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Permalink

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Journal

Inhalation Toxicology, 29(10)

ISSN

0895-8378

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Publication Date

2017-08-24

DOI

10.1080/08958378.2017.1392655

Peer reviewed

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Author manuscript

Inhal Toxicol. Author manuscript; available in PMC 2018 August 29.

Published in final edited form as:

Inhal Toxicol. 2017 August ; 29(10): 435–442. doi:10.1080/08958378.2017.1392655.

Perinatal exposure to environmental tobacco smoke is associated with changes in DNA methylation that precede the adult onset of lung disease in a mouse model

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Abstract

Prenatal and early-life environmental tobacco smoke (ETS) exposure can induce epigenetic alterations associated with inflammation and respiratory disease. The objective of this study was to address the long-term epigenetic consequences of perinatal ETS exposure on latent respiratory disease risk, which are still largely unknown. C57BL/6 mice were exposed to prenatal and early-life ETS; offspring lung pathology, global DNA, and gene-specific methylation were measured at two adult ages. Significant alterations in global DNA methylation and promoter methylation of *IFN-γ* and *Thy-1* were found in ETS-exposed offspring at 10–12 and 20 weeks of age. These sustained epigenetic alterations preceded the onset of significant pulmonary pathologies observed at 20 weeks of age. This study suggests that perinatal ETS exposure induces persistent epigenetic alterations in global DNA, as well as *IFN-γ* and *Thy-1* promoter methylation that precede the adult onset of fibrotic lung pathology. These epigenetic findings could represent potential biomarkers of latent respiratory disease risk.

Keywords

Inflammation; early life; environmental tobacco smoke; methylation; prenatal; respiratory disease

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Declaration of interest

None declared.

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Introduction

Prenatal and early-life environmental tobacco smoke (ETS) exposure causes premature death and can instigate chronic respiratory diseases, thus continuing to be an immense public health issue (DiFranza et al., 2004). A dynamic and regulated coordination of organogenesis occurs in prenatal and early life that, if disrupted by adverse environmental insult, can permanently alter physiology and immune function, predisposing individuals to lifelong diseas (Maritz and Harding, 2011; Renz et al., 2011).

Immunological and respiratory harm can manifest in the early years of life (Joad et al., 2009; Lovasi et al., 2010; Vardavas et al., 2016). Perinatal exposures are identified as risk factors for an increased incidence of allergic asthma, lower respiratory and ear infections, as well as decreased lung growth in young children and adolescents (Lee et al., 2003; DiFranza et al., 2004; Dietert and Zelikoff, 2008; Thacher et al., 2014). Animal studies have shown that immunological disruption from prenatal and early-life ETS exposure is sustained over time and prenatal ETS exposure can persistently skew Th2 polarization (Basta et al., 2000; Wang et al., 2007; Singh et al., 2011). Therefore, ETS exposures in perinatal development may be related to an increased incidence of adult onset asthma and other respiratory harm, including chronic obstructive pulmonary disease (COPD), chronic wheezing, and breathlessness (Larsson et al., 2001; Janson, 2004; Penn et al., 2007; Xiao et al., 2012).

The developmental origins of health and disease (DOHaD) hypothesis proposes that the human respiratory system is highly susceptible to lifelong reprogramming through adverse environmental exposures during perinatal development, potentially through epigenetic mechanisms (Barker, 2004; Pinkerton and Joad, 2006; Swanson et al., 2009; Feil and Fraga, 2011). Epigenetic mechanisms, which can be heritable and sensitive to exogenous influence, are independent of the consequences of changes in DNA sequence for regulating gene expression (Feil and Fraga, 2011). Epidemiological and experimental studies have reported an association between prenatal smoke exposure and altered epigenetic methylation patterns in both elementary school-aged children and 30 days post-birth mice (Breton et al., 2009; Meyer et al., 2017). DNA methylation alteration is the process of adding or removing methyl groups to DNA, particularly within gene promoter regions, which can then influence gene regulation, without altering the genetic sequence (Smith and Meissner, 2013). This epigenetic process has been suggested as a potential mechanism behind the altered immune responses observed in offspring exposed to perinatal ETS (Ho, 2010).

