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CIGARETTE SMOKE INDUCES PROTEASOMAL-MEDIATED DEGRADATION OF DNA METHYLTRANSFERASES AND METHYL CpG-/CpG DOMAIN-BINDING PROTEINS IN EMBRYONIC OROFACIAL CELLS

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Abstract

Orofacial clefts, the most prevalent of developmental anomalies, occur with a frequency of 1 in 700 live births. Maternal cigarette smoking during pregnancy represents a risk factor for having a child with a cleft lip and/or cleft palate. Using primary cultures of first branchial arch-derived cells (1-BA cells), which contribute to the formation of the lip and palate, the present study addressed the hypothesis that components of cigarette smoke alter global DNA methylation, and/or expression of DNA methyltransferases (Dnmts) and various methyl CpG-binding proteins. Primary cultures of 1-BA cells, exposed to 80 μg/ml cigarette smoke extract (CSE) for 24 hrs, exhibited a >13% decline in global DNA methylation and triggered proteasomal-mediated degradation of Dnmts (DNMT-1, and - 3a), methyl CpG binding protein 2 (MeCP2) and methyl-CpG binding domain protein 3 (MBD-3). Pretreatment of 1-BA cells with the proteasomal inhibitor MG-132 completely reversed such degradation. Collectively, these data allow the suggestion of a potential epigenetic mechanism underlying maternal cigarette smoke exposure-induced orofacial clefting.

Keywords

Cigarette smoke; Cleft lip/palate; Mouse; Branchial arch; Proteasome; DNA methylation; DNA methyltransferases; CpG-binding proteins

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The authors declare that there are no conflicts of interest.

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1. INTRODUCTION

Orofacial clefting (cleft lip with or without cleft palate, CL/P, or cleft palate only, CPO), is currently the most prevalent of congenital anomalies, with a frequency of 1 in 700 live births [1, 2]. During week four of human gestation, the upper lip forms via fusion of the maxillary process (MxP) of the first branchial arch with the medial nasal process (MNP) [3]. Between weeks 7–10 of gestation, the secondary palate develops from the oral aspect of each maxillary process and eventually gives rise to the roof of the oral cavity [4]. Thus, both the upper lip and the palate develop from cells derived from the first branchial arch. While a wide range of genetic and environmental factors can adversely affect formation of the lip or palate [5], maternal cigarette smoking [6–10], as well as passive smoking during pregnancy [11–13], has been well documented as risk factors for orofacial clefting. Studies have also documented abnormal mandibular development following prenatal cigarette smoke exposure [14]. This is of note since certain abnormalities of the mandible, also derived from the first branchial arch, can secondarily cause clefts of the palate. Precise genetic and/or epigenetic mechanisms underlying *in utero* cigarette smoke exposure-induced orofacial clefting remain, however, largely undefined. Recent studies demonstrating prenatal cigarette smoke exposure-induced alterations in DNA methylation in offspring [15–18], suggest that teratogenic effects of cigarette smoke could be partly mediated by alteration of gene expression through modulation of DNA methylation.

Methylation of mammalian DNA is primarily catalyzed by three active DNA methyltransferases (DNMTs). Several studies have highlighted the indispensability of both the “maintenance” methyltransferase, DNMT-1, and the *de novo* methyltransferases, DNMT-3a and DNMT-3b, during embryogenesis. Targeted disruption of the genes encoding *each* of these enzymes led to embryonic lethality and dysmorphologies [19–23]. Specific developmental roles for these enzymes are suggested by the association of inadequate DNMT-1-mediated maintenance of DNA methylation and abnormal embryogenesis [24], as well as by evidence that Dnmt-3a functions as a molecular switch mediating the neural tube-to-neural crest fate transition [25]. In addition, a mutated *DNMT3B* gene is associated with the occurrence of immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome in humans [26, 27]. Interestingly, 5-azacytidine, a powerful inhibitor of DNA methylation, is capable of inducing cleft palate in developing fetuses following *in utero* exposure [28].

Based on their ability to bind methylated DNA, the methyl-CpG-binding domain (MBD) proteins operate as “interpreters” of the DNA methylation mark and thus, as crucial modulators of diverse epigenetic processes [29]. MBD-1, MBD-2, MBD-4, and MeCP-2, have been identified as transcriptional repressors that recruit Dnmts, histone deacetylases and chromatin remodelers to methylated DNA [30]. In this regard, methylation-dependent genomic targeting by MBD has been shown to regulate expression of 5-hydroxymethylcytosine marked genes [31, 32]. While the lethality of *Mbd3*-null embryos suggests a critical role for this gene during embryogenesis [33, 34], the precise mechanisms by which MBD proteins govern normal embryonic development remain to be defined [35, 36].

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Maternal exposure to heavy metal teratogens, such as, arsenic and cadmium, has also been documented to trigger developmental dysmorphogenesis via alteration of global DNA methylation and modulation of expression of DNMTs and MBDs. Significant downregulation of *Dnmt3a* and *Dnmt3b*, significant decline in global DNA methylation and developmental dysmorphogenesis (e.g. ventral body wall defects) were reported in embryos exposed *in utero* to cadmium [37]. Another study documented arsenic-induced altered expression of DNMTs and MBDs, a significant decrease in S-adenosylmethionine (SAM) (the source of the "methyl" group for DNA methylation), and global DNA hypomethylation - resulting in neural tube defects in exposed embryos [38].

Prenatal cigarette smoke exposure is thought to target cells of the developing midfacial complex to cause orofacial anomalies (e.g. CL/P). Thus, primary cultures of 1-BA (first branchial arch) cells were utilized to determine the effects of maternal cigarette smoke exposure on the expression of several DNMTs and MBDs. We show that exposure to cigarette smoke extract (CSE) led to global DNA hypomethylation, and reveal for the first time, significant proteasomal degradation of two DNA methyltransferases (DNMT-1 and DNMT-3a), as well as two methyl CpG-binding proteins, MeCP-2 and MBD-3, in 1-BA cells. Moreover, pretreatment of these cells with the proteasomal inhibitor, MG-132 completely reversed the CSE-induced degradation of Dnmts and Mbds.

