Overexpression of MECP2 attenuates cigarette smoke extracts induced lung epithelial cell injury by promoting CYP1B1 methylation

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ABSTRACT — MECP2 (Methyl-CpG-binding protein 2) has been shown to have a critical role in regulating DNA methylation against smoke exposed lung injury. However, the biological function of MECP2 and the underlying molecular mechanism remains elusive. Human bronchial epithelial (16HBE) and alveolar type II epithelial cells (AECII) were exposed to increasing concentrations of cigarette smoke extracts (CSE) solution to establish CSE-induced lung epithelial cell injury models. Our findings revealed that MECP2 was down-regulated, while CYP1B1 was up-regulated in CSE-induced lung epithelial cell injury models by quantitative real time PCR, western blotting and immunofluorescence staining. Down-regulated CYP1B1 was ascribed to the demethylation of its promoter by methylation-specific PCR (MSP). The in vitro experiments further showed that MECP2 overexpression significantly attenuated CSE-triggered cell growth attenuation, cell cycle arrest, apoptosis and ROS generation in lung epithelial cells by CCK-8 and flow cytometry assays. In molecular level, we further demonstrated that MECP2 overexpression obviously suppressed the expression of CYP1B1 through elevating its methylation status. Therefore, our data suggest that MECP2 protects against CSE-induced lung epithelial cell injury possibly through down-regulating CYP1B1 expression via elevating its methylation status.

Key words: Cigarette smoke extracts, Lung injury, MECP2, CYP1B1, DNA Methylation

INTRODUCTION

Cigarette smoke extracts (CSE) contain high concentrations of toxins that are reported to be the primary risk factor for the progression of lung diseases including asthma, bronchitis, emphysema, chronic obstructive pulmonary disease (COPD) and even malignancy (Husari et al., 2016; Clark and Molloy, 2017; Mariani, 2016). Recently, evidence is accumulating that CSE could generate chemotactic molecules into the lung by activating a majority of lung epithelial cells and alveolar macrophages, which in turn induce oxidative stress and apoptosis in lung (Sundar et al., 2013; Somborac-Bacura et al., 2013; Jorgensen et al., 2008). However, the molecular mechanism by which inhalation of CSE induced lung injury remains largely unknown.

DNA methylation regulates chromosomal stability and gene expression and is considered as the most common genome epigenetic modification (Ehrlich and Lacey, 2013). Altered DNA methylation has been shown to play a key role in the development of lung diseases (Qiu et al., 2015; Duruisseaux and Esteller, 2018; Reynolds et al., 2015), which is strongly modified by cigarette and tobacco smoke (Barcelona et al., 2019; Tsaprouni et al., 2014). The cytochrome P450 family 1 subfamily B polypeptide 1 (CYP1B1), a dioxin inducible member of the CYP supergene family gene, has garnered wide attention for its actions as a carcinogenic factor in various human malignancies (Tokizane et al., 2005). Alteration of DNA methylation in the promoter regions of CYP1B1 has been reported in multiple types of cancer including prostate cancer (Tokizane et al., 2005), acute lymphoblastic leukemia (DiNardo et al., 2013) and gastric carcinoma (Kang et al., 2008). An in vitro study from Kwon et al. revealed that CYP1B1 could reduce apoptotic cell death in human breast cancer and leukemia cells through involvement of
DNA methylation-mediated death receptor 4 suppression (Kwon et al., 2018). In colorectal cancer, treatment of the cells with 5-aza-2′-deoxycytidine resulted in significant up-regulation of CYP1B1 mRNA levels (Habano et al., 2009). In addition to human malignancies, there was a clear increase in CYP1B1 promoter CpG island methylation level in isoniazid-induced liver injury in rats (Li et al., 2018). Interestingly, bioinformatics analyses by Cao et al. identified CYP1B1 as a potential target in smoking-mediating malignancies (Cao et al., 2015). Experimental analysis further demonstrated that hypo-methylated CYP1B1 was up-regulated in current smokers (Tsai et al., 2018). Overexpression of CYP1B1 is considered to be aggressive therapeutic target in advanced non-small cell lung cancer (Su et al., 2009). These evidences might reveal an interaction between hypo-methylated CYP1B1 and CSE induced lung injury.

