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Interleukin 1β and Prostaglandin E2 affect expression of DNA methylating and demethylating enzymes in human gingival fibroblasts

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ABSTRACT

Periodontitis is a common chronic inflammatory condition that results in increased levels of inflammatory cytokines and inflammatory mediators. In addition to oral disease and tooth loss, it also causes low-grade systemic inflammation that contributes to development of systemic conditions including cardiovascular disease, pre-term birth, diabetes and cancer. Chronic inflammation is associated with epigenetic change, and it has been suggested that such changes can alter cell phenotypes in ways that contribute to both ongoing inflammation and development of associated pathologies. Here we show that exposure of human gingival fibroblasts to IL-1β increases expression of maintenance methyltransferase DNMT1 but decreases expression of *de novo* methyltransferase DNMT3a and the demethylating enzyme TET1, while exposure to PGE2 decreases expression of all three enzymes. IL-1β and PGE2 both affect global levels of DNA methylation and hydroxymethylation, as well as methylation of some specific CpG in inflammation-associated genes. The effects of IL-1β are independent of its ability to induce production of PGE2, and the effects of PGE2 on DNMT3a expression are mediated by the EP4 receptor. The finding that exposure of fibroblasts to IL-1β and PGE2 can result in altered expression of DNA methylating/demethylating enzymes and in changing patterns of DNA methylation suggests a mechanism through which inflammatory mediators might contribute to the increased risk of carcinogenesis associated with inflammation.

1. Introduction

Periodontal disease is a common cause of tooth loss in the US and around the world [1,2], and is recognized as a contributing factor in a variety of systemic conditions [3–6]. Bacteria are required to initiate the disease, but host factors are critical for the development of chronic inflammation, which is characterized by an imbalance between inflammatory and anti-inflammatory cytokines. Increased levels of inflammatory cytokines, especially IL-1β, activate gingival fibroblasts to produce matrix metalloproteinases, chemokines and other inflammatory mediators, including prostaglandin E2 (PGE2), which contribute to both continuing inflammation and to degradation of tissues supporting teeth [5–8]. The presence of periodontitis has been associated with development of serious health conditions, including cardiovascular disease [9], pre-term birth [10], diabetes [11] and several types of cancer [12–15]. Periodontal pathogens have been found in distant tissues and could be directly involved in development or progression of some of these conditions [3,16–18], but the presence of chronic, low-grade systemic inflammation associated with periodontal disease may also be important [19,20].

Modification of CpG DNA by methylation (producing 5-methylcytosine, 5mC), constitutes an important epigenetic signal that has vital roles in maintaining genomic stability and regulating gene expression. DNA methylation is established by *de novo* methyltransferases DNMT3a and 3b, and maintained during DNA replication by the maintenance methylase DNMT1. The Ten Eleven Translocation (TET1-3) family of dioxygenase enzymes can reverse DNA methylation through successive oxidation of 5mC to 5 hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can then be removed passively or actively through DNA repair pathways. Although DNA methylation is especially important for stable suppression of gene expression during development and differentiation, recent evidence

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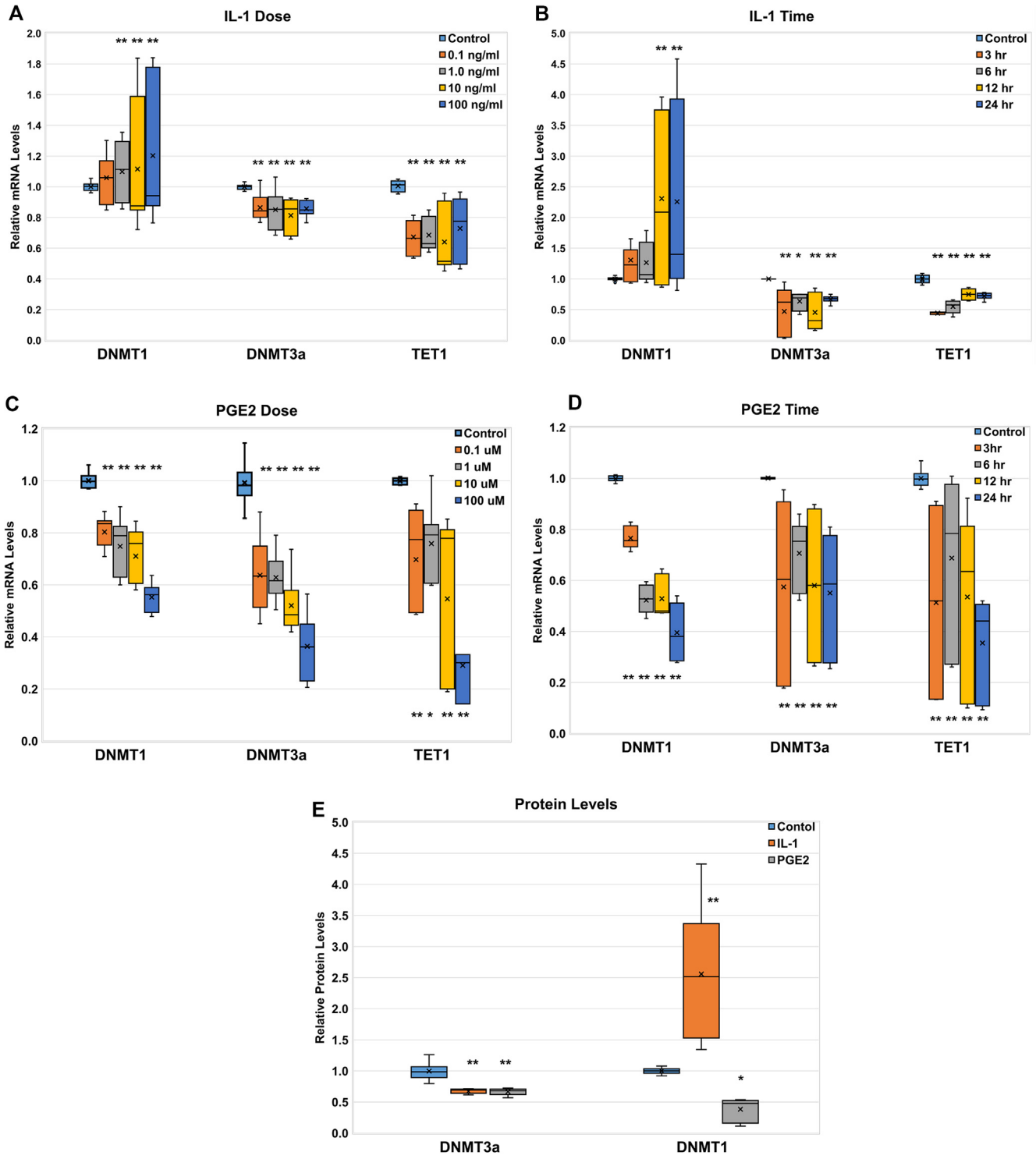