2. MATERIALS AND METHODS

2.1 Establishment of primary cultures of murine first branchial arch-derived (1-BA) cells

ICR mice (Harlan Laboratories, Indianapolis, IN) were housed under climate-controlled conditions at a temperature of 22°C with an alternating 12-h dark–light cycle and were provided access to food and water *ad libitum*. Mature male and female mice were mated overnight and the presence of a vaginal plug the following morning (day 0 of gestation) was considered as evidence of mating. Pregnant mice were euthanized on day 10.5 of gestation, and embryos removed from pregnant dams. First branchial arches were microdissected (Figure 1) from the embryos in sterile, cold phosphate-buffered saline (PBS), minced and dissociated with 0.05% trypsin/0.1% EDTA in PBS for 10 min at 37°C with constant shaking. Trypsin was inhibited by the addition of Opti-MEM medium (Life Technologies, Grand Island, NY, USA) containing 5% fetal bovine serum (FBS) (Sigma; St. Louis, MO, USA). Cells were seeded in 100 mm tissue culture dishes at a density of $2X10^5$ cells/dish in Opti-MEM containing Earle's salts, 25 mM HEPES buffer and supplemented with 2 mM glutamine, 5% FBS, 150 μg/ml streptomycin, and 100 U/ml penicillin (complete medium) (Life Technologies) and maintained at 37°C in an atmosphere of 95% air/5% CO2, with medium replaced every 48 h. Each experiment was performed using cells derived from first branchial arch tissues dissected from a single litter of embryos.

2.2 Cigarette Smoke Extract

Cigarette smoke extract (CSE), purchased from Murty Pharmaceuticals (Lexington, KY), was prepared by burning 1R3E standard cigarettes and extracting the condensate into dimethyl sulfoxide (DMSO) to generate a 40 mg/ml stock solution.

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2.3 Global DNA Methylation Assay

First branchial arch-derived (1-BA) cells were re-seeded into 60 mm tissue culture dishes (Nalge Nunc International; Rochester, NY) at an initial density of 1.5 x 10^4 cells/dish. Cells were grown to confluence in the growth medium (as described in section 2.1), washed with phosphate-buffered saline (PBS) and treated with either vehicle (PBS) or cigarette smoke extract (CSE) at a final concentration of 80 μg/mL for 24 h. 1-BA cell cultures, similarly grown, were treated with 10 μM 5-Azacytidine (Sigma; St. Louis, MO) (a DNA methylation inhibitor used as a positive control) for 24 hrs. Cells (both treated and control) were incubated for 24 hr at 37°C followed by genomic DNA extraction and assessment of Global DNA methylation as previously described [39].

2.4 TaqMan® Quantitative Real-Time PCR (qRT-PCR)

Total RNAs from control or CSE-treated 1-BA cells were extracted using the RNeasy Protect Mini Kit (Qiagen) following the manufacturer's recommendations. The quality and quantity of extracted total RNAs were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) and spectrophotometric UV absorbance at 260/280 nm. cDNA synthesis, TaqMan qRT-PCR analysis and processing of raw data were accomplished as previously described [39].

2.5 Preparation of Nuclear Extracts and Western Blotting

First branchial arch-derived (1-BA) cells were exposed to vehicle (PBS) or cigarette smoke extract (CSE) and maintained for 24 hr as described under "Global DNA Methylation Assay". Nuclear-enriched protein extracts were prepared from CSE- or vehicle (PBS)-treated 1-BA cells using the NE-PER kit (Pierce; Rockford, IL) following the manufacturer's recommendations. Steady state levels of various DNMT and MBD proteins were determined by Western (immuno) blotting using commercially available primary and secondary antibodies as previously described [39].

2.6 Proteasome Inhibitor

First branchial arch-derived (1-BA) cells, were grown to confluence in 60 mm tissue culture dishes (Nalge Nunc International), and pre-treated with either vehicle (DMSO) or 0.5-, 1.0- or 1.5-μM of the proteasome inhibitor MG-132 (Sigma) for 3 hr. The inhibitor was then removed, and fresh medium containing DMSO (vehicle), or CSE (various concentrations) was added, and the cells maintained for an additional 24 hr as described under "Global DNA Methylation Assay". Control experiments, in which cells were treated with only 1.5-μM MG-132 (for 3 hr) were also performed. Subsequently, nuclear protein extracts were prepared and examined by western analysis as detailed in the previous paragraph.

2.7 DNMT-1 ELISA

Nuclear extracts from 24-hr vehicle-, or CSE (alone or after 3-hr MG-132 pre-treatment) - treated 1-BA cells, were assayed for DNMT-1 protein using the Epiquik DNMT-1 assay kit (Epigentek) as previously described [39].

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2.8 Densitometric Analysis of Western Blots

Densitometric analyses of DNMT-1, -3a, -3b, MeCP-2, MBD-2, MBD-3 and β-actin protein bands were accomplished using Image J (version 1.38) software as previously described [39].

2.9 Statistical Analyses

Statistical analyses were performed using R version 3.1.2 (http://www.R-project.org). Effects of CSE on (1) percent global DNA methylation, (2) normalized protein band intensities (relative to steady state levels of proteins), and (3) normalized Ct values (ΔC_t) from qRT-PCR for gene expression were all analyzed using one-way ANOVA models. For the global methylation experiments, control samples were compared to samples treated with 20-, 40- and 80 μg/mL CSE and 10 μM Azacytidine. For experiments #2 (protein band intensity) and #3 (C_t values) control samples were compared to samples with increasing CSE exposure (20 μg/mL, 40 μg/mL, and 80 μg/mL CSE). Means and standard deviations from three independent experiments, followed by differences or fold-changes relative to control samples were presented with simultaneous 95% confidence limits. P-values of <0.05 were considered statistically significant.

