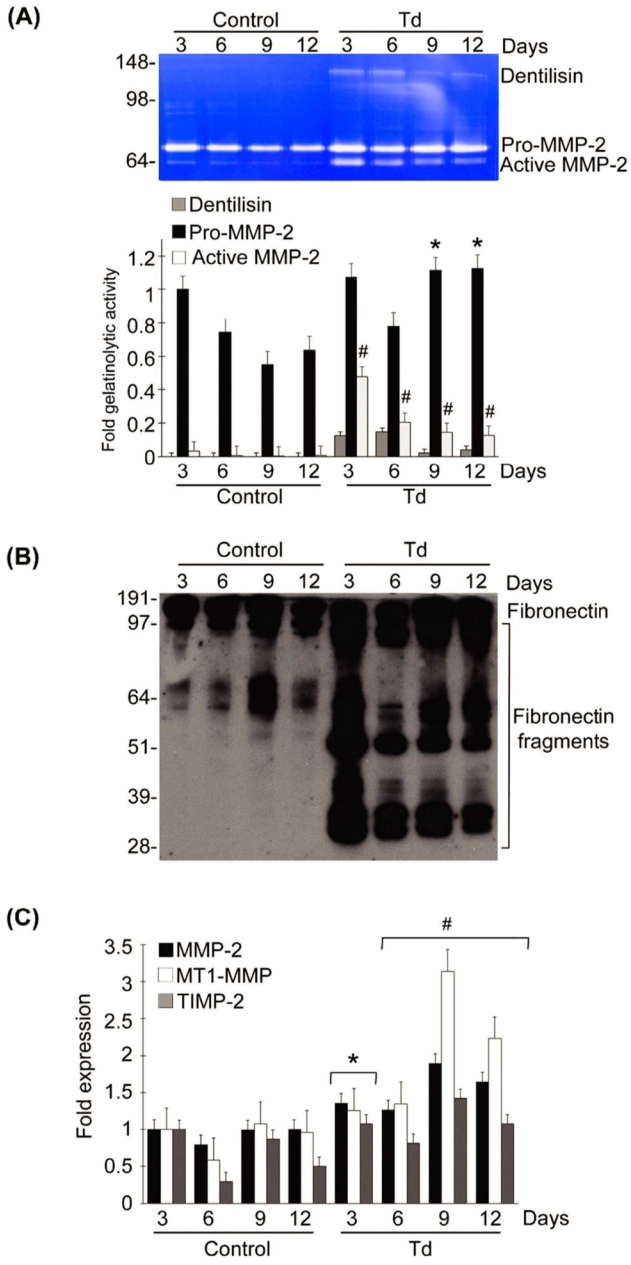

Figure 1. *T. denticola* mediates chronic expression and activation of MMP-2, MT1-MMP, and TIMP-2 in PDL cells, with subsequent fragmentation of cellular fibronectin

Cultured PDL cells were challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours, then incubated for 3, 6, 9, and 12 days with daily medium changes. The experiments were repeated three times in triplicate. Data were analyzed using one-way ANOVA. (*) represents $p \leq 0.05$ compared to the same time point in the control group. (#) represents $p \leq 0.001$ compared to the same time point in the control group.

Panel A: A representative gelatin zymogram of PDL cell conditioned medium showing the gelatinolytic activity of pro-MMP-2 (72-kDa), active MMP-2 (64-kDa), and *Td* dentilisin (100-kDa). The left 4 lanes represent the control unchallenged PDL cells and the right 4

lanes represent the *Td*-challenged PDL cells. The bar chart below represents the densitometric analysis of gelatinolytic activity in the zymograms using ImageJ™-NIH software. The Y-axis represents fold-gelatinolytic activity of the pro-MMP-2, active MMP-2, and dentilisin relative to unchallenged control at day 3. The X-axis represents different time points.

Panel B: A representative immunoblot of PDL cell culture supernatants probed with a polyclonal anti-fibronectin antibody showing FN fragmentation in conditioned medium from *Td*-challenged PDL cells.

Panel C: Transcript levels of MMP-2, MT1-MMP, and TIMP-2 in PDL cells at different time points after challenge with *Td* or media control assayed by qRT-PCR. The Y-axis represents fold-expression level of each gene relative to unchallenged control at day 3. The X-axis represents different time points.