Fig. 1. IL-1β and PGE2 affect expression of DNMT1, DNMT3a and TET1. (A and C) Total RNA was isolated from untreated HGF cells (control) or cells treated for 24 h with IL-1β or PGE2 at the indicated doses. (B and D) RNA was isolated at the indicated times after addition of IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM). mRNA levels of DNMT1, DNMT3a and TET1 were determined by real-time PCR. (E) Whole cell lysates were isolated from HGF cultures that were untreated (control) or treated for 24 h with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM). DNMT1 and DNMT3a protein levels were determined by ELISA. Graphs show mRNA or protein levels expressed relative to untreated controls as box and whisker plots showing interquartile ranges, with the mean marked by X. At least three different HGF cell lines derived from different donors were used in each experiment. Statistical significance (*$p < 0.05$, **$p < 0.01$) vs untreated control was determined by One-way ANOVA followed by Dunnett's test for multiple comparisons.

shows that 5mC and 5hmC can also play important roles in more dynamic types of gene regulation, including in some cases transcriptional activation (reviewed in [21]).

Changes in patterns of DNA methylation are a common feature of cancer cells, with 5mC levels decreased globally but increased in the promoters of certain genes. Global hypomethylation contributes to genomic instability, while hypermethylation in promoters generally inhibits transcription and is a common mechanism for inactivation of

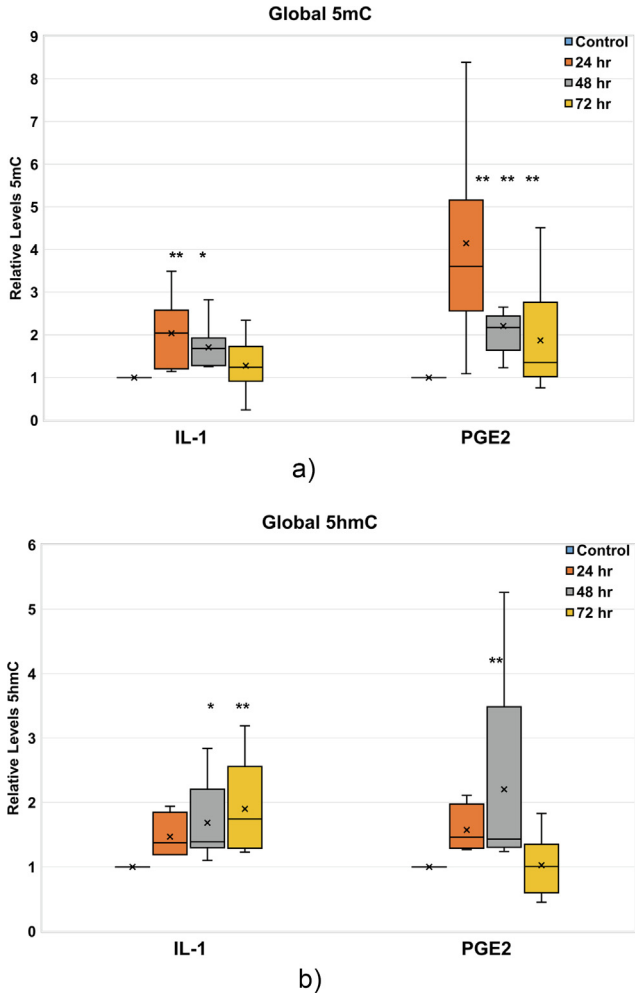

Fig. 2. IL-1β and PGE2 affect global levels of 5mC and 5hmC. Global levels of 5mC (A) and 5hmC (B) were determined by MethylFlash ELISA-like assays using DNA isolated from HGF that were untreated (control) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24, 48 or 72 h. Graphs show percent 5mC or 5hmC expressed relative to untreated controls, as box and whisker plots showing interquartile ranges, with the mean marked by X. Five different HGF cell lines derived from different donors were used. Statistical significance (*$p < 0.05$, **$p < 0.01$) vs untreated control was determined by One-way ANOVA followed by Dunnett's test for multiple comparisons. Global levels of 5hmC were lower as compared to 5mC, but in the range expected based on other studies [75,79].

tumor suppressor genes. The finding that DNA methylation patterns are similarly (though less severely) disrupted in chronically inflamed tissues [22–26], has led to the hypothesis that inflammatory signals create an "inflammatory milieu" that over time might result in epigenetic changes in affected tissues, causing altered gene expression patterns and contributing to both the persistence of inflammation and to the predisposition to cancer [27,28]. However, it is still unclear whether or how inflammatory signals initiate the epigenetic changes.

The current series of experiments was undertaken to determine whether exposure of primary cultures of human gingival fibroblasts (HGF) to inflammatory cytokine IL-1β, or inflammatory mediator PGE2, results in altered expression or activity of enzymes involved in DNA methylation/demethylation.

2. Materials and methods

2.1. Cell culture

Human gingival tissue samples were obtained with informed consent and without any identifying information from patients receiving treatment for periodontitis. Samples were processed by enzymatic dispersion to produce primary cell cultures, as described previously [29,30]. Cells were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (penicillin, streptomycin, amphotericin; Gibco/BRL). Cells between passages 3 and 5 were used for experiments. At least three different HGF primary cell lines, each derived from a different donor, were used for each experiment. Cells were treated with IL-1β (0.1–100 ng/ml (5.7×10^{-3}–5.71 μM); Sigma), or PGE2 (0.1–100 μM; Sigma) for the indicated times. COX-2 inhibitor NS398 (0.1 μM, Sigma) was added to cells 1 h prior to addition of IL-1β and incubation for another 24 h. Agonists to prostanoid receptors EP1-4 (Iloprost, Butaprost, Sulprostone, and CAY10598) were obtained from Cayman Chemicals and used at 0.1 or 10 μM for 24 h. EP4 receptor antagonist ONOAE3208 (0.01 μM, R&D Systems) was added 1 h prior to addition of IL-1β or PGE2, followed by incubation for another 24 h.