Previously, we reported altered global and gene-specific methylation in 6-week old mice exposed to prenatal ETS that corresponded with alterations in pulmonary inflammation and airway hyperreactivity, suggesting that prenatal ETS exposure can increase disease susceptibility through altered DNA methylation (Lee et al., 2015). However, whether the epigenetic effects of these exposures resolve with age and to what extent these effects may alter the mechanisms involved in adult onset lung pathologies remained unaddressed. In order to address the impact of significant methylation changes observed in early life on the susceptibility to adult respiratory disease, this study aimed to determine whether perinatal ETS exposure contributes to persistently altered DNA methylation, particularly for genes relevant to inflammation and fibrosis, and to address whether these patterns contribute to

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adult lung disease. Interferon (*IFN)-γ* and thymus cell antigen-1 (*Thy-1*) genes were selected as genes relevant to the inflammatory immune response and respiratory fibrosis pathology (Miller and Ho, 2008; Sanders et al., 2008). IFN-γ in particular is a major Th1 cytokine that is mechanistically understood to have an important anti-fibrotic function (Borthwick et al., 2013). IFN-γ dysregulation with decreased pulmonary Th1 cytokine production in response to tobacco smoke exposure is associated with chronic pulmonary inflammation (Ouyang et al., 2000; Tebow et al., 2008). Similarly, a loss of Thy-1 expression attributable to DNA hypermethylation was found in patients with idiopathic pulmonary fibrosis (Sanders et al., 2012), suggesting that these can be mechanistically important for fibrogenesis (Hagood et al., 2005). We hypothesized that perinatal exposure to ETS would persistently alter methylation of *IFN-γ* and *Thy*-1 gene promoters and precede indications of latent pulmonary fibrotic disease in adult offspring.

Methods

Animals

C57BL/6 mice were maintained in pathogen-free conditions (22 ± 2 °C, 30–40% humidity, 12 h light/12 h dark cycles) and offered food and water *ad libitum* in the animal facilities of the University of California, Davis (UCD, Davis, CA) and the University of Montana (UM, Missoula, MT). All experiments met the approval of the University of Montana Institutional Animal Care and Use Committee (IACUC).

Breeding and ETS exposure

Breeding and ETS exposure were conducted as previously described (Lee et al., 2015; Brown et al., 2016). Briefly, two female mice were paired with one male mouse to create a timed-pregnant exposure scenario. Twelve virgin female and 6 male mice (8–9 weeks old) were used for breeding. Following verification of a vaginal plug at day 0 of gestation, four and eight female mice were exposed to either filtered air (FA) or ETS throughout gestation, respectively. For the control group, time-mated dams were exposed to FA only for 24 h 7 d/week for the duration of the study as shown in Figure 1. For the ETS-exposed groups, time-mated dams were exposed daily to approximately 1.0 mg/m^3 of tobacco smoke for 6 h/day for 7 d/week in a smoke exposure system (UC-Davis), with collection of both side-stream and mainstream cigarette smoke from 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY). A concentration of 1.0 mg/m^3 smoke is considered a low-dose since it represents a no-observable-effect level in rats (Rajini et al. 1994); therefore, the total concentration of suspended particulates was maintained at 1.01 ± 0.03 mg/m^3 for this study. This concentration was also similar to those reported in prior ETS exposure studies in mammals (Seymour et al., 2002; Slotkin et al., 2006). The burn rate was two cigarettes consumed per 10 min with a 35-mL puff volume per 2 s, once per min. The carbon monoxide and nicotine levels were 6.01 ± 0.44 ppm and 266 ± 48 μg/m^3, respectively, and the average temperature was 21.1 °C. Following birth, four dams and their offspring were moved out of the ETS chamber and subsequently exposed to FA only (Pre ETS group); another four dams and offspring continued to receive ETS exposure under identical conditions for 3 consecutive weeks (Pre/Post ETS group). Following this additional period of ETS or FA exposure, the dams and pups were exposed to FA only until weaning

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and then shipped to the University of Montana via air. Upon arriving at the University of Montana, the dams and their offspring were quarantined for 3–4 weeks.

Mean litter size per FA and ETS-exposed dams was 6.9 ± 0.3. Litter size and sex ratio were not significantly different among groups (data not shown). ETS exposure did not induce any spontaneous losses in mice. Offspring mice in each group were randomly selected across litters from 4 dams under the same conditions in an equal-sex ratio at 10- to 12- and 18- to 20-week old intervals and euthanized via intraperitoneal injection of 0.1 mL sodium pentobarbital (Euthasol™). All analyses included 3–6 pups per group. Lungs from offspring mice were either prepared for histological analyses (10–12 and 18–20 weeks old) or snap-frozen and stored at −80°C for later epigenetic analyses (10–12 and 20 weeks old).