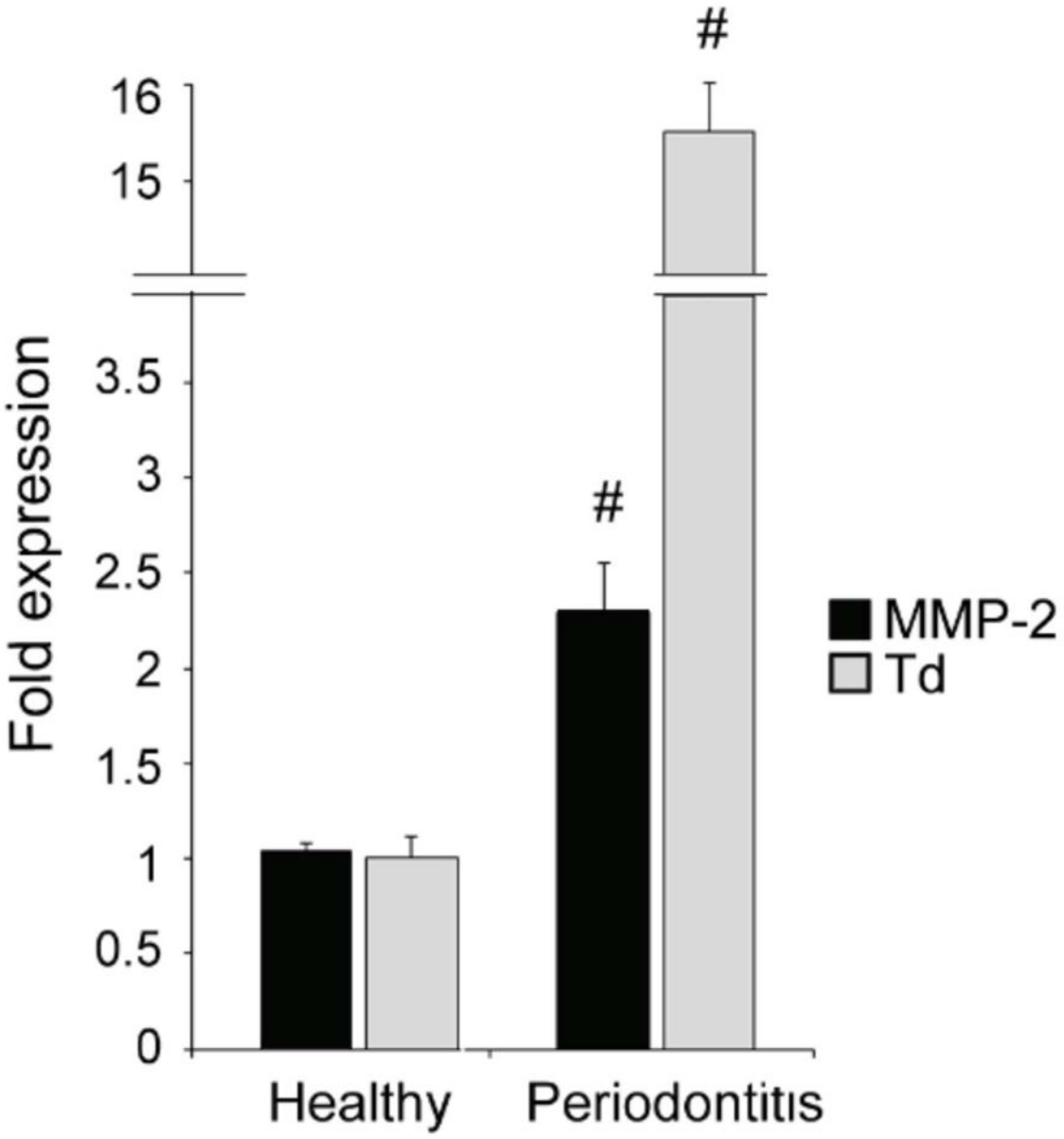

Figure 2. Elevated MMP-2 mRNA and *T. denticola* in diseased periodontal tissue

Levels of *T. denticola* 16S rRNA and MMP-2 mRNA in tissue specimens from periodontally diseased or healthy sites were assayed by qRT-PCR. The Y-axis represents levels of each RNA in periodontitis specimens relative to healthy specimens. The X-axis represents the source of tissue specimens. Data were analyzed using Student's t-test. (#) represents $p \leq 0.001$ compared to healthy tissue.