As an abundantly present mammalian protein, MECP2 (Methyl-CpG-binding protein 2) contains two main methyl-CpG-binding domain (MBD) and transcriptional repression domain (TRD) (Vieira et al., 2015). MECP2 has been shown to act mostly as a master gene repressor by binding to methylated DNA or gene promoters (Nan et al., 1998). Based on its capability to bind methylated DNA, MECP2 could recruit DNA methyltransferases, histone deacetylases and chromatin remodelers to methylated DNA (Miremadi et al., 2007). Of great interest to us is that MECP2 is a key regulator of DNA methylation against smoke exposed lung injury (Gong et al., 2015). Furthermore, microarray gene expression profile GDS534 from Gene Expression Omnibus (GEO) database identified that MECP2 is significantly down-regulated, while CYP1B1 expression is up-regulated in active smokers compared with non-smokers. These above evidences prompted us to make an assumption that MECP2 might exert crucial role in CSE induced lung injury by regulating CYP1B1 methylation.

In the present work, we first constructed CSE-induced lung epithelial cell injury models and then determined the expression of MECP2 and CYP1B1, as well as CYP1B1 methylation status. Furthermore, we performed gain-of-function assay to test whether MECP2 affected CSE-induced lung epithelial cell injury through regulating the expression of CYP1B1 via altering its methylation status. The findings of the current study may provide unique insights into the molecular mechanisms underlying CSE induced lung injury and help develop useful intervention strategies.

**MATERIALS AND METHODS**

**Experimental materials**

Human bronchial epithelial (16HBE) and alveolar type II epithelial cells (AECII) were purchased from American type culture collection (ATCC, Manassas, VA, USA). RPMI-1640 medium and fetal bovine serum (FBS) was obtained from Gibco (Carlsbad, CA, USA). The kits of PI staining and Annexin V-FITC apoptosis detection were obtained from BD Biosciences (San Diego, CA, USA). Primary antibodies against MECP2, CYP1B1 and GAPDH were from Abcam (Cambridge, UK) and ProteinTech Group, Inc. (Chicago, IL, USA). Reactive oxygen species (ROS) assay kit were obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Enhanced chemiluminescence reagent was purchased from Pierce Biotech, Inc., (Rockford, IL, USA). Those of other chemicals and reagents were reagent from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stipulated.

**CSE preparation**

CSE was prepared according to previously described method (Slebos et al., 2007; Benedikter et al., 2017). Briefly, 3R4F Research Cigarettes (University of Kentucky, USA) were smoked using a peristaltic pump machine (Fisher Scientific, Pittsburgh, PA, USA). Under the temperature of 25°C, the smoke was bubbled into phosphate buffered saline (PBS, 15 mL) in a 100 mL graduated cylinder with a diameter of 28 mm. The solution of CSE was set to a pH 7.4 and sterilized by filtration. The resultant CSE solution was diluted with culture medium and the experiments were conducted within 30 min.

**Cell culture and treatment**

Human 16HBE and AECII cells were maintained in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) under a humidified 5% CO₂ atmosphere at 37°C. Subsequently, 16HBE or AECII cells were incubated with various concentrations of CSE solution (0, 50, 100, and 200 μg/mL) for 2 hr.

**Cell transfection**

The eukaryotic overexpression vector pcDNA3.1-MECP2 was constructed by Guangzhou VipotionBio Co., Ltd. (Guangzhou, China). For the in vitro experiments, 16HBE or AECII cells were divided the following four groups: (1) Blank group: Without CSE solution treatment; (2) CSE group: Cells were incubated with 200 μg/mL CSE for 2 hr; (3) CSE + NC group: Cells were transfected with empty vector pcDNA3.1 for 48 hr,
followed by incubation with 200 μg/mL CSE; (4) CSE + MECP2 group: Cells were transfected with pcDNA3.1-MECP2 for 48 hr before the addition of 200 μg/mL CSE. All cell transfection was performed with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

CCK-8 assay

After different treatments, the 16HBE or AECII cells were plated into 96-well microplates at a seeding density of 4,500 cells/well, followed by proliferation detection using Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). After the indicated time points (24, 48, and 72 hr, respectively) of incubation, the cells were treated with 10 μL CCK-8. Two hours later, the optical density values at 450 nm were measured on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Cell cycle and apoptosis analysis

Treated cells were harvested, seeded into 6-cm culture dishes at $2 \times 10^5$ cells/well and then washed two times in cold PBS. In order to determine cell cycle distribution, cells were fixed in ice-cold 70% ethanol overnight at 4°C and then stained with propidium iodide (PI, 20 mg/mL) in darkness for 20 min. Finally, cells were washed and cell cycle profiles were measured using flow cytometer (BD Biosciences, San Diego, CA, USA). For apoptosis assay, cells were re-suspended in 200 μL Binding Buffer, incubated with Annexin V/PI staining (BD Biosciences), and then detected by flow cytometry.

