ALINE CRISTIANE PLANELLO

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Faculdade de Odontologia de Piracicaba
Universidade Estadual de Campinas

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas - UNICAMP como parte dos requisitos para obtenção do Título de Doutora em Biologia Buco Dental, na área de Histologia e Embriologia.

Orientadora: Dra. Ana Paula de Souza Pardo

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RESUMO

A inflamação crônica é conhecida por induzir alterações epigenéticas, em particular, alterações da metilação do DNA. A ilha CpG do gene *SOCS1* tem sido observada hipermetilada em diferentes tipos de câncer e em doenças associadas à inflamação. No entanto, pouco se sabe sobre essas alterações epigenéticas associadas à periodontite crônica. A fim de abordar essa questão, nós investigamos a metilação do DNA na Ilha CpG do *SOCS1* e sua relevância funcional para a periodontite crônica em 90 tecidos gengivais usando a técnica de de comparação de melting (MS-HRM). Nós encontramos uma região no exon 2 do *SOCS1* hipermetilada quando comparada com amostras de indivíduos saudáveis, sem alterações em sua expressão. Investigações posteriores da sequencia do *SOCS1* revelaram marcadores de enhancer na região. Analise de sítios hipersensíveis a Dnase I (Dnase I hipersensitivity site – DHS) mostraram um DHS dentro do exon2 do *SOCS1*, que cobre a mesma região estudada na técnica MS-HRM, correlacionado com vários promotores na vizinhança do *SOCS1*, os genes alvos. Fragmentos cobrindo a região encontrada com diferença de metilação no *SOCS1* foram clonados em um plasmídeo repórter que possui um promotor e é livre de qualquer CpG. Foram testados fragmentos desmetilados e metilados. Os fragmentos desmetilados aumentaram a atividade da luciferase, demonstrando a atividade de enhancer da região. Já os fragmentos metilados, além de não exercerem atividade de enhancer, foram capazes de reprimir a função do promotor. Corroborando essas informações uma correlação negativa entre a metilação no *SOCS1* e a expressão de genes alvo foi observada na inflamação crônica. Os dados apresentados indicam que a função principal da metilação do DNA em um enhancer é controlar sua função regulatória e consequentemente os níveis de transcrição dos genes alvo, o que pode ser evidenciado pela hipermetilação do exon do *SOCS1* na inflamação crônica.

Palavras Chave: *SOCS1*, metilação DNA, enhancer, inflamação crônica.

ABSTRACT

Chronic inflammation is known to induce epigenetic alterations, in particular alterations in DNA methylation. *SOCS1* CpG island (CGI) has been demonstrated hypermethylated in many types of cancer and inflammation-associated diseases. However, little is known about the epigenetic changes associated with chronic periodontitis. In order to address this question, we investigated DNA methylation of *SOCS1* CGI and its functional relevance to chronic periodontitis in 90 gingival tissue samples using methylation sensitive high resolution melting (MS-HRM). We found a region of exon 2 SOCS1 hypermethylated when compared with healthy controls with no changes in gene expression. Further investigations showed enhancer marker at *SOCS1* region. Dnase I hypersensitivity site (DHS) correlation analysis showed a DHS inside exon2 SOCS1 correlated with several promoter in the neighboring region. Fragments harboring the *SOCS1* fragment found to be differentially methylated, enhanced the promoter activity of a CpG free-Luc reporter, when unmethyalted. Strikingly, not only the exon2 of *SOCS1* CGI presented enhancer activity but also it had its activity disrupted by DNA methylation. Accordingly, negative correlation between SOCS1 methylation and expression of neighboring genes was observed in chronic inflammation.

The data indicate that the primary function of enhancer DNA methylation is to control its regulatory function and the transcription levels of enhancer target genes, which can be evidenced by exon 2 *SOCS1* CGI hypermethylation on chronic inflammation.

Key-words: *SOCS1*, DNA methylation, enhancer, chronic inflammation.

SUMÁRIO

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INTRODUÇÃO

A família SOCS (*Supressor Of Cytokine Signaling*) é composta por proteínas intracelulares que participam da regulação da resposta de células imunes ao estímulo de várias citocinas pró-inflamatórias. Pelo menos oito proteínas membros da família SOCS já foram descritas: SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7 E CIS (Yoshimura et al., 2007).

SOCS1 em específico é forte inibidor da via de sinalização intracelular JAK/STAT (JAK (*Janus kinase*) / STAT (*Signal Transducer e Activator of Transcription*), uma das mais importantes e estudadas vias de sinalização de transdução de sinal que ocorrem na inflamação, uma vez que é ativada pela ligação de citocinas secretadas durante o processo inflamatório ao receptor de membrana que dispara a via (Alexeer e Hilton, 2004). Recentemente foi mostrado que SOCS1 desempenha importante papel na modulação da sinalização do receptor Toll-like (TLR) (Yoshimura et al., 2012), podendo assim afetar a diferenciação, maturação e função das células (Palmer e Restifo, 2009). Além disso, SOCS1, quando presente no núcleo celular, interage diretamente com o supressor de tumor TP53, através de seu domínio central SH2, contribuindo para a ativação desse gene, o que faz a proteína SOCS1 ser requerida para a senescência celular de maneira dependente de TP53 (Calabrese et al., 2009). A importância desses mecanismos que apontam a proteína SOCS1 como importante regulador da proliferação, diferenciação e transformação celular tem aumentado o número de estudos sobre os mecanismos controladores da sua expressão, sendo a investigação a respeito da regulação epigenética um dos campos de maior interesse da atualidade. A expressão do gene *SOCS1* é fortemente regulada por múltiplos mecanismos, estando sua expressão alterada em diferentes doenças (Chan et al., 2014; Choi et al., 2013; Isomaki et al., 2007; Sasi et al., 2010). Alterações epigenéticas no gene *SOCS1*, como alterações no padrão de metilação do DNA, já foram demostradas em diferentes tipos de câncer (Inagaki-Ohara et al.,

2013; Souma et al., 2012) e em lesões pré-malignas muitas vezes associadas à inflamação crônica (Yoshida et al., 2004). O gene *SOCS1* é relativamente pequeno, com 1765 pares de base contendo apenas dois exons e um intron. Chama a atenção a extensão da ilha CpG que cobre praticamente quase todo gene e se estende desde a região promotora até quase o final do exon 2.

Os dinucleotides CpG esparsos pelo genoma estão predominantemente metilados em genomas de vertebrados. A metilação dos dinucleotides CpG consiste na adição de um radical metil a citosina (5mC) que precede a guanina. Consequentemente, CpG estão presentes em número reduzido nestes genomas devido a mutações que transformam as metilcitosinas em timinas, nas células germinativas (Bird, 2002). Entretanto, certas regiões enriquecidas em CpG, denominas ilhas CGs, são regiões do DNA densas em dinucleotideos CpG e que estão frequentemente desmetilados. Assim, as ilhas CpGs estão isoladas no genoma como frações homogêneas em sequencias ricas em C + G (Deaton e Bird, 2011). Aproximadamente 70% dos promotores se associam a uma ilha CpG sendo prevalentes na região de inicio de transcrição (*transcription start site* -TSS) de genes *housekeeping* e reguladores do desenvolvimento (Smith e Meissner, 2013).

Os efetores da metilação do DNA são as enzimas DNA metiltransferases (DNMTs), sendo as responsáveis por catalisar a metilação de novo, as DNMT3A e 3B ou manter a metilação prévia da fita hemimetilada pós-replicação, a DNMT1. Recentemente foi proposto que as DNMT3A/B participam em conjunto com a DNMT1 na manutenção da metilação do DNA (Sharma et al., 2011). Também sõa conhecidas as enzimas efetoras da desmetilação, as *Ten-Eleven Translocation* (TET) que promovem a desmetilação dos dinucleotideos CpG (Arioka et al., 2012). Essas enzimas são responsáveis pela perda ativa da 5mC, de maneira independente da divisão celular mas dependente do aparato de reparo do DNA nuclear. A 5mC é convertida em 5-hidroximetilcitosina (5hmC), uma forma intermediaria durante a desmetilação ativa do DNA. Após, a 5hmC sofre

deaminação por enzimas (Arioka et al., 2012) ou oxidação natural (Maiti e Drohat, 2011) o que é seguida por reparação de base por excisão (BER), resultando na remoção da 5hmC e recolocação de citosina não metilada.

Em condições de normalidade celular, os dinucleotídeos CpG encontrados nas regiões que formam as ilhas CpG estão geralmente desmetilados devido a mecanismos moleculares que tendem a minimizar as taxas de metilação nas ilhas CpGs (Jones, 2012). Porém, em condições de quebra da homeostasia celular por fatores diversos, como o desenvolvimento de tumores malignos ou a instalação de processos inflamatórios acompanhados ou não por infecção, alterações no perfil de metilação do DNA nas ilhas CpGs podem ocorrer, levando a quadros de hipermetilação destas ilhas CpGs (Deaton e Bird, 2011; Jones, 2012; Kulis e Esteller, 2010).