3. RESULTS

3.1 Effect of Cigarette Smoke Extract on 1-BA Cell Global DNA Methylation

In a separate study (unpublished observation), we have assessed the effect of increasing doses of CSE (20-, 40-, 80- and 100 μg/mL) on the survival and proliferation of 1-BA cells in primary culture. Results from those experiments revealed that 24-hr exposure to CSE at doses higher than 80 μg/mL significantly reduces the survival as well as proliferation of 1-BA cells. Based on these findings we first tested the impact of the highest, tolerable dose (80 μg/mL) of CSE, on global DNA methylation as well as on the expression of various DNMTs and Methyl CpG-binding proteins, within 1-BA cells. Results from these experiments demonstrated significant reduction in global DNA methylation as well as significant downregulation of genes encoding various Dnmts and methyl CpG-binding proteins (see below). Finally, we repeated the aforementioned experiments assessing the effect of 20- (low dose), 40- (intermediate dose), and 80- (high dose) μg/mL of CSE on 1-BA cells (i.e. performed a dose response).

Cigarette Smoke Extract (80 μg/mL) significantly (>13%) diminished global DNA methylation in 1-BA cells (Table 1). While treatment with 20- or 40 μg/mL CSE had no appreciable impact on global DNA methylation (Table 1), exposure to 5-azacytidine – a powerful inhibitor of DNA methylation – effected a >20% reduction in global DNA methylation in 1-BA cells (Table 1).

3.2 Effect of Cigarette Smoke Extract on Expression of DNA Methyl Transferases and Methyl CpG/CpG Domain Binding Proteins in Nuclear Extracts of 1-BA Cells

Expression of DNMT-1, -3a, -3b, MeCP-2, MBD-2 and MBD-3 proteins were assessed by immunoblotting of vehicle- or CSE-exposed 1-BA cell nuclear extracts. Two major bands in the molecular weight range of 200–190 kDa and 140–120 kDa were detected for DNMT-1

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and DNMT-3a, respectively, while a single major ~110 kDa band was detected for DNMT-3b, on immunoblots of nuclear extracts of 1-BA cells treated for 24h with either vehicle or 20- or 40 μg/mL CSE (Figure 2a,b,c; upper panels). Under similar conditions, a single major 75 kDa band for MECP-2, a 49 kDa band for MBD-2, and two 34–30 kDa bands for MBD-3 were detected (Figure 2d,e,f; upper panels). Exposure to 80 μg/mL CSE for 24 hr resulted in a marked reduction in steady state levels of DNMT-1, DNMT-3A, and MeCP-2 proteins (Figure 2a,b,d, upper panels; and Table 2). The 20- or 40 μg/mL CSE dose also caused reduction in steady state levels of these three proteins (Figure 2a,b,d, upper panels; and Table 2). This dose-dependent reduction was verified via densitometric analysis of film-detected chemiluminescent signals (Table 2). While steady state levels of the 34 kDa MBD-3 protein were markedly reduced following exposure to 80 μg/mL CSE (Figure. 2f, upper panel; and Table 2), steady state levels of the 30 kDa MBD-3 protein increased in a dose-related manner after 24 hr treatment with CSE (Figure. 2f, upper panel; and Table 2). Notably, the CSE-induced effects were protein-specific in that steady state levels of DNMT-3B and MBD-2 proteins were unaffected at all exposure doses of CSE (Figure 2c,e, upper panels;). The presence of multiple bands in the blots corresponding to some of the DNMTs and MBDs (Fig 2, upper panels;) is most likely due to the presence of multiple isoforms and/or post-transcriptionally modified forms of the proteins, as reported earlier [36]. In order to ensure equal loading of proteins, and for sample normalization during densitometric analysis, all blots were reprobed with an antibody recognizing the housekeeping protein β-actin (Figure 2a,b,c,d,e,f; lower panels).

3.3 Cigarette Smoke Extract Differentially Altered Expression of Genes Encoding DNMT and Methyl CpG/CpG Domain Binding Proteins in 1-BA Cells

Effect of 24 hr, 20-, 40- or 80 μg/mL CSE exposure on the expression of genes encoding the three DNMTs and MBDs were assessed by TaqMan qRT-PCR and cycle threshold (Ct) values for each gene were compared [40]. Results from the qRT-PCR analyses revealed significant downregulation of mRNA expression of all three DNMTs and MBDs in 1-BA cells exposed to 40- or 80 μg/mL CSE, whereas 20 μg/mL CSE (or the vehicle) did not alter the expression of these genes (Table 3). Expression of *Dnmt-1*, *Dnmt-3a*, *Dnmt-3b*, *Mbd-2*, *MeCP-2* and *Mbd-3* was significantly decreased 1.9- and 2.2-fold, 1.7- and 1.85-fold, 1.6- and 1.8-fold, 1.45- and 1.40-fold, 2.06- and 2.2-fold and 1.4- and 1.5-fold, following exposure to 40- or 80 μg/mL CSE, respectively (Table 3). Expression of the housekeeping gene encoding 18S rRNA (as well as those encoding Hsp-90 and Hprt; data not shown) remained unaltered under all treatment conditions. All these experiments were repeated three times using unique RNA extracts prepared from cultures of CSE- or vehicle-treated 1-BA cells.