2.2. RNA isolation and analysis

Total RNA was isolated from HGF cells using the RNeasy Plus Micro Kit (Qiagen). Two ug total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) and a TC300 Thermal Cycler (Techne). Two μl cDNA was used with TaKaRa Premix Ex Taq (Clonetech), and probes for detection of DNMT1 (FAM-MGB Hs00945875_m1), DNMT3a (FAM-MGB Hs01027166_m1), TET1 (FAM-MGB Hs04189347_g1), IL-8 (FAM-MGB Hs00174103_m1), CD40 (FAM-MGB Hs01002915_g1), or TNFRSF10C (FAM-MGB Hs00182570_m1), along with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, product limiting, VIC-MGB PL Hs0275891_g1); all obtained from Applied Biosystem. Real Time PCR was performed using the Applied Biosystem StepOne Plus Real Time PCR system. Reactions were performed in quadruplicate with both the target gene and endogenous control in the same well. Results were calculated as $2^{-\Delta CT}$, normalizing to GAPDH, and expressed relative to untreated control.

2.3. Determination of DNMT1 and DNMT3a protein levels

Whole cell lysate isolated from HGF that were untreated (Controls) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24 h were analyzed using the Human DNMT3a or DNMT1 ELISA kits (LifeSpan BioSciences). The colorimetric assays were read using the BioRad iMark MicroPlate Reader at 450 nm.

2.4. Determination of global levels of 5mC and 5hmC

DNA was isolated from HGF that were untreated (Controls) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24, 48 or 72 h using the FitAmp Blood and Cultured Cell DNA Extraction Kit (EpiGenTek). The MethylFlash Methylated DNA 5-mC Quantification Kit or the MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit (EpiGenTek) were used to quantitate 5mC and 5hmC, respectively. The colorimetric assays were read using the BioRad iMark MicroPlate Reader at 450 nm.

2.5. Determination of 5mC levels at specific CpG

DNA was isolated from two different HGF cell lines which were untreated (Controls) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 48 h using the FitAmp Blood and Cultured Cell DNA

Table 1
Effect of IL-1β or PGE2 treatment on DNA methylation at specific CpG.

Gene	CpG#	Percent Methylated DNA					
		HGF1			HGF2		
		Control	IL-1β (fold)	PGE2 (fold)	Control	IL-1β (fold)	PGE2 (fold)
CD40	34	4.0	4.1 (1.0)	5.8 **(1.5)**	8.9	14.3 **(1.6)**	14.8 **(1.7)**
	33	4.1	3.7 (0.9)	5.8 **(1.4)**	5.7	5.2 (0.9)	5.9 (1.0)
	32	1.0	2.2 **(2.2)**	2.8 **(2.8)**	2.2	1.8 (0.8)	4.8 **(2.2)**
	31	3.2	3.2 (1.0)	3.8 **(1.2)**	9.6	12.9 **(1.3)**	16.1 **(1.7)**
	30	2.9	3.7 **(1.3)**	5.8 **(2.0)**	4.3	4.9 (1.1)	6.1 **(1.4)**
	29	0.4	1.3 **(3.3)**	1.4 **(3.5)**	2.6	3.5 **(1.3)**	2.4 (0.9)
	28	4.8	8.2 **(1.7)**	9.8 **(2.0)**	7.6	7.4 (1.0)	6.6 (0.9)
	27	3.3	2.6 **(0.8)**	1.7 **(0.5)**	3.7	4.5 **(1.2)**	3.7 (1.0)
	26	5.5	4.4 **(0.8)**	4.0 **(0.7)**	13.6	8.9 **(0.7)**	11.1 **(0.8)**
IL-8	5	26.4	25.2 (1.0)	25.6 (1.0)	37.0	39.6 (1.1)	40.7 (1.1)
	4	4.6	6.4 **(1.4)**	8.0 **(1.7)**	10.5	9.8 (0.9)	10.9 (1.0)
	3	3.8	2.4 **(0.6)**	2.1 **(0.6)**	0.9	0.3 **(0.3)**	1.4 **(1.6)**
TNFRSF10C	20	57.7	61.7 (1.1)	57.4 (1.0)	47.6	46.0 (1.0)	41.8 (0.9)
	19	26.9	37.0 **(1.4)**	30.0 (1.1)	26.8	31.9 **(1.2)**	30.9 **(1.2)**
	18	28.5	28.0 (1.0)	25.3 (0.9)	15.2	19.8 **(1.3)**	22.6 **(1.5)**
	17	36.4	40.6 (1.1)	41.9 **(1.2)**	46.8	51.7 (1.1)	47.6 (1.0)
	16	45.8	51.6 (1.1)	60.3 **(1.3)**	55.8	59.0 (1.1)	56.6 (1.0)
	15	24.1	25.1 (1.0)	27.5 (1.1)	37.5	36.4 (1.0)	36.5 (1.0)
	14	16.6	17.7 (1.1)	17.3 (1.0)	32.4	35.4 (1.1)	29.7 (0.9)
	13	31.0	31.2 (1.0)	32.4 (1.0)	26.0	29.5 (1.1)	32.0 **(1.2)**
	12	13.9	16.0 **(1.2)**	11.8 **(0.8)**	23.4	27.5 **(1.2)**	22.5 (1.0)
	11	9.6	10.9 (1.1)	10.4 (1.1)	12.3	12.3 (1.0)	14.5 **(1.2)**
MMP13	4	19.5	25.1 **(1.3)**	26.2 **(1.3)**	25.5	27.7 (1.1)	27.9 (1.1)
	3	46.2	52.2 (1.1)	49.4 (1.1)	36.7	41.4 (1.1)	40.0 (1.1)
	2	48.8	52.6 (1.1)	50.5 (1.0)	45.2	49.3 (1.1)	49.31.1)

Two different HGF cell lines were left untreated (Control) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 48 h. Isolated DNA was analyzed by targeted next-generation bisulfite sequencing. Methylation levels were calculated by dividing the number of methylated reads by the total number of reads. Only CpG sites covered by at least 100 reads were considered. The table shows genes from the analysis that had a least one site exhibiting a change in methylation of at least 20% in treated vs untreated control cells. Changes of 20% or more are bold.