Estando a maior parte dos promotores de genes associados às ilhas CpGs, a metilação do DNA representa uma forma de regulação da expressão genica (Deaton e Bird, 2011). De fato, a hipermetilação das ilhas CpGs tem sido associada à diminuição da taxa de expressão do gene (Deaton e Bird, 2011; Kulis e Esteller, 2010). O efeito da metilação em regiões do genoma conhecidas como elementos regulatórios *cis* como, por exemplo, os *enhancers* também têm sido estudados.

Enhancers desempenham um papel central guiando a expressão genica que gera especificidade celular sendo capazes de ativar a transcrição de seus genes alvos a grandes distancias o que pode variar de algumas centenas a milhares de pares de bases (Calo e Wysocka, 2013). A característica principal dos *enhancers* é sua habilidade de funcionar como uma plataforma integrada de ligação dos fatores de transcrição. Os *enhancers* têm comumente 200 – 500 bp de comprimento e contem *clusters* de sítio de ligação para diferentes fatores de transcrição. Geralmente, a ativação do *enhancer* requer a presença de múltiplos fatores, que incluem os fatores específicos de linhagem e os efetores das vias

celulares sequencia-dependente, garantindo a integração de vias intrínsecas e extrínsecas nesses elementos (Spitz e Furlong, 2012).

Para ativarem a transcrição no *template* de cromatina, os fatores de transcrição são dependentes de proteínas co-ativadoras que não apresentam sitio de ligação sequencia-específica no DNA (Weake e Workman, 2010). No entanto, elas funcionam como modificadores das histonas, remodeladores da cromatina ou mediadores de interações a longa distancia com a maquinaria do promotor. *Enhancers* ativam a transcrição dos genes através da entrega de importantes fatores acessórios para o promotor (Calo e Wysocka, 2013). Tanto a condensação da cromatina (pela formação das unidades de nucleossomas) (John et al., 2008) como a metilação do DNA são capazes de inibir a atividade do *enhancer* (Ko et al., 2013).

A inflamação crônica tem sido associada com o desenvolvimento de canceres, como o gástrico, câncer de colon e hepatocarcinoma (Kaz et al., 2011; Yoshida et al., 2004). A metilação aberrante do DNA tem sido observada em inflamações associadas ao câncer (Kulis e Esteller, 2010; Yoshida et al., 2004) sendo a inflamação crônica responsável por induzir alterações na metilação do DNA nestes casos (Suzuki et al., 2009; Wu et al., 2013). Embora as alterações epigenéticas em processos inflamatórios venham sendo bastante estudadas, pouco se sabe sobre as alterações epigenéticas associadas à periodontite crônica.

A periodontite crônica é caracterizada por infecção bacteriana associada à inflamação dos tecidos que ancoram os dentes nos alvéolos e, se não controlada, promove a destruição dos tecidos periodontais (ligamento periodontal e cemento) e osso alveolar (Armitage, 2004). Além da infecção bacterina, há fatores de risco associados que predispõe o indivíduo ao desenvolvimento da periodontite crônica, sendo estes fatores ambientais ou intrínsecos do individuo (Page et al., 1997). A periodontite crônica pode apresentar efeitos sistêmicos que levam ao aumento da concentração de marcadores inflamatórios na circulação. A severidade da doença

pode ser diretamente correlacionada com os níveis desses marcadores no sangue (Moutsopoulos e Madianos, 2006).

O gene *SOCS1* exerce um papel na modulação da periodontite e é menos expresso na periodontite crônica quando comparado com a gengivite (Garlet et al., 2006). Desta forma, o objetivo deste estudo foi analisar o padrão de metilação da ilha CpG do gene *SOCS1* em amostras de tecido gengival de indivíduos afetados e não afetados pela periodontite, correlacionar os níveis de metilação observados com a expressão de *SOCS1* e identificar a relevância funcional desse evento epigenético para a patogênese dessa doença.

CAPITULO 1

DNA Methylation-Mediated Disruption of a Transcriptional Enhancer in Chronic Inflammation

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ABSTRACT

Chronic inflammation is known to induce epigenetic alterations, in particular alterations in DNA methylation. *SOCS1* CpG island (CGI) has been demonstrated hypermethylated in many types of cancer and inflammation-associated diseases. However, little is known about the epigenetic changes associated with chronic periodontitis. In order to address this question, we investigated DNA methylation of exon 2 of *SOCS1* CGI and its functional relevance to chronic periodontitis in 90 gingival tissue samples using methylation sensitive high resolution melting (MS-HRM). We found this region to be hypermethylated when compared with healthy controls. No changes in gene expression were observed. Integrated genome –wide chromatin landscape identified an enhancer within exon 2 of *SOCS1* CGI. The Dnase I hypersensitivity site (DHS) correlation analysis showed this region correlated with several promoter in the neighboring region. The fragment mapping to this enhancer and found to be differentially methylated, enhanced the promoter activity of a CpGfree-Luc reporter, when unmethylated. Importantly, not only the exon2 of *SOCS1* CGI presented enhancer activity but also it had its activity disrupted by DNA methylation. Accordingly, negative correlation between *SOCS1* methylation and expression of genes found on the DHS analysis was observed in chronic inflammation. The data indicate that the primary function of enhancer DNA methylation is to control its regulatory function, and thus the transcription levels of enhancer target genes, which can be evidenced by exon 2 *SOCS1* CGI hypermethylation on chronic inflammation.

INTRODUCTION

Epigenetics refers to heritable changes in gene expression patterns that are independent of primary DNA sequence (1). Naturally, the DNA in eukaryotic cells is wrapped around an octamer of core histones, giving rise to an organized and dynamic protein/DNA complex, the chromatin.

The transcription factor (TF) binding and initialization of gene expression depend on chromatin accessibility which characterizes the region as regulatory elements or not (2). The mechanisms that create and maintain these accessible chromatin regions are not well understood but it is clear they are central to the regulation of tissue selective function. Accessible chromatin is controlled by post-translational modifications of the histone tails and cytosine methylation (3).

DNA methylation is one of the best studied epigenetic modifications. In differentiated mammals cells it is primary restricted to the CpG context. Normally, genomes are globally CpG depleted with the exception of the CpG islands (CGIs) which are CG dense regions that can be found as isolate fractions often associated with transcription start sites (TSSs) of housekeeping and developmental regulator genes (4). Although the CGIs are susceptible to DNA methylation because of its inherent sequence, they are very resistant to this mechanism, which makes CGIs to be normally found unmethylated (4, 5).

During tumorigenesis CGIs suffer some methylation changes going from unmethylated to hypermethylated. Hypermethylation of the CGIs in the promoter regions of TSS is recognized as a major event in the origin of many types of cancers (6). Although the specific mechanism by which aberrant DNA methylation is induced in cancer remains unclear, it is well established that gene hypermethylation is strongly associated with both aging and chronic inflammation

(7). Specifically, tumors of the gastrointestinal tract show chronic inflammation as effector of DNA methylation changes that precede the tumor development (8, 9).

The SOCS1 is a member of SOCS (Suppressor Of Cytokine Signaling) family that acts as classic negative feedback inhibitors of JAK/STAT pathway and is key physiological regulator of both innate and adaptive immunity (10) *SOCS1* is a small gene, containing a CGI that lies from promoter region until almost all gene body. The *SOCS1* CGI is hypermethylated in many different types of cancer (11, 12) and it is one the genes used to detect the of CpG island methylator phenotype (CIMP) in colon cancer (13) . *SOCS1* hypermethylation leads to gene silence (11, 14) but, in some cases, it does not change its own gene expression (12, 15, 16). The hypermethylation of *SOCS1* CGI is also seen in chronic inflammation (17). Even though methylation changes have been widely studied little is known about this epigenetic event in chronic periodontitis.

Chronic periodontitis represents an infection-associated inflammation that affect the periodontium, the connective tissue that attaches the surface of root teeth to alveolar bone (18). *SOCS1* has been show to play a potential role in modulating periodontitis (19) and it is down-regulated in chronic periodontitis when compared with chronic gingivitis (20).