3.4 Cigarette Smoke Extract Diminishes Cellular Levels of DNA methyltransferases and Methyl CpG/CpG Domain Binding Proteins in 1-BA Cells via the Proteasome Pathway

Reduction in protein levels after exposure to CSE (observed in case of DNMT-1, DNMT-3a, MeCP-2 and MBD-3 proteins; Figure 3a,b,c,d) as well as appearance of lower molecular weight bands (seen in immunoblots of DNMT-1, DNMT-3a, MeCP-2 and MBD-3; Figure 3a,b,c,d) could be the result of CSE-induced proteomic degradation of these proteins. This notion is reinforced by the reversal of CSE-induced reduction in protein levels for all four

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proteins (Figure 3a,b,c,d; upper panels), and by the reduction (Fig 3b; upper panel) or elimination (Fig. 3a and 3c; upper panels) of the lower molecular weight bands in immunoblots of DNMT1, DNMT-3a and MeCP-2 proteins respectively, following pretreatment with the proteasome inhibitor, MG-132 (1.5 μM). Twenty four hour exposure of 1-BA cells to only MG-132 (1.5 μM) did not result in any degradation of the aforementioned proteins (Figure 3a,b,c,d; upper panels). A dose-dependent (0.5-, 1.0- and 1.5 μM) effect of MG-132 in rescuing the proteasomal degradation of the two DNMTs and MBDs was also observed. A representative Western blot, documenting the dose-dependent rescue of the MeCP2 protein is shown in Figure 4a. Equal abundance of β-actin in various lanes was also confirmed (Figure 4b).

3.5 Determination of DNMT-1 Protein Levels in 1-BA Cells

Treatment of 1-BA cells with 80 μg/mL CSE for 24 hr revealed a significant reduction in DNMT-1 protein level (*P< 0.05, one-way analysis of variance [ANOVA]). Pre-treatment of 1-BA cells with 1.5 μM of the proteasomal inhibitor MG-132 resulted in total prevention of CSE-induced DNMT-1 protein degradation (*P< 0.05, one-way ANOVA) (Figure 5). This verified the involvement of the proteasome pathway in CSE-induced degradation of DNMT and MBD proteins.

4. DISCUSSION

Orofacial clefting (CL/P, or CPO), including clefts associated with exposure to maternal cigarette smoke, is a prevalent category of human congenital anomalies with significant adverse medical, financial and social consequences to those affected. In depth knowledge of the cellular and molecular mechanisms governing morphogenesis of the midfacial region is essential for any consideration given to therapeutic intervention of this birth defect. An array of conditions such as maternal exposure to cigarette smoke, alcohol, heavy metals (e.g. arsenic and cadmium), various drugs, microbial infection, and deficiency of essential nutrients (e.g., trace elements and folate) have been implicated in the etiology of CL/P, or CPO [6, 9, 41–44]. Although, a number of studies have documented increased occurrence of CL/P with maternal active and passive cigarette smoking [6, 9, 12, 13], the precise molecular underpinnings directing *in utero* tobacco smoke exposure-induced orofacial clefting are still obscure. One potential mechanism that could account for the pathogenesis of such orofacial defects is altered expression of crucial genes due to aberrant methylation of their regulatory regions. Support for this notion comes from recent studies demonstrating modulation of global and gene-specific DNA methylation in offspring subsequent to maternal exposure to cigarette smoke [15, 17]. In a recent study, infants exposed *in utero* to cigarette smoke demonstrated elevated methylation at a differentially methylated region (DMR) regulating Insulin-like Growth Factor 2 (*IGF2*) [17]. Inasmuch as *IGF2* represents a gene essential for normal embryonic growth and development [45, 46], this suggests that the plasticity of this DMR may represent a crucial mechanism underlying *in utero* adjustments to environmental toxicants/teratogens, such as cigarette smoke [17].

Global hypomethylation was also reported in cord blood DNA of newborns of mothers who smoked [16]. This observation is consistent with data from the present study which demonstrated a significant global DNA hypomethylation in 1-BA cells following exposure

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to CSE. Underscoring the possibility that exposure to cigarette smoke contributes to adverse developmental outcomes via altered methylation, is the demonstration that *in utero* tobacco smoke exposure resulted in altered placental methylation of a number of loci within the RUNX3 gene [47], known to be critical for embryonic growth and development [48, 49]. Association of maternal smoking with anomalous placental epigenome-wide DNA methylation [50] and altered placental expression of *CYP1A1* due to hypomethylation of CpG sites proximal to its regulatory region [51] have been reported. One role for *CYP1A1*, which encodes a member of the cytochrome P450 superfamily of enzymes, is metabolism of polycyclic aromatic hydrocarbons found in cigarette smoke [52]. Moreover, fetuses exhibiting polymorphic variants of NAT1, an enzyme involved in phase II detoxification of components of cigarette smoke, possess an increased risk for orofacial clefts [53]. Collectively, these studies allow the suggestion that *in utero* cigarette smoke exposure can epigenetically modulate gene expression in a manner that adversely affects the developing fetus.

In the present study, exposure to cigarette smoke extract (CSE) significantly altered global DNA methylation, and diminished both mRNA and protein levels of DNMT-1 and DNMT-3A. DNMT-3B was reduced only at the mRNA level with no significant change in protein levels. These findings are consistent with previous studies demonstrating CSE-induced genomic hypomethylation and diminished DNMT-1 expression in small airway- and bronchial epithelial cells [54]. Cigarette smoke extract has also been shown to trigger a significant decrease in DNMT-1 mRNA and protein levels in frontal cortex and GABAergic neurons of mice injected with nicotine and to downregulate Dnmt3b mRNA in lung cancer cells [55, 56]. Benzo[a]pyrene (BaP), another major toxic constituent of cigarette smoke, downregulated both DNMT-3a and DNMT-3b in mouse embryonic fibroblast cells, and led to a significant decrease in global and gene specific DNA methylation during embryogenesis [57, 58].