Extraction Kit (EpiGenTek). Targeted Next Generation Bisulfite Sequencing was performed by EpigenDx, Inc., Hopkinton, MA using their pre-validated immunology panel, with the addition of CpG targets in IL-8, MMP-1, MMP-3 and MMP-13 promoters. The multiplex PCR conditions were optimized for the new targets, then seven multiplex PCRs were performed on the DNA templates provided, which had been treated with bisulfite using the EZ DNA methylation Kit (Zymo Research). Products were pooled and purified using QIAquick PCR Purification columns (Qiagen), then libraries were made using the KAPA Library Preparation Kit for Torrent Platforms and In Xpress Barcode adaptors (Thermo Fisher). Library molecules were purified using Agencourt AMPure XP beads and quantified using the Qiagen QIAxcel Advanced system. Barcoded samples were pooled in an equimolar fashion and templates prepared and enriched using the Ion Chef system (Thermo Fisher). Template-positive library molecules were sequenced on the Ion S5 sequencer using Ion 530 sequencing chips (Thermo Fisher). FASTQ files from the Ion Torrent S5 server were aligned to the local reference database using Bismark Bisulfite Read Mapper and the Bowtie2 program. Methylation levels were calculated as a percentage of methylated reads as compared to total number of reads. Only CpG sites covered by a minimum of 100 total reads were considered. An R-square value of > 0.9 was required for validation.

2.6. Determination of PGE2 levels

PGE2 levels in conditioned media from untreated control cultures or cultures treated with IL-1β (10 ng/ml, 0.57 μM) and/or NS398 (0.1 μM) for 24 h were determined using the Prostaglandin E2 Parameter Assay Kit (R&D Systems) according to the manufacturer's suggested protocol. The colorimetric assay was read at 450 nm in a BioRad iMark microplate reader and values were determined from the standard curve.

2.7. Statistical analysis

Statistical significance was determined by One-way or Two-way analysis of variance (ANOVA) followed by Dunnett, Bonferroni or Tukey tests for multiple comparisons using GraphPad Prism 7.05 software. Comparisons with adjusted p values less than 0.05 were considered statistically significant.

3. Results

3.1. IL-1β and PGE2 affect expression of DNMT1, DNMT3a and TET1

Treatment of HGF with IL-1β for 24 h caused statistically significant increased expression of DNMT1 mRNA, while decreasing expression of DNMT3a and TET1 (Fig. 1A). In order to show the degree of variation among the different HGF cell lines used (at least three for each experiment, and each derived from a different donor), the data was plotted to show the interquartile range of results, with X marking the mean. This shows that the response of DNMT1 mRNA to IL-1β showed greater variation among cell lines as compared to DNMT3a and TET1, in which the magnitude of response sometimes varied but the direction was always the same (with some exception at the lowest doses). Ten ng/ml (0.57 μM) IL-1β was used for further study because it gave consistent results across multiple HGF cell lines without signs of toxicity, and was used previously to study the effects of IL-1β on gene expression in HGF [29,31–35]. Incubation of HGF cell cultures with 10 ng/ml IL-1β over time showed that, on average, IL-1β significantly increased DNMT1 mRNA within 12–24 h, with a concomitant decrease in expression of DNMT3a and TET1 which began as early as 3 h (Fig. 1B).

Since IL-1β is known to increase production of PGE2 through the action of cyclo-oxygenase 2 (COX-2) in HGF [29,30], and since PGE2