To understand whether or not epigenetic changes occur and thereby potentially contribute to chronic periodontitis, we assessed the DNA methylation profile of exon 2 of *SOCS1* CGI in 90 gingival tissue samples divided into chronic periodontitis and healthy groups. We found this region be hypermethylated in chronic inflamed tissues when compared with healthy ones. We also assessed the RNA expression of *SOCS1* and no difference was found between groups. Also, no correlation with DNA methylation was observed. Nevertheless, ENCODE data set shows markers on exon 2 of *SOCS1* consistent with a role of regulatory region. Bioinformatics analysis of Dnase I hypersensitivity sites (DHS), which is a mark of regulatory region, showed correlation of a DHS within exon 2 of *SOCS1* with some

promoters of genes that are down-regulated on chronic periodontitis. Gene reporter assay was performed for methylated and unmethylated studied DNA fragment and showed that exon 2 of *SOCS1* gene presented enhancer activity, which was disrupted by DNA methylation. A negative correlation between the methylation studied region on *SOCS1* and gene expression of the target genes was also observed. Hence, our key finding of this study is the enhancer activity disruption by DNA methylation of *SOCS1* CGI in chronic inflammation.

RESULTS

DNA methylation of exon 2 *SOCS1* CGI in chronic periodontitis

Hypermethylation of *SOCS1* CGI has been seen in different types of cancer (11, 12, 14, 15), chronic inflammation-associated disease (17) and LPS-stimulated macrophage (21). To assess the DNA methylation of exon 2 of *SOCS1* in chronic periodontitis, gingival tissue from 90 age matched individuals (Supplementary Table 1), 46 with chronic periodontitis diagnosis (CP group) and 44 with no sign or symptoms of CP (healthy group), were used for the Methylation sensitive – High resolution melting (MS-HRM) which allows for the detection of DNA methylation levels (22) (Supplementary Figure S1A) . We found the region of exon 2 of *SOCS1* CGI to be hypermethylated in 50% (23/46) of CP group and 9% (4/44) of healthy group. From the 23 samples hypermethylated in chronic periodontitis group, 18 were 6-10% methylated, 4 were 11-25% methylated and 1 was 25-50% methylated. In the control group, the 4 samples found to be hypermethylated they were 6-10% methylated. The samples that were 0-5% were considered unmethylated (Figure 1A). The heterogeneity of cells in the gingival tissue samples could represent a limitation of this experiment. Gingival tissue represents a heterogeneous population of cells, with inflammatory cells being over represented in CP samples when compared to the healthy ones. However, a study using DNA methyl array on peripheral blood cells from healthy donors (23) shows the probes

that are placed on the studied *SOCS1* region are unmethylated in all types of blood cells (Figure 1D). This data evidences that the methylation seen on CP samples is not a consequence of cell heterogeneity in the tissue but certainly that the cells gained methylation due to chronic inflammation.

In order to verify the effect of the hypermethylation, we performed qPCR gene expression of *SOCS1* in 20 samples of CP group and 25 of Healthy group with no significant difference between groups (Figure 1B). No correlation between hypermethylation and downregulation of *SOCS1* gene was observed (Figure 1C).

Three enzymes, DNA methyltranferase 1 (DNMT1), DNMT3A and DNMT3B catalyze and maintain the covalent addition of methyl groups to cytosines (4). Three other enzymes, Ten-eleven translocation methylcytosine dioxygenase 1 (TET1), TE2 and TET3 are responsible for erasing the methylation from cytosine and are essential for normal development (5). Given there was DNA methylation change occurring on *SOCS1* gene, we also examined the expression of *DNMT1*, *3A*, *3B* and *TET1* and *TET2* using the same samples for *SOCS1* expression and found *DNMT1* and *TET1* to be upregulated in CP group (Supplementary Figure S1B). Overexpression of DNMT1 is implicated with genomic hypermethylation (24). Differently, TET enzymes promote DNA demethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine (5hmC). In addition, a recent study showed TET1 expression is regulated during inflammation both in THP-1 and in primary dendritic cells (25). Thus, the overexpression of DNMT1 and TET1 in CP samples corroborate with observation that epigenetic events were in progress in chronic inflamed tissue.

As CGI exon 2 hypermethylation of *SOCS1* did not regulated its own mRNA transcription levels, we hypothesized whether *SOCS1* CGI methylation could present some other functional relevance. Thus, we decided to further investigate the genome sequence of *SOCS1* region.

Evaluation of epigenetic markers associated with enhancer activity

Mammal's genome contains a selection of unique combinations of coding and regulatory elements. Some region of DNA may impact upon the expression of distant genes by acting as enhancer, requiring looping of the chromatin and interaction of enhancer-promoter (26). Many enhancers regions contain binding sites for transcription factors and are identifiable by the presence of epigenetic histone modifications such H3K4me1 and H3K27Ac (26, 27). In addition, recent evidence showed DNase I Hipersensitivity site (DHS) as a marker of regulatory region (16).

The publicly available ChIP-seq and DHS data sets from ENCODE project was explored in order to investigate histone modification marks and regulatory region marked by DHS throughout *SOCS1* gene. The exon 2 was found enriched for enhancer marks in human cells (Figure 2A). Further evidence for a regulatory function of exon 2 of *SOCS1* is provided by the observation that its sequence is bound by CTCF protein that has been shown to cooperate with cohesion to promote the formation of chromatin loops at some loci facilitating the interaction between regulatory elements such as enhancer and promoter (28, 29). Observations of ENCODE strong suggested the exon2 of *SOCS1* to be a regulatory region.

Exon 2 of *SOCS1* DHS to Promoter DHSs connections

Chromatin access dictates an important constraint for TF interactions with the DNA template (30). Regulatory elements can become available by localized remodelling of nucleosome structures, and these perturbations can be detected by an increased susceptibility to DNaseI digestion. DNaseI hypersensitive (DHS) sites thus serve as hallmarks of regulatory protein interactions at the template as well as sites of chromatin remodeling (31). The DHS profile is highly cell specific,

implicating cell-selective organization of the chromatin landscape as a critical determinant of tissue-selective receptor function (31). Many known cell-selective enhancers become DHSs synchronously with the appearance of hypersensitivity at promoter of their target gene (16).

We examined the DHS profile across many human cell types from ENCODE project on ±500kb surrounding the DHS anchor site that contained our region of interest in the exon 2 of *SOCS1* (chr16:11,348,911-11,349,051). We calculated the DHS correlation in a cell type-specific fashion restricting the analysis to only DHS sites identified within AG09313 (gingival fibroblast), HEEpiC (esophageal epithelial), Th1, Th2 and CD4+cells. We observed several promoters to correlate with the anchor region. The sites were ranked accordingly to the correlation value with *SOCS1* CGI, proximity to transcriptional start sites (TSSs) and down-regulation in chronic periodontitis by meta-analysis (32)

We found the promoter region of *RSL1D1(CSIG), SNN, RMI2, CLEC16A, GSPT1* genes to be correlated with exon 2 of *SOCS1* and every one of these genes are downregulated in chronic periodontitis (Figure 2B). These genes identified here are related to DNA repair (33), cell proliferation (34), apoptosis (35, 36) and immune inflammatory regulation (37).

Evaluation of *SOCS1* enhancer activity by gene reporter assay

Based on the regulatory predictions, we measured the enhancer activity of exon 2 *SOCS1* with a luciferase assay using a reporter plasmid (human EF-1α promoter) completely devoid of CpG dinucleotides with cloning site in place of the enhancer. Four fragments were cloned. Two covering the region of *SOCS1* that was found hypermethylated on MS-HRM assay (A and B), one downstream from these two and still in exon 2 at the end of CGI (C), and one that was the junction of A and B (AB) (Figure 3A). A fragment which lies in a repressed chromatin region was

cloned as a negative control (NC) (Supplementary Figure S1C). The constructs were transfected into HEK 293 cells and the luciferase activity measured 24 hours after transfection. All the results were normalized and relative to empty vector (just promoter).

As shown in Figure 3B, all the fragments, except the negative control significantly enhanced the activity of basal (CpG-free) promoter. The fragment AB showed the highest level of luciferase activity. Probably, this was a consequence of the fragment length as it is larger than A, B and C allowing a higher number of transcription factors binding in this sequence. Another possibility is that when A and B are separated an important transcription binding site can be disrupted, which explains the lower activity of A and B when compared to AB fragment. We also observed activity for fragment C which reflects that regulatory region on *SOCS1* can be extended to a larger region on exon 2.

Enhancers are cis-acting elements capable of regulating transcription in a distance and orientation-independent manner. These results indicate an enhancer activity for the *SOCS1* exon 2.

DNA methylation-mediated disruption of a transcriptional enhancer

Constitutively accessible chromatin regions (pre-programmed enhancers) are enriched by CpG dinucleotides and it is clear DNA methylation play a key role regulating those regions (38) as CpG sites hypomethylation correlates with chromatin accessibility (16, 38-40). In addition, DNMT1-depleted cells moderately increase chromatin accessibility (38, 40). The accessible chromatin facilitates the TF binding and a negative correlation between transcription factor expression and binding site methylation at DHSs exists.(16).