We also demonstrate that cigarette smoke extract (CSE) significantly diminished both mRNA and protein levels of the two methyl CpG-binding proteins, MeCP-2, and MBD-3, in 1-BA cells. As with DNMT-3b, CSE caused a decrease in MBD-2 mRNA levels without any measurable change in protein levels. Currently, almost nothing is known regarding the effect(s) of cigarette smoke exposure, on the expression of the methyl CpG-binding proteins - key regulators of the epigenome as well as the transcriptome. Our results represent the first evidence of the impact(s) of CSE exposure on the expression of MeCP-2, MBD-2 and MBD-3 within an embryonic cell type. Collectively, these data support the premise that CSE, via modulation of expression of the DNMTs and the MBDs, can modulate cellular DNA methylation and gene expression. The data provide support for the possibility that the teratogenic effect(s) of components of cigarette smoke may be epigenetically mediated.

We have further shown that CSE-induced diminution of DNMT-1, DNMT-3a, MeCP-2 and Mbd-3 was due to extensive proteasome-mediated nuclear degradation. Pre-treatment of 1-BA cells with the proteasome inhibitor MG-132 inhibited CSE-induced degradation of each of the DNMT and MBD proteins. This supports our hypothesis that *CSE-induced alteration of the DNA methylation machinery (DNA methyltransferases and methyl CpG binding proteins) in cells of the developing first branchial arch is mediated in part by the ubiquitin/*

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proteasomal degradation pathway and in part by the alteration in the transcription/ translation machinery. Further support for this hypothesis comes from numerous additional studies demonstrating proteasome-mediated protein degradation by components of cigarette smoke**,** and resultant adverse effects on crucial cellular processes [18, 59–61]. Our data support the conclusion that proteasomal degradation of crucial effectors/modulators of DNA methylation (such as DNMT-1, -3a, MeCP-2, and MBD-3) represents a potential epigenetic mechanism by which exposure to cigarette smoke may result in an orofacial cleft.

Taken in isolation, results of the current study indeed do not demonstrate that 1-BA cells are any more, or less, sensitive than cells from other regions of the embryo that apparently develop normally. However, these studies are based on data from our laboratory that 1) *In utero* CSE exposure in mice results in an increased frequency of orofacial clefting and dysmorphology of tissues derived from 1-BA cells used in the present study, 2) Whole genome sequencing of CSE-exposed 1-BA tissue demonstrates detectable alterations in DNA methylation, and 3) Prenatal exposure to CSE resulted in hypomethylation of the *Peg3* gene in cells of the 1-BA in mice. These data, which support rationale for our design and conclusions, are unpublished observations from our laboratory and are being incorporated into manuscripts in preparation.

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Hightlights

- We demonstrated that cigarette smoke extract (CSE) reduces global DNA methylation in primary cultures of first branchial arch-derived cells (1-BA cells), which contribute to the formation of the lip and palate.
- Documented significant CSE-induced alteration of genes encoding two DNMTs and two Methyl CpG-binding proteins (Mbds) in 1-BA cells.
- CSE induced *degradation* of the DNMTs and the MBD proteins in 1-BA cells by the proteasomal pathway.
- Proteasomal degradation could be completely rescued by pre-treatment of 1-BA cells with the proteasomal inhibitor, MG-132.
- We reported a potential epigenetic molecular mechanism underlying maternal cigarette smoke exposure-induced orofacial clefting.

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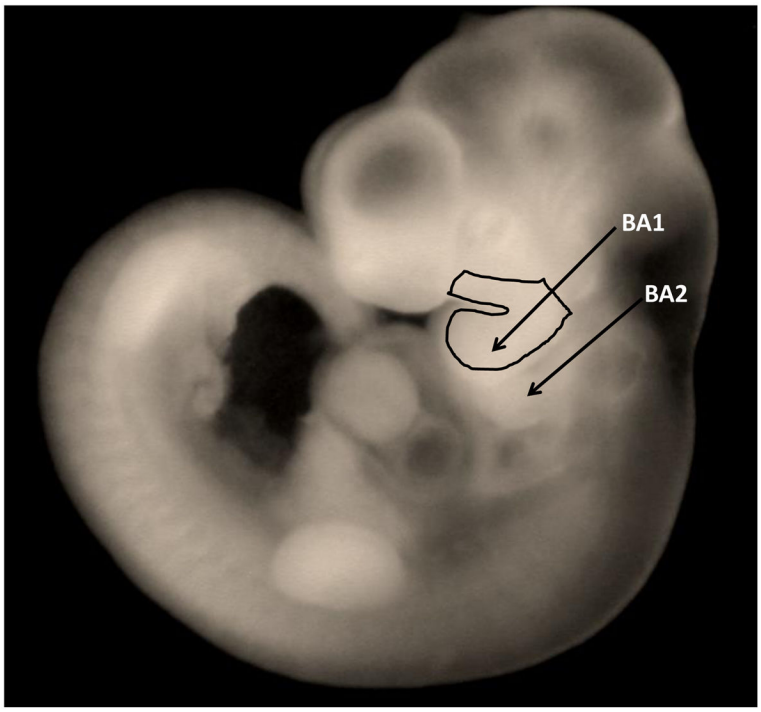

Figure 1.

Photomicrograph of a representative GD 10.5 embryo. The region demarcated by the black line was microdissected and utilized to establish primary cultures of first branchial arch-derived (1-BA) cells. BA1 = First branchial arch; BA2 = Second branchial arch.