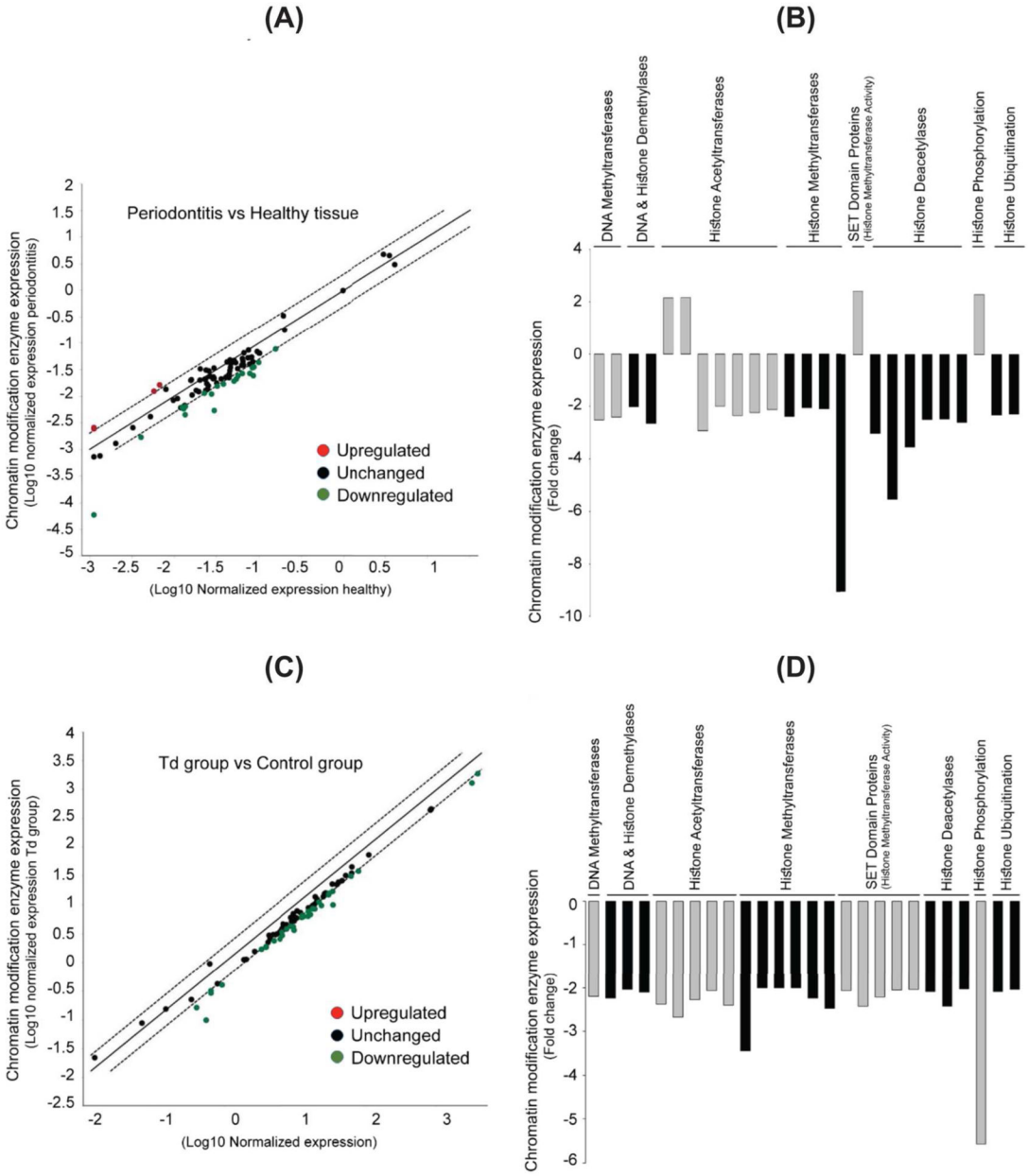

Figure 3. Transcription of multiple chromatin modification enzymes is altered in both periodontally diseased tissue and in *T. denticola*-challenged PDL cells

Transcription levels of 87 chromatin modification enzymes were examined by qRT-PCR using RNA extracted from healthy and diseased periodontal tissues (Panels A and B, respectively) and from PDL cell lysates collected 9 days after *Td* challenge as described in Fig. 1 (Panels C and D, respectively).

Panels A and C: Scatter blots showing the transcriptional level of chromatin modification enzymes in periodontally diseased tissues relative to healthy tissue controls (Panel A) and in *Td*-challenged PDL cells relative to unchallenged control cells (Panel C). The Y-axis represents expression level of different enzymes relative to healthy tissue (Panel A) or

unchallenged control cells (Panel C). The X-axis represents normalized expression of the respective control group.