Measurements of ROS

Intracellular ROS was analyzed using FACS Calibur flow cytometer (BD Bioscience) in conjunction Cell-Quest Pro software. Briefly, treated cells were seeded into six-well culture dishes (1 × 10^5 cells/well) and grown overnight. After being washed three times with PBS, cells were incubated with 20 μM of 2’, 7’-dichlorofluorescin diacetate (Beijing Solarbio Science & Technology Co., Ltd) for 20 min at 37°C. The fluorescence intensity was acquired with BD FACS calibur (BD Biosciences) and the amount of ROS was calculated using Cell Quest Pro software.

Quantitative real time PCR analysis

Total RNA was obtained by using TRIzol reagent (Takara, Dalian, China) according to the manufacturer’s specifications. Reverse transcription for cDNA synthesis was performed using M-MuLV reverse transcriptase and Oligo (dT) primers (Sangon Biotech). The expression of MECP2 and CYP1B1 was determined using a Platinum

| Table 1. Primer sequences for quantitative real time PCR. |
| Gene | Sequence (5’ to 3’) | PS (bp) |
|------|---------------------|---------|
| GAPDH | F: TGTTCGTCATGGGTGTGAAC  
R: ATGGCATGAGCTGTAGTCAT | 154 |
| MECP2 | F: TGACCGGGAACCCATGTAT  
R: CTCACCTTTAGAAGCGAAGG | 145 |
| CYP1B1 | F: AAGTTCTTTGAGGCACGTGCGAA  
R: GGCCGTTACGTCCTCCTCAAAT | 144 |

F: forward; R: reverse; PS: product size

SYBR Green PCR Kit (Invitrogen) on an ABI 7500 system (Thermo Fisher Scientific). Depending on the method of $2^{-\Delta\Delta CT}$, relative gene expression profiles were normalized to GAPDH expression. The primer sequences used for PCR were listed in Table 1.

Western blot analysis

After being washed twice with PBS, the treated cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China). The mixture was centrifuged at 12,000 rpm at 4°C for 15 min, cell supernatants were harvested and protein amount was analyzed using BCA protein assay kit (Beyotime). Approximately 30 μg of total protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% fat-free milk in PBS-Tween-20 (0.1%) and incubated with primary antibodies (1:1000) against MECP2, CYP1B1 and GAPDH overnight at 4°C, followed by 2 hr incubation with HRP-conjugated secondary antibody at room temperature. Then the proteins were detected using an enhanced chemiluminescence (ECL) reagent (Pierce Biotech, Inc.).

Immunofluorescence staining

Treated cells were inoculated on sterilized coverslips placed in 6-well dishes and fixed in 4% formaldehyde for 15 min. After 0.2% Triton X-100 permeabilization, those cells were blocked using 3% BSA and then incubated with MECP2 and CYP1B1 antibodies (1:300) overnight at 4°C. Subsequently, those cells were incubated with Alexa Fluor® 594-conjugated secondary antibody and the nuclei of cells were then counterstained with DAPI (Sigma-Aldrich). Image acquisition and further analysis were performed under a confocal laser microscope (Olympus, Tokyo, Japan).

Methylation-specific PCR (MSP)

The methylation status of CpG island in treated cells was screened at the CYP1B1 gene promoter regions by MSP. In brief, cellular DNA was prepared using
Get pure DNA Kit (Dojindo Molecular Technologies, Kumamoto, Japan) and treated with sodium bisulfite. Then bisulfite-modified DNA were subjected to PCR amplification an ABI3700 automated sequencing system (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA), followed by 2% agarose gel electrophoresis. As positive and negative controls, we used methylated and non-methylated human DNAs, respectively. The primer sequences of methylated and un-methylated CYP1B1 presented in Table 2 were designed by Methyl primer Express v1.0.

**Statistical analysis**

All of the statistical analyses were provided by the SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). Quantitative data are expressed as mean ± standard deviation (SD) from six separate experiments. Differences were evaluated using one-way analysis of variance, followed by Dunnett’s test or Tukey’s test for among groups. Statistical significance was accepted when p-values less than 0.05.