In order to examine if DNA methylation would change the enhancer activity unmetylated fragments we also tested *in vitro* methylated fragments. As the

plasmid used for this study was completed devoid of CpG sequence it assured the DNA methylation was carried out only in the enhancer fragment (inserted DNA). Not only all the constructs had their enhancer activity abolished when the plasmids were methylated but they also repressed the promoter activity of the reporter gene (Figure 3B). The repression of the promoter could be explained by the repression model which involves proteins that are attracted to methyl-CpG. The methyl-CpG-binding domain (MBD) MeCP2 is involved in turning off gene and have been implicated in methylation-dependent repression of transcription (for review, see Bird, 2002) (41).

In the next approach, we wanted to explore the functional effect of DNA methylation of exon2 of *SOCS1* enhancer in chronic inflammation. We sought to investigate the gene expression of *SOCS1* and found the DNA methylation observed in MS-HRM assay presented a negative correlation with target genes expression in corresponding samples (Figure 3C).

In summary, we demonstrated that exon 2 of *SOCS1* gene acts as regulatory element which is disrupted by DNA methylation. The negative correlation of *SOCS1* methylation and target genes expression suggests chronic inflammation contributes to the regulation of genes important for cellular control through DNA methylation changes in a cis- regulatory manner.

DISCUSSION

In this report, we highlight the role of DNA methylation as an integral component of transcriptional regulation mediated by enhancer activity. Using MS-HRM we found *SOCS1* CGI to be differently methylated in chronic periodontitis and despite this epigenetic event has been associated with gene expression silence, we did not see difference in expression for this gene. In fact, we observed a negative correlation between methylation of exon 2 *SOCS1* CGI and transcriptional levels of

neighboring genes on chronic periodontitis. *In vitro* assay showed DNA methylation as a repressor event for *SOCS1* enhancer activity.

The differential methylation of *SOCS1* CGI is observed in chronic inflammation of the liver (17). Our findings show the hypermethylation of exon 2 of *SOCS1* CGI in chronic periodontitis without changes in gene expression. The hypermethylation of the CGIs has been shown as a mechanism to gene silence however, some studies failure to show this association. In certain types of cancer like melanoma (12) and breast cancer (15), the hypermethylation of *SOCS1* did not silent the gene expression (42, 43). Hypermethylation of a specific locus is not always perfectly associated with gene expression but it might be clinically relevant if it is placed in a *cis*-regulatory region that regulates others genes. In fact, at least in a normal development context, most of dynamic changes in DNA methylation are seen in regulatory region, particularly enhancers (44).

Classically, CpG islands are short regions containing CpG dinucleotides at high density that map to promoter regions. Unexpectedly, the ENCODE project identified a correspondence between unmethylated genic CGIs and binding by P300, a histone acetyltransferase linked to enhancer activity (16). Our study highlights a region in a CGI within the body of *SOCS1* as regulatory region with enhancer activity. CGIs have unstable nucleosome as its signature and the chromatin is intrinsically accessible (45, 46). *In vitro* nucleosome assembly indicated that a set of these CGIs is significantly more reluctant to assemble into nucleosomes than other genomic DNA (46). This CGI inherent chromatin state facilitates the transcription factor accessibility to CGIs, probably allowing this region to function as regulatory element other than a promoter.

We also found that the enhancer activity of *SOCS1* region is under DNA methylation control which is disrupted by hypermethylation. This observation is in agreement with genome wide-analyses that show strong anti-correlation between enrichment of active enhancer marks and DNA methylation density (16, 47). Due

to the observation that *SOCS1* enhancer lies on a CpG island and the islands are normally maintained unmethylated, we propose DNA methylation as a mechanism to control CGI enhancer activity besides controlling CGI promoter. Most studies have focused in the CGI promoters known for their predominantly unmethylated biology, however it has become apparent that CGIs remote from annotated transcription start sites (TSSs) located either between genes or within the body of a transcription unit, exhibit a high degree of tissue-specific methylation (48) a characteristic of distal enhancers.

The differential methylation observed in the chronic periodontitis samples is placed on the enhancer region of *SOCS1* CGI and presents a negative correlation to promoter region of some genes located at its neighborhood in the chr16p13. On a multiple sclerosis study, the authors showed correlated expression of the genes from the chr16p13 region with independent single polymorphisms (SNPs) effect and suggested *SOCS1* region contains a regulatory domain that influences expression of multiple genes in this region (49). Accordingly, our data suggest the hypermethylation of exon 2 at *SOCS1* CGI in chronic periodontitis control the gene expression of a several genes located at chr16p13 through disruption of its enhancer activity, which is supported by a recent study that shows DNA methylation of distal regulatory sites is closely related to gene expression levels across the genome (50).

To our knowledge, this is the first time the DNA methylation of *SOCS1* CGI is shown to control the expression of other genes in an enhancer/promoter interaction fashion. The mechanism exposed here will help to elucidate the changes on *SOCS1* DNA methylation commonly seen in chronic inflammation and tumorogeneses. Taken together the data indicate clear evidence that the hypermethylation of *SOCS1* gene represents a new and important pathway in the pathophysiology of chronic inflammation.

MATERIALS AND METHODS

Samples from chronic periodontitis volunteers and healthy volunteers

Fresh gingival biopsies were obtained from 46 patients diagnosed with chronic periodontitis (18, 51) volunteers and 44 healthy volunteers exhibiting no signs of periodontal disease and who had surgery for esthetical reason and/or 3nd molar excision. Inclusion criteria were non-smoking individuals, non-systemic disorder that could affect the periodontal condition, no antibiotics and no anti-inflammatory medication within the past six months, non-current pregnancy nor lactation. The samples were obtained from School of Dentistry of Piracicaba, University of Campinas (FOP/UNICAMP). Written informed consent was obtained from all individuals and the study was approved by FOP/UNICAMP Ethics Committee.

Gingival biopsies were obtained from a single tooth of subjects undergoing periodontal surgery for periodontal related disease (Chronic periodontitis group), and non-disease related reasons (Healthy group). The biopsies were composed by junction epithelia and connective tissue.

All samples were collected immediately after surgery and stored in a nucleic acid conserver (RNA holder, Bioagency, São Paulo, SP, Brazil) and frozen to minus 80 °C, until the moment of nucleic acid extraction.

RNA and DNA extraction and bisulfite treatment

The RNA was extracted and purified using TRIZOL reagent protocol (Invitrogen, Carlsband, CA, USA). After RNA purification the remained phases contained proteins and DNA were subjected to a new step of phenol/chloroform/ethanol protocol to purify DNA.

DNA was bisulfite converted using the MethylSEQr Bisulfite Conversion (Life, Carlsbad, CA, USA) following the manufacturer´s protocol. The samples were short time storage and used for MS-HRM.

Methylation Sensitive High Resolution Melting (MS-HRM)

Real-time PCR followed by HRM was carried out in Light Cycler 480 II (Roche, Mannheim, Germany). The sequence of the primer set used is shown in Supplementary Table 2. The design of the primer followed the guidelines proposed by Wojdacz (22).The reaction mixture consisted of 18 ng of bisulfite-converted DNA, 1x LightCycler®480 HRM Master Mix (Roche, Mannheim, Germany), 150 nM of each primer, 3 mM of MgCl+2 in a final volume of 20 µl. The reaction conditions were an initial cycle of 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 10 seconds, 55 °C for 4 seconds and 72 °C for 10 seconds. MS-HRM analyses were performed at the temperature ramping and fluorescence acquisition settings recommended by the manufacturer, 1 minute at 95 °C, a hold at 70 °C for 1 minute to allow reanneling of all PCR product, then the acquisition step started ramping from 70 °C to 95 °C, rising by 0,2 °C/s with 25 acquisitions per °C.

To estimate the methylation level of the samples, converted fully methylated and fully unmethylated DNA (EpiTect PCR Control DNA Set (Qiagen) were used to prepare dilution series as controls. The dilution series of relevant methylated DNA in a background of unmethylated DNA were prepare in the concentration of 5%, 10%, 25%, 50%, 75% and 100% of methylation to build standards curves. PCR bias toward unmethylated DNA was reversed following the guideline published by Wojdacz (22). Standard curves and no template controls were included in each experimental run. As a negative control, genomic unconverted DNA from a pool of 3 healthy individuals was tested once with each primer. Initial assays were first run using 50% and 0% controls until the proportionality of amplification was achieved. MS-HRM data were normalized with the Light Cycler 480 II analysis software to

compensate for varying starting fluorescence levels. The amplicon melting profile of each sample was compared to standard curves, and based on that, samples of each individual was classified into different methylation categories; 0-5, 6-10, 11-25, 26-50, 51-75 and 76-100%.