Panels B and D: Bar-charts showing the significantly downregulated chromatin modification enzymes in periodontally diseased tissues relative to healthy tissue controls (Panel B) and in *Td*-challenged PDL cells relative to unchallenged control cells (Panel D). The Y-axis represents the fold-expression/downregulation level of enzymes relative to unchallenged controls. The X-axis represents different chromatin modification enzymes, with enzyme type grouped by color.

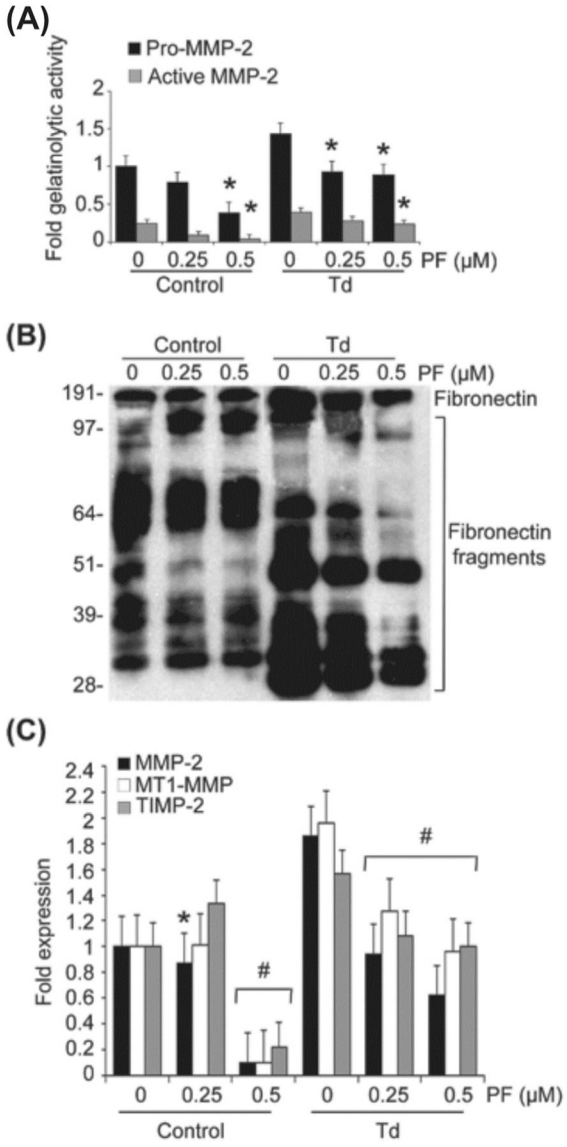

Figure 4. Inhibition of histone phosphorylation using PF-03814735 results in decreased expression and activation of MMP-2, MT1-MMP and TIMP-2, and decreased fibronectin fragmentation in *T. denticola*-challenged and control PDL cells

Cultured PDL cells were pre-treated with 0.25 or 0.5 μM PF-03814735 (PF) for two days. The cells were then challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours then incubated for three days. The conditioned medium and cell lysates were collected for zymography, western blotting, and qRT-PCR. The experiments were repeated three times in triplicate. Data were analyzed using one-way ANOVA. (*) represents $p \leq 0.05$ compared to the "0" concentration in the same group. (#) represents $p \leq 0.001$ compared to the "0" concentration in the same group.

Panel A: Densitometric analysis of pro-MMP-2 (72kDa) and active MMP-2 (64kDa) detected by gelatin zymography. The X-axis represents different concentrations of histone kinase/phosphorylation inhibitor, PF-03814735 (PF) in the control and *Td* groups. The Y-

axis represents fold-gelatinolytic activity of the pro-MMP-2 and active MMP-2 relative to unchallenged and untreated controls.

Panel B: A representative immunoblot of PDL cell culture supernatants probed with a polyclonal anti-fibronectin antibody showing FN fragmentation in conditioned medium from *Td*-challenged PDL cells, control and PF-treated.

Panel C: Bar chart showing transcript levels of MMP-2, MT1-MMP and TIMP-2 in the control and PF-treated PDL cells as determined by qRT-PCR. The Y-axis represents fold-expression level of each gene relative to unchallenged and untreated controls. The X-axis represents different concentrations of PF in the control and *Td* groups.