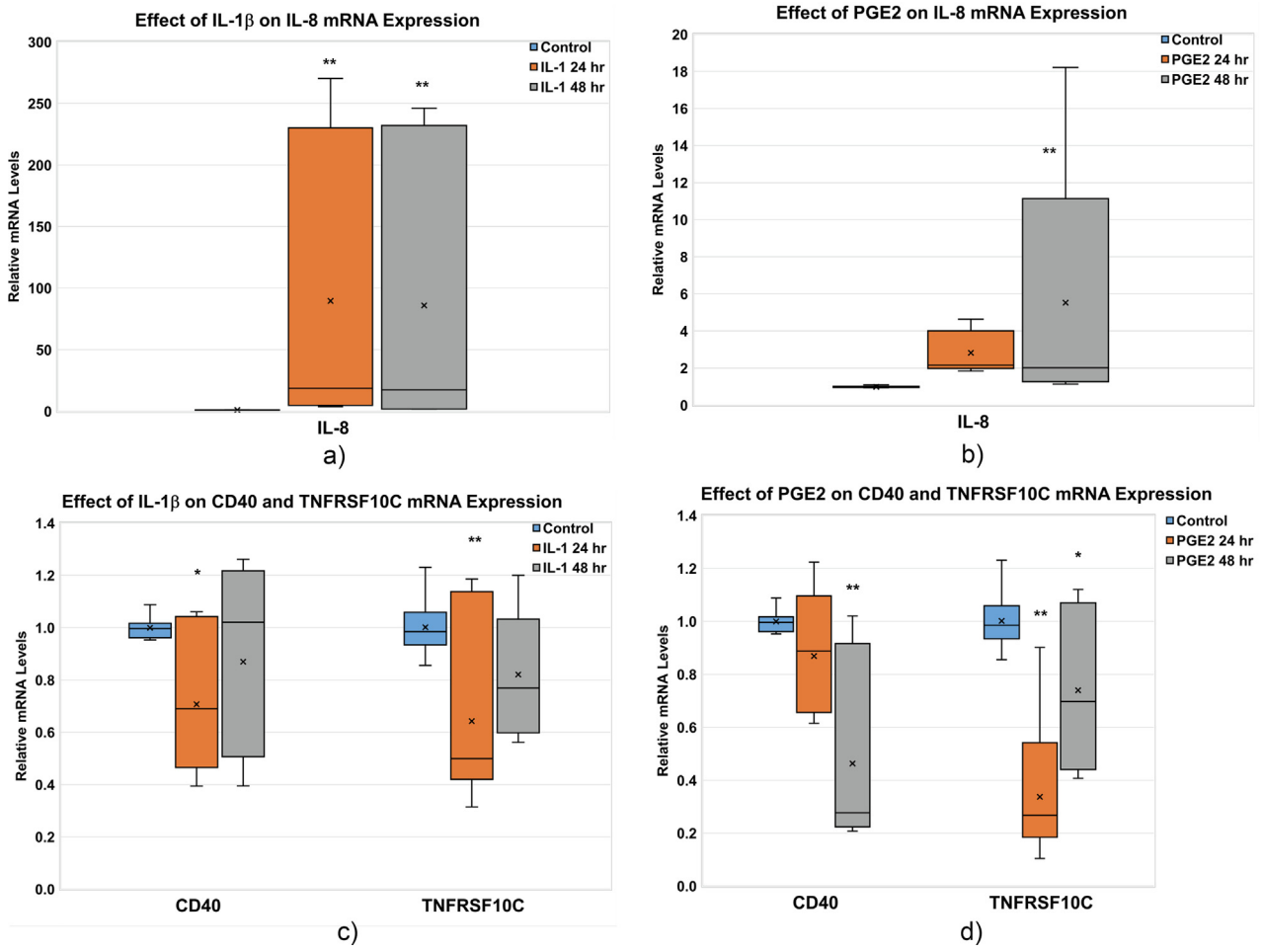

Fig. 3. Effects of IL-1β and PGE2 on mRNA expression of genes that showed changes in methylation at specific CpG in their promoters. Total RNA was isolated from HGF cultures that were untreated (control) or treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24 or 48 h. mRNA levels of IL-8 (A and B) and CD40 and TNFRSF10C (C and D) were determined by real-time PCR and expressed relative to levels in untreated controls. Graphs show data from three different HGF cell cultures derived from three different donors, with mRNA levels expressed relative to untreated controls and depicted in box and whisker plots showing interquartile ranges, with the mean marked by X. Statistical significance (*$p < 0.05$, **$p < 0.01$) vs untreated control was determined by One-way ANOVA followed by Dunnett's test for multiple comparisons.

has been shown to affect expression of DNMTs in other systems [36,37], the effect of PGE2 on expression of these enzymes was also assessed. Results (Fig. 1C and D) show that PGE2 caused a decrease in expression of both DNMT1 and DNMT3a, as well as TET1 mRNA. One hundred μM PGE2 gave consistent results across multiple HGF cell lines without signs of toxicity and was used for further study. Although the magnitude of response varied among the cell lines, all three genes were consistently and significantly decreased, and this occurred as early as 3 h. In accordance with the mRNA results, protein levels of DNMT1 were increased by IL-1β but decreased by PGE2, and DNMT3a was decreased by both IL-1β and PGE2 at 24 h (Fig. 1E), though again the magnitude of response of DNMT1 to IL-1β was more variable. Protein levels of TET1 were below the limit of detection by this method and could not be accurately determined.

3.2. IL-1β and PGE2 affect global levels of 5mC and 5hmC, as well as methylation of specific sites in the promoters of inflammation-associated genes

In order to determine whether changes in levels of the enzymes were functionally significant, global levels of DNA methylation and hydroxymethylation were assessed in HGF cells treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24, 48 or 72 h. Global levels of 5mC were significantly increased by both IL-1β and PGE2, but the magnitude of the response, especially to IL-1β, was decreased by 72 h (Fig. 2a). Global levels of 5hmC were also increased by both IL-1β and PGE2, but whereas the IL-1β increase was maintained for at least 72 h, the effects of PGE2 was more transient (Fig. 2B).

Targeted next generation bisulfite sequencing was performed in order to determine whether specific changes in DNA methylation in the promoters of inflammation-associated genes occurred in response to IL-1β and/or PGE2 (Epigendx). Two different HGF cell lines were treated with IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 48 h, and levels of DNA methylation at specific CpG sites were compared to untreated controls. Results of this analysis are shown in Table 1. Basal levels of DNA methylation at the specific CpG tested often differed between the cell lines, but the changes in response to IL-1β and PGE2 were usually similar. Among the genes showing changes in DNA methylation of at least 20% at one or more CpG in both cell lines were: CD40, IL-8, and TNFRSF10C. DNA methylation at the following sites was increased or decreased by 20% or more in both cell lines by IL-1β and/or by PGE2: CpG 34, 32, 31, 30, 29, and 26 in the CD40 promoter; CpG3 in the IL-8 promoter; and CpG 19 and 12 in the TNFRSF10C promoter. Methylation at most of these sites was increased, but methylation of CpG 26 of CD40 and CpG3 of IL-8 was decreased. Genes included in the analysis that did not show changes in methylation at the CpG sites examined included: CDKN2A, IL1B, IL2, IL4, IL7, IL7R, IL13, TNFA, TNFRSF25, MMP1 and MMP3.

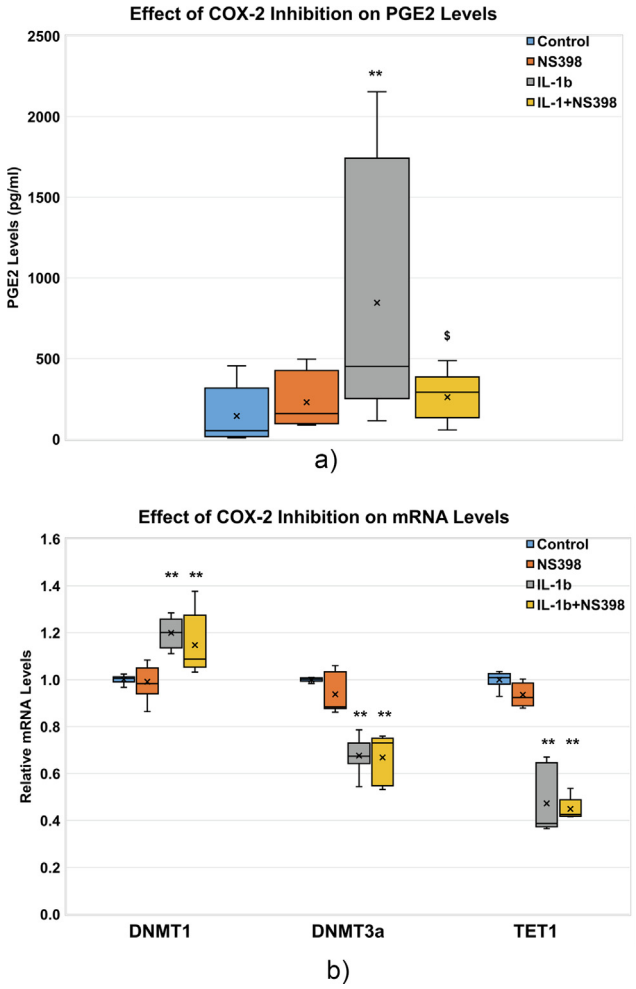

Fig. 4. IL-1β effects on gene expression are not altered by inhibition of COX-2 mediated PGE2 production. HGF cell cultures were untreated (control) or pre-treated with NS398 (0.1 μM) for 1 h before addition of IL-1β (10 ng/ml, 0.57 μM). Conditioned media and total RNA were harvested 24 h after addition of IL-1β. (A) PGE2 levels in conditioned media were determined by Prostaglandin E2 Parameter Assay Kit (R&D Systems). (B) DNMT1, DNMT3a and TET1 mRNA levels were determined by real-time PCR and expressed relative to untreated control. Graphs show data from three to five different HGF cell lines derived from different donors, expressed relative to untreated controls, as box and whisker plots showing interquartile ranges, with the mean marked by X. Statistical significance (**$p < 0.01$ vs untreated control, $$p < 0.05$ vs IL-1β alone)) was determined by Two-way ANOVA followed by Tukey's test for multiple comparisons. There was no statistical significance detected in comparison of PGE2 levels in IL-1 + NS398 vs control or vs Ns398 alone; and also no significant difference in mRNA levels between IL and 1 + NS389 vs IL-1 alone.