Expression Analyses

One microgram of total RNA was treated with DNase I, RNase free (Life, Carlsband, CA, USA) and used for cDNA synthesis with First strand (Roche, Mannheim, Germany) following the manufacturer´s recommendations. Real Time qPCR mRNA analyses were performed in Light Cycler 480 II (Roche, Mannheim, Germany). The reaction was carried out in a total volume of 20 µl containing 1x LightCycler®480 Sybr Green (Roche, Mannheim, Germany), 250 nM of each primer and 2 µl of cDNA. PCR conditions were: 95 °C 10 min, 40 cycles at 95 °C for 10 seconds, appropriate temperature for each primer (Supplementary Table 3) for 10 seconds, 72 °C for 10 seconds. Determination of relative levels of gene expression was performed using the cycle threshold (Ct) method in reference to GAPDH.

Bioinformatics

Thurman et al (16) observed that highly correlated DNaseI-Seq signal intensities ($r > 0.7$) calculated across a panel of 79 different cell types were enriched within chromatin interactions identified through 5C or ChIA-PET. We applied this approach to predict chromatin interactions at the chr16:11,348,911-11,349,051 locus. We calculated the Pearson correlation coefficient (r) between DNaseI hypersensitivity signal intensities from all ENCODE cell-lines with DNaseI-Seq data available. We calculated the correlation in a cell type-specific fashion restricting the analysis to only DHS sites identified within AG09313, HEEpiC, Th1, Th2 and

CD4+cells. In addition, we restricted our analysis to ±500kb surrounding the DHS anchor site that contained our region of interest. The DNaseI-Seq data was download from the ENCODE website (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeUwDnase/). (32).

In vitro analysis of *SOCS1* enhancer and the influence of DNA methylation on its activity.

Insert amplification

Genomic DNA was isolated from human venous blood using the QIAamp DNA Blood Kit (Qiagen, Hilden). The exon 2 of SOCS1 region (chr16:11348911-11349051) was amplified by PCR using Kapa Hifi PCR kit (Kapa Biosystems, Massachusetts, USA) and 50 ng of gDNA (Supplementary Table S4). The cloned fragments were, two covering the region of SOCS1 that was found hypermethylated on MS-HRM assay, A (chr16:11348973-11349115) and B (chr16:11348872-11348999). One downstream from these two and still in exon 2 at the end of CGI, C (chr16:11348544-11348676) and one that was the junction of A and B, AB (chr16:11348872-11349115) fragment (Figure 3A). A fragment which lies in a repressive chromatin state was cloned as a control, NC (chr15:67610118-67610254) (Supplementary Figure S1B). PCR steps were performed as followed: 95°C 5 min; 98°C 20 sec, 60°C 15 sec, 70°C 30 sec (30 cycles). The fragments were gel purified using MinElute Gel Kit (Qiagen).

Cloning of Luciferase expression vectors

BamHI e ScaI HF-linearized pCpGfree-promoter-Lucia (human EF-1α promoter) coelenterazine-utilizing luciferase (like Renilla) reporter plasmid (Invivogen) was used to clone the fragments with adapter sequences in place of the enhancer by

recombination using InFusion HD Enzyme (Clontech Laboratories Inc., Mountain View, CA). The ratio was 50 ng vector : 50 ng insert. Competent *E. coli* GT115 (Invivogen) were transformed with 2.5 µL recombined plasmid and plated on LB-Agar Fast-Media Zeo Agar (Invivogen). A few grown colonies were transferred to 3 mL de Fast-media Zeo TB (Invivogen) and plasmids from an aliquot were isolated using the Pure link HiPure plasmid kit Miniprep (Life). After verifying the plasmids containing the fragments by restriction digestion of the plasmids with BamHI and ScaI, the transformed bacterias were transferred to 50 ml of Fast-media Zeo TB (Invivogen), and the plasmids were isolate and purified with Pure link HiPure plasmid kit Midiprep (Life).

In vitro-Methylation of *SOCS1* enhancer constructs.

All the Fragments (3 µg), including the empty vector, were incubated (4h 37°C) with SssI DNA methyltransferase (16U; New England Biolabs, Ipswich, MA) in buffer containing 640 µM S-adenosylmethionine (New England Biolabs, Ipswich, MA), followed by heat inactivation ate 65°C for 20 minutes following the manufacture instructions. DNA was purified with phenol/chloroform/ethanol protocol.

Transfection and Luciferase-based reporter gene assays

Human Embryonic Kidney 293 (ATCC, Wesel) was cultured in DMEMs supplemented with 10% FBS, 2 mM of glutamine without antibiotics. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in atmosphere.

Lipofectamine LTX was used according to manufacturer's protocol. 1.25 x 10^5 HEK 293 cells / well were plated in 24 well plates 24 hours before transfection. For transfection, medium was removed and replaced by 100 ul of Opti-MEM reduced serum media (Life) without antibiotic containing the plasmids and 0.75 ul of lipofectamine LTX. For each fragment, including empty vector, different

concentration of unmethylated or methylated plasmids were used per well. 10 ng, 50 ng and 100 ng of the test plasmid were co-transfected with 1 ng of the firefly PG13 promoter plasmid to enable normalization for transfection control. After 24 hour incubation, cells were lysed and luminescence was measured using the Dual Luciferase Reporter Assay System (Promega, Madison) and the GloMax Multi+ Luminometer (Promega, Madison), according to the manufacturer's protocol. The luciferase luminescence was normalized by plasmid amount.

Acknowledgements

We are sincerely grateful to Dr. Tomasz K Wojdacz for helpful discussion regarding MS-HRM technique and Dr. Marisi Aidar for helping with sample collection. We thank FAPESP for the Brazilian scholarship and CAPES for internship abroad.