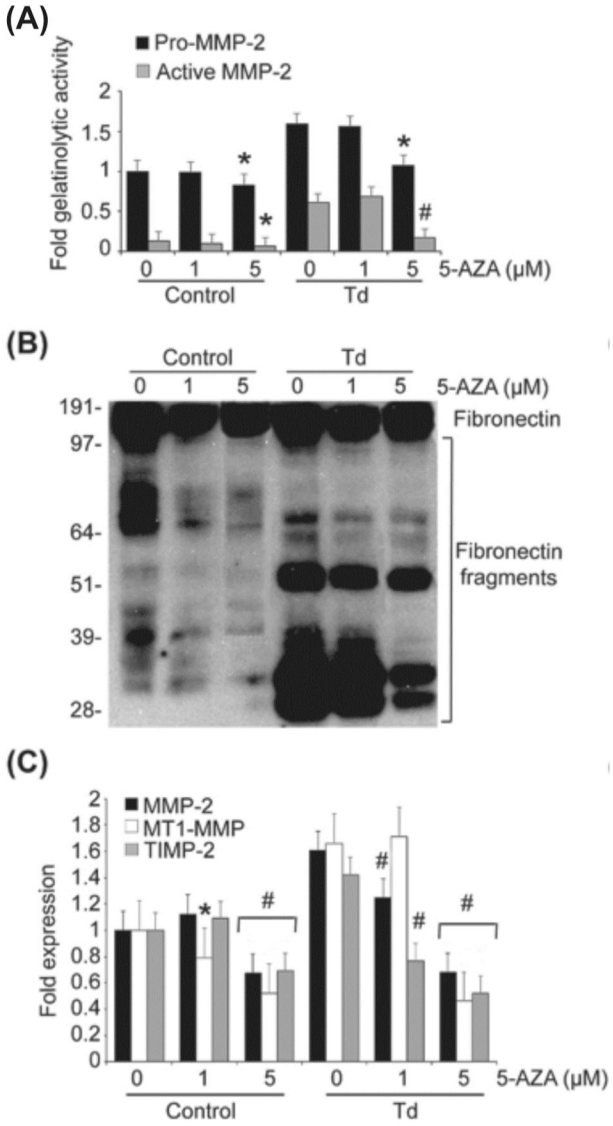

Figure 5. Inhibition of DNA methyltransferases using AZAcytidine (AZA) result in decreased expression and activation of MMP-2, MT1-MMP and TIMP-2, and decreased fibronectin fragmentation in *T. denticola*-challenged and control PDL cells

Cultured PDL cells were pre-treated with 1 or 5 μM AZA for four days. The cells were then challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours then incubated for three days. The conditioned medium and cell lysates were collected for zymography, western blotting, and qRT-PCR. The experiments were repeated three times in triplicate.

Panel A: Densitometric analysis of pro-MMP-2 (72kDa) and active MMP-2 (64kDa) detected by gelatin zymography. The X-axis represents different concentrations of DNA methyltransferases inhibitor Azacytidine (AZA) in the control and *Td* groups. The Y-axis represents fold-gelatinolytic activity of the pro-MMP-2 and active MMP-2 relative to unchallenged and untreated controls. Data were analyzed using one-way ANOVA. (*)

represents $p \leq 0.05$ compared to the "0" concentration in the same group. (#) represents $p \leq 0.001$ compared to the "0" concentration in the same group.

Panel B: A representative immunoblot of PDL cell culture supernatants probed a polyclonal anti-fibronectin antibody showing FN fragmentation in conditioned medium from *Td*-challenged PDL cells, control and AZA-treated.

Panel C: Bar chart showing transcript levels of MMP-2, MT1-MMP and TIMP-2 in the control and AZA-treated PDL cells as determined by qRT-PCR. The Y-axis represents fold-expression level of each gene relative to unchallenged and untreated controls. The X-axis represents different concentrations of AZA in the control and *Td* groups.