The effects of IL-1β and PGE2 stimulation on expression of IL-8, CD40 and TNFRSFC10 mRNA in HGF was examined in order to determine whether the changes in methylation of their promoters could be correlated with changes in gene expression. IL-8 mRNA expression in HGF was increased by both IL-1β and PGE2 (Fig. 3 A and B) as has been previously reported [38–40], while expression of CD40 and TNFRSF10C was decreased (Fig. 3 C and D).

3.3. Effects of IL-1β are not dependent on COX-2-mediated increased production of PGE2

IL-1β is known to induce COX-2-mediated production of PGE2 in many cell types, including HGF [29,30]. In order to determine whether the effects of IL-1β on expression of DNMTs and TET1 are mediated by PGE2, cells were pre-treated with COX-2 inhibitor NS-398 prior to addition of IL-1β. As expected based on previous data [29,30], IL-1β significantly increased production of PGE2 in HGF, though the amount at baseline and the magnitude of increase varied among cells from different donors. Treatment with NS-398 inhibited the IL-1β induction and resulted in PGE2 levels not significantly different from untreated controls (Fig. 4A). However, the effects of IL-1β on expression of DNMT1, DNMT3a and TET1 mRNA were unchanged by addition NS-398 (Fig. 4B). These results suggest that IL-1β can affect expression of these enzymes independently of its ability to increase PGE2 production.

3.4. Effects of PGE2 on DNMT1, DNMT3a and TET1 expression in HGF are mimicked by an agonist of the EP4 receptor

The effects of PGE2 on gene expression are often cell-type and condition specific, due in part to differing levels of expression of its four receptors, EP1-4, which activate different signal transduction pathways by activating different G proteins [41,42]. HGF cell cultures were treated with agonists of each of the four receptors to determine which EP receptor(s) are involved in PGE2-mediated inhibition of DNMTs and TET1 expression (Fig. 5). Only the EP4 agonist CAY10598 resulted in decreased expression of all three genes. DNMT3a expression was significantly decreased in response to the EP4 agonist CAY10598, but unaffected by the other three agonists (Fig. 5). Expression of DNMT1 and TET1 were also decreased by the EP4 agonist, but their patterns were somewhat more complicated. DNMT1 inhibition by CAY10598 required a higher dose, and DNMT1 mRNA expression was also significantly increased by the EP3 agonist sulprostone. TET1 mRNA levels were decreased by 1 μM CAY10598 to a level similar to that seen with PGE2, but significantly increased by 10 μM, and TET1 expression was also decreased by the higher dose of sulprostone.

In order to confirm the role of the EP4 receptor in mediating the effects of PGE2 and the PGE2-independent effects of IL-1β on DNMT3a expression, HGF were pre-treated with EP4 receptor antagonist ONOAE3208 prior to addition of IL-1β or PGE2. As expected, the EP4 receptor antagonist prevented PGE2 inhibition of DNMT3a mRNA expression, but had no effect on decreased expression caused by IL-1β (Fig. 6). The antagonist also did not alter the effect of IL-1β on DNMT1 or TET1 expression. However, it abrogated the effects of PGE2 in only one of four HGF cell lines (data not shown).

4. Discussion

Results of these experiments show that IL-1β and PGE2 can not only affect expression of epigenetic modifiers (Fig. 1), but they can also affect both global and site-specific patterns of DNA methylation (Fig. 2 and Table 1). These results are consistent with the idea that epigenetic changes play an important role in periodontitis pathology, and potentially in the increased risk of cancer associated with it. A recent study by Planello et al [26] found that the DNA methylation patterns in gingival tissue of patients with chronic periodontitis were significantly different from healthy controls, but had much in common with the DNA methylation alterations found in oral squamous cell carcinomas. The authors suggest that the epigenetic changes seen in the chronically inflamed tissues represent a pre-neoplastic state. The finding that IL-1β and PGE2 are able to affect expression of enzymes that regulate DNA methylation, as well as levels of 5mC and 5hmC in gingival fibroblasts, supports this notion and provides a clue to how these changes might be initiated.

Treatment of HGF cultures with IL-1β caused a transient increase in global levels of 5mC with a more sustained increase in 5hmC levels (Fig. 2). Effects of PGE2 were similar, although levels of 5mC were still significantly above control levels at 72 h. Since IL-1β caused an increase in DNMT1 expression while PGE2 caused a decrease, these results suggest that levels of DNMT1 may not be the most important factor in determining global levels of 5mC. It is important to remember,