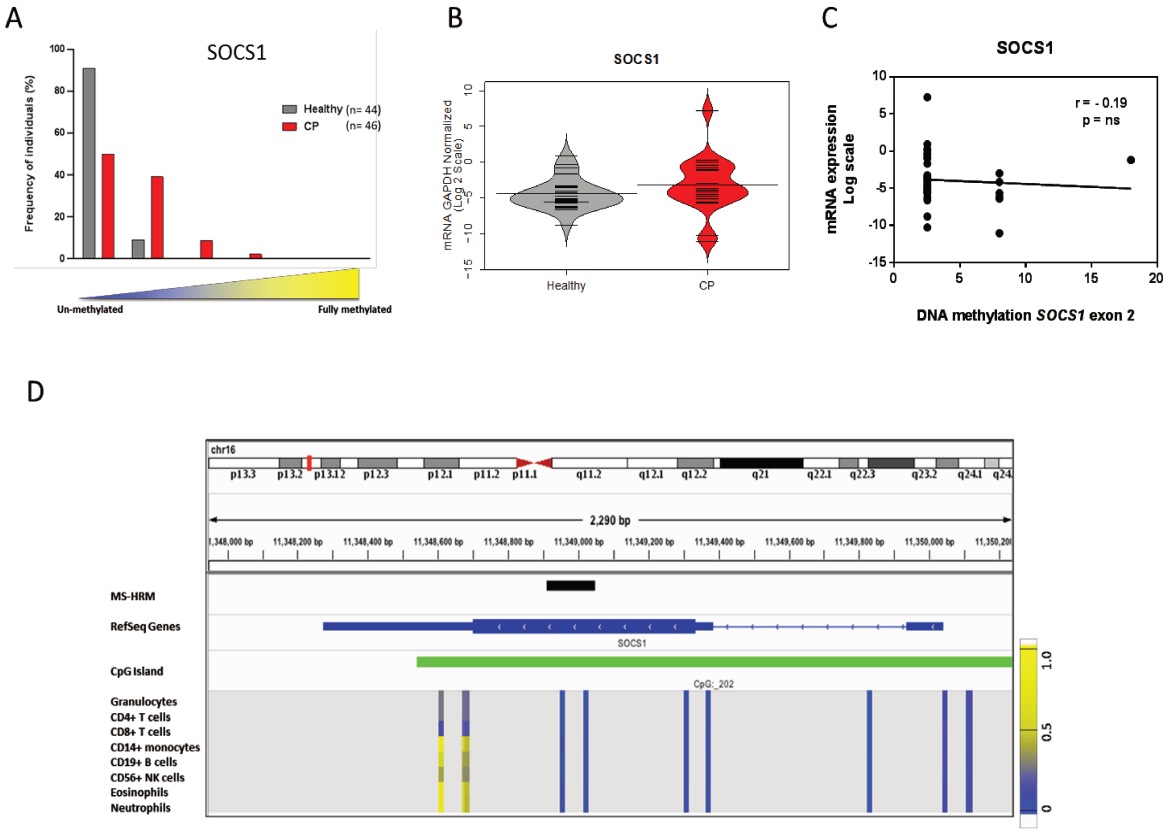
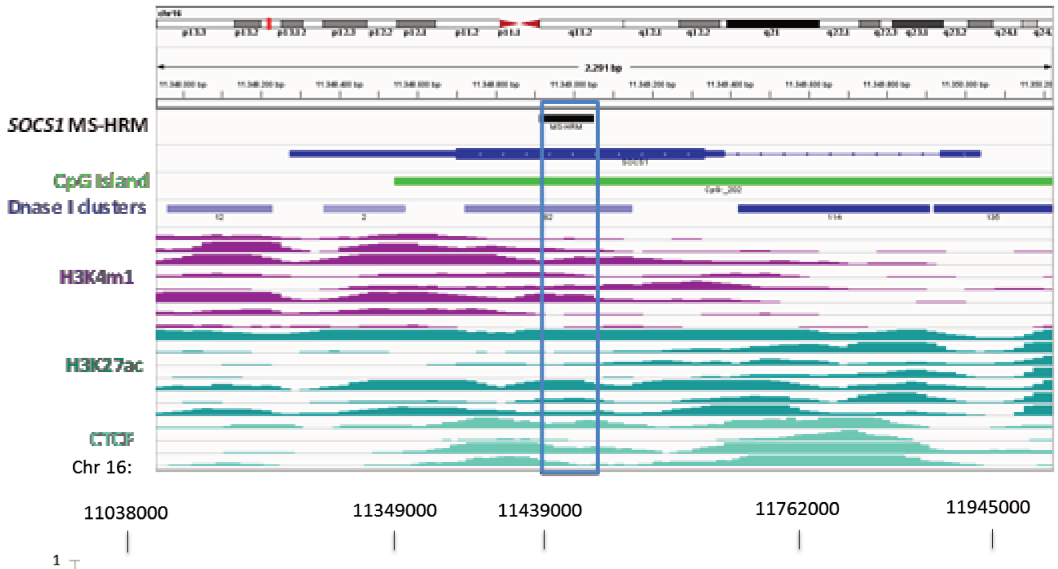
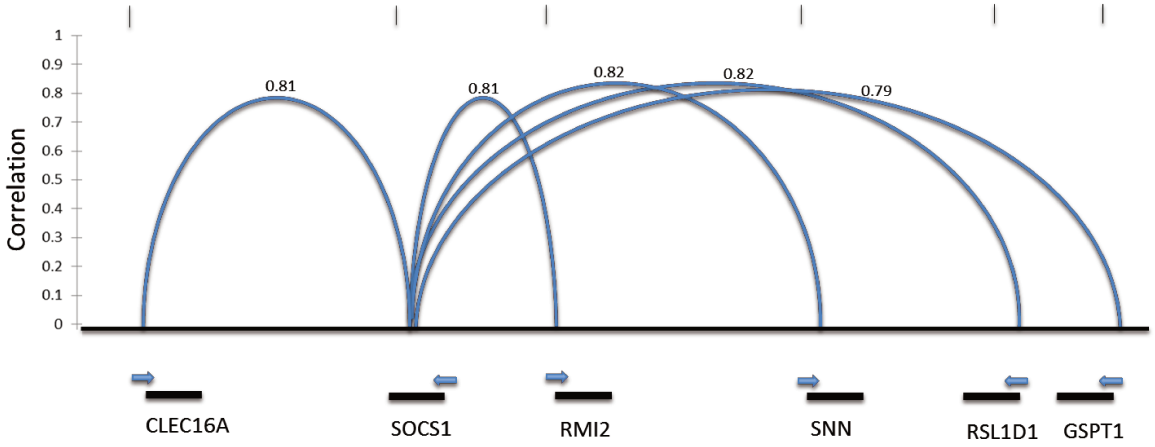


Figure 1- SOCS1 exon2 is hypermethylated in Chronic Periodontitis and this is not associated with change in gene expression. **A)** DNA methylation of exon 2 *SOCS1* region (chr16:11,348,911-11,349,051) in chronic periodontitis n=46 (red) and healthy groups n=44 (gray) using MS-HRM. The results were categorized in groups showing methylation level, 0-5, 6-10, 11-25, 26-50% methylation and are show in the y axis as the percentage of individuals in each category. Groups were compared using Fisher exact test. **B)** Expression levels of *SOCS1* gene using qPCR. Data is transformed and normalized to GAPDH expression. Values are shown in log2 scale and groups were compared using Mann Whitney U test: n=20 (CP), n=25 (Healthy). NS, non-significant. **C)** No correlation between *SOCS1* methylation and gene expression. Methylation values are the mean of each MS-HRM group (0-5% = 2.5%). **D)** Exon2 *SOCS1* region investigated in MS-HRM, RefSeq gene and CpG Island from UCSC. DNA Methylation (Infinium array 450K) of peripheral blood cells from healthy donnors (23) data shows no methylation in the studied region in any blood type cells. The data is available under at GEO 35069 number (n= 6). Intensity values were converted to beta-values where the value of 0 (blue) represents unmethylated and the value of 1 (yellow) represents fully methylated (displayed using Integrative Genomic viewer – IGV).

A



B (i)



B (ii)

DHS site position			Gene name	Correlation to DHS exon 2 of *SOCS1*	Expression Fold change CP/Healthy	*p*-Value expression.
chr16	11944889	11945770	RSL1D1	0.82	-0,3939	3,51E-07
chr16	11761847	11762798	SNN	0.82	-0,244	5,09E-06
chr16	11438775	11439418	RMI2	0.81	-0,2	2,60E-05
chr16	11038018	11039127	CLEC16A	0.81	-0,1085	0,03191
chr16	12009460	12010142	GSPT1	0.79	-0,1801	0,0001701

Figure 2- SOCS1 exon2 locus is connected to neighboring promoters A) Chromatin modification and CTCF binding patterns across SOCS1 gene locus in different human cells. H1-hESC(H1), GM128878 (GM), HSMM (HS), Huvec (HU) K562 (K), NHEK (EK), NHLF (LF). DnaseI clusters, Dnase hypersensitivity sites. (ENCODE project, displayed using Integrative Genomic viewer – IGV). **B)** (i) Exon2 *SOCS1* DHS correlation to DHS in the promoter region of *CLEC16*A, *RSL1D1*, *RMI2*, *SNN*, *GSTP*1 . (ii) Table shows DHS chromosome position, gene name, correlation value, gene expression fold change of chronic periodontitis relative to healthy tissue and p-value of gene expression. RNA expression data was obtained from GEO depository for chronic periodontitis and healthy tissue GSE10334 (n=90).