**RESULTS**

**CSE attenuated cell proliferation, triggered cell cycle arrest, apoptosis and oxidative stress in lung epithelial cells**

Human 16HBE and AECII cells were grown to approximately 90% confluence and treated with increasing concentrations of CSE (0, 50, 100, and 200 μg/mL). Cell proliferation was determined by measuring the optical density values and found to decrease in both 16HBE (Fig. 1A) and AECII (Fig. 1B) cells in a dose-dependent manner. Moreover, we evaluated the effects of increasing concentrations of CSE on cell cycle distribution using flow cytometry with PI staining (Fig. 1C). Statistical analysis indicated that exposure to CSE elevated the percentage of cells in G0/G1 phase and decreased that in S phase, indicating G0/G1 phase arrest in 16HBE and AECII cells in a dose-dependent manner (Fig. 1C). With double Annexin V/PI staining, we further found CSE significantly increased the apoptosis rate of 16HBE and AECII cells in a dose-dependent manner in (Fig. 1D). In addition, the 2’, 7’-dichlorofluorescin diacetate probe was used to examine intracellular ROS levels. The results demonstrated that the intracellular ROS levels were significantly increased compared with control cells with increasing concentration of CSE exposure in 16HBE and AECII cells (Fig. 1E).

The dose-dependent regulatory effect of CSE exposure on the profile of MECP2 and CYP1B1

Next, we analyzed the expression of MECP2 and CYP1B1 levels in 16HBE and AECII cells after exposure to CSE for 2 hr. From Fig. 2A we can see that, the mRNA expression levels of MECP2 showed a significant decreased tendency, while CYP1B1 was gradually increased as the concentration of CSE was increasing in 16HBE and AECII cells. The protein levels of MECP2 and CYP1B1 were measured by western blotting (Fig. 2B) and immunofluorescence staining (Fig. 2C), which were consistent with the quantitative real time PCR data. To better explain the aberrant increased expression of CYP1B1, we detected the DNA methylation status of CYP1B1. Data from our MSP analysis (Fig. 2D) displayed that the methylation status of CYP1B1 promoter region was obviously decreased in 16HBE and AECII cells after exposure to 100, and 200 μg/mL CSE. We thus speculated that the up-regulation of CYP1B1 by CSE exposure might be associated with decreased DNA methylation.

**MECP2 overexpression attenuated CSE-induced lung epithelial cell injury**

The above results indicated that 200 μg/mL CSE had severe cytotoxic effects on 16HBE and AECII cells, which was thus selected as optimal dose for the following experiments. As MECP2 was lower expressed after CSE exposure, we next carried out gain-of-function assays to investigate its biological functions in CSE treated 16HBE and AECII cells. CCK-8 assay showed that MECP2 overexpression significantly attenuated CSE-induced cell growth inhibition in 16HBE (Fig. 3A) and AECII (Fig. 3B) cells. Moreover, the proportion of CSE treat-

### Table 2. Methylated or un-methylated specific primer sequences used for MSP.

| Gene | Primer sequence 5’-3’ | PS (bp) |
|------|-----------------------|---------|
| CYP1B1 | M-F: GTTTTTTGGGAAGTAAGTTTAAGTC  
       | M-R: ATACCCACGTTTCCATTATAGAT | 189     |
| CYP1B1 | U-F: GGTTTTTTGGGAAGTAAGTTTAAGTT  
       | U-R: AATACCCACATTCCATTATACAT | 191     |

M-F: methylated forward; M-R: methylated reverse; U-F: un-methylated forward; U-R: un-methylated reverse; PS: product size
MECP2 down-regulates CYP1B1 to attenuate lung cell injury

Fig. 1. CSE-induced lung epithelial cell injury in a dose-dependent manner. 16HBE and AECII cells were exposed to an increasing concentration of CSE solution (0, 50, 100, and 200 μg/mL) for 2 hr. (A-B) Cell proliferation was evaluated using the CCK-8 assay for the indicated time points. (C) Cell cycle analysis was measured using flow cytometry with PI staining. (D) Cell apoptosis was detected using flow cytometry with Annexin V/PI double staining. Cells in quadrants (Annexin V+/PI+ and Annexin V+/PI-) were considered to be apoptotic. (E) ROS were detected using 2′, 7′-dichlorofluorescin diacetate, and the percentage of ROS-positive cells was determined. Each sample was analyzed six times. Data are expressed as mean ± standard deviation (SD). Differences were evaluated using one-way analysis of variance, followed by Dunnett’s test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with 0 μg/mL.
ed 16HBE and AECII cells were increased and decreased at G0/G1 phase and S phase, respectively and partially restored by MECP2 overexpression (Fig. 3C). Additionally, MECP2 overexpression significantly attenuated the increased cell apoptosis (Fig. 3D) and ROS generation (Fig. 3E) in 16HBE and AECII cells after exposure to CSE.