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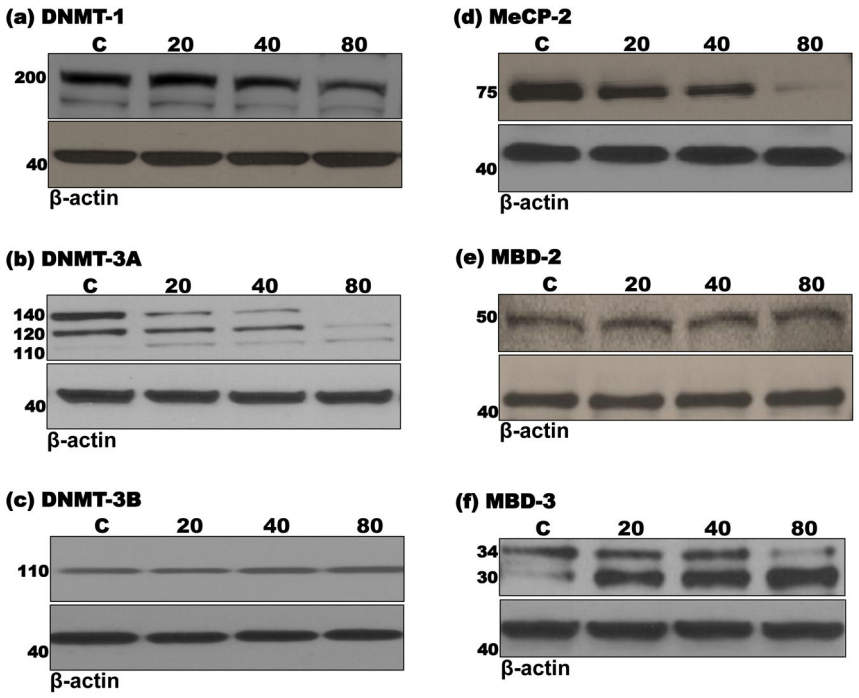

Figure 2.

Immunoblots (upper panels) demonstrating steady-state levels of DNMT-1 (a), DNMT-3a (b), DNMT-3b (c), MeCP-2 (d), MBD-2 (e) and MBD-3 (f) proteins in nuclear extracts derived from murine first branchial arch-derived (1-BA) cells following treatment (24 hr) with either 20-, 40- or 80 μg/mL Cigarette smoke extract (CSE) or vehicle control (DMSO). Equal amounts of protein (20 μg) were resolved by SDS-PAGE on 12% polyacrylamide bis-tris gels, transferred to PVDF membranes, probed with specific antibodies and immunoreactive species detected by chemiluminescence, as detailed in the Materials and Methods section. Molecular weights are indicated to the left of each panel. The lower panels (a,b,c,d,e,f) depict immunoblots of the normalization loading control, β-actin. Each immunoblot is representative of no less than three independent blots from three unique sets of extracts from control and CSE treated 1-BA cells.

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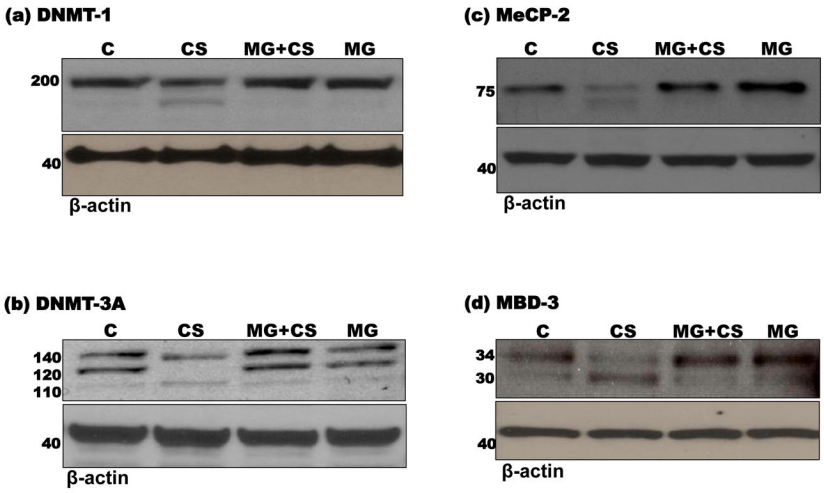

Figure 3.

Immunoblots (upper panels) demonstrating steady-state levels of DNMT-1 (a), DNMT-3a (b), MeCP-2 (c), and MBD-3 (d), proteins in nuclear extracts derived from murine first branchial arch-derived (1-BA) cells. These cells were pre-treated (3 hr) with either DMSO (vehicle/control) or 1.5 μM of the proteasomal inhibitor, MG-132, followed by a 24-hr treatment with either 80 μg/mL CSE or vehicle control (DMSO), as detailed in the Materials and Methods section. Equal amounts of protein (20 μg) were resolved by SDS-PAGE on 12% polyacrylamide bis-tris gels, transferred to PVDF membranes, probed with specific antibodies and immunoreactive species detected by chemiluminescence as detailed in the Materials and Methods section. Molecular weights are indicated to the left of each panel. The lower panels (a,b,c,d) depict immunoblots of the normalization control, β-actin. Each immunoblot is representative of no less than three independent blots from three unique sets of extracts from control, inhibitor and CSE treated 1-BA cells. **C**: pre-treatment with DMSO + treatment with DMSO; **CS**: pre-treatment with DMSO + treatment with 80 μg/mL CSE; **MG+CS**: pre-treatment with 1.5 μM MG-132 + treatment with 80 μg/mL CSE; **MG**: pre-treatment with 1.5 μM MG-132 + treatment with DMSO.

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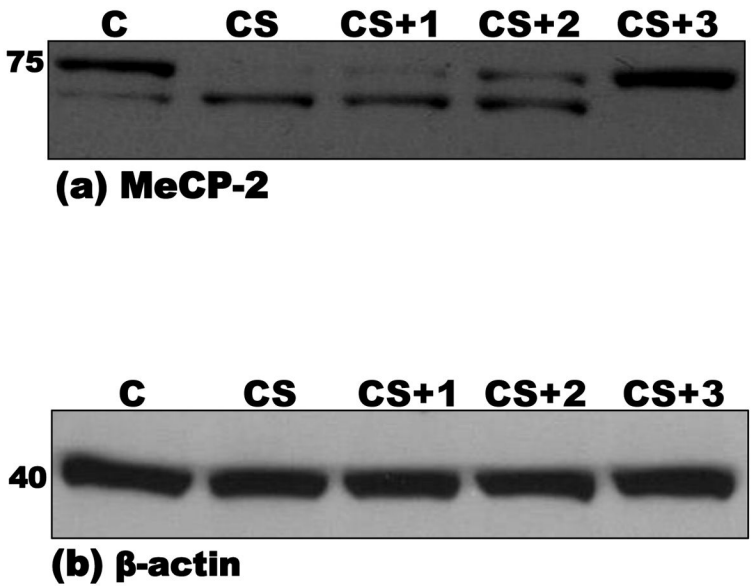

Figure 4.