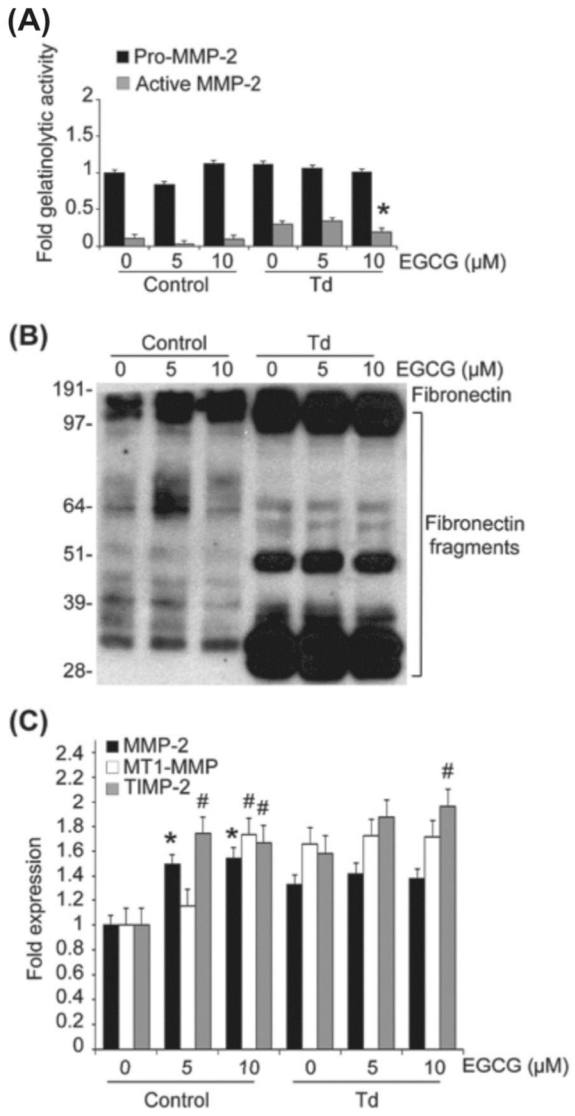

Figure 6. Inhibition of DNA methyltransferases using epigallocatechin gallate (EGCG) mediate a decrease in MMP-2 activation, an increase in TIMP-2 transcription and minimal decrease in fibronectin fragmentation in *Td*-challenged PDL cells

Cultured PDL cells were pre-treated with 5 or 10 μM EGCG for two days before being challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours, then incubated for three days. The conditioned medium and cell lysates were collected for zymography, western blotting, and qRT-PCR. The experiments were repeated three times in triplicate. Data were analyzed using one-way ANOVA. (*) represents $p \leq 0.05$ compared to the "0" concentration in the same group. (#) represents $p \leq 0.001$ compared to the "0" concentration in the same group.

Panel A: Densitometric analysis of pro-MMP-2 (72kDa) and active MMP-2 (64kDa) detected by gelatin zymography. The left 3 lanes represent the control group (unchallenged PDL cells) and the right 3 lanes represent the Td group (*Td*-challenged PDL cells). The cells in both groups were treated with indicated concentrations of EGCG. The X-axis represents different concentrations of EGCG in the control and *Td* groups. The Y-axis represents fold-

gelatinolytic activity of pro-MMP-2 and active MMP-2 relative to unchallenged and untreated controls.

Panel B: A representative immunoblot of PDL cell culture supernatants probed with a polyclonal anti-fibronectin antibody showing FN fragmentation in conditioned medium from *Td*-challenged PDL cells, control and EGCG-treated.

Panel C: Bar chart showing transcript levels of MMP-2, MT1-MMP and TIMP-2 in the control and ECGC-treated PDL cells as determined by qRT-PCR. The Y-axis represents fold-expression level of each gene relative to unchallenged and untreated controls. The X-axis represents different concentrations of ECGC in the control and *Td* groups.