Histological preparation

Lungs were prepared using protocols as previously described (Beamer et al., 2010; Hamilton et al., 2012). Briefly, lungs were inflation-fixed in 1 mL 3% paraformaldehyde-phosphate buffered saline (PBS) overnight at 4°C. Samples were then rinsed three times with PBS, placed into labeled cassettes, and submerged in 70% ethanol (EtOH). A Leica ASP 300 tissue processor (Buffalo Grove, IL) was used on a 7.25-h program: 30 min in 70% and 95% EtOH; two 1-h changes in 100% EtOH; three changes of xylene for 30 min each; and three paraffin changes for 45 min in the first bath, and 1 h each in the second and third bath under vacuum. A Leica RM2235 microtome (Buffalo Grove, IL) was used to prepare tissue sections 5–6 microns thick. The tissue sections were stained with both hematoxylin and eosin (H&E) and trichrome using a Shandon 24–4 autostainer (GMI, Ramsey, MN). For the H&E program, Mayer's hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) and alcoholic eosin (Thermo Shandon Limited, Runcorn, UK) were used. Weigert's hematoxylin (Electron Microscopy Sciences, Hatfield, PA) and Gomori trichrome (Harleco, EMD, VWR Randor, PA) were used for trichrome staining.

Collagen deposition

A Thor Labs (formerly CompuCyte) iCys Laser Scanning Cytometer (Sterling, VA) was used to analyze trichrome stained lung tissues with a protocol designed to quantitatively assess collagen deposition. A low-resolution scan of the entire tissue at 20X was first conducted for each tissue section. Using the incorporated iCys Workstation computer software (version 3.4) tools, we randomly hand-selected six interstitial areas and six airways for high-resolution scans. "Phantom" contours were used to divide tissues into very small (8-μm diameter) circles as previously described (Brown et al., 2015; Brown et al., 2016). Briefly, a blue staining contour was considered a collagen-positive event via trichrome stain. Positive contour percentage over total tissue contours were determined to compare one tissue to the next. All airways and blood vessels were removed from the calculation of total collagen deposition. Two sections from one lung lobe 21–28 microns apart were analyzed and averaged. We analyzed three mice per exposure condition.

Lung pathology scoring

H&E stained lung tissues from 10- to 12- and 18- to 20-week old offspring were imaged at 100X using a Zeiss Axioskop attached to a Zeiss digital camera and processed with Zeiss

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Axiovision software (Thornwood, NY). As previously described, two experienced observers blinded to the experimental conditions independently scored the degree of visible inflammatory lung disease of the left and right lobes considering the parameters of cellularity, airway thickening, and structure distortion using a 5-point scale (0–4), with zero as no effect and 4 as the most severe pathology evident (Hamilton et al., 2012). There were three exposed mice per condition. Values shown are the median of both scorers' median values per condition.

Global DNA methylation by LUminometric methylation assay (LUMA)

Genomic DNA was extracted from lung tissues according to the manufacturer's protocols using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Global DNA methylation was measured via LUMA as previously described (Karimi, Johansson and Ekstrom, 2006; Karimi et al., 2006; Lee et al., 2015). The extent of cleavage within the digested samples was quantified by means of bioluminometric polymerase extension using pyrosequencing on a Pyromark Q96 MD (Qiagen). A HpaII/MspI ratio was used to calculate percentage of 5-methylcytosine (5-mC). Samples were run in duplicates on plates with positive, negative, and water controls.

Gene-specific methylation by pyrosequencing assay

Methylation levels in the promoter regions of selected genes (*IFN-γ* and *Thy-1*) were measured using pyrosequencing assay with primers designed as previously described (Brown et al., 2015). Briefly, genomic DNA was modified via bisulfite conversion of unmethylated cytosine to uracil using Zymo EZ DNA Methylation kits according to the manufacturer's protocols (Zymo Research, Orange, CA). Samples (50 ng) of bisulfite-treated DNA were PCR amplified using Pyromark PCR kits (Qiagen). A Pyromark Q96 Vacuum Workstation with Streptavidin Sepharose® High Performance Beads (GE Healthcare, Piscataway, NJ) was used for sample immobilization, and a Pyromark Q96 MD was used for all subsequent pyrosequencing (Qiagen). Samples were processed in duplicate on plates with water controls. Percent methylation of a sample was calculated by averaging all of the interrogated CpG sites.