Immunoblots demonstrating steady-state levels of MeCP-2 (a), protein in nuclear extracts derived from murine first branchial arch-derived (1-BA) cells. These cells were pre-treated (3 hr) with either DMSO (vehicle/control) or 1-, 3- or 5 μM of the proteasomal inhibitor, MG-132, followed by a 24-hr treatment with either 80 μg/mL CSE or vehicle control (DMSO), as detailed in the Materials and Methods section. Equal amounts of protein (20 μg) were resolved by SDS-PAGE on 12% polyacrylamide bis–tris gels, transferred to PVDF membranes, probed with specific antibodies and immunoreactive species detected by chemiluminescence as detailed in the Materials and Methods section. Molecular weights are indicated to the left of each panel. The lower panel (b) depicts one representative immunoblot of the normalization control, β-actin. Each immunoblot is representative of no less than three independent blots from three unique sets of extracts from control, inhibitor and CSE treated 1-BA cells. **C**: pre-treatment with DMSO + treatment with DMSO; **CS**: pre-treatment with DMSO + treatment with 80 μg/mL CSE; **CS+1**: pre-treatment with 0.5 μM MG-132 + treatment with 80 μg/mL CSE; **CS+2**: pre-treatment with 1.0 μM MG-132 + treatment with 80 μg/mL CSE; **CS+3**: pre-treatment with 1.5 μM MG-132 + treatment with 80 μg/mL CSE.

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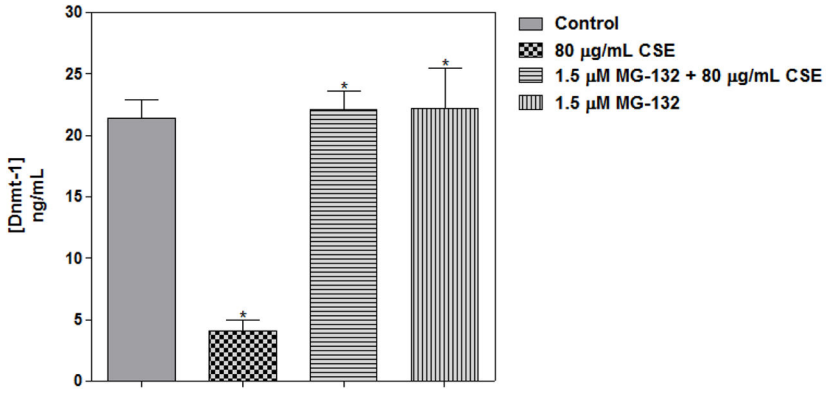

Figure 5.

Histogram depicting DNMT-1 protein levels in murine first branchial arch-derived (1-BA) cells. Nuclear extracts from 24-hr, vehicle-, or 80 µg/mL CSE (alone or after 3-hr MG-132 pre-treatment)-treated 1-BA cells, were assayed for DNMT-1 protein using the Epiquik DNMT-1 ELISA assay kit (Epigentek). A standard curve was generated utilizing protein standards of known concentrations (2, 5, 10, and 20 ng). The amount of DNMT-1 protein was estimated as: DNMT protein (ng/ml) = (Sample OD – blank OD/standard slope) x sample dilution.

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Table 1

Effect of CSE on Global DNA Methylation in First Branchial Arch-derived (1-BA) Cells.

Sample Type[1]	% Global DNA Methylation[2,3]	%Change (vs. Control)
Control	3.33 ± 0.14	-
20 μg/mL CSE	3.35 ± 0.05	-
40 μg/mL CSE	3.27 ± 0.61	1.8% decrease
80 μg/mL CSE	2.90 ± 0.11 **	13.0% decrease
10 μM Azacytidine	2.65 ± 0.08 ***	20.4% decrease

[1] 1-BA cells were treated with vehicle (control) or CSE (80 μg/mL), or 10 μM azacytidine for 24 hrs as described in the “Materials and Methods”.

[2] Global DNA methylation was quantified using the Methylamp Global DNA Methylation Quantification Ultra Kit as described in the “Materials and Methods”.

[3] Data represent mean ± standard deviation, obtained from three independent experiments.

** $P < 0.01$ vs. control.

*** $P < 0.001$ vs. control.

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Table 2

Densitometric Analysis of DNMT and Methyl CpG/CpG Domain Binding Protein Immunoblots of First Branchial Arch-derived (1-BA) Cell Nuclear Extracts.

Protein[1]	Treatment[2]	Fold increase or decrease in protein level (vs. Vehicle)[2]
DNMT-1	Vehicle (Control)	Ref
	20 μg/mL CSE	+1.03 ± 0.06
	40 μg/mL CSE	−1.35 ± 0.06 *
	80 μg/mL CSE	−1.88 ± 0.20 **
DNMT-3a	Vehicle (Control)	Ref
	20 μg/mL CSE	−3.76 ± 0.42 *
	40 μg/mL CSE	−5.64 ± 1.42 **
	80 μg/mL CSE	−32.04 ± 9.51 **
DNMT-3b	Vehicle (Control)	Ref
	20 μg/mL CSE	+1.09 ± 0.12
	40 μg/mL CSE	+1.17 ± 0.08
	80 μg/mL CSE	+1.12 ± 0.08
MeCP-2	Vehicle (Control)	Ref
	20 μg/mL CSE	−1.38 ± 0.09
	40 μg/mL CSE	−1.63 ± 0.06
	80 μg/mL CSE	−9.22 ± 2.65 **
MBD-2	Vehicle (Control)	Ref
	20 μg/mL CSE	+1.10 ± 0.13
	40 μg/mL CSE	+1.13 ± 0.06
	80 μg/mL CSE	+1.10 ± 0.05
MBD-3	Vehicle (Control)	Ref
	20 μg/mL CSE	+1.09 ± 0.05
	40 μg/mL CSE	+1.15 ± 0.07
	80 μg/mL CSE	+5.63 ± 1.29 **

[1] Steady state levels of DNMT-1, DNMT-3a, DNMT-3b, MeCP-2, MBD-2 and MBD-3 proteins (the uppermost band) were determined by immunoblotting (Fig. 2) of nuclear extracts of 1-BA cells treated (24 hr) with either vehicle (DMSO), 20-, 40- or 80 μg/mL Cigarette Smoke Extract (CSE).