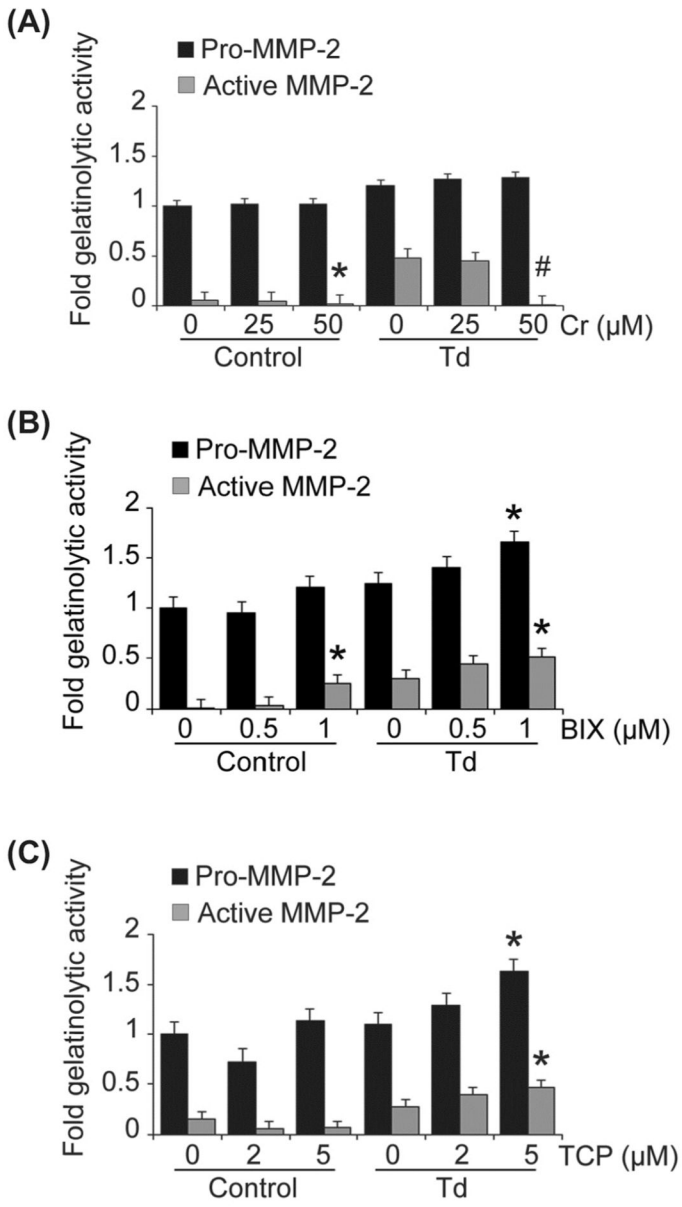

Figure 7. Inhibition of different chromatin modification enzymes mediate alterations in levels of expression and activation of MMP-2 in *Td*-challenged and control PDL cell cultures

PDL cells were pre-treated with enzyme inhibitors at the indicated concentrations as follows: curcumin (histone acetyltransferase inhibitor) for two hours **(A)**, 0.5 μM and 1 μM BIX-01294 (histone methyltransferase inhibitor) for two days **(B)**, and 2 μM and 5 μM tranylcypromine/TCP (histone demethylase inhibitor) for four days **(C)**. The cells were then challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours, then incubated for three days. The conditioned medium and cell lysates were collected for zymography. Shown are densitometric analyses of pro-MMP-2 (72kDa) and active MMP-2 (64kDa) detected by gelatin zymography. The X-axis represents different concentrations of enzyme inhibitors in the control and *Td* groups. The Y-axis represents fold-gelatinolytic activity of the pro-MMP-2 and active MMP-2 relative to unchallenged and untreated

controls. Panels: A, curcumin (Cr); B, BIX-01294 (BIX); C, tranylcypromine (TCP). The experiments were repeated three times in triplicate. Data were analyzed using one-way ANOVA. (*) represents $p \leq 0.05$ compared to the "0" concentration in the same group. (#) represents $p \leq 0.001$ compared to the "0" concentration in the same group.