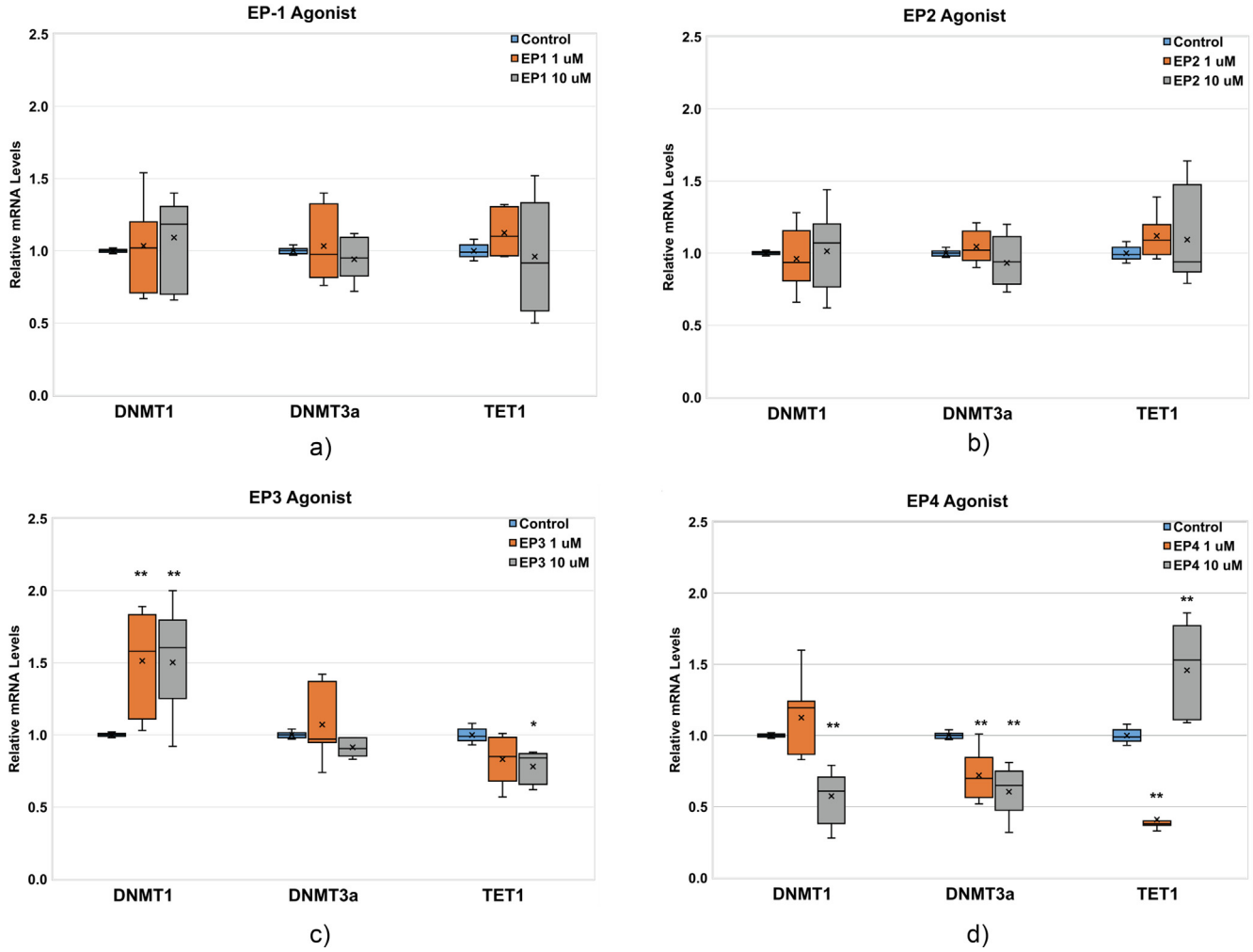

Fig. 5. EP receptor agonists affect expression of DNMT1, DNMT3a and TET1. HGF cultures were left untreated (control) or treated for 24 h with the indicated doses (0.1 or 10 μM) of selective agonists to (A) EP1 (Iloprost), (B) EP2 (Butaprost), (C) EP3 (Sulprostone), and D) EP4 (CAY10598). Levels of DNMT1, DNMT3a, and TET1 mRNA were determined by real-time PCR and expressed relative to untreated controls. Graphs show data expressed relative to untreated controls, as box and whisker plots showing interquartile ranges, with the mean marked by X. At least three different HGF cell lines from different donors were used for each experiment. Statistical significance (*$p < 0.05$, **$p < 0.01$) vs untreated control was determined by Two-way ANOVA with post-hoc Tukey test for multiple comparisons.

however, that global levels of DNA methylation/hydroxymethylation are determined by the combined effects of multiple epigenetic effectors. For example, it has been suggested that one of the roles of TET1 is to protect CpG sites from inappropriate methylation [43]. This might provide a partial explanation both for the increase in global DNA methylation and for the fact that most of the site-specific methylation changes observed in response to IL-1β or PGE2 were increased methylation. Interestingly, a recent study by O'Neill et al [44] showed that depletion of DNMT1 in normal human fibroblasts resulted in increased methylation of a substantial number of sites. In addition, inflammation is also associated with changes in histone modifications, which may be involved in the disruption of DNA methylation patterns. In a mouse colitis model, changes in patterns of H3K27me3 were seen in colonic epithelial cells exposed to inflammatory conditions, and some of those changes were linked to later changes in DNA methylation [45].

Despite different effects on DNMT1 levels, IL-1β and PGE2 affected DNA methylation at mostly the same sites, and usually in the same direction (Table 1). Of the inflammation-related genes examined, three (CD40, IL-8, TNFRSF10C) had CpG sites whose levels of methylation changed at least 20% following treatment with IL-1β or PGE2 for 48 h in both cell lines tested. Methylation of CpG 19 in the TNFRSF10C promoter was increased 20–40% by IL-1β, and nearly 20% by PGE2. This gene encodes Decoy Receptor 1 (DCR1, TRAILR3), a negative regulator of apoptosis induced by TRAIL (tumor necrosis factor-related apoptosis-induced ligand) [46]. Its expression is decreased in a variety of cancer cells due to hypermethylation [47–50] and this was linked to increased binding of DNMT1 and DNMT3a to the promoter in malignant melanoma cells [49]. Consistent with these findings, both IL-1β and PGE2 decreased expression of TNFRSF10C in HGF (Fig. 3).

CD40 is a co-stimulatory molecule required for activation of antigen presenting cells, and binding of CD40L in those cells induces expression of cytokines and chemokines [51]. CD40 is also expressed on endothelial cells and fibroblasts, and its expression in fibroblasts was shown to be increased by IFNγ and related to cell proliferation [52]. In HGF, CD40 crosslinking was shown to inhibit both basal and IL-1β induced production of matrix metalloproteinases 1 and 3 [53]. As shown in Fig. 3, IL-1β and PGE2 both decreased mRNA expression of CD40 (Fig. 3). However, since treatment with IL-1β and PGE2 increased DNA methylation of some CpG sites in the CD40 promoter while decreasing methylation of others, further study would be needed to determine whether changes in mRNA expression can be linked to specific changes in DNA methylation. Interestingly, several of the sites that showed increased methylation are in transcription factor binding sites [54–57]. Although many transcription factors bind preferentially to hypomethylated sites, some prefer methylated binding sites [58].