[2] The relative levels of DNMT-1, DNMT-3a, DNMT-3b, MeCP-2, MBD-2 and MBD-3 on immunoblots (Fig. 2) were analyzed by densitometry using the ImageJ (version 1.38) software, as described in the “Materials and Methods”. Densitometric analysis of protein steady state levels was conducted on no less than three independent blots of nuclear extracts of 1-BA cells treated with either vehicle, 20-, 40- or 80 μg/mL CSE. β-actin was used as an internal control for sample normalization. The densitometric data for each protein band was normalized to that of β-actin in that lane. Fold change was determined as follows: intensity of the DNMT-1 band (from 20-, 40- or 80 μg/mL CSE sample) / intensity of the DNMT-1 band (from Vehicle-treated sample). The data are presented as the mean±standard deviation from three independent experiments. (+) indicates higher protein levels in CSE treated samples in comparison to vehicle-treated samples, whereas (−) indicates lower protein levels in CSE treated samples in comparison to vehicle-treated samples.

* $P < 0.05$ vs. control.

** $P < 0.01$ vs. control.

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Table 3

Effect of Cigarette Smoke Extract (CSE) on the expression of genes encoding various DNMT and Methyl CpG/CpG domain binding proteins in First Branchial Arch-derived (1-BA) cells.

Gene	Treatment[a]	ΔCt[b,c]	Fold change[d] ($2^{-\Delta\Delta Ct}$) (95% confidence limits)
DNMT-1	Control	8.13 ± 0.05	
	20 μg/mL CSE	8.17 ± 0.12	−1.03 (−1.15,1.09)
	40 μg/mL CSE	9.05 ± 0.14	−1.9 (−2.13,−1.7)***
	80 μg/mL CSE	9.27 ± 0.21	−2.21 (−2.47,−1.97)***
DNMT-3a	Control	8.82 ± 0.12	
	20 μg/mL CSE	8.89 ± 0.11	−1.05 (−1.15,1.04)
	40 μg/mL CSE	9.59 ± 0.11	−1.71 (−1.87,−1.56)***
	80 μg/mL CSE	9.71 ± 0.12	−1.85 (−2.03,−1.69)***
DNMT-3b	Control	9.53 ± 0.12	
	20 μg/mL CSE	9.62 ± 0.06	−1.06 (−1.18,1.05)
	40 μg/mL CSE	10.21 ± 0.13	−1.6 (−1.77,−1.44)***
	80 μg/mL CSE	10.38 ± 0.19	−1.8 (−2,−1.62)***
MeCP-2	Control	11.34 ± 0.1	
	20 μg/mL CSE	11.48 ± 0.06	−1.1 (−1.21,−1)
	40 μg/mL CSE	12.38 ± 0.1	−2.06 (−2.26,−1.88)***
	80 μg/mL CSE	12.62 ± 0.18	−2.43 (−2.67,−2.21)***
MBD-2	Control	7.62 ± 0.07	
	20 μg/mL CSE	7.72 ± 0.06	−1.07 (−1.18,1.03)
	40 μg/mL CSE	8.16 ± 0.11	−1.45 (−1.61,−1.31)***
	80 μg/mL CSE	8.21 ± 0.21	−1.5 (−1.66,−1.36)***
MBD-3	Control	7.74 ± 0.09	
	20 μg/mL CSE	7.85 ± 0.1	−1.08 (−1.21,1.04)
	40 μg/mL CSE	8.22 ± 0.12	−1.4 (−1.57,−1.25)***
	80 μg/mL CSE	8.34 ± 0.23	−1.52 (−1.7,−1.36)***

[a] cDNA samples were prepared from Control, 20-, 40- or 80 μg/mL CSE treated 1-BA cells and subjected to TaqMan® quantitative real-time PCR (QRT-PCR) for each target gene. Analyses were performed in triplicate using data from three independent experiments.

[b] C_t values represent the number of cycles during the exponential phase of amplification necessary to reach a predetermined threshold level of PCR product as measured by fluorescence. The more template present at the start of a reaction, the fewer the cycles required to synthesize enough fluorescent product to be recorded as statistically above background. All data were normalized to the amplification signal from the housekeeping gene, 18S rRNA. The ΔCt values represent these normalized signals, $\Delta C_t = C_{t\ \text{sample}} - C_{t\ 18S\ rRNA}$. Data presented represent mean $\Delta C_t \pm$ standard deviation for three replicates.

[c] Negative methodological control reactions, which lacked reverse transcriptase, did not amplify any detectable product.

[d] Fold-change (FC) values were determined according to the relationship: $FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t$ is the difference in ΔC_t values between CSE treated and control samples [37]. Statistical analysis comparing the control with the three CSE treatment groups was done using one-way ANOVA of the ΔC_t values. 95% confidence intervals for the FC were calculating by taking the appropriate transformation of the 95% confidence limits for

the estimated difference in ΔC_t values. A negative fold-change value indicates down regulation of gene expression relative to control samples and a positive fold-change value indicates up regulation.

*** $P < 0.001$ vs. control.

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