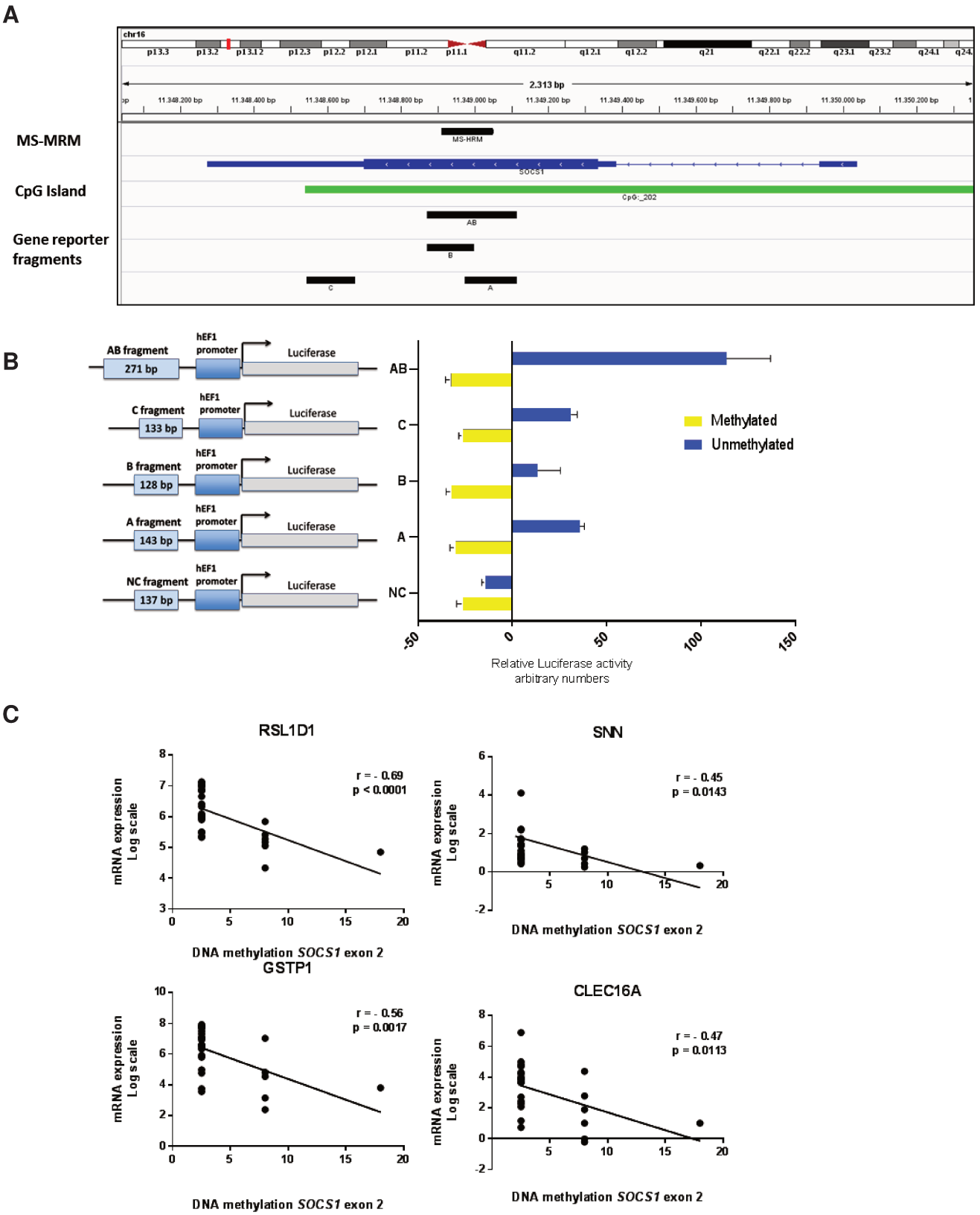
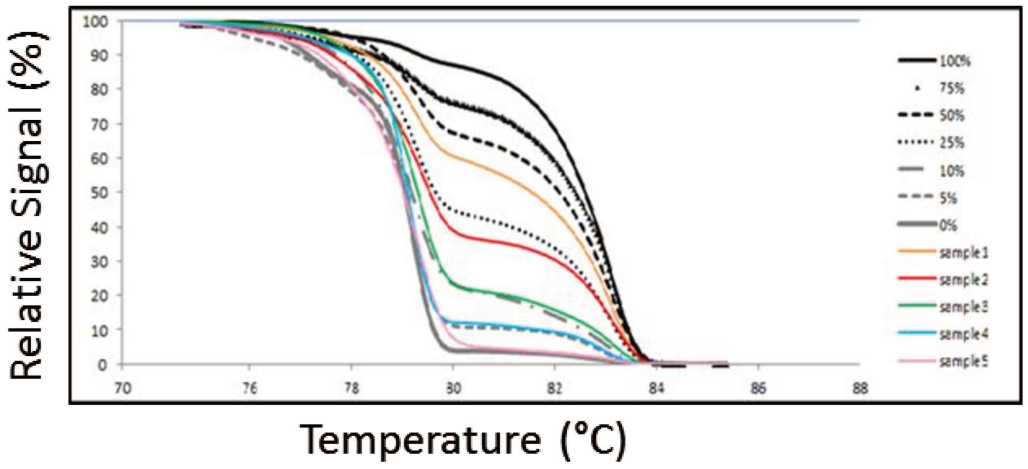

A
MS-MRM
CpG Island
Gene reporter fragments
2.313 bp
B
AB fragment
271 bp
C fragment
133 bp
B fragment
128 bp
A fragment
143 bp
NC fragment
137 bp
hEF1 promoter
Luciferase
Methylated
Unmethylated
Relative Luciferase activity
arbitrary numbers
C
RSL1D1
r = - 0.69
p < 0.0001
SNN
r = - 0.45
p = 0.0143
GSTP1
r = - 0.56
p = 0.0017
CLEC16A
r = - 0.47
p = 0.0113
mRNA expression
Log scale
DNA methylation SOCS1 exon 2

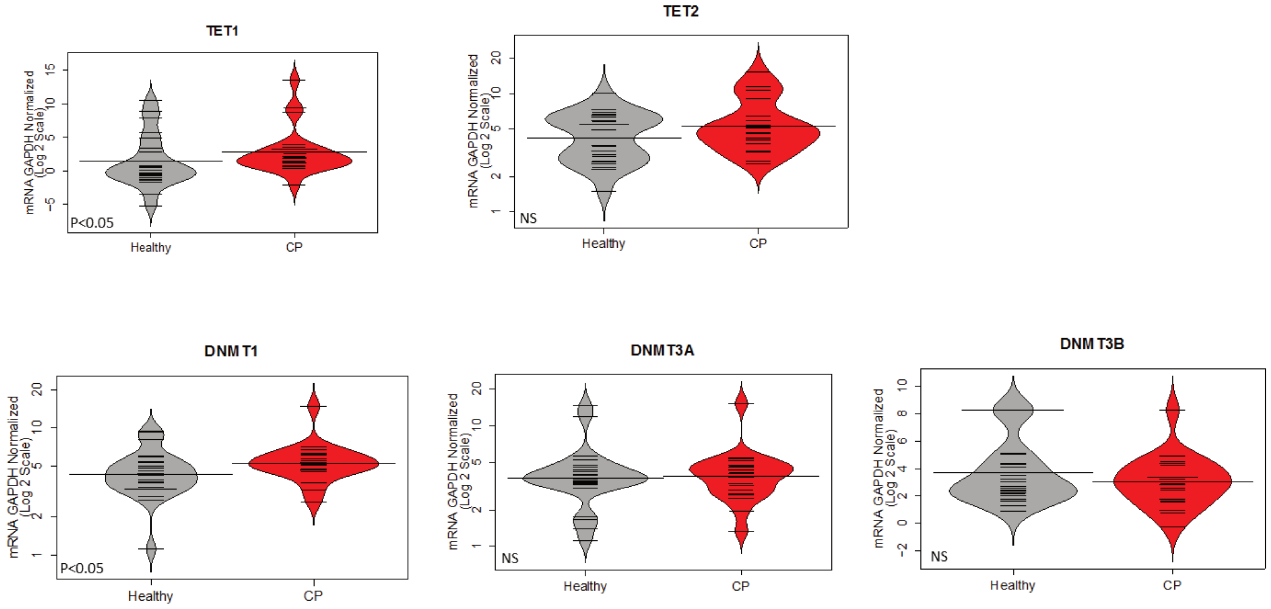
Figure 3- CpG methylation-dependent enhancer activity of SOCS1 exon 2 locus A) Fragments used for the gene reporter constructs: A (chr16:11348973-11349115), B (chr16:11348872-11348999), C (chr16:11348544-11348676) and AB (chr16:11348872-11349115). **B**) Luciferase assay was performed in HEK292 cells. Unmethylated and methylated pCpGfree-promoter-Lucia (Renilla) plasmids harboring the fragments mentioned above in place of an enhancer were used. A sequence from a repressed region (chr15:67610118-67610254) was used as a negative control (NC) (Supplementary Figure S1C). Relative luciferase units were normalized to firefly signal. The luciferase expression level for each fragment is relative to the empty vector (just promoter). The replicates were 10, 50 and 100 ng of the test plasmid and the luciferase levels were normalized by transfected plasmid amount. All the constructs were compared using T test with pooled SD, adjustment method: bonferroni; (*) $p \leq 0.05$; (**) $p \leq 0.01$. A= *. B= ns. C=*. AB= ** **C)** Correlation of exon2 SOCS1 methylation and gene targets. Spearman correlation. Expression levels were assessed using qPCR

Supplementary Figures

S1A



S1B



S1C

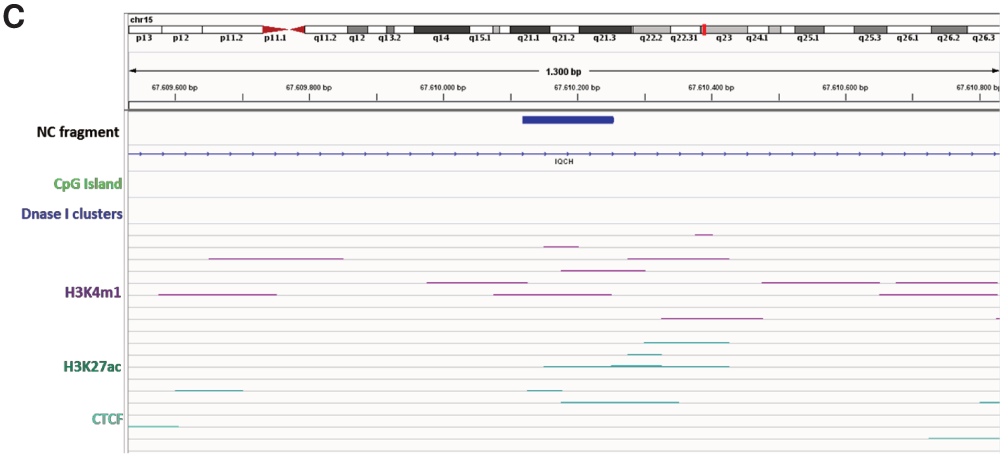


Figure S1 - A) MS-HRM methylation groups and some example samples. **B)** IGV snapshot of repressed chromatin region containing NC fragment used in the luciferase assay. **C)** Gene expression levels of DNA methyltransferases and TET enzymes using qPCR. Data is transformed and normalized to GAPDH expression. Values are shown in log2 scale and groups were compared using Mann Whitney U test: n=20 (CP), n=25

Table S1- Mean and SD age of CP and Healthy groups.