**MECP2 overexpression suppressed the expression of CYP1B1 by promoting DNA methylation**

To further explore whether MECP2 regulated the expression of CYP1B1 in CSE treated 16HBE and AECII cells, we first confirmed the expression of MECP2 after transfection using quantitative real time PCR. As shown in Fig. 4A, remarkably down-regulated MECP2 and up-regulated CYP1B1 mRNA expression levels in CSE treated 16HBE and AECII cells were notably reversed by MECP2 overexpression vector transfection. We further used western blotting (Fig. 4B) and immunofluorescence staining (Fig. 4C) to detect the protein expression levels of MECP2 and CYP1B1. As expected, the same protein trend to quantitative real time PCR was also acquired. Interestingly, MSP assay (Fig. 4D) demonstrated that MECP2 overexpression promoted the methylation status of CYP1B1 promoter region in CSE treated 16HBE and AECII cells after exposure to CSE.

**Fig. 2.** Effects of CSE exposure on the expression of MECP2 and CYP1B1 in a dose-dependent manner. 16HBE and AECII cells were exposed to an increasing concentration of CSE solution (0, 50, 100, and 200 μg/mL) for 2 hr. (A) The mRNA expression levels of MECP2 and CYP1B1 were determined by quantitative real time PCR. Data are expressed as mean ± standard deviation (SD). Each sample was analyzed six times. Differences were evaluated using one-way analysis of variance, followed by Dunnett’s test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with 0 μg/mL; (B) The protein expression levels of MECP2 and CYP1B1 were evaluated using western blotting. (C) Immunofluorescence staining demonstrated the labeling intensity of MECP2 (red staining) and CYP1B1 (green staining). Magnification, ×200. (D) Methylation-specific PCR (MSP) analysis of CYP1B1 promoter region methylation in 16HBE and AECII cells after exposed to an increasing concentration of CSE solution (0, 50, 100, and 200 μg/mL) for 2 hr. U, unmethylation; M, methylation.
MECP2 down-regulates CYP1B1 to attenuate lung cell injury

Fig. 3. MECP2 overexpression attenuated CSE-induced lung epithelial cell injury. 16HBE or AECII cells were divided into Blank group (Without CSE solution treatment), CSE group (Cells were treated with 200 μg/mL CSE), CSE + NC group (Cells in CSE group were transfected with empty vector pcDNA3.1) and CSE + MECP2 group (Cells in CSE group were transfected with pcDNA3.1-MECP2). (A-B) Cell proliferation was assessed using the CCK-8 assay. (C) Cell cycle analysis was measured using flow cytometry with PI staining. (D)Cell apoptosis was detected using flow cytometry with Annexin V/PI double staining. Cells in quadrants (Annexin V+/PI+ and Annexin V+/PI-) were considered to be apoptotic. (E) ROS were detected using 2', 7'-dichlorofluorescin diacetate, and the percentage of ROS-positive cells was determined. Each sample was analyzed six times. Data are expressed as mean ± standard deviation (SD). Differences were evaluated using one-way analysis of variance, followed by Tukey’s test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with Blank; *p < 0.05, **p < 0.01, ***p < 0.001, compared with NC+CSE.
AECII cells. These data suggested that MECP2 overexpression might attenuate CSE-induced lung epithelial cell injury partially through promoting CYP1B1 methylation status.

**DISCUSSION**

In this study, we observed that CSE suppressed MECP2, but enhanced CYP1B1 expression in human bronchial epithelial and alveolar type II epithelial cells. Importantly, MECP2 overexpression attenuated CSE-induced epithelial cell injury, as confirmed elevated cell proliferation and reduced apoptosis and ROS generation. Moreover, MECP2 overexpression suppressed the expression of CYP1B1 by promoting DNA methylation in CSE-induced epithelial cell injury models. These findings suggest that targeting CYP1B1 by MECP2 possibly linked with regulating CSE-induced lung injury by relieving ROS and apoptosis.

MECP2 is an epigenetic regulator and necessary for normal neuronal maturation and glial cell function (Ezeonwuka and Rastegar, 2014). During embryonic development, a key role for MECP2 in organizing structure in neuronal nucleoli has been revealed (Singleton et al., 2011). Our data showed that MECP2 overexpression significantly elevated cell proliferation,
MECP2 down-regulates CYP1B1 to attenuate lung cell injury

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Conflict of interest---- The authors declare that there is no conflict of interest.

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