Statistics

Statistical analyses were performed using Graphpad Prism 7.0 (Graphpad Software, San Diego, CA). In cases where multiple variables were simultaneously compared, statistical significance was tested using one-way ANOVA, followed by Tukey's multiple comparisons *post hoc* analysis. Non-parametric one-tailed Mann-Whitney testing was used in certain cases of two comparisons. Ordinal data were analyzed by non-parametric Kruskal-Wallis testing, followed by Dunn's *post hoc* analysis for pairwise median comparisons. All statistical significance was defined as the probability of type I error occurring at less than 5% ($p < 0.05$).

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Results

Histology and collagen deposition of ETS-induced alterations in lung morphology

Abnormal collagen deposition in the interstitium was observed in both pre ETS and Pre/Post ETS groups as shown in the representative photomicrographs in 18- to 20-week old offspring (Figure 2). The visual observations indicate stable phenotypic alterations in lung morphology associated with the combined pre- and postnatal ETS exposures. Interstitial collagen deposition increased in a dose-dependent manner in the 18- to 20-week old offspring; however, the changes were not statistically significant (3A).

Average pathology scores from 18- to 20-week old mice increased across the FA, Pre ETS, and Pre/Post ETS groups (Figure 3B). There were significant differences in pathology scores between the FA and Pre/Post ETS groups. However, no significant pathology was observed in 10- to 12-week Pre- and/or Post-ETS-exposed groups and controls. In contrast, pathology was more noticeable in 18- to 20-week Pre/Post ETS than that in the 10- to 12-week tissues from corresponding ETS exposed groups (Supplementary Figure 1).

Prenatal and early-life ETS exposure vs. methylation alterations at two stages of adulthood

To determine the long-term epigenetic impact of prenatal and early-life ETS exposure, global DNA and gene promoter methylation was measured in 10- to 12-week (Figure 4A) and 20-week old adult offspring (Figure 4B).

Global DNA methylation was significantly hypomethylated in lung tissues from 10- to 12-week old offspring exposed to combined Pre/Post ETS in a dose-dependent manner, which corresponded to the ETS exposure (Figure 4A). As an indication of stable global hypomethylation, a similar trend was observed in 20-week old offspring (Figure 4B). There were significant differences between the global DNA methylation of FA and Pre/Post ETS in both 10- to 12- ($p < 0.01$) and 20-week old offspring ($p < 0.05$).

We also measured genomic DNA methylation among dams that were directly exposed to ETS at the same time as the 20-week old offspring; however, there were no significant changes in lung tissue methylation in these dams (Supplementary Figure 2), indicating that the early-life stage can be uniquely sensitive to epigenetic reprogramming associated with ETS exposure.

For gene specific methylation, hypermethylation of *IFN-γ* was observed in 10- to 12-week old offspring (Figure 4A), compared across FA, Pre ETS, and Pre/Post ETS groups. A corresponding trend of persistent hypermethylation of *IFN-γ* was observed in 20-week old offspring (Figure 4B). There were significant differences in *IFN-γ* hypermethylation between the FA and Pre/Post ETS groups in both 10- to 12- ($p < 0.01$) and 20-week old offspring ($p < 0.05$).

In addition, *Thy-1* hypermethylation was observed in 10- to 12-week old offspring across the FA, Pre ETS, and Pre/Post ETS groups in a dose-dependent manner (Figure 4A). Dose-dependent trends in *Thy*-1 hypermethylation were also found in 20-week old offspring (Figure 4B). Significant differences of *Thy*-1 methylation levels between the FA and Pre/

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Post groups were observed in 10- to 12-week ($p < 0.001$) and 20-week old ($p < 0.05$) offspring. Taken together, persistent *Thy-1* hypermethylation corresponded to combined prenatal and early-life ETS exposure.

Discussion

Very few studies have reported the epigenetic status following prenatal and early-life ETS exposure in adult mice; therefore, we performed this study as an extended investigation into their effects on adult offspring respiratory health. To determine if the exposures induced distinct, persistent methylation patterns associated with fibrotic lung disease, we assessed methylation changes in lung tissue DNA from adult offspring mice at 10- to 12- and 20-week old intervals. The exposure dose was designed to be relevant to ambient smoke that active smokers and young children and pregnant mothers could encounter if living with a tobacco smoker (Slotkin et al. 2006); active smokers can attain particulate levels as high as 2.0 mg/m^3 (Jinot and Bayard, 1992). Human and animal model studies have indicated that offspring exposed to prenatal and early-life ETS have lifelong adverse respiratory deficits (Gilliland et al., 2000; Li et al., 2000; Pugmire et al., 2014; Fernandez-Plata et al., 2016). Further evidence shows that combined prenatal and early-life ETS exposure into adolescence may induce the most severe degree of lung deficits in offspring, as the exposure follows the full duration of lung development (Gibbs et al., 2016). Our study was designed to determine both the adult physiological effects of combined prenatal and early-life ETS exposure and the correlation of those effects to preceding epigenetic alterations.

Epidemiological and laboratory studies have found distinct respiratory deficits in offspring associated with ETS exposure timing, suggesting that age at exposure is an important factor in respiratory outcomes (Wang and Pinkerton, 2008). Our results are in line with those studies, as the pathology scores in the 18- to 20-week old offspring were higher in the Pre ETS group than in the FA group and continued to increase and reach significance in the Pre/Post ETS group. Since the presence of lung pathology was not visually evident in tissue examined from 10- to 12-week old ETS exposed offspring, it appears that the methylation changes reported could have preceded the manifestations of adult fibrotic lung disease that were observed in this study: increased collagen burden and significant pathology in 18- to 20-week old Pre/Post ETS offspring. Similarly, global DNA and gene specific methylation alteration patterns in the Pre/Post ETS groups were significantly different from those in the FA groups. Furthermore, these methylation patterns persisted into the later stage of adulthood in ETS-exposed offspring only, suggestive of a stable epigenetic alteration induced in early life.

Genomic DNA hypomethylation has been identified as a biomarker sensitive to tobacco smoke exposure that is associated with the respiratory diseases of COPD (Qiu et al., 2012) and cancer (Liu et al., 2010). Epidemiological and animal model studies of tobacco smoke exposure have reported significant hypomethylation in genomic DNA associated with prenatal exposure (Wilhelm-Benartzi et al., 2012), including our own (Lee et al., 2015). In this study, global DNA hypomethylation at two adult ages was observed in pre- and/or postnatal ETS exposed offspring that was not observed in the directly exposed maternal dams of the same exposure duration, suggesting that age at exposure could be a crucial

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factor for inducing stable epigenetic alterations that can increase risk of developing chronic respiratory disease.

Prenatal and early-life ETS exposure can augment the typical immune response, compromising its role in mitigating the effects of exposure to environmental agents in adulthood (Penn et al., 2007; Brown et al, 2016). Normally occurring, early-life immunological alterations in gene promoter methylation are a coordinated series of dynamic events that when disrupted, can lead to persistent skewed Th2 phenotypes and a greater risk for asthma and atopy into adulthood (Prescott et al., 1998; Lee et al., 2002; Siegle et al., 2011). Exposure to particles and chemical components from ETS could therefore disrupt these normal developmental immune system patterns in prenatal and early life. There is evidence that disruption can manifest in the form of environmentally-influenced Th2 polarization, in which T-cells become induced to proliferate cytokines towards an adaptive T-cell inflammatory phenotype of T-helper 2 (Th2), coinciding with suppression of the Th1 phenotype and IFN-γ cytokine secretion via promoter hyper-methylation (Miller and Ho, 2008; Singh et al., 2011). Further, suppression of IFN-γ production (contributing to a prevailing Th2 phenotype) may be associated with the progression of pulmonary fibrosis (Wynn, 2004; Borthwick et al., 2013). A human study reported that ETS exposure is a high-risk factor in sustained early-life Th2 polarization in children (van der Velden et al., 2001).

Gene promoter hypermethylation of *IFN-γ*, a Th1 cytokine, has been implicated in Th2 polarization associated with allergic asthma (Miller and Ho, 2008). Altered *IFN-γ* promoter methylation has been experimentally associated with differences in gene expression (Yano et al., 2003; de Araujo-Souza et al., 2015). In a primate study, altered *IFN-γ* expression in offspring lung tissue was associated with perinatal ETS exposure (Wang et al., 2007). We previously confirmed that hypermethylation of the *IFN-γ* promoter is associated with a corresponding decrease in IFN-γ cytokine production in BALF (Lee et al., 2015). Here, we observed continued *IFN-γ* hypermethylation in both adult-aged ETS exposed offspring—an effect that was not observed in their FA-only counterparts. Taken together with our 6-week old offspring observations (Lee et al., 2015), we suggest that prenatal and early-life ETS exposure-associated *IFN-γ* hypermethylation contributes to Th2 polarized respiratory disease.

A persistently skewed Th2 inflammatory profile is known to increase the risk of developing pulmonary fibrosis (Stampfli and Anderson, 2009). *Thy-1* promoter hypermethylation has been proposed as a mechanism involved in development of the fibrotic phenotype (Sanders et al., 2008; Sanders et al., 2012). In our study, *Thy-1* was significantly hypermethylated in both 10- to 12- and 20-week old offspring of prenatal and early-life ETS-exposed dams. We previously confirmed that *Thy-1* hypermethylation in murine lung tissue corresponds to decreased mRNA expression (Brown et al., 2015). Given that *Thy-1* was persistently hypermethylated and preceded the apparent onset of adult lung disease (concluded from collagen deposition and histopathology data), we suggest that prenatal and early-life ETS exposure induces latent fibrotic lung disease and that altered *Thy-1* expression via promoter hypermethylation is involved in this adult disease process.

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Our observations are consistent with the DOHaD hypothesis, since the sustained methylation patterns associated with prenatal and early-life ETS exposure preceded the indicators of disease in adulthood. Since epigenetic regulation is a complex, multi-factorial process, future studies are warranted to determine causality between *IFN-γ* and *Thy-1* promoter methylation patterns and onset of adult respiratory disease. Furthermore, the association between DNA methylation changes in *IFN-γ* and *Thy-1* and mRNA expression levels was not directly confirmed and relied upon our previous publications; therefore, this is a limitation of this study.

In this study, we found lower level/small changes in the percent of methylation between ETS- and FA-exposed mice, but they were statistically significant. These results agree with other previous studies measuring LINE-1 methylation and T-cell–relevant genes, including IL-13 (Guerrero-Preston et al., 2010; Lee et al., 2013). Given that the DNA samples were collected from whole lung tissue and comprised multi-cellular populations, the methylation profiles reported in this study represent a diverse heterogeneity of cells. Therefore, even relatively small differences in percent methylation can denote important methylation profiles in which future directions should be explored at the single-cell level. Furthermore, our findings here support the emerging paradigm that even small environmentally-cued differences in methylation percentages can be associated with significant phenotypic changes, particularly in the context of development, which is observable in human studies (Leenen et al., 2016).

Additionally, there are limitations in using a murine model to acquire information relevant to human respiratory health that should be considered. Though lung development spans pre- and postnatal life in both humans and mice, the timing of these stages of growth are distinctly different (Wang and Pinkerton, 2008). Therefore, a study in which the methylation pattern changes can be correlated to precise timing of developmental ETS exposures (distinguishing between the contributions of combined prenatal and early life exposure and early life exposure alone, as well as direct inhalation and indirect prenatal exposure) could yield further insight into disease susceptibility. Furthermore, since alterations in methylation patterns can be biomarkers of human respiratory disease susceptibility and diagnostics, future studies could determine whether these observations can be translated into potential biomarkers for adult onset respiratory disease in humans for early interventions to improve health outcomes.

Conclusions

Significant and stable alterations in methylation patterns of *IFN-γ* and *Thy-1* promoters were observed in 10- to 12-week and 20-week old offspring mice after perinatal ETS exposure. Our histopathological findings together with our methylation data suggest that combined prenatal and early-life ETS exposures lead to adult onset fibrotic lung disease through epigenetic mechanisms. Our results identify potential epigenetic biomarkers of perinatal ETS exposure and support the importance of interventions to prevent prenatal and early childhood ETS for reducing the lifelong risk of developing asthma and other chronic respiratory diseases.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Virginia Porter, Mary Buford, Melisa Schelvan, Forrest Jessop, Pamela Shaw, Lou Herritt, Zeina Jaffar, Maria Ferrini, the Fluorescence Cytometry Core, and the Molecular Histology and Fluorescence Imaging Core, (Center for Environmental Health Sciences at UM), Dale Uyeminami, and Shanie McCarty (Center for Health and the Environment at the University of California, Davis) for their varied expert technical assistance with different aspects of this manuscript.

Funding

This work was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health (grant number P30GM103338) and an S10 Shared Instrument Grant (grant number S10RR026325–01). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, NIGMS, or NIEHS.

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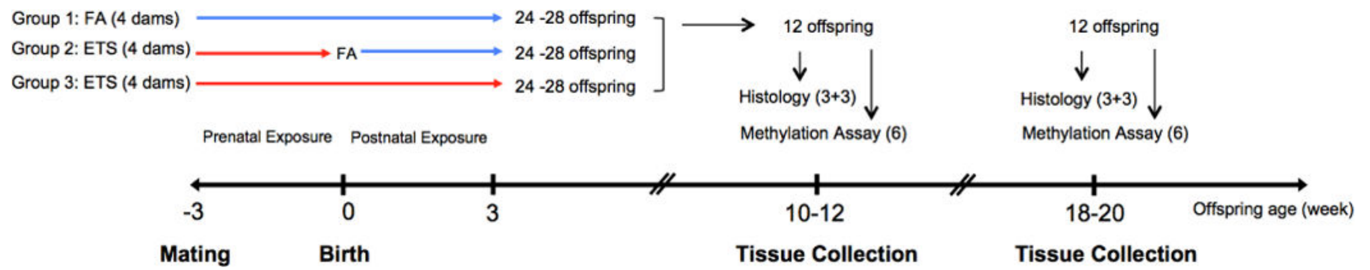

Figure 1.

Timeline of experiment. Upon confirmation of a vaginal plug, female mice were exposed to either FA only or 1.0 mg/m^3 ETS 6h/day, 7 days/week for the duration of pregnancy. Dams and pups were exposed to FA only (pups born from FA and Pre ETS) or continued to receive ETS exposure (Pre/Post ETS) for three weeks post-birth. In equal sex-ratio, mice lungs were harvested at 10–12 weeks of age and again at 18–20 weeks of age.

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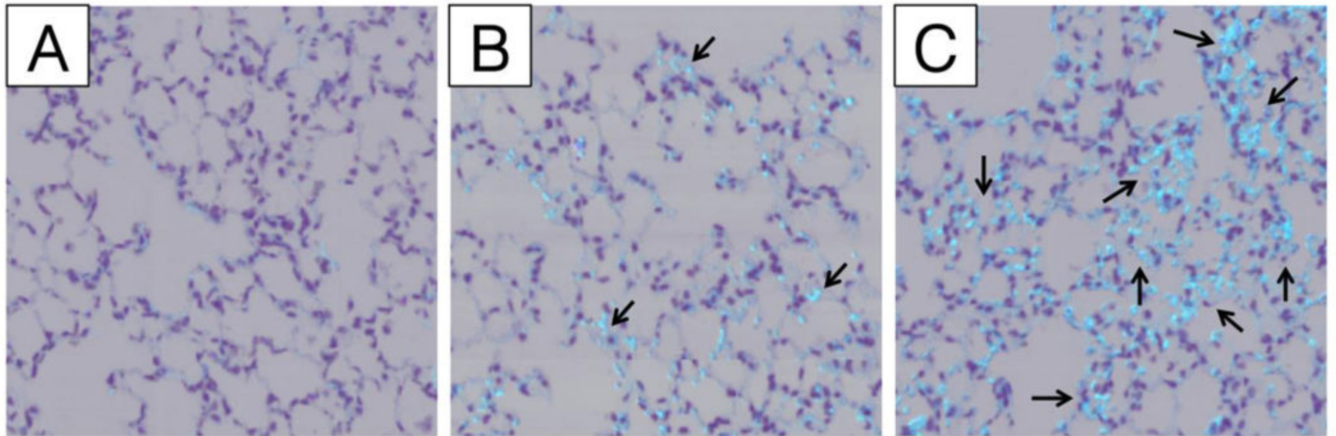

Figure 2.

Representative pictures of interstitial collagen deposition of 20 week aged offspring for each group. Collagen (arrowed) was stained in light blue. (A) FA, (B) Pre ETS, and (C) Pre/Post ETS

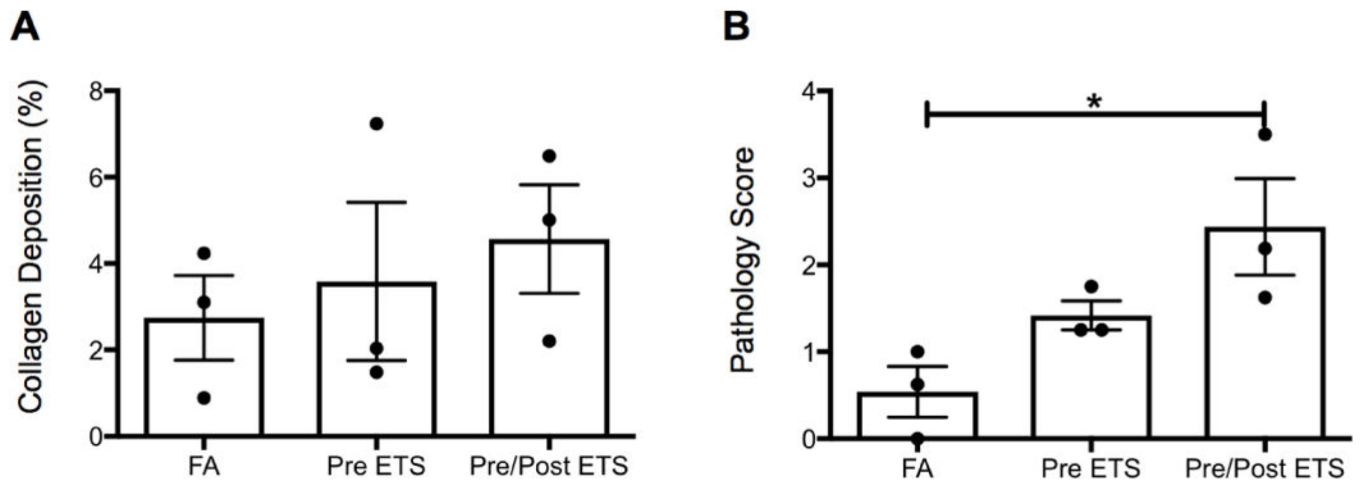

Figure 3.

Lung collagen deposition and pathology scores. (A) Lung interstitium collagen deposition percentage from 18- to 20-week old offspring, n = 3 mice per group, presented as means ± SEM. (B) Lung pathology scoring from 18–20 week old offspring, n = 3 mice per group, presented as medians ± interquartile range for all scores, $*p < 0.05$.

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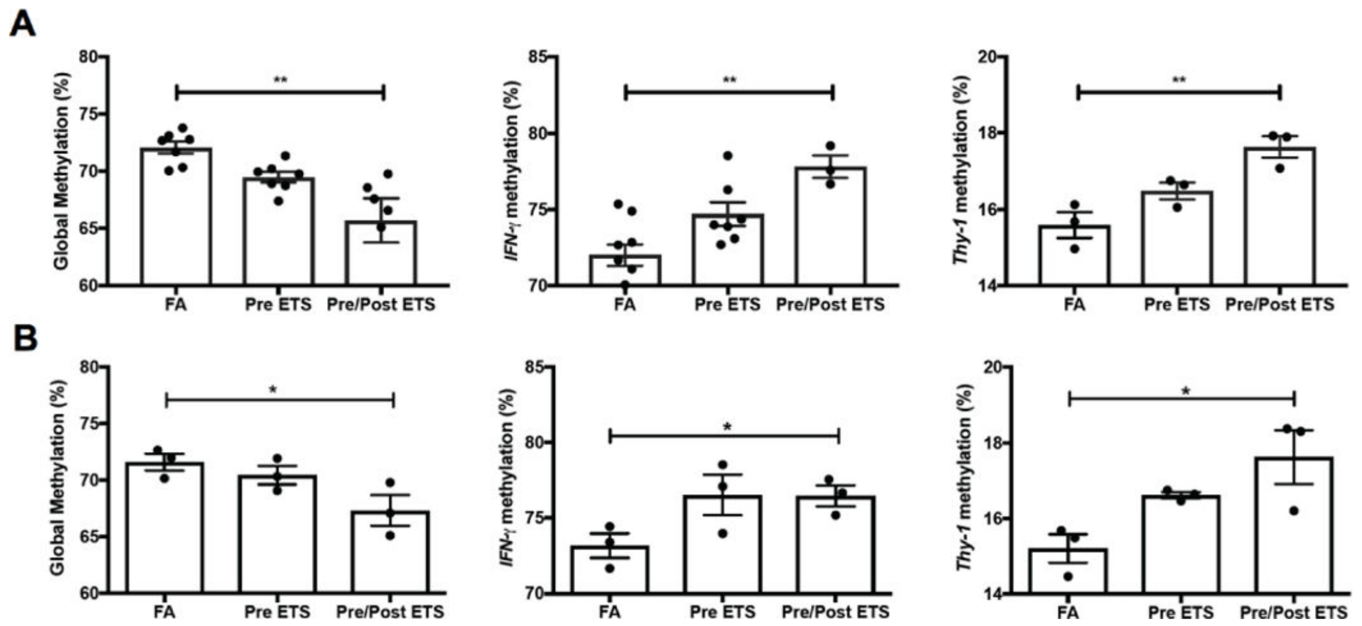

Figure 4.

(A) Global and promoter methylation of each group in 10- to 12-week old offspring. Data shown as means ± SEM percent methylation, $n = 3–6$ per group, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$. (B) Global and promoter methylation of each group in 20-week old offspring. Data shown as means ± SEM percent methylation, $n = 3$ per group, $**p < 0.01$, $*p < 0.05$.