	MS-HRM	
	Healthy	CP
Male/ Fem (%)	36,3/63,3	45,6/54,3
Age mean	42,54 SD 11,94	47,17 SD 11,31

Table S2- Primer sequence for MS-HRM

	Primer Sequence	Chromosome Position	Amplicon size
SOCS1	F 5'TCGCGGTTGTTATTTAGGTGAAAG 3'	Chr 16	140 bp
	R 5'CGAACCCGTAAACACCTTCCTA 3'	11348911- 11349051	

Table S3 – Primer sequence for RNA expression

Genes	Primer sequence	Annealing temperature	Amplicon size
GAPDH	F 5′ CCACTCCTCCACCTTTGAC 3’	56	103 bp
	R 5′ ACCCTGTTGCTGTAGCCA 3′		
SOCS1	F 5′ CTGGGATGCCGTGTTATTTTG 3′	58	224 bp
	R 5′ TAGGAGGTGCGAGTTCAGGTC 3’		
DNMT1	F 5′ACCTGGCTAAAGTCAAATCC 3′	60	80 bp
	R 5′ATTCACTTCCCGGTTGTAAG 3′		
DNMT3A	F 5′GAAGGACTTGGGCATTCAGGT 3′	60	105 bp
	R 5′CCGACGTACATGATCTTCCC 3′		
DNMT3B	F 5′CGAGTCCTGTCATTGTTTGATGG 3′	60	60 bp
	R 5′ GCGACGTACTTTCCTACCTTTATG 3′		
TET1	F 5' AAAGATTCTGAACTGCCCACCTG 3	60	122 bp
	R 5' TTCTCCATGATTTCCCTGACAGC 3'		
TET2	F 5' TGGAGAAAGACGTAACTTCGGG 3'	60	116 bp
	R 5' CTTGGGCTACAGAACTCACAGAT 3'		
RSL1D1	F 5' CGTATTGGTCACGTTGGAATGC 3'	60	93 bp
	R 5' CCACTTCTCTGGCAATTTTTCTG 3'		

SNN	F 5' CTGCTGGTGCAGTATTCGG 3'	60	68 bp
	R 5' CCGTTGGGAGTCATCAGCTTG 3'		
CLEC16A	F 5' ATGCTGCACTACATCCGAGAT 3'	60	86 bp
	F 5' TCGAGTTCGATCACATGGCTC 3'		
GSTP1	F 5' TGGACGCACATTTGATGCC 3'	60	104 bp
	R 5' CCACCTCCTCAATACAGGTATGA 3'		

Table S4- Primer sequence of *SOCS1* region for reporter assay.

Fragment Luc assay	Primer sequence 5'- 3'	Annealing temperature
A	F **CCTAGGATGCATAGT**CGAGGCCATCTTCACGCTA	60
	R **GTTACATGTTGGATC**TGGACGCCTGCGGATTCTA	
B	F **CCTAGGATGCATAGT**ACGTAGTGCTCCAGCAGCT	60
	R **GTTACATGTTGGATC**TTCGCCCTTAGCGTGAAGATG	
C	F **CCTAGGATGCATAGT**GCCTCGTCTCCAGCCGAG	60
	R **GTTACATGTTGGATC**AGCATTAACTGGGATGCCGTGT	
AB	F **CCTAGGATGCATAGT**ACGTAGTGCTCCAGCAGCT	60
	R **GTTACATGTTGGATC**TGGACGCCTGCGGATTCTA	
NC	F **CCTAGGATGCATAGT**AGAGGAAGGATTCTGTAGAGAAGTG	60
	R **GTTACATGTTGGATC**CCTAAGGGAAGCCGTGTGTAG	

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CONCLUSÃO

Nós concluímos que a metilação do DNA no exon 2 do gene *SOCS1* é capaz de controlar a expressão de outros genes através da interação enhancer/promotor. O mecanismo exposto neste estudo é novo e trás avanços sobre o entendimento do papel da metilação no gene *SOCS1,* evento epigenético frequentemente observado na inflamação crônica e em determinados tumores malignos. Os dados apresentados mostram que a hipermetilação do gene *SOCS1* pode representar um evento com importância ainda maior do que o até o momento entendido na patogênese da inflamação crônica.

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COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise de metilação no promotor dos genes SOCS1, SOCS2 E SOCS3 em células epiteliais da mucosa bucal e células do tecido gengival de indivíduos afetados pela periodontite crônica: Associação com a expressão de transcritos destes genes"**, protocolo nº 050/2010, dos pesquisadores Ana Paula de Souza Pardo, Aline Cristiane Planello e Luciana Souto Mofatto, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/09/2013.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"DNA methylation analysis of SOCS1, SOCS2 and SOCS3 gene promoters in oral epithelial cells and gingival tissue cells from individuals with chronic periodontitis: association with gene transcription"**, register number 050/2010, of Ana Paula de Souza Pardo, Aline Cristiane Planello and Luciana Souto Mofatto, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 09/12/2013.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO2

Assunto:	Fwd: Your manuscript has been accepted for publication in principle.
De:	Daniel De Carvalho (ddecarv@uhnresearch.ca)
Para:	alinep_fisio@yahoo.com.br; rajitz@gmail.com; javier.alfaro.4@gmail.com;
Data:	Quinta-feira, 30 de Janeiro de 2014 12:32

congratulations!

-------- Original Message --------
Subject: Fwd: Your manuscript has been accepted for publication in principle.
Date: Thu, 30 Jan 2014 09:26:24 -0500
From: Nizar Batada <nizar.batada@gmail.com>
To: Daniel De Carvalho <ddecarv@uhnresearch.ca>, Jun Feng Ji <JunFeng.Ji@oicr.on.ca>, vivek sharma <viveksharma_bt@yahoo.com>, "Christoph Bock" <cbock@cemm.at>

---------- Forwarded message ----------
From: CELL REGENERATION EDITORIAL
Date: Thursday, January 30, 2014
Subject: Your manuscript has been accepted for publication in principle.
To: Dr Nizar Batada

Authors: Aline C Planello, Junfeng Ji, Vivek Sharma, Rajat Singhania, Faridah Mbabaali, Fabian Muller, Javier Alfaro, Christoph Bock, Daniel D
De Carvalho and Nizar N Batada
Title : Aberrant DNA methylation reprogramming during iPS generation is
dependent on the choice of reprogramming factors
Journal: Cell Regeneration
MS : 5756878811589754

Dear Dr Batada,

Peer review of your manuscript (above) is now complete and we are delighted to accept the manuscript for publication in Cell Regeneration.

Before publication, our production team needs to check the format of your manuscript, to ensure that it conforms to the standards of the journal. They will get in touch with you shortly to request any necessary changes or to confirm that none are needed.

Anexo 3

Assunto:	PLOS ONE: Notification of co-authorship on manuscript [High-throughput DNA Analysis Shows the Importance of Methylation in the Control of Immune-Inflammatory Genes Transcription in Chronic Periodontitis.] - [EMID:2c333cd4dc21a1c6]
De:	PLOS ONE (plosone@plos.org)
Para:	alinep_fisio@yahoo.com.br;
Data:	Quarta-feira, 29 de Janeiro de 2014 10:34

Dear Dra. Aline Planello,

You are receiving this email because you have been listed as an author on a manuscript recently submitted to PLOS ONE and entitled "High-throughput DNA Analysis Shows the Importance of Methylation in the Control of Immune-Inflammatory Genes Transcription in Chronic Periodontitis.".

The corresponding author for the submission process is: Dr. Ana Paula de Souza Pardo

The full author list for the submission is: Ana Paula de Souza Pardo, Ph.D.; Aline Cristiane Planello; Daniel Diniz De Carvalho; Sergio Roberto Peres Line

If you are not aware of this submission, or if you should not be listed as a co-author, then please contact the journal office at plosone@plos.org.

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