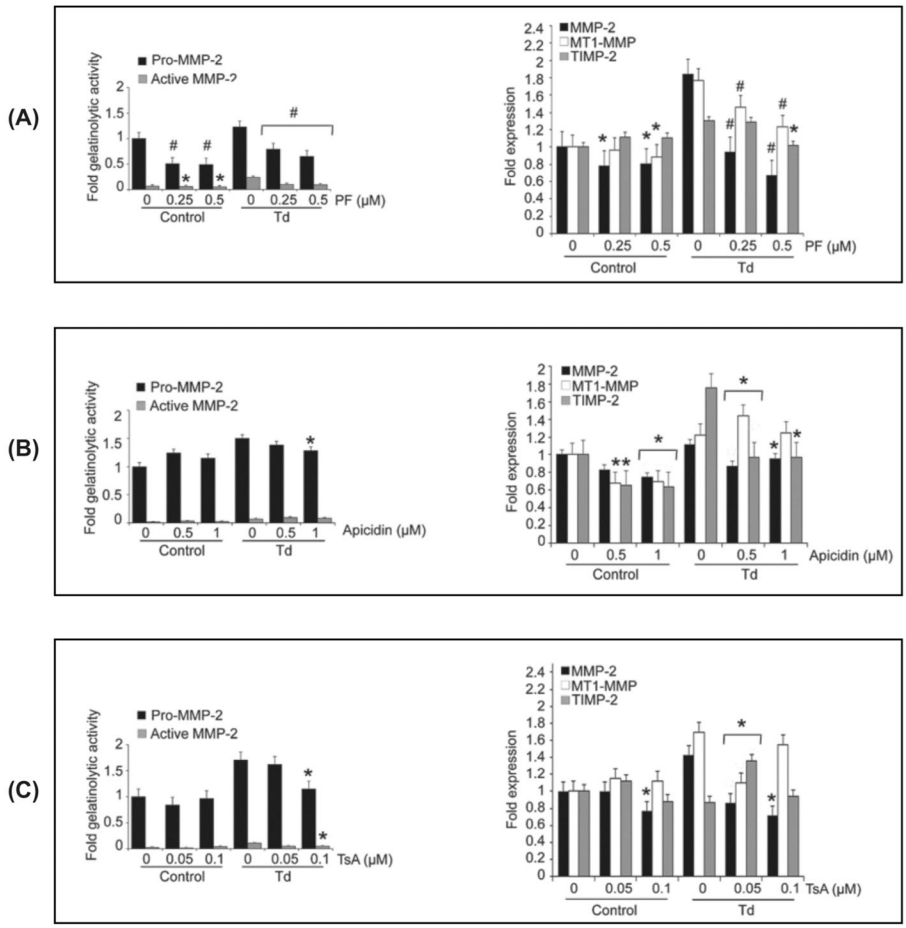

Figure 8. Inhibitors of histone phosphorylases and deacetylases reverse *T. denticola*-mediated increases in MMP-2 activity and expression in PDL cells

Cultured PDL cells were challenged with *T. denticola* (*Td*) at MOI = 100 or media control for two hours, incubated for three days in serum- and antibiotic-free MEM-α with daily culture medium refreshment, then treated with the following enzyme inhibitors: (Panel A) 0.25 or 0.5 μM PF-03814735 (PF) for two days; (Panel B) 0.5 or 1 μM apicidin for one day; (Panel C) 0.05 or 0.1 μM trichostatin A (TsA) for four days. The conditioned medium and cell lysates were collected for zymography, western blotting, and qRT-PCR. The left portion of each panel shows densitometric analysis of pro-MMP-2 (72kDa) and active MMP-2 (64kDa) detected by gelatin zymography. The X-axis represents different concentrations of the indicated inhibitor in the control and *Td*-challenged cultures. The Y-axis represents fold-gelatinolytic activity of the pro-MMP-2 and active MMP-2 relative to unchallenged and untreated controls. The right panel shows transcript levels of MMP-2, MT1-MMP and TIMP-2 in the control and inhibitor-treated PDL cells as determined by qRT-PCR. The Y-axis represents fold-expression level of each gene relative to unchallenged and untreated controls. The X-axis represents different concentrations of PF in the control and *Td* groups. The experiments were repeated three times in triplicate. Data were analyzed using one-way ANOVA. (*) represents $p \leq 0.05$ compared to the "0" concentration in the same group. (#) represents $p \leq 0.001$ compared to the "0" concentration in the same group.

Table 1

Chemical agents used to target chromatin modification enzymes.

Compound	Target enzyme	Concentrations(μM)		Treatment period	Source	References
		Low	High			
Inhibition of DNA methyltransferases (DNMT)						
5-Azacytidine	• DNMT	1	5	4 days	*	(Chernov *et al.*, 2009, Zhang *et al.*, 2011, Hassler *et al.*, 2012)
(−)-Epigallocatechin gallate (EGCG)	• DNMT • HAT: – P300, CBP, PCAF, TIP60	5	10	2 days	*	(Achour *et al.*, 2013, Saldanha *et al.*, 2014)
Inhibition of Histone Deacetylases (HDACs)						
Trichostatin A	• HDAC	0.05	0.1	4 days	*	(Mogal *et al.*, 2006, Chang *et al.*, 2012)
Apicidin	• HDAC	0.5	1	1 day	*	(Ahn *et al.*, 2012, Bauden *et al.*, 2015)
Inhibition of Histone phosphorylation						
PF-03814735	• AURK A • AURK B	0.25	0.5	2 days	*	(Hook *et al.*, 2012)
Inhibition of Histone Acetyltransferases (HATs)						
Curcumin	• HATs	25	50	2 hours	*	(Balasubr amanyam *et al.*, 2004, Ahn *et al.*, 2012)
Inhibition of Histone Methyltransferases (HMTs)						
BIX 01294	• HMT G9a	0.5	1	2 days	*	(Kubicek *et al.*, 2007)
Inhibition of Histone Demethylases (HDMs)						
Tranylcypromine, HCl (TCP)	• Monoamine oxidase A/B Histone LSD1/2	2	5	4 days	#	(Nebbioso *et al.*, 2012)

*Sigma-Aldrich, St. Louis, MO, USA

#EMD Millipore, Temecula, CA, USA