Increased expression of IL-8 [38,40] is an established part of the

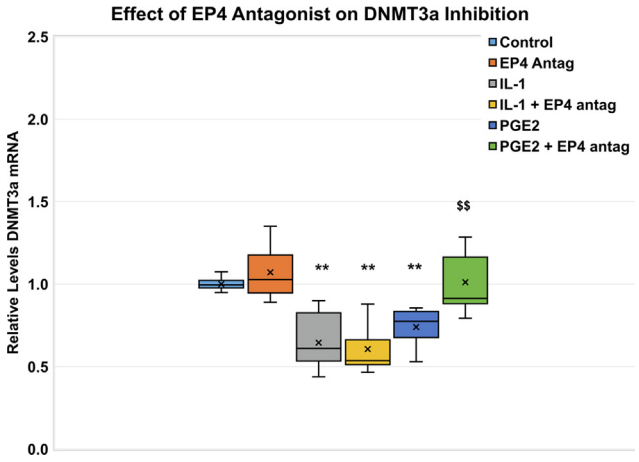

Fig. 6. An EP4 receptor antagonist blocks the effect of PGE2 on expression of DNMT3a. HGF cultures were pre-treated with EP4 receptor antagonist ONOAE3208 (0.01 μM) for one hour before addition of IL-1β (10 ng/ml, 0.57 μM) or PGE2 (100 μM) for 24 h. Levels of DNMT3a mRNA were determined by real-time PCR and expressed relative to untreated controls. Four different HGF cell lines derived from different donors were used. The graph shows data in box and whisker plots showing interquartile ranges, with the mean marked by X. Statistical significance (**$p < 0.01$ vs untreated control, $$ $p < 0.01$ vs PGE2 alone) was determined by Two-way ANOVA with post-hoc Tukey. No statistical significance was detected between IL-1 + antagonist vs IL-1 alone, or between control vs PGE2 + antagonist.

response of gingival fibroblasts to inflammatory mediators. Our results confirmed that both IL-1β and PGE2 increased expression of IL-8 mRNA (Fig. 3A and B). IL-1β increased IL-8 expression to a variable extent, ranging from 4 to over 200-fold compared to the untreated control, while PGE2 caused a more modest but consistent increase. The finding that methylation of CpG 3 in the IL-8 promoter was decreased (Table 1) is consistent with other studies showing that the IL-8 promoter is hypomethylated in periodontitis [59,60]. Also, Venza et al. [57] showed that PGE2 caused decreased methylation at CpG3 (−83) in astrocytoma cells, and this was linked to increased binding of CEBPβ and decreased association of DNMT1 and DNMT3a to the site.

This is, to our knowledge, the first report of TET1 mRNA expression in HGF. However, the mRNA levels were quite low and protein levels could not be easily detected, so the relevance of this finding is unclear. Both IL-1β and PGE2 did consistently decrease TET1 mRNA levels in all HGF cultures tested, however. They also caused a significant increase in global levels of 5hmC (Fig. 2), although in the case of PGE2 this effect was transient. It has been reported that TET1 and TET2 act preferentially on 5mC residues as compared to 5hmC or 5fC [61]. Thus, the initial increase in 5hmC may reflect a stabilization of 5hmC due to inability to completely oxidize it to 5fC and 5caC when TET levels are decreased. The subsequent decrease at 72 h might then reflect passive loss of 5hmC over continued time in culture.

Although the role of 5hmC in gene regulation is still not well understood [43,62], decreased levels of TET1 and 5hmC are frequently seen in tumors, and these changes are considered poor prognostic indicators in several types of cancer [63–65]. Some evidence suggests that decreased TET activity may be involved in initiation of carcinogenesis [66–68], including a study in transgenic mice that linked COX-2 overexpression, which was associated with increased production of PGE2 and decreased TET1 levels, with spontaneous development of hepatocellular carcinoma [66].

Since IL-1β and PGE2 resulted in similar decreases in DNMT3a and TET1 expression (Fig. 1), and since previous results from this laboratory [29,30] and others [69,70] have shown that IL-1β induces COX-2 mediated PGE2 production in HGF, it was important to determine whether the effects of IL-1β were determined by its ability to induce production of PGE2. Although inhibition of COX-2 did result in decreased levels of PGE2 production, there was no change in the ability of IL-1β to cause changes in DNMT1, DMT3a or TET1 expression (Fig. 4). These results suggest that PGE2 and IL-1β are operating through different, though possibly overlapping pathways. Although treatment of HGF with PGE2 itself caused decreased expression of DNMT1, as did stimulation of the EP4 receptor with CAY10958 (albeit at a higher dose than required for DNMT3a), the EP3 receptor agonist increased DNMT1 mRNA expression (Fig. 5). Thus it appears likely that the final net effect of PGE2 on DNMT1 expression includes both stimulation via EP3 and suppression via EP4. This complexity probably also explains the inability of the EP4 antagonist to consistently abrogate the effect of PGE2 on DNMT1 expression. The fact that suppression by EP4 seems to dominate most of the time might be explained by differences in the relative levels of the receptors and/or by different affinities of the receptors for PGE2. The affinities of recombinant EP receptors for PGE2 have been reported as EP3 > EP4 > EP2 > EP1 [71], but levels of EP3 on HGF are reported to be low [8,71]. Although cell surface expression of the receptors was not formally addressed in this study, mRNAs encoding all four receptors were easily detected (not shown). The ability of an EP4 receptor antagonist to abrogate the effects of PGE2 on expression of DNMT3a (Fig. 6) confirm the importance of EP4 at least in this case, but further research is needed to fully elucidate the signaling pathway(s) that are activated.

The effects of IL-1β and PGE2 on expression of the DNMTs and TET might be mediated at the transcriptional or post-transcriptional level. Transcription factors known to affect expression of DNMT1, DNMT3a and/or TET include: NF-κB, AP-1, p53, RB/E2F, STAT3 and Sp1 and Sp3 (reviewed in [72,73]). DNMT1 induction by IL-1β and TNFα in adipocytes [74], and IL-1β inhibition of TET1 expression in chondrocytes [75] was linked to NF-κB. However, levels of all three enzymes can also be controlled at the post-transcriptional level by micro-RNAs [72,73] and some of these have been reported to be up-regulated in periodontitis-affected tissues [76]. For example, miR-29 directly targets TET1 and DNMT3a [77], and is up-regulated in gingival tissue in periodontitis [78]. Further experiments are needed to determine the molecular mechanisms involved in regulation of these enzymes by inflammatory mediators.

Menendez and Alarcon [28] suggested that cells respond to infection or stress by activating an inflammatory response that creates a transient state of “epigenetic plasticity” that allows cells to make temporary changes to their phenotype in order to restore tissue homeostasis. However, if the state of epigenetic plasticity goes on too long, it can interfere with the repair process and create a cancer-like state. Our findings suggest that exposure of fibroblasts to inflammatory cytokines and mediators such as PGE2 might contribute to this plasticity by affecting the expression and/or activity of enzymes directly involved in regulating epigenetic signals.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105920.

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