ENVIRONMENTAL GENOMICS AND EPIGENOMICS: RESPONSE, DEVELOPMENT AND DISEASE

EDITED BY: Qiang Zhang, Timothy M. Barrow, Hyang-Min Byun and Yanqiang Li

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ENVIRONMENTAL GENOMICS AND EPIGENOMICS: RESPONSE, DEVELOPMENT AND DISEASE

Topic Editors:
Qiang Zhang, Tianjin Medical University, China
Timothy M. Barrow, University of Sunderland, United Kingdom
Hyang-Min Byun, Newcastle University, United Kingdom
Yanqiang Li, Harvard Medical School, United States

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EDITORIAL
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Editorial: Environmental Genomics and Epigenomics: Response, Development and Disease

Yanqiang Li[1,2*], *Hyang-Min Byun*[3], *Timothy M. Barrow*[4] *and Qiang Zhang*[5,6*]

[1] Basic and Translational Research Division, Department of Cardiology, Boston Children's Hospital, Boston, MA, United States, [2] Department of Pediatrics, Harvard Medical School, Boston, MA, United States, [3] Population Health Sciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [4] Faculty of Health Sciences and Wellbeing, University of Sunderland, Sunderland, United Kingdom, [5] Department of Occupational and Environmental Health, School of Public Health, Tianjin Medical University, Tianjin, China, [6] Center for International Collaborative Research on Environment, Nutrition and Public Health, School of Public Health, Tianjin Medical University, Tianjin, China

Keywords: environmental exposures, DNA methylation, histone modification, epigenetics, disease

Editorial on the Research Topic

Environmental Genomics and Epigenomics: Response, Development and Disease

With the rapid process of industrialization and the growth of the human population to unprecedented levels, individuals are increasingly exposed to multiple pollutants known to have an adverse impact upon human health, such as heavy metals, endocrine disruptors, tobacco smoke, polychlorinated biphenyls, diesel exhaust particles, pesticides, and other indoor and outdoor pollutants. Multiple non-communicable diseases (NCDs), including stroke, heart disease, cancers and chronic respiratory disease, now amount to nearly two-thirds of the total deaths caused by unhealthy environments. Considering this global burden, it is vital to understand the mechanisms underpinning the associations between environmental exposures and NCDs. Many studies have demonstrated that the epigenome can be altered by the environmental exposures, but the functional role of these changes in the processes of diseases have not been fully elucidated. This Research Topic has collated high-quality studies in the form of five reviews and four original research papers covering the genetic and epigenetic response to a range of environmental exposures.

Phthalates, esters of phthalic acid, are widely used as a plasticizer for a range of consumer products, but their presence in the environment has become a public health concern as they have potential effects on reproduction, development and obesity. Dutta et al. present a comprehensive review of the epigenetic changes associated with phthalate exposures. They summarized the metabolism of phthalates, and the effect of phthalates on different tissues including embryonic stem cells, peripheral blood, placenta, cord blood, and germ cells. They also described the impact of phthalate exposure across the lifespan, and specifically reviewed the epigenetic effects associated with childhood asthma, lipid metabolism disorders, obesity, men's reproductive health, allergies, and cancer. In addition, the authors summarized differential expression of microRNAs associated with phthalate exposure in gestational diabetes, female fertility, and the placenta.

Bisphenol A (BPA) is used in the production of a range of plastics, but it has a well-established impact as an endocrine disruptor. Qin et al. reviewed the recent progress in the study of epigenetic changes following BPA exposure. DNA methylation changes have now been revealed in multiple genes that are involved in brain development, cancer progression and other key cellular signaling pathways. Histone modifications also change significantly at some loci upon BPA exposure. Lastly, Qin et al. reviewed the recent studies of the toxic-epigenomics approach to study the epigenetic effect of BPA exposure at the genome-wide level.

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Edited and reviewed by:
Douglas Mark Ruden, Wayne State University, United States

****Correspondence:***
Yanqiang Li
yanqiang.li@childrens.harvard.edu
Qiang Zhang
qiangzhang@tmu.edu.cn

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Nano silicon dioxide (Nano-SiO_2) has systematic toxicity, which is due to the induction of oxidative stress and inflammation in multiple organs. However, the carcinogenicity of nano-SiO_2 has not yet been established. Lou et al. investigated carcinogenicity of nano-SiO_2 exposure *in vitro*, finding that exposure can induce malignant transformation and global DNA hypomethylation of human bronchial epithelial cell lines. In particular, demethylation of CpG islands within the NRF2 promoter region results in increased gene expression, and this gene may have a functional role in nano-SiO_2-associated carcinogenesis.

Wang et al. investigated the tissue- and sex-specific DNA methylation changes resulting from lead exposure in mice by applying ERRBS to the liver and blood of male and female mice. The authors identified hundreds of differentially methylated regions (DMRs) following lead treatment, with the majority of these proven to be tissue- and sex-specific. They also identified differential methylation of several mouse imprinted genes common to both tissues and genders. Together, these findings reveal the diversity in epigenetic changes resulting from lead exposure and could form the basis of future studies in humans.

Pulczinski et al. utilized a mouse model of allergic lung disease to examine the effects of pre- and perinatal house dust mite (HDM) allergen exposure across three generations. Their data suggests that maternal HDM exposure (F0) may act synergistically with adult HDM exposure, leading to enhanced airway hyper-responsiveness (AHR) and lung inflammation when compared to mice exposed solely in adulthood. These findings indicate that maternal allergen exposures are capable of enhancing either susceptibly to or severity of allergic airway disease. They explored the mechanistic basis of the role of epigenetics in the multigenerational inheritance by utilizing genome-wide MeDIP (Methylated DNA Immunopreciptation)-seq and hMeDIP-seq analyses to identify genes differentially methylated (DMG) and hydroxy-methylated (DHG), which serves to help elucidate the mechanisms governing multigenerational inheritance of asthma risk.

Many metals can induce Alzheimer's disease (AD), which is the most prevalent movement disorder and the second most common neurodegenerative disease. Cai et al. reviewed the changes in DNA methylation and histone modifications involved in AD and the contribution of the environment to its development, especially through epigenetic mechanisms. Specifically, they described the epigenetic impact of the plumbum, arsenic, and aluminum to AD. This review summarizes the relationships between Alzheimer's disease and different metals, revealing them to be an important factor in AD development and suggesting a direction for further study to develop potential epigenetic therapeutics. In parallel, Wei et al. provide a mini review summarizing the metals regulating epigenetics in Parkinson's disease. Specifically, they described the impact of lead (Pb), mercury (Hg), copper (Cu), manganese (Mn), aluminum (Al), iron (Fe), and zinc (Zn) on the epigenome, and particularly changes in DNA methylation, suggesting an important role of metal exposures in the risk of developing PD.

Most PD cases cannot be explained by genetic variation alone, indicating that epigenetic changes may have contributed to PD pathogenesis. CTCF is a chromatin architecture protein and its binding to DNA is regulated by DNA methylation and histone modifications. Freeman and Wang investigated the changes in DNA methylation and H3K27 acetylation within CTCF binding sites around several PD-associated genes whose expression was altered by exposure to rotenone, a PD toxicant. They observed a global decrease of 5-methylcytosine and increase of H3K27ac level under rotenone treatment. Local abundance of H3K27 acetylation and CTCF binding was also changed at several CTCF-binding sites in PD-associated genes. Their study has implications for the understanding of gene-environment interactions and epigenetic regulation in PD.

To understand the tool to investigate neurodevelopmental disorders, Xie et al. summarized the impact of developmental exposure on neurodevelopment, highlighting the importance of using *in vitro* neural cell system to study neurotoxicity. The authors discuss recent progress using cell culture to recapitulate brain complexity: composition, structural, and functional complexity are discussed, respectively. Finally, how to mimic developmental exposure and assess toxicity using cell culture models are discussed in the context of elucidating their role in neurodevelopmental disorders.

To summarize, our special topic covers endocrine disruptors, neuro toxins, inorganic metals in investigating DNA methylation, histone modifications and miRNAs, linking embryonic development, cancer progress, neurodegenerative diseases, using human, animal, and *in vitro* studies. These research studies complemented by review articles that summarize our current understanding of environmental epigenetics and the role of such epigenetic alterations in the development of non-communicable diseases. We hope that our Research Topic will serve to educate, stimulate debate, and guide future research.

AUTHOR CONTRIBUTIONS

YL and QZ prepared the manuscript with extensive help from H-MB and TB. All authors contributed to the article and approved the submitted version.

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REVIEW
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Phthalate Exposure and Long-Term Epigenomic Consequences: A Review

Sudipta Dutta[1], *Diana K. Haggerty*[2†], *Daniel A. Rappolee*[3,4] *and Douglas M. Ruden*[3,5*]

[1] Department of Obstetrics and Gynecology, University of Nebraska Medical Center, Omaha, NE, United States, [2] Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI, United States, [3] Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility, CS Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI, United States, [4] Reproductive Stress, Inc., Grosse Pointe Farms, MI, United States, [5] Institutes for Environmental Health Science, Wayne State University School of Medicine, Detroit, MI, United States

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Edited by:
Yanqiang Li,
Houston Methodist Research Institute,
United States

Reviewed by:
Tomokazu Tomo Fukuda,
Iwate University, Japan
Dan Lou,
Johns Hopkins University,
United States

***Correspondence:**
Douglas M. Ruden
douglasr@wayne.edu

†Present address:
Diana K. Haggerty,
Human Nutrition, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI, United States

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Phthalates are esters of phthalic acid which are used in cosmetics and other daily personal care products. They are also used in polyvinyl chloride (PVC) plastics to increase durability and plasticity. Phthalates are not present in plastics by covalent bonds and thus can easily leach into the environment and enter the human body by dermal absorption, ingestion, or inhalation. Several *in vitro* and *in vivo* studies suggest that phthalates can act as endocrine disruptors and cause moderate reproductive and developmental toxicities. Furthermore, phthalates can pass through the placental barrier and affect the developing fetus. Thus, phthalates have ubiquitous presence in food and environment with potential adverse health effects in humans. This review focusses on studies conducted in the field of toxicogenomics of phthalates and discusses possible transgenerational and multigenerational effects caused by phthalate exposure during any point of the life-cycle.

Keywords: phthalates, epigenomics, DNA methylation, DOHAD, gestational exposure

INTRODUCTION

Phthalates or diesters of phthalic acid are a group of ubiquitous synthetic compounds commonly found in a variety of consumer products like plasticizers since the 1930s (Koch et al., 2013). Historically, the most prevalent one is a high molecular weight (HMW) phthalate, di-(2-ethylhexyl) phthalate (DEHP), which is used in the manufacture of PVC plastics found in items like containers, food packaging, vinyl flooring, furniture, and medical devices (Cirillo et al., 2013). Low molecular weight (LMW) phthalates, including diethyl phthalate (DEP) and butylbenzyl phthalate (BBzP), are used as solvents in the manufacture of daily use personal care products (e.g., perfumes, lotions, cosmetics, shampoo), paints, and adhesives (Buckley et al., 2012). Phthalates are not covalently attached to their substrates and can actively leach into the environment, food, and drinks and thereby enter the human body either through inhalation, digestion, or dermal absorption (Araki et al., 2014; Dewalque et al., 2014) (**Figure 1**). The persistence of phthalates in the environment makes them an emerging public health concern, as they have potential effects on reproduction, development, obesity, and other public health problems. In animals, phthalates are lipid soluble enabling easy storage in adipose tissue for long periods (Gutierrez-Garcia et al., 2019). In this review, we document several studies that suggest that phthalate exposure in humans, at real-world levels, might have some toxicological risk, with a focus on epigenetic changes associated with phthalate exposures.

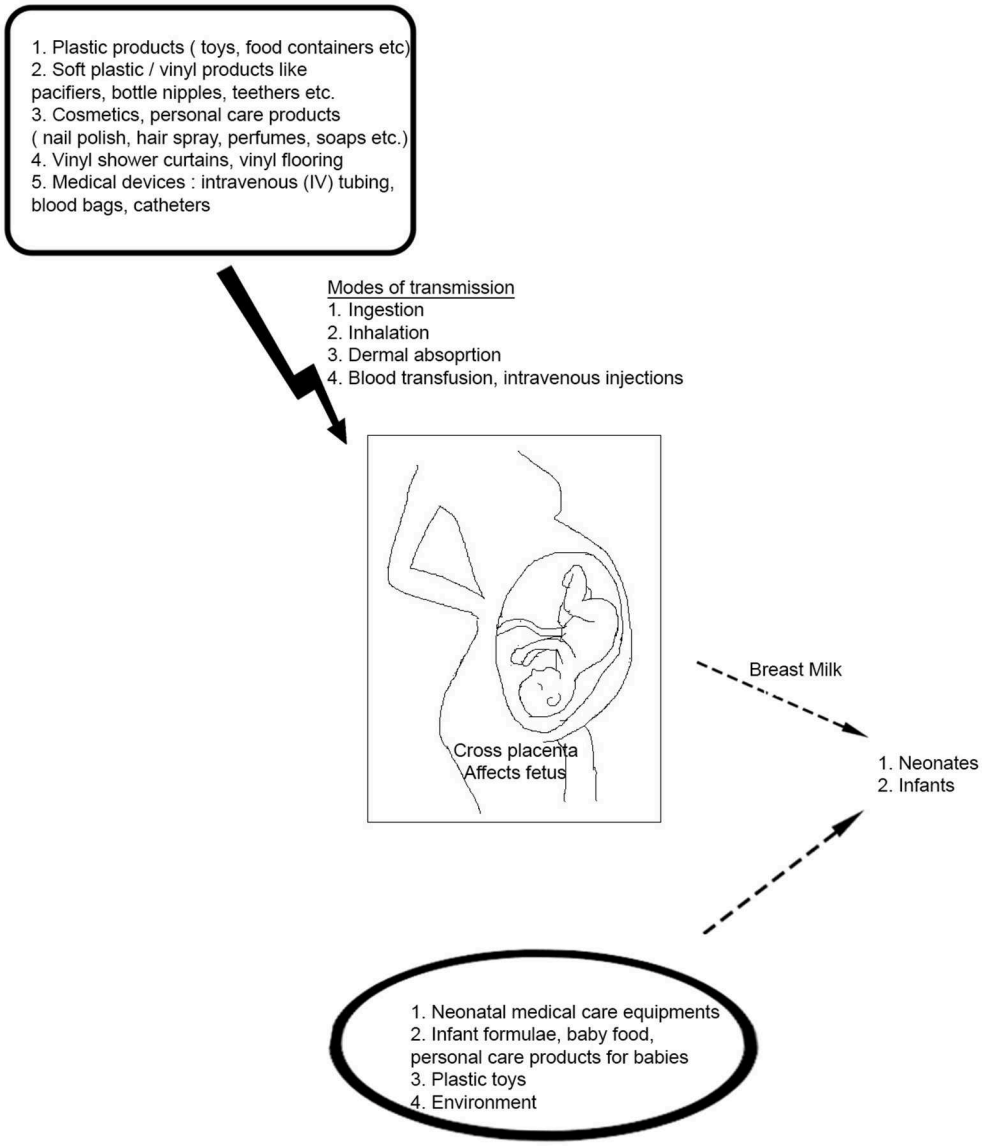

FIGURE 1 | Potential Sources of Phthalate Exposure in daily life. Phthalates have widespread applications in consumer products- they are used in a wide range of daily use household and personal care items starting from soaps, body lotions, and plastic containers to blood transfusion units. They can enter the human body through different routes like ingestion of foods, air inhalation, dust ingestion or dermal absorption. Phthalates can also cross the placenta and affect the developing fetus in a pregnant woman. Infants and neonates are also subjected to phthalate exposure via breast milk and from infant toys like pacifiers, bottle nipples, teethers, and neonatal medical care units.

Metabolism of Phthalates

Phthalates, on human exposure get hydrolyzed to their monoesters and then converted by P450 enzymes to their oxidative metabolites. The metabolites can also be transformed to glucuronide conjugates and released in the urine and feces (ATSDR, 1995, 2001, 2002; Silva et al., 2004). Phthalates which are low molecular weight (LMW) are mostly converted to their monoesters and excreted (ATSDR, 1995, 2001; Silva et al., 2004). DEHP, a commonly found phthalate, is hydrolyzed to its monoester, diethyl phthalate (DEP) and further metabolized in a multi-step pathway to oxidative metabolites which are detected in the urine (ATSDR, 2002). Recent studies suggest that dibutyl phthalate (DBP) and benzylbutyl phthalate (BzBP) are excreted in urine mostly as glucuronidated monoesters like monobutyl phthalate glucuronide (mBP-glu) and monobenzyl phthalate glucuronide (mBzP-glu). DEP is mostly excreted as free mono ethyl phthalate (MEP) (Silva et al., 2003, 2004) and DEHP is excreted as the glucuronidated form of its oxidative metabolites (Kato et al., 2004) (**Figure 2**). The 10 phthalates most commonly used in consumer products are dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), benzylbutyl phthalate (BzBP), dicyclohexyl

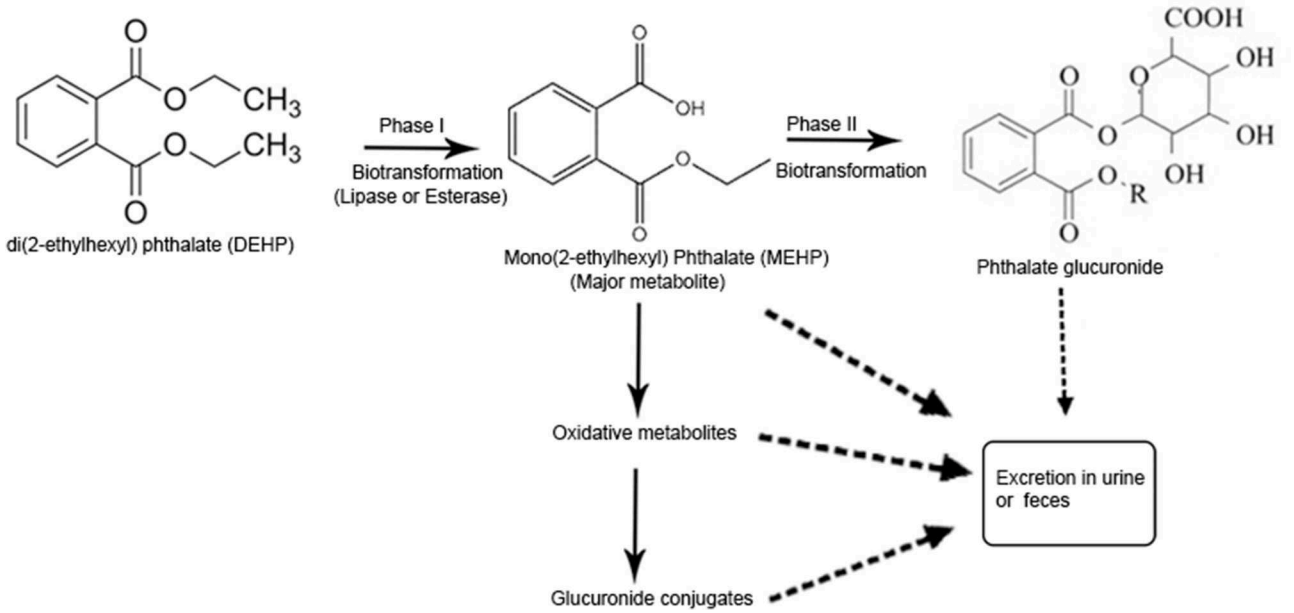

FIGURE 2 | Pathway of phthalate metabolism in human body. LMW phthalates are mainly excreted in urine and feces as a monoester, no further metabolism is required. During phase I hydrolysis, diester phthalates are hydrolyzed by the enzymes like esterases and lipases in the intestine and parenchyma to their respective monoesters. High molecular weight (HMW) phthalates such as diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), and dipropylheptyl phthalate (DPHP) have 9–13 carbon atoms in their chemical backbone and undergo further metabolism from monoesters via hydroxylation or oxidation and produce several oxidative metabolites which are excreted in urine within 24 h of exposure. Oxidative metabolites can also undergo phase II conjugation to form hydrophilic glucuronide conjugates which are excreted. Urinary phthalate metabolite is the most important biomarker for phthalate exposure [Adapted from the article, Metabolism of phthalates in humans by (Frederiksen et al., 2007)].

phthalate (DCHP), DEHP, di-n-octyl phthalate (DnOP), di-isononyl phthalate (DiNP), and di-isodecyl phthalate (DiDP) (Wang et al., 2019). Some of the major phthalate diesters and their metabolites are shown in **Figure 3**.

Overview of Epigenetics

This review focuses on the epigenetic changes associated with phthalate exposure which can potentially be heritable, such as DNA methylation of specific genes in germline cells. DNA methylation of specific genes can alter the expression of these genes without any change of the underlying DNA sequence in cell lineages that differentiate from the germline cells (Das et al., 2008). Epigenetic changes such as DNA methylation are thought to be at the interface of genetics and environment controlling fetal growth and development (Bestor et al., 1988; Okano et al., 1999; Kriaucionis and Heintz, 2009). Genomic DNA methylation occurs at the 5th position of cytosine to give rise to 5-methylcytosine (5mC), in the dinucleotide CpG on both DNA strands. Usually, >70% of CpGs are constitutively methylated in somatic tissues (Wu and Zhang, 2017; Meehan et al., 2018). The cumulative action of DNA methyltransferases (DNMTs) and DNA demethylation pathways help to propagate and maintain the DNA methylation patterns during development creating an unique epigenetic "landscape" to promote genome integrity and maintain cell type specific gene regulatory networks, imprinted gene activity, and repression of transposon activity (Wu and Zhang, 2017; Meehan et al., 2018). DNA methylation reprogramming can take place due to inhibition of DNMTs or *de novo* DNMT activity (Meehan et al., 2018).

In humans and other mammals, it is generally recognized that only stem cells contain the enzymes that can alter the DNA methylation profile of a cell. Embryonic stem cells (ESCs) originate from the inner cell mass at the blastocyst stage of a preimplantation embryo and can differentiate into the three germ layers of the embryo: the ectoderm, endoderm, and the mesoderm (Das et al., 2008; Jeon et al., 2017). While most, if not all cells contain the maintenance DNA methyltrasferase, Dnmt1 (Bestor et al., 1988), it is generally the case that only stem cells contain the "writers" of DNA methylation - Dnmt3a, and Dnmt3b (Okano et al., 1999). Therefore, the putative effects of phthalates on DNA methylation probably involves affecting a writer or an eraser in one of the stem cells in the lineage of the cells being investigated, such as the hematopoietic stem cells from which PBMCs (peripheral blood mononuclear cells) are derived.

Usually, stem cells and a few neuronal cells, contain the "erasers" of DNA methylation known as ten-eleven translocation (TET) family—Tet1, Tet2, and Tet3 (Kriaucionis and Heintz, 2009; Cimmino et al., 2011). Tet1/2 are present in embryonic stem cells and Tet3 is found in the germ line and zygote. All three TET proteins are expressed in blastocysts (Ito et al., 2010). Tet1 preferentially causes promoter demethylation while Tet2/3 act on enhancers (Hon et al., 2014; Huang Y.

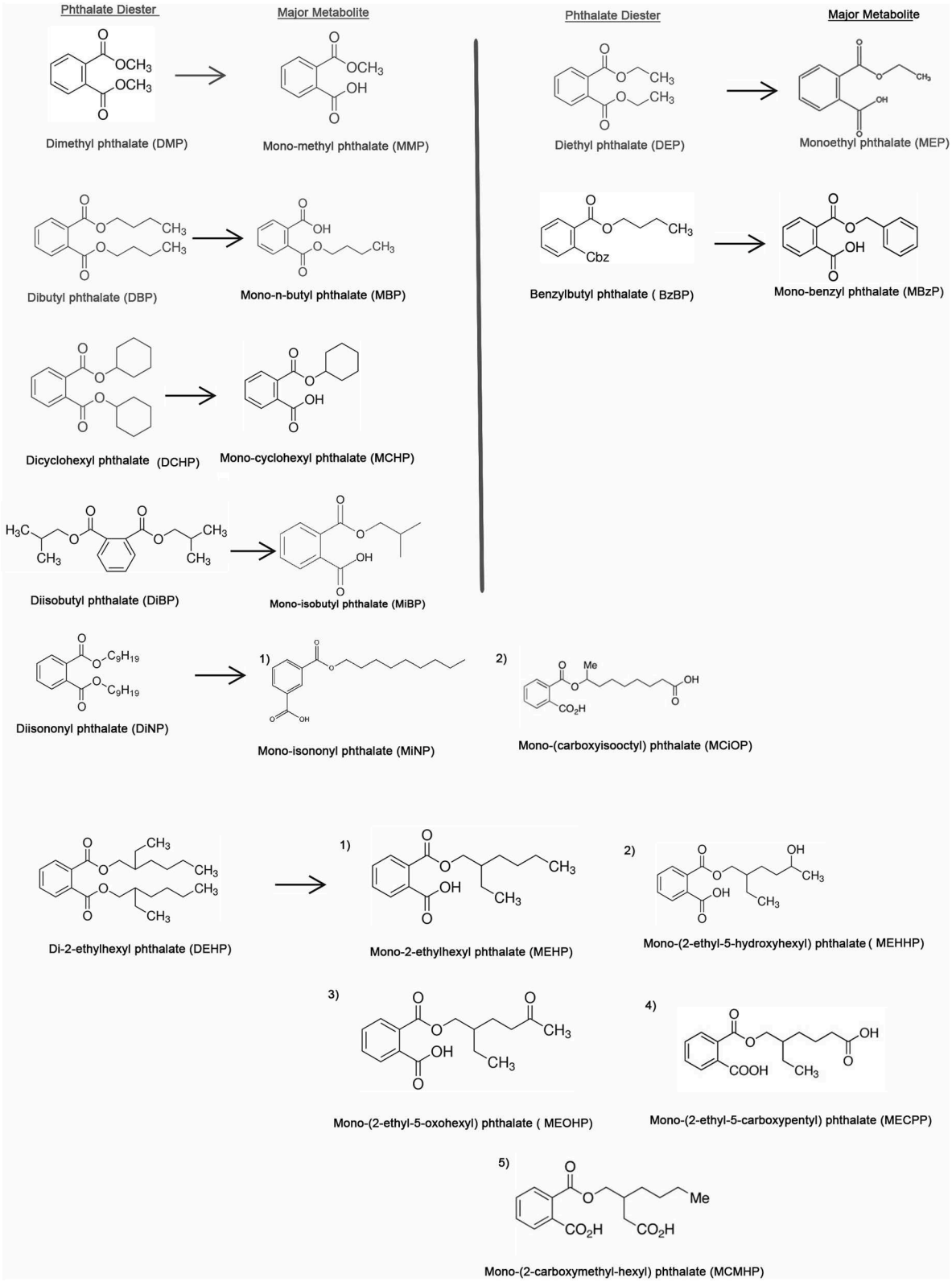

FIGURE 3 | Chemical structures of the top 10 major phthalates and their corresponding metabolites [adapted from the article, A Review of Biomonitoring of Phthalate Exposures by (Wang et al., 2019)].

et al., 2014).TET enzymes participate in DNA methylation dynamics by oxidation of 5mC to 5 hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) as intermediates in DNA demethylation pathways (Meehan et al., 2018). Tet2 is responsible for the vast majority of 5hmC generation (Lio and Rao, 2019). These DNA modifications serve as unique epigenetic signals (Nestor et al., 2012; Meehan et al., 2018). The profiles of 5hmC, 5fC, and 5caC are determined by active gene transcription and enhancer activity. They are less abundant than 5mC but are susceptible to environmental signals and can be used to identify the state of the cell (Meehan et al., 2018).

The most commonly used assays to analyze global DNA methylation levels due to environmental exposures in human cells are the Illumina Human Methylation 450K BeadChip (HM450K) and Illumina Human Methylation 850K BeadChip (EPIC). The HM450K array measures DNA methylation levels at over ∼450,000 CpG dinucleotides throughout the genome and has been replaced in 2016 by the EPIC array that measures over >850,000 CpG dinucleotides and and overlaps with about 90% of the sites represented by HM450K chip (Pidsley et al., 2016; Zhou W. et al., 2017). The HM450K and EPIC arrays have been used to study the effects of phthalates on DNA methylation in humans in PBMCs, whole blood, placental tissues, and sperm. In addition to DNA methylation changes induced by phthalates, we also discuss changes in small RNAs such as microRNAs which are thought to be epigenetically transmitted across generations.

EFFECT OF PHTHALATES ON DIFFERENT TISSUES

Embryonic Stem Cells (ESCs)

A cost-effective alternative to using laboratory animals in developmental and reproductive toxicity (DART) studies is using embryonic stem cells (ESCs) in so-called embryonic stem cell tests (ESTs). ESCs can make organismal decisions and ultimately give rise to the three main lineages of the embryo—the ectoderm, mesoderm, and endoderm. Human embryonic stem cell (huESC) models serve as *in vitro* models to analyze the epigenetic effects of phthalates on embryonic development since the huESCs form embryoid bodies, which is like an early stage of embryogenesis (Singh and Li, 2012b). *In vitro* differentiation of ESCs is regulated by specific culture conditions. Treatment of culture media with phthalates cause changes in specific gene expression profiles that are predictive of embryotoxicity (van Dartel et al., 2009). Specifically, the top three categories for phthalate toxicity are cardiotoxicity, hepatotoxicity, and nephrotoxicity and the most commonly caused diseases are cardiovascular, liver, urologic, endocrine, and genital diseases (Singh and Li, 2011).

Proper growth and full-term development of the fetus requires a healthy intrauterine environment. In a previous study, a significant relationship was observed between early pregnancy loss ($n = 48$) and elevated periconceptional mono-2-ethylhexyl phthalate (MEHP) exposure (mean: 23.4 ng/mL) (Toft et al., 2012). Since ESCs give rise to all three germ layers-ESCs especially huESCs, serve as an excellent *in vitro* platform to study developmental toxicity of phthalates during pregnancy (Shi et al., 2013). Several labs hypothesize that exposing ESCs to phthalates in culture and determining how this affects their epigenome and differentiation into different lineages might help us to understand the phthalate concentrations which are toxic. In one study, it was revealed that treatment of mouse embryos with a final concentration of 10^{-3} M mono-n-butyl phthalate (MBP) affected the developmental competency, and exposure to 10^{-4} MBP resulted in the delay of progression from embryo to blastocyst (Chu et al., 2013).

Several phthalate esters have been shown to exert developmental toxicity as determined by *in vivo* tests in animals and also by *in vitro* tests such as whole embryo culture (WEC) and embryonic stem cell tests (ESTs) (Shi et al., 2013). In one study (Shi et al., 2013), the authors tested the toxic effects of MEHP on two cell lines of huESCs—CH1, established in their lab with a Chinese female genetic background and H1 with Caucasian male genetic background. MEHP in low concentrations (25 μmol/L or 4,103 ng/mL) after 8 days of treatment in culture did not cause any cytotoxicity but did cause changes the gene expression pattern of several differentiation genes. MEHP in a high concentration (1,000 μmol/L or 164,110 ng/mL) decreased the expression of genes related to mesoderm and the primary germ cells, induced cytotoxicity, and reduced cell proliferation and viability (Shi et al., 2013). Studies examining the effects on *in vitro* expansion of human hematopoietic cells from umbilical cord blood found that four phthalates—DBP, benzyl butyl phthalate (BBP), DEP, and DEHP decreased the cell expansion with DBP being the most cytotoxic (Gutierrez-Garcia et al., 2019).

Peripheral Blood Mononuclear Cells and Whole Blood Are the Cells-of-Choice to Study the Epigenetic Effects of Environmental Exposures

An ideal *in vitro* research model to study the exposure of xenobiotics in humans is human peripheral blood. After exposure to an environmental toxicant, peripheral blood samples express the hallmarks of epigenetic dysregulation within hours (Baccarelli and Ghosh, 2012). A study (Sicinska, 2019) was conducted to evaluate the effect of DBP, BBP, and their metabolites: MBP, mono-benzylphthalate (MBzP) on apoptosis in human peripheral blood mononuclear cells (PBMC)s after incubation periods of 12 h and/or 24 h. The concentrations tested were in the range of 1 to 100 μg/mL similar to the levels detected in general population exposure (0.02–8 μg/mL) (Chen et al., 2008; Lin et al., 2008; Wan et al., 2013). It was demonstrated that there was a reduction in cell viability after 12 h incubation of PBMCs with phthalates- first by BBP followed by DBP. In the 24 h incubation group, DBP exerted the earliest changes in cell viability, followed by BBP, and by both metabolites (MBP and MBzP) (Sicinska, 2019). To elucidate the mechanism of programmed cell death induced by phthalate exposure, the changes in the level of calcium ions (Ca^{2+}), transmembrane mitochondrial potential ($\Delta\Psi m$) and caspase −8,−9,−3 activity were determined. It was seen that phthalates particularly DBP

and BBP increased levels of Ca^{2+} and reduced $\Delta\Psi m$ of the PBMCs. Phthalates also increased the activity of the caspases, the most significant being caspase−9 (Sicinska, 2019).

A study (Glue et al., 2002) was carried out to estimate the immunotoxicological effects of monophthalates, specifically the cytokine production profiles of MBP, monobenzyl phthalate (MBEP), MEHP, mono-n-octyl phthalate (MOP), mono-isononyl phthalate (MINP), mono-iso-decyl phthalate (MIDP) which are major metabolites of some commonly used phthalates. The monophthalates were used at a concentration of 400 mg/mL. The studies demonstrated that MBP is the only phthalate that lead to an increase in the gene expression of IL-4 with no concomitant increase in the gene expression of IL-5 and IFN-γ. When data was grouped from all phthalate stimulations, there was a significant increase in gene expression of several inflammatory cytokines like IL-4, IL-5, and INF- γ gene (Glue et al., 2002).

Dendritic cells are critical in the development of allergic diseases (von Bubnoff et al., 2001). PBMCs contain the precursors for DC and are frequently exposed to various environmental toxicants. Thus, DCs derived from PBMCs are an excellent model to study immunological responses due to phthalate exposure (Ito et al., 2012). It was seen that only DEHP (not MEHP) at a concentration of 10 μM significantly reduced the expression of markers of maturation and differentiation in DC like CD11c, CD40, CD80, CD86, and CD205 in PBMC-derived DCs of NC/Nga mice. The effects of DEHP on PBMC-derived DCs were partially restored when the cells were treated with an estrogen receptor (ER) antagonist—ICI 182,780 (Ito et al., 2012). Taken together, these findings infer that DEHP attenuated the maturation of PBMC-derived DCs through ER activation.

A study was conducted to compare HM450K and EPIC BeadChips to measure DNA methylation at birth and adolescence. The study used whole blood samples from Mexican-American newborns and 14-year old children ($n = 109$ and $n = 86$, respectively) residing in Salinas Valley, California (Solomon et al., 2018). The overall per-sample correlations analyzed on HM450K and EPIC in both samples was strong ($r > 0.99$), though correlations of individual CpG sites with low variance of methylation were modest (median $r = 0.24$). There was also a subset of CpG sites that had large differences in the mean methylation beta-estimates between the two platforms (Solomon et al., 2018). Finally, the estimates of cell type proportion prediction by the two platforms showed strong correlations in both samples, and differences in boys and girls were successfully replicated across the two platforms (Solomon et al., 2018).

Placenta

The placenta plays a pivotal role in maintaining the appropriate intrauterine environment by delivering nutrients and oxygen to the developing fetus. It also serves as an important endocrine organ by secreting several hormones plus signaling molecules essential for maintaining the maternal physiology during pregnancy and regulating fetal growth. The placenta is essential to the Developmental Origins of Health and Disease (DOHaD) hypothesis, which posits that *in utero* events program our responses to the environment after birth, and the placenta is important to this process (Gillman et al., 2007; Strakovsky and Schantz, 2018). Any perturbations to the maternal intrauterine environment negatively impacts the long term health status of an infant by acting as a risk factor for adulthood diseases such as cardiovascular disease, obesity, and cancer (Barker, 1997; Nilsson et al., 2012; Zoeller et al., 2012; Radford et al., 2014).

Studies of DNA Methylation in Placental Tissue

A recent study investigated how phthalates impair human placental function by epigenetic regulation of critical placental genes (Grindler et al., 2018). The authors looked at epigenome-wide DNA methylation and gene expression using the Agilent whole human genome array and found associations between phthalate exposures during the first trimester of pregnancy and 39 genes with altered DNA methylation and gene expression in the group of women who were highly exposed to phthalates. Further analysis determined epidermal growth factor receptor (EGFR) to be a critical candidate gene that mediates the relationship between exposure to phthalates and early placental function (Grindler et al., 2018). Thus, phthalates may alter the expression of placental genes by epigenetic regulation and thereby affect its regular activity.

The level of phthalates in the third trimester urine of pregnant women was associated with reduced placental long interspersed nucleotide elements (LINE-1) methylation and low birth weight. Placental LINE-1 methylation might serve as a biomarker for environmental exposure causing adverse fetal growth as fetal programming is regulated by appropriate methylation patterning (Zhao et al., 2015).

Genomic imprinting is an epigenetic phenomenon by which genes are methylated to reflect parent of origin expression. The paternally expressed gene Insulin-like growth-factor 2 (IGF2) and maternally expressed H19 both on chromosome 11 are two reciprocally critical imprinted genes and play significant roles in fetal and embryonic growth. Inverse associations were observed between *IGF2* and *H19* differentially methylated regions (DMRs) in placenta and prenatal exposure to HMW phthalate metabolites. Abnormal IGF2/H19 methylation in placenta suggests the fact that the developing fetus may be exposed to an adverse intrauterine environment (LaRocca et al., 2014).

It was demonstrated in at least two studies using human placenta that phthalate exposure during pregnancy was inversely associated with DNA methylation on selected candidate genes like H19 and insulin-like growth-factor 2 (*IGF2*) which are important in embryonic growth and development. These associations were very predominant in fetal growth restriction (FGR) newborns as opposed to normal neonates (LaRocca et al., 2014; Zhao et al., 2016). The placenta persists from the earliest stages of pregnancy through delivery and is responsible for nutrient and gas exchange, waste elimination and thermo-regulation of the developing fetus via mother's circulation. The epigenetic markers in the placenta from an uncomplicated pregnancy/birth vs. a complicated one can thus serve as good indicators of exposures both from intrauterine and extrauterine environments (Rossant and Cross, 2001; Nelissen et al., 2011).

Maternal exposure to phthalates also leads to fetal exposure, as these chemicals can diffuse through the placental barrier and thereby modulate the intrauterine environment (Latini et al., 2003a). In a study designed to investigate the *in utero* effects of human exposure of DEHP and its main metabolite, MEHP, it was observed that phthalate exposure decreased the duration of pregnancy resulting in preterm birth (Latini et al., 2003b). In a sample of 84 newborns, which included 11 preterm births, three very low birthweight newborns, four small-for-gestational-age (SGA) newborns—DEHP, MEHP, or both were found in 88.1% of the cord blood samples, and DEHP and MEHP were individually found in 77.4% of the samples (Latini et al., 2003b). Mean concentrations of DEHP and MEHP in cord blood samples were 1.19 ± 1.15 and 0.52 ± 0.61, μg/mL respectively. Moreover, MEHP-positive newborns showed a significantly decreased gestational age as opposed to MEHP-negative infants ($p = 0.033$) (Latini et al., 2003b).

It has been hypothesized that phthalates may elicit an intrauterine inflammatory response leading to shortened gestation (Goncalves et al., 2002; Latini et al., 2003b). A structural similarity has been observed between DEHP and the proinflammatory mediators like prostaglandins and thromboxanes (Maroziene and Grazuleviciene, 2002). There were also reports of DEHP-induced interleukin-1 secretion in mononuclear cells and in babies born to mothers who suffered from prenatal infection and inflammation (Calo et al., 1993; De Felice et al., 1999, 2002; Yang et al., 2000). Case-control studies to study preterm birth in pregnant women revealed that increased levels of ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) family-ADAMTS4, ADAMTS5 and proinflammatory cytokines like interleukin (IL)-6, and tumor necrosis factor-α (TNF-α) in mid-trimester amniotic fluid associated with spontaneous preterm delivery (Ronzoni et al., 2018; Melekoglu et al., 2019). These data suggest that the toxicity of periconception phthalate exposure and phthalates' capacity to disrupt pregnancy and birth via endocrine and inflammatory pathways impact development and health across the lifespan.

Cord Blood

Umbilical cord blood (representing fetal blood) and whole blood samples for 9-year olds were examined to investigate the relationship between *in utero* phthalate exposure and methylation of repetitive elements, Alu and LINE-1. A consistent inverse relationship was observed between prenatal concentrations of MEHP and cord blood methylation of Alu repeats for early and late pregnancy, and a similar association was observed with LINE-1 methylation (Huen et al., 2016). In a longitudinal pregnancy cohort study from California that recruited Mexican-American women and their children, increases in prenatal urinary concentrations of DEHP metabolites gave rise to decreased methylation of Alu repeats. Pyrosequencing of bisulfite-treated DNA was used to analyze the methylation of Alu and LINE-1 (Huen et al., 2016). This observation suggests that prenatal phthalate exposure leads to differences in methylation of repetitive elements and thus epigenetics may be the mechanism by which phthalates exert their transgenerational effect.

A birth-cohort study involving cord blood samples from 64 infant–mother pairs at Taiwan measured DNA methylation levels using the HM450K array was discussed in the introduction section (Chen et al., 2018). Only 25 CpG sites in cord blood with altered methylation levels were significantly associated with DEHP exposure during perinatal period. Gene-set enrichment analysis (GSEA) identified androgen response genes, estrogen response genes, and spermatogenesis genes among the genes enriched via changes in DNA methylation after prenatal DEHP exposure (Chen et al., 2018). Inverse associations were found between maternal phthalate metabolite levels and gestational age, birth weight, birth length, and BMI. Taken together, these studies demonstrate that phthalate exposure *in utero* may impact DNA methylation in cord blood. These changes in DNA methylation may be candidates for biomarkers used to ascertain maternal exposure to phthalates during pregnancy and potential candidates for studying the underlying mechanisms of the long-term effects of phthalates and also the ways phthalates may impact health throughout the life course (Chen et al., 2018).

Effect of Phthalates as Endocrine Disrupting Chemicals on Embryonic Stem Cells and *in utero*

Phthalates can act as endocrine disrupting chemicals (EDCs) by exerting strong antiandrogenic (Doyle et al., 2013; Martino-Andrade et al., 2016) and weak estrogenic (Lee et al., 2012; Huang P. C. et al., 2014) effects. Though, EDC exposure can be harmful at any stage of human life, the developing human fetus in a phase of rapid proliferative growth *in utero* may be particularly vulnerable (Gutierrez-Garcia et al., 2019). The *in utero* environment or the preconception time period has been regarded as the most vulnerable period of growth and development to environmental insults (Chapin et al., 2004). Additionally, *in utero* phthalate exposure has been associated with pre-term birth (Ferguson et al., 2014), pre-eclampsia (Cantonwine et al., 2016), reduced birth size (Whyatt et al., 2009), sex-specific changes to childhood growth and high blood pressure (Valvi et al., 2015), deficits in neuro-endocrine development (Engel et al., 2010; Kim et al., 2011; Factor-Litvak et al., 2014), and impaired male reproductive health (Cai et al., 2015; Swan et al., 2015). Phthalates can also affect the thyroid hormone balance (Araki et al., 2014) which leads to metabolic dysfunction in adults. Optimal maternal thyroid function during early pregnancy is essential for proper fetal brain development (Chen and Xue, 2018; Ghassabian and Trasande, 2018; Levie et al., 2018; Prezioso et al., 2018).

Phthalates are endocrine disrupting chemicals (EDCs) that mimic the natural hormones found in the human body and thus interfere or impair normal hormonal activity (Grindler et al., 2018). The primary female sex hormones, estrogen (E_2), and progesterone (P_4), play important roles in regulating the menstrual cycle, pregnancy, and embryogenesis in humans and other species (Bouman et al., 2005; Hong et al., 2016; Jeon et al., 2017). Apart from that, they also have an effect in regulating the pluripotency of huESCs. E_2 and P_4 treatment on human ESCs in a feeder-free culture protocol decreases the pluripotency

of human ESCs by inhibiting the expression of pluripotency-associated markers like POU class 5 homeobox 1 (*POU5F1), sex determining region Y-box 2 (SOX2)*, and *NANOG* homeobox genes at both transcriptional and translational levels. The cells growing in control culture media without any hormones assumed the form of tightly packed cells growing in a monolayer with clean and defined edges and showed no signs of differentiation. These cells also expressed several markers specific for undifferentiated ES cells including POU5F1, SOX2, and NANOG (Jeon et al., 2017).

The female sex hormones E_2 and P_4 also alter the protein expression of markers for the epithelial-to-mesenchymal transition (EMT). E_2 or P_4 treatment increases the protein expression levels of N-cadherin, Snail and Slug, which are highly expressed in mesenchymal cells and decreases E-cadherin expression, which is highly expressed in epithelial cells. Phthalates exert weak estrogenic effects (Lee et al., 2012; Huang P. C. et al., 2014) and thus they can perturb normal pregnancy by decreasing the pluripotency of the stem cells in developing pre-implantation embryos (Jeon et al., 2017). Further validation studies involving treatment of E_2 and P_4 in combination with estrogen and progesterone receptor inhibitors (ICI 182,780 and RU486 respectively) found the effects of hormones on EMT and pluripotency of ES cells were restored to control levels. These findings indicate that E_2 and P_4 regulation of EMT and pluripotency of human ES cells are mediated by their receptors (Jeon et al., 2017). Bone marrow (BM) hematopoietic stem progenitor cells express functional receptors for follicle-stimulating hormone (FSH), and luteinizing hormone (LH). *In vitro and in vivo*, pituitary sex hormones such as FSH, LH, and prolactin (PRL) stimulate hematopoietic stem progenitor cells to proliferate. These cells also proliferate in response to gonadal sex hormones like androgen, estrogen, and progesterone (Carreras et al., 2008; Maggio et al., 2013; Nakada et al., 2014; Mierzejewska et al., 2015). These data elicit an interesting avenue where phthalates can interrupt normal embryonic growth and development by impairing the pluripotency of ESCs and by causing a misregulation of the EMT.

Germ Cells (Eggs and Sperm) and Ano-Genital Distance

Epigenetic Changes in Sperm

Current research findings suggest that several adulthood diseases are programmed *in utero* as a result of maternal exposures to endocrine disruptors like DEHP. Those diseases are linked with critical genes which are epigenetically modified by DNA methylation or modification of histone tails (Martinez-Arguelles et al., 2009; Wu et al., 2010; Anderson et al., 2012; Strakovsky and Pan, 2012; Ayala-Garcia et al., 2013). The first reprogramming event occurs in the genome of the germ cell precursors while they colonize the embryo's urogenital crest. This imprinting event creates an epigenetic memory on the gamete's genome that represents the environment around them while they are committed to the gamete lineage (Jaenisch et al., 2004; Surani et al., 2004). In sexually mature males, a second round of gamete epigenetic reprogramming occurs during differentiation to give rise to spermatozoa. The spermatogonial populations can constantly reedit the epigenetic information, which enables them to inherit an updated epigenetic print that reflects the varying environmental situations. It is hypothesized that such epigenetic reprogramming permits the spermatozoa of several generations to provide updated information about the environment during consecutive periods of fertilization and transmit this information to the offspring. The next event of epigenetic reprogramming of both the paternal and maternal chromosomes occurs shortly after fertilization. Thus, experiments which involve prenatal and postnatal exposure to phthalates may unravel mechanism underlying the epigenetic modulation of gene expression for phenotypic variability between individuals and across species (Ayala-Garcia et al., 2013).

One study in rats reports that *in utero* exposure to DEHP impaired testicular function through changes in DNA methylation (Sekaran and Jagadeesan, 2015). It was observed in a separate study that *in utero* exposure to DEHP in rats was associated with both transgenerational DNA methylation in sperm and testicular and prostate diseases (Manikkam et al., 2013), while another study in rats reported that *in utero* exposure to DEHP alters DNA methylation throughout the epigenome, particularly in CpG islands (Martinez-Arguelles and Papadopoulos, 2015).

Various animal studies have established the fact that *in utero* phthalate exposure gives rise to transgenerationally inherited reproductive defects by altering sperm DNA methylation (Manikkam et al., 2013; Iqbal et al., 2015; Prados et al., 2015). To address the relevance of epigenetic reprogramming of sperm in humans, a study was conducted to examine the relationship of pre-conception urinary phthalate with sperm DNA methylation profiles in men undergoing fertility treatment in IVF clinics (Wu et al., 2017a). In a study performed by HM450K analyses, 131 sperm DMRs were correlated with at least one preconception urinary metabolite. The DMRs were typically clustered with genes responsible for growth and development and other functions like cellular movement and cytoskeleton structure. Most sperm DMRs were associated with phthalate metabolites like MEHP, mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), MBP and cyclohexane-1, 2-dicarboxylic acid-monocarboxy isooctyl (MCOCH), which are anti-androgenic. Furthermore, 13% of sperm DMRs could determine reproductive success and were attributable for diminished quality blastocyst-stage embryos after *in vitro* fertilization (IVF) (Wu et al., 2017a,b).

Ano-Genital Distance

The anogenital index (AGI) is defined as anogenital distance (AGD) divided by weight at examination [AGI = AGD/weight (mm/kg)]. The rationale for measuring AGD is that males have a shorter AGD than females and consequently, changes in this distance are a measure of feminization or masculinization of the reproductive organs. In a study that aimed to correlate prenatal exposure to phthalates in amniotic fluid, maternal urine, and health of newborns in humans, it was found that *in utero* exposure to MBP was associated with a shortened AGI in female newborns, although no correlation was found between prenatal phthalate exposure *in utero* and AGI in male

newborns (Huang et al., 2009). In a study that involved analysis of 106 boys aged around 12.8 months in the population, genital measurements (including AGD) were measured in relation to concentrations of phthalate metabolites in maternal prenatal urine samples. AGD was significantly and inversely related to maternal urinary concentrations of metabolites of DEHP. Incomplete testicular descent was also observed in those boys (Swan et al., 2005; Swan, 2008). These findings demonstrate that phthalate exposure may disrupt human male genital development. Females may experience reproductive toxicity due to phthalates as severe as that observed in males (Benjamin et al., 2017). Recent reports suggest that high urinary phthalate concentrations were linked with delayed attainment of puberty in girls (Frederiksen et al., 2012), increased endometriosis risk (Cobellis et al., 2003; Masuyama et al., 2003; Upson et al., 2013), low yield of oocytes (Hauser et al., 2016), increased incidences of infertility (Du et al., 2016), and increased clinical pregnancy loss (Mu et al., 2015; Hauser et al., 2016). To model some of these effects of phthalates seen in humans in mammalian models, there are several reports that prenatal phthalate exposure affects testicular function and is responsible for decreasing anogenital distance (AGD) in male rodents (Mylchreest et al., 1998; Wolf et al., 1999; Gray et al., 2000; Ema et al., 2003; Tyl et al., 2004; Carruthers and Foster, 2005; Andrade et al., 2006).

PHTHALATE EXPOSURE ACROSS THE LIFESPAN

Phthalate Exposure in Neonatal Intensive Care Units

DEHP is primarily used to soften PVC plastic in medical apparatus like blood bags, or bags used for intravenous administration of nutrients, drugs, and fluids. DEHP can leach out from the plastic that is used to make all of these bags to enter the patient's circulation during procedures like transfusion, heart bypass surgery, or the administration of intravenous fluids (Latini, 2000; Tickner et al., 2001). Based on a report by the Center for Devices and Radiological Health, U.S. Food and Drug Administration, devices used in Neonatal Intensive Care Units (NICU) are a prime concern because newborns undergoing procedures using these medical devices may be exposed to DEHP levels ranging from 130 to 6,000 μg/kg bw/day (Hillman et al., 1975; Plonait et al., 1993; Latini and Avery, 1999; Latini, 2000; Loff et al., 2000; Food U. S. Drug Administration, 2001; Tickner et al., 2001). The NICU babies are exposed to plastic tubing, blood bags, IV drips, etc. Particularly in a NICU setting, neonates would be small for two main reasons: (i) they might be preterm which makes them very underweight since they were born early; (ii) neonates who had intrauterine growth restriction (IUGR) develop many health complications and may be small for their gestational age (SGA). Children, due to their low body weights and their underdeveloped organs, are at a higher risk than adults to phthalate exposure. In fact, people of all ages who undergo such medical procedures are exposed to fairly high levels of DEHP (Shelby, 2006).

Asthma

An epidemiological study linked phthalate exposure to lower DNA methylation of TNFα which is an inflammatory cytokine that may increase asthma risk in children (Wang et al., 2015). Differential methylation patterns have been observed in three genes-*androgen receptor* (*AR*), *TNF*α, and *IL-4* causing asthma in children (Wang et al., 2015). TNFα 5'CGI is a potential epigenetic biomarker for uncovering a phthalate mechanism in childhood asthma research. Hypomethylation of TNFα 5'CGI gives rise to increased TNFα protein levels which may give rise to allergic inflammation (Wang et al., 2015). TNFα is present in high concentrations in bronchoalveolar fluid derived from the airways of asthma patients (Broide et al., 1992; Berry et al., 2006). A study involving a transgenerational asthma model in mouse demonstrated that maternal exposure to BBP could cause allergic airway inflammation in the offspring over 2 generations (F2). In the offspring, BBP induced global DNA hypermethylation in CD^{4+} T cells (Jahreis et al., 2018).

Lipid Metabolism

It was observed that exposure to DEHP *in utero* in pregnant mice induces excessive visceral fat accumulation, affects lipid metabolism and adipogenesis in their F1 offspring (Gu et al., 2016). The study was conducted with pregnant C57BL/6J mice who were administered with DEHP (0.05 mg/kg/day) from gestational days 1-19, the pups had significantly higher levels of serum leptin, insulin, lipid, and fasting glucose concentrations than the control pups. DEHP-exposed pups also had excessive visceral fat accumulation compared to control pups. These metabolic disorders were hypothesized to be induced by elevated levels of mRNA expression of T-box 15 (*Tbx15*) and glypican 4 (*Gpc4*) in subcutaneous and visceral adipose tissues respectively. *Tbx15 and Gpc4* are known to be developmental genes which play a role in obesity and body fat distribution in mice (Gesta et al., 2006; Gu et al., 2016).

In a longitudinal study involving 250 Mexican American children, the relationship between perinatal exposure to phthalates and adiposity during the peri-adolescence (between ages 8 and 14 years) was evaluated (Bowman et al., 2019). Among girls, adiposity was associated with exposure to MBP, MiBP, and MBzP. First trimester maternal urine concentrations of MiBP were associated with increased values for skinfold thickness, BMI-for-age, and waist circumference in girls ($p < 0.01$) as opposed to control samples. *H19* methylation was positively associated with skinfold thickness in girls. There were sex-specific differences in exposure outcomes- among boys, adiposity was inversely associated with second trimester and adolescent MBzP (Bowman et al., 2019).

Lipid metabolism is important for synthesis of steroid hormones and phthalate-driven aberrant lipid metabolism disrupts the normal metabolic and reproductive processes (Moody et al., 2019). In this study (Moody et al., 2019), it was demonstrated that when pregnant Long-Evans rats were administered mixture of phthalates 0 (CON), 200 (LO), or 1,000 (HI) mg/kg body weight/day during the perinatal period-the male offspring for both groups at PND90 had higher body weights than control. Sterol regulatory element binding proteins

(SREBPs) have been hypothesized to play a pivotal role in phthalate-induced metabolic dysregulation (Johnson et al., 2011; Zhang et al., 2017). In both testis and adipose tissue of males belonging to the HI phthalate dosage, gene expression of lipid metabolism pathways were dysregulated. *Srebf1* expression was reduced in testis whereas *Srebf2* was upregulated in adipose tissue. DNA methylation was increased at two loci in testis of HI rats and reduced at another site surrounding Srebf1 transcription start site. Simultaneously, in rats belonging to the HI phthalate dosage group- in the adipose tissue increased DNA methylation at one region was observed within the first intron of *Srebf2* (Moody et al., 2019). Thus, phthalate exposure impairs metabolism of lipids by DNA methylation through tissue-specific changes in gene expression.

Adulthood Diseases

Obesity

Phthalates are associated with a number of diseases in adults due to their endocrine-disrupting abilities (Gore et al., 2015). One of the common disorders associated with EDCs is obesity in children and adults (Biemann et al., 2014; Di Ciaula and Portincasa, 2019). Obesity is recognized as a public health epidemic in both developed and developing countries. As per convention, for adults (individuals above the age of 18 years), overweight is defined as having a body-mass index (BMI) greater than or equal to 25 and lower than 30 and obesity is defined as having a BMI greater than or equal to 30 (Ng et al., 2014). A statistic from 188 countries indicates that between 1980 and 2013, combined percentage of overweight and obesity has increased by 27.5% for adults and 47.1% for children (Ng et al., 2014). For both developed and developing countries, the proportion of adults with a BMI of 25 or greater increased from 28.8% in 1980 to 36.9% in 2013 for men and from 29.8% to 38% for women (Ng et al., 2014). The peroxisome proliferator-activated receptor (PPAR)ϒ, a nuclear receptor is regarded as the master regulator of adipogenesis and regulates the expression of metabolic genes during differentiation (Janesick and Blumberg, 2011; Stel and Legler, 2015).

Obesogenic EDCs have the ability to stimulate adipogenesis and fat storage and increase the chances for obesity by activating PPARϒ (Stel and Legler, 2015). PPARϒ acts on the differentiation pathway connecting multipotent stromal stem cells to mature adipocytes (Janesick and Blumberg, 2011; Watt and Schlezinger, 2015). PPARϒ regulates histone deacetylation, changes in DNA methylation and modulates a series of mechanistic pathways leading to increase in adipocyte formation and fat storage (Tabb and Blumberg, 2006; Blumberg, 2011; Janesick and Blumberg, 2012; Rajesh and Balasubramanian, 2014; Stel and Legler, 2015; Watt and Schlezinger, 2015). One study using a primary mouse bone marrow culture model demonstrated that phthalates interact with PPARs and regulate the expression of genes involved in adipocyte differentiation, adipogenesis, and metabolic processes like lipid and glucose homeostasis (Desvergne et al., 2009; Grygiel-Gorniak, 2014; Watt and Schlezinger, 2015). The Developmental Origins of Health and Disease (DOHaD) hypothesis is a paradigm in which prenatal and perinatal exposure to environmental factors plays a pivotal role in determining life-long patterns of health and disease (Gluckman and Hanson, 2004). The Newcastle thousand families study, which consisted of 932 members of thousand families 1947 birth cohort, aimed to track whether being overweight in childhood increases the risk of adult obesity (Wright et al., 2001). The study examined 412 subjects at age 50 and found that BMI at age 9 years was significantly correlated with BMI at age 50 and only children who were obese at 13 had an increased risk of obesity during adulthood (Wright et al., 2001). Maternal urinary levels of mono-3-carboxypropyl phthalate (MCPP), a non-specific metabolite of multiple phthalates, caused childhood obesity in a study that recruited 707 children from three prospective cohort studies in the USA between 1998 and 2006 (Buckley et al., 2016). The study explored the relationship between maternal urinary phthalate metabolite concentrations during pregnancy with weight and height of children at ages 4 to 7 years (Buckley et al., 2016). Metabolites of DEP and DEHP were associated with sexually dimorphic effects on BMI and ΣDEHP was inversely related with BMI z-scores among girls, but no association was noted in boys (Buckley et al., 2016). Children's Health and Environmental Chemicals in Korea (CHECK) Study recruited 128 healthy pregnant women and their newborns (65 boys and 63 girls)—levels of DEHP metabolites were measured in maternal blood, urine, placenta, and cord blood samples as well as newborns' urine (Kim et al., 2016). The study revealed that DEHP exposure may decrease ponderal index (PI) and increase triglyceride (TG) levels in newborn infants especially boys (PI, $\beta = -0.13$, $p = 0.021$; and TG, $\beta = 0.19$, $p = 0.025$) causing increase in body mass in early life. This observation also suggested that *in utero* exposure to DEP and DEHP was positively associated with body mass change of the newborns during first 3 months after birth (Kim et al., 2016).

Men's Reproductive Health

The major male reproductive anomaly associated with phthalates is "testicular dysgenesis syndrome" which is characterized by hypospadias, cryptorchidism, undescended testes, reduced anogenital distance, reduction in sperm count and quality, sterility, and occurrence of testicular cancer (Sharpe and Skakkebaek, 2008; Swan, 2008). Anogenital distance (distance between anus and genitalia) is the most sensitive marker for estimating the impact of phthalates in human males; this anomaly is associated with prenatal exposure of the male fetus to phthalates while in the womb (Swan et al., 2005; Marsee et al., 2006; Suzuki et al., 2012). Phthalates bind to histone tails thus regulating the extent of DNA enclosed by it and thereby alter the availability of genes which can be activated (Wu et al., 2010; Manikkam et al., 2013). One study (Wu et al., 2010) showed that in mice, maternal exposure of DEHP caused testicular dysfunction which was mediated by DNA hypermethylation, leading to increased expression of DNA methyltransferases and downregulated production of insulin like hormone-3, a gene responsible for testosterone production. Phthalate monoesters like mono-n-butyl phthalate (mBP), mono-ethyl phthalate (mEP) found in human breast milk have a positive correlation with postnatal surge of hormones like serum hormone binding globulin (SHBG) in newborn boys. Phthalates like mono-methyl

phthalate (mMP), mono-ethyl phthalate (mEP), and mono-n-butyl phthalate (mBP) were directly related with the ratio of LH:free testosterone and mono-isononyl phthalate (miNP) with luteinizing hormone (LH). mBP was negatively correlated with free testosterone and these hormonal imbalances could be a sign of testicular dysgenesis (Main et al., 2006). Testicular dysgenesis syndrome can lead to impaired spermatogenesis and is associated with testicular cancer in adult men (Virtanen et al., 2007).

Allergies and Asthma

High molecular weight phthalates like DEHP, BBP, and their monoesters have been associated with allergies, asthma, wheezing, hay fever, itchy rashes, and eczema in adults. These phthalates are hypothesized to affect disease of the airways through increased levels of oxidative stress and secretion of several inflammatory cytokines like IL-4, IL-5, and INF- γ gene (Glue et al., 2002; Braun et al., 2013; Hoppin et al., 2013; North et al., 2014). DEHP and BBP have been shown to interfere with immunity against infection and alter the response of T helper type 2 (Th2) to increase allergic responses by acting on human plasmacytoid DCs (pDCs) by suppressing IFN-α/IFN-β expression and regulating the ability to elicit T-cell responses (Kuo et al., 2013).

Cancer

Phthalates have been implicated in the development of several types of cancer because of their xenoestrogenic properties-breast cancer in women and liver, skin, and gastrointestinal cancers in general population (Ardies and Dees, 1998; Lopez-Carrillo et al., 2010). A study that included 233 women residing in northern Mexico found exposure to DEP (the parent compound of MEP) was associated with increased risk of breast cancer with phthalate metabolites detected in at least 82% of the women (Lopez-Carrillo et al., 2010). MEP urinary concentrations were positively associated with breast cancer [odds ratio (OR), highest vs. lowest tertile = 2.20; 95% confidence interval (CI), 1.33–3.63; p for trend < 0.01] (Lopez-Carrillo et al., 2010).

Phthalates damage DNA in animal and human mammary epithelial cells which causes genomic instability in the breast tissue (Konduracka et al., 2014). Phthalates act as agonists for PPARs and activate the BARC gene through molecular signaling (Guyton et al., 2009; Rusyn and Corton, 2012; Sarath Josh et al., 2014). DEHP at high doses (100 and 500 μM) impaired the efficacy of camptothecin (CPT), an antitumor agent and reduced CPT- induced formation of reactive oxygen species (ROS) in ERα-positive MCF-7 cells (Chou et al., 2019). The impaired response of CPT in DEHP- exposed MCF-7 cells was mediated by epigenetic changes. MCF-7 cells after 48 hrs of exposure to 100 μM DEHP displayed considerable changes in patterns of DNA methylation, including hypermethylation of 700 genes and hypomethylation of 221 genes (Chou et al., 2019). In a Danish nationwide cohort of 1.12 million women who were followed for 10 years, 84% of breast cancers were ER-positive, and high level DBP exposure (≥10,000 mg) was directly related with a 2-fold increase in the rate of estrogen receptor- positive breast cancer risk (Ahern et al., 2019). This *in vivo* observation is consistent with *in vitro* evidences of DBP induced increases in proliferation and viability in an ER-dependent MCF-7 breast cancer cell line (Hong et al., 2005; van Meeuwen et al., 2007; Chen and Chien, 2014; Chen et al., 2016).

PHTHALATES AND microRNAs

MicroRNAs (miRNAs) are single-stranded, non-coding RNA molecules (sncRNAs) which are evolutionary-conserved and are involved in the regulation of gene expression at the posttranscriptional level (Ambros, 2004; Macfarlane and Murphy, 2010). miRNAs are ∼22 nucleotides long and can base-pair with complementary sequences of the 3' untranslated region (UTR) of messenger RNAs (mRNA) and thereby cause repression of translation and/or degradation of mRNA (Bird, 2007; Goldberg et al., 2007; Berger et al., 2009; Zhang and Ho, 2011).

One hypothesis is that the long-term reproductive defects associated with phthalate exposure is exerted through the action of non-coding miRNAs (Scarano et al., 2019). In that study, pregnant rats were dosed with phthalate mixture in the following proportion: 21% DEHP, 35% DEP, 15% DBP, 8% DiBP, 5% BBzP, and 15% DiNP. This proportion of phthalate mixture was based on proportion of phthalates metabolites detected in urine samples from pregnant women (Zhou C. et al., 2017; Scarano et al., 2019). To examine whether exposure to the phthalate mixture is capable of altering gene expression during prostate development of the filial generation, levels of mRNAs and miRNAs genome-wide were analyzed by RNA-seq (Scarano et al., 2019). The period of treatment was from gestational day 10 (DG10) to postnatal day 21 (DPN21) as development of urogenital tract especially the prostate occurs during this period (Vilamaior et al., 2006; Prins and Putz, 2008; Zhou C. et al., 2017; Scarano et al., 2019). Results indicated that the phthalate mixture induced changes in phenotypic parameters such as the AGD on PND1 and PND22 and prostate weight and testosterone levels at PND22 (Scarano et al., 2019). miR-184 was upregulated in all treated groups as opposed to control and miR-141-3p was upregulated only at the lowest dose. RNA sequencing analyses indicated that 120 genes were downregulated at the lowest dose with several of these genes associated with development, differentiation, and oncogenesis. A considerable number of the downregulated genes were predicted to be targets of miR-141-3p and miR-184, and the genes were induced at the lower exposure doses (Scarano et al., 2019). It was concluded that differentially expressed genes (DEG)s were under negative regulation either by the miRNAs which are upregulated or other mechanisms causing gene suppression (Scarano et al., 2019).

Gestational Diabetes

Several circulating miRNAs are dysregulated in patients diagnosed with gestational diabetes mellitus (GDM) during pregnancy (Zhao et al., 2011; Zhu et al., 2015). A study sought to identify the association of BPA and phthalate exposure measured in serum with the expression of circulating miRNAs related to GDM (miR-9-5p, miR-16-5p, miR-29a-3p, and miR-330-3p) revealed higher levels of miR-9-5p, miR-29a-3p, and miR-330-3p of patients with GDM compared to non-diabetic

subjects (Martinez-Ibarra et al., 2019). Phthalate metabolites like MBP, mono-isobutyl phthalate (MiBP), mono-benzyl phthalate (MBzP), and MEHP were detected in 97–100% of urine samples and Bisphenol-A (BPA) in only 40% of samples (Martinez-Ibarra et al., 2019). Thus, phthalates and BPA may play a role in the development of metabolic diseases like GDM via epigenetic regulatory mechanism such as miRNA regulation.

Female Fertility

A cross-sectional study was carried out to assess whether biomarkers of phenols and phthalates in urine of women undergoing IVF treatment are correlated with expression of extracellular vesicles (EV)-miRNAs in their follicular fluid. The urine samples were collected from participants during ovarian stimulation and the day oocyte was retrieved (Martinez et al., 2019). Results indicated that hsa-miR-125b and hsa-miR-15b were positively related with DEHP, while levels of hsa-miR-106b, and hsa-miR-374a were inversely related with DEHP. MBP was positively associated with levels of hsa-miR-24. *hsa-let-7c* was positively associated with urinary concentrations of mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), and DEHP (Martinez et al., 2019). EV-miRNAs are associated in cellular communication (both intra- and inter-) within the ovarian follicle and thus their dysregulation after EDC exposure can impact follicular growth, ovarian function and thus fertility rates.

microRNAs and Placenta

Changes in mRNA levels by phthalates have also been correlated with placental function. In a population study composed of 179 pregnant women–newborn dyads, an analysis was conducted to investigate the association between 8 phenol and 11 phthalate metabolites measured in first trimester urine and expression of 29 candidate miRNAs in placenta (LaRocca et al., 2016; Strakovsky and Schantz, 2018). Three miRNAs—miR-142-3p, miR15a-5p, and miR-185 were significantly associated with phenol or phthalate levels and potential mRNA targets of these microRNAs were linked with several biological pathways like regulation of protein serine/threonine kinase activity (LaRocca et al., 2016). Another small study comprising of 10 twin pregnancies suggested that several maternal urinary phthalate metabolites, including mono(carboxy-isononyl) phthalate (MCNP), MEHP, MEHHP, MECPP, mono-2-ethyl-5-oxohexyl phthalate (MEOHP), MBzP, mono(carboxy-isooctyl) phthalate (MCOP), mono-hydroxyisobutyl phthalate (MHiBP), and MiBP were positively correlated with placental long non-coding RNAs (lncRNAs) (Machtinger et al., 2018).

Placenta-derived EV-miRNAs are released by the placenta into the maternal circulation during pregnancy, and are responsible for regulating the endocrine environment to facilitate pregnancy and fetal growth (Mitchell et al., 2015). An exploratory study revealed that maternal exposure to phthalates and parabens can alter the profile of circulating EV-miRNAs (Zhong et al., 2019). miR-518e is highly expressed in women with elevated urinary levels of monobenzyl phthalate and methyl paraben. miR-373-3p had the lowest expression in women exposed to high levels of methyl paraben while miR-543 showed significant downregulation in women with high levels of paraben metabolites (Zhong et al., 2019). miR-518e, a member of the C19MC family is restricted to the placenta and the reproductive system and has high expression levels in the placentas of women having preeclampsia (Yang et al., 2015; Vashukova et al., 2016).

BIOMONITORING AND COMPARATIVE TOXICOGENOMICS DATABASE (CTD)

Biomonitoring Phthalate Levels in Humans

Single spot urine samples comprising of excreted urinary metabolites contribute most of the data collected for biomonitoring of human phthalate exposure (McKee et al., 2004; Hauser and Calafat, 2005; Wormuth et al., 2006; Frederiksen et al., 2007). Short-branched phthalates are mainly excreted as its monoester phthalates via urine (Frederiksen et al., 2007). The long-branched phthalates undergo further hydroxylation and oxidation and are excreted in urine and feces as phase II conjugated compounds. The phase II conjugates can be catalyzed by the enzyme uridine 5'-diphosphoglucuronyl transferase to form the hydrophilic glucuronide conjugate and is excreted in urine (Silva et al., 2003; Koch et al., 2005). A single urine sample for measurement of phthalate metabolites is also not an accurate estimation for an individual's long-term exposure level (Meeker et al., 2009). There have been scarce reports so far about phthalate levels in fetal cord blood and amniotic fluid. Also, cord blood or placenta may not correctly represent fetal exposure during the vulnerable period (Latini et al., 2003b). Phthalates diesters and their metabolites have been measured in breast milk, cord blood, and other pregnancy related specimens in humans (Adibi et al., 2003; Latini et al., 2003b; Main et al., 2006). Amniotic fluid is primarily formed from fetal urination and metabolized fetal cells. The phthalate metabolite concentrations in amniotic fluid varies based on metabolic activities of both mother and fetus and placental transfer but none of these metabolic parameters have been so far characterized for phthalate metabolites (Huang et al., 2009). Routine amniocentesis is usually performed at 16–20 weeks of gestation and amniotic fluid obtained during that time may provide accurate fetal exposure assessment during a period of reproductive differentiation and organogenesis (Silva et al., 2004). In the same study, among 10 phthalate metabolites analyzed-mEP, mBP, and mEHP were detected in 18.5% of the amniotic fluid samples taken from 54 anonymous donors (Silva et al., 2004). Infact, mEP, mBP, and mEHP were also major phthalate metabolites detected in serum samples from a multiethnic population (Silva et al., 2003). In one Italian study of 84 newborns, it was observed that MEHP in the cord blood of the newborns was associated with shorter gestations (Latini et al., 2003b).

The Comparative Toxicogenomics Database (CTD)

The Center for the Evaluation of Risks to Human Reproduction (CERHR) was established by the National Toxicology Program (NTP) in 1998 (National Toxicology Program, 2019). CERHR

provides a public resource for information regarding adverse health effects caused by exposure to various environmental and occupational chemicals. The chemicals are nominated for evaluation based on extent of public concern, production volume, potential of human exposure from environmental sources and availability of database on reproductive and developmental toxicity studies of the chemical (National Toxicology Program, 2019). CERHR selected DEHP based on the fact that general population of the United States is exposed to DEHP levels ranging from 1 to 30 μg/kg bw/day and it has been estimated that infants are exposed to DEHP through medical procedures and exposure can be as high as 6,000 μg/kg bw/day (National Toxicology Program, 2019). The Phthalates Expert Panel completed the first CERHR panel evaluation of DEHP in 2000. CEHR selected DEHP because of widespread public and government interest in its adverse health outcomes and availability of several toxicity papers at that time (Singh and Li, 2011; National Toxicology Program, 2019).

The Comparative Toxicogenomics Database (CTD) is a curated database that aids in understanding the effects of various environmental chemicals on human health. Biocurators at CTD use information from the literature to manually curate chemical-gene interactions, chemical-disease relationships and gene-disease relationships and construct chemical-gene-disease networks (Davis et al., 2009). Biocurators at CTD maintain toxicogenomic data and curation focuses on environmental chemicals. It is composed of data collected from 270 species with over 116,000 interactions between 3,900 chemicals and 13,300 genes/proteins, 5,900 gene/protein– disease direct relationships, and 2,500 chemical–disease direct relationships (Singh and Li, 2011).

In the CTD database, five most frequently curated phthalates (DEHP/MEHP and DBP/BBP/MBP) along with BPA have 1,232 and 265 interactions with unique genes/proteins, respectively (Singh and Li, 2011, 2012a,b). In one study (Singh and Li, 2011), in order to understand the health impact of the five most abundantly found phthalates, the authors downloaded the curated interactions between the five most common phthalates and the genes /proteins from CTD. From this database, 249 phthalate-interacting genes/proteins were fully analyzed for their Gene Ontology (GO) pathways, networks, and human diseases inferred by the phthalate–gene/protein–disease relationships. This analysis has ascertained that the pathways and networks of the top 34 genes were very similar to those of the 249 unique genes. Thus, the top 34 genes may be regarded as molecular biomarkers of phthalate toxicity (Singh and Li, 2011). The developmental effects of DBP/BBP/MBP depend primarily on two different factors- the duration of exposure and age of the embryo at the time of exposure. In an attempt to study the embryo-toxicant MBP, ESCs were exposed from the early embryoid body stage to 24 h post exposure and RNA was collected after 6, 12, and 24 h of exposure to study gene expression profile (van Dartel et al., 2009). There were a total number of 43 genes that were upregulated in the study and those were functionally related to cardiomyocyte differentiation (van Dartel et al., 2009).

No-Observable-Effect-Level (NOEL)/No-Observable-Adverse-Effect-Level (NOAEL)/Lowest-Observed-Adverse-Effect Level (LOAEL)

Regulatory agencies like U.S. Environmental Protection Agency (EPA), the National Toxicology Program (NTP) typically include three doses while testing chemicals such as environmental endocrine disruptors (EEDs) like phthalates for the purposes of non-clinical risk assessment: (i) No-Observable-Adverse-Effect-Level (NOAEL): the highest dose/exposure given to an organism found by experiment or observation that has no observed toxic or adverse effect on traditional toxicological endpoints compared to an appropriate control (ii) No-Observable-Effect-Level (NOEL): the highest dose or exposure level that produces no observable effect in the animals tested when compared with its appropriate control (iii) Lowest-Observed-Adverse-Effect Level (LOAEL): the lowest concentration of a substance that causes toxic or biochemical effects in animal studies (Vandenberg et al., 2012). The term "adverse effect" designates any harmful anatomical, biochemical, or functional changes caused in test subjects due to administration of the particular chemical used in that study (Kerlin et al., 2016).

Traditionally, NOAELs, NOELs, and LOAELs are calculated by first determining the maximum tolerated dose of a chemical, and then adjusting the dose downward until no adverse effects are observed (Vandenberg et al., 2012). This approach fails to identify non-monotonic dose responses that may be found in lower doses of endocrine disrupting chemicals like phthalates. A review of low-dose effects and non-monotonic dose curves of endocrine disrupting chemicals is available (Vandenberg et al., 2012). The endocrine system has evolved to respond to very low concentrations of unbound physiologically active hormones (Welshons et al., 2003). Natural hormones can affect their targets with serum levels in the nano and picomolar range. Likewise, EDCs often exert effects at doses in the nano to micromolar range, resulting in non-monotonic dose-responses that fail to align with predictions from higher doses (Vandenberg et al., 2012).

A dose response curve is termed non-monotonic when the slope of the curve changes sign one or more times within the range of doses examined (Vandenberg et al., 2012). Non-monotonic dose-response curves (NMDRCs) are either U-shaped, indicating that maximum responses of the measured endpoint are observed at low and high doses, or inverted U-shaped, indicating maximal responses are observed at intermediate doses (Vandenberg et al., 2012). NMDRCs are generated by a variety of mechanisms—(i) hormones though toxic at high doses can affect biological endpoints at very low doses (ii) two or more monotonic responses can overlap affecting a common endpoint in opposite directions via different pathways (iii) differences in receptor affinity at low *vs.* high doses (iv) downregulation of receptor and receptor desensitization (e.g., decrease in response to a hormone occurs due to biochemical inactivation of a receptor) (v) receptor competition, in that the mixture of endogenous hormones and EDCs creates an

environment that gives rise to NMDRCs (Vandenberg et al., 2012).

The epigenome varies by cell, tissue and stage of development, and EDCs may impact different cells and tissues differently, and findings that identify differential methylation resulting from phthalate exposure may be confounded by cell composition of the biological sample (Breton et al., 2017). Further complicating the identification of NOAELs of phthalates in human epigenetic studies is the fact that humans are chronically exposed to low doses as opposed to the acute high-dose exposures used in animal studies (CDC, 2019). This makes the traditional NOAEL calculations challenging to apply to human studies. Two critical areas of research for identifying NOAELs of phthalates in human epigenetic research are the identification of EDC induced changes in the epigenome across varying tissues and the characterization of the mechanisms through which phthalates impact the epigenome. There are several factors that determine the success of high-throughput epigenome-wide association scans (EWAS) like Illumina Infinium HumanMethylation450 (Illumina 450K) e.g., sample size, statistical power, epigenetic risk effect size and differentially methylated regions (DMRs) (Tsai and Bell, 2015).

The effects of acute doses of phthalates often fail to manifest in the dosed generation but are noted in one to two generations after the original generation is dosed, and multigenerational animal studies are generally designed to evaluate such effects. Low-powered studies and low number of widely spaced dose groups can give rise to inaccurate NOAEL values (Barnes et al., 1995; Sand et al., 2002; Hotchkiss et al., 2008). One study (Blystone et al., 2010) designed to evaluate the effect of DEHP on male reproductive malformations (RTM)s in male Sprague-Dawley rats used more than three traditional dose groups plus control and a bigger than usual sample size of F1 and F2 male rats until adulthood to define the DEHP NOAEL for male RTMs and also to evaluate the shape of dose-response curve. The *in utero* exposures for F1 and F2 were the same and the NOAEL for F1 and F2 RTM combined data were 100 ppm (4.8 mg/kg/day), and the lowest observed adverse effect level (LOAEL) was 300 ppm or 14 mg/kg/day (Blystone et al., 2010).

Furthermore, NOAELs vary according to the different endpoint parameters studied (Zhang et al., 2004). A particular study designed to evaluate the effect of DBP on reproductive and developmental toxicity during gestational day (GD1) to postnatal day (PND21) on F1 male rats showed that the NOAEL was 250 mg/kg/day when endpoint measured was number of live pups per litter whereas the NOAEL was 50 mg/kg/day when endpoint measured was birth weight of live pups (Zhang et al., 2004). These multigenerational animal models demonstrate that the calculation of NOAELs requires specific endpoints to delineate adverse effect. DNA methylation can be viewed as a process that results from the exposure and contributes to the eventual expression of an endpoint but is not the endpoint itself. As DNA methylation is not an endpoint in health research but is an intermediary between phthalate exposure to other endpoints such as gene-expression, determination of NOAELs for epigenetic research will need to reflect the degrees to which changes in methylation adversely affect these end-points, which are yet unknown. Perhaps a more important question to answer is do we need to establish NOEALs for epigenetic research if the changes in DNA methylation are not the ultimate outcomes we seek to understand.

DISCUSSION

This review is expected to inspire future research endeavors in environmental epigenetics to investigate the effects of the endocrine disruptors at different life stages from the perspective of transgenerational and multigenerational epigenetic inheritance (**Table 1**). There is substantial evidence from ESTs and rodent models that phthalates disrupt healthy growth and development of a fetus. There is more limited human evidence, though there is a body of literature that demonstrates associations between phthalate exposure and disrupted growth and development. Experimental evidence also demonstrates that the effects of phthalate exposure may not be realized until later in the life-cycle or in subsequent generations. Experimental and observational data demonstrate that exposure to phthalates modifies gene expression through epigenetic changes such as DNA methylation at CpG sites. One of the main areas of concern is maternal exposure of phthalates to fetus and infants via placenta and breast milk (Latini et al., 2003a; Calafat et al., 2006).

Environmental exposure to phthalates causes developmental and reproductive toxicity in rodent studies, though such relationships are difficult to demonstrate in humans. Life in all mammals occurs in cycles: production of germ cells (sperm and eggs) followed by fertilization, gestational development of the embryo, birth, postnatal growth followed by puberty leading to sexual maturity and the ability to reproduce. Though the terms "developmental" and "reproductive" toxicities are two separate entities, there is substantial overlap between them (Shelby, 2006). Toxicologists in recent years prefer to conduct their studies in a life-cycle specific manner because it is a common occurrence that chemical exposure at one stage of the life cycle may lead to observable effects at a later stage (Akingbemi et al., 2001). The adverse effect of phthalates on the early development of male reproductive tract affecting expression of genes involved in testis development and steroid hormone synthesis has been of particular interest (Wong and Gill, 2002; Shelby, 2006; Sekaran and Jagadeesan, 2015). Any chemical-induced epigenetic defects in eggs or sperm in a sexually mature individual might not affect the individual but may be transmitted to its progenies. That effect of the chemical might be embryonic lethality, miscarriage, stillbirth or the offspring might be born with a developmental disorder.

Most of the evidences we currently have for transgenerational epigenetic inheritance is in animals. In humans, because of our long lifespan and diverse genetics, it is complicated to conduct studies for 3 to 4 generations (Calo et al., 1993). From this perspective, the zebrafish provides an ideal model as it has a short time to sexual maturity (∼3–4 months). Moreover, zebrafish eggs get fertilized externally in water and thus are at exposed environment and so F0 fish is equivalent to F1 mice. This enables us to examine the effect of the exposed environmental toxin directly at F0 (De Felice et al., 2002).

TABLE 1 | Research studies of the epigenetic impact of those phthalates and the long-term health consequences of exposure to phthalates by model.

Model	Life-cycle timing of impact of phthalate exposure	Impact of exposure	Epigenetic dysregulation associated with exposure	Associated phthalate di-esters or monoesters	Analysis method	References
Embryo and embryonic stem cell models						
Embryonic stem cells (Murine)	Embryonic stage	Inhibition of mesoderm-derived cardiomyocyte differentiation	Upregulated gene expression of 43 genes	MBP	Microarray analysis & Gene Set Enrichment Analysis (GSEA)	van Dartel et al., 2009
Embryonic stem cells (Human)	Embryonic stage	Cytotoxic and affected the development of hESCs	Changed gene expression patterns in embryoid bodies (EB)	MEHP	Gene expression patterns analyzed by real-time PCR	Shi et al., 2013
Embryo (Murine)	Embryonic stage	Impaired developmental competency, delayed progression of preimplantation, increase in reactive oxygen species, increased apoptosis	Decreased DNA methylation	MBP	Immunofluorescent staining& quantification of immunofluorescent intensity	Chu et al., 2013
Placenta models						
Placenta (human)	Fetal stage	Placental function	Altered methylation and gene expression in human placenta	Total urinary phthalate concentration	Illumina Infinium HM 850k BeadChip	Grindler et al., 2018
Placenta, Cord blood	Fetal stage	No association with fetal length or birthweight	Decreased methylation *H19* in women with high levels of total urinary phthalate concentrations Total phthalates and low molecular weight phthalates associated with decreased methylation of *IGF2*DMR0	11 phthalate metabolites (MBzP, MEHP, MEHHP, MECPP, MEOHP, MnBP, MiBP, MBzP, MEP, MCOP, MCPP, MCNP).	Methylation of differentially methylated regions (DMRs) were assessed by pyrosequencing of *H19*, *IGF2*DMR0, and *IGF2*DMR2	LaRocca et al., 2014
Placenta (Human)	Fetal & neonatal stages	Fetal growth restriction (FGR) newborns	Inverse association of urinary phthalate concentrations with *IGF2* DNA methylation in human placenta	MEHHP MEOHP	PCR & pyrosequencing	Zhao et al., 2016
Placenta (Human)	Placental and fetal growth	Gene ontology (GO) identified biological pathways to health outcomes	Three miRNAs were significantly associated with phthalate levels (miR-185, miR-142-3p, miR15a-5p)	11 phthalate metabolites (MBzP,MEHP, MEHHP,MECPP, MEOHP, MnBP, MiBP, MBzP, MEP, MCOP, MCPP, MCNP).	qPCR	LaRocca et al., 2016
Placenta (Human)	Newborn stage	long non-coding RNAs (lncRNA)s play an important role in regulating genomic imprinting	lncRNAs	MCNP,MEHP, MECPP, MEOHP, MBzP, MCOP, MHiBP, MiBP, MMP, MCPP,MEP, MNP, MnBP, MHBP	Real-time PCR	Machtinger et al., 2018
Blood-based models						
Peripheral Blood Mononuclear Cells (human), monocytic cell line THP-1	Adult birch-pollen allergic and non-allergic individuals	Increased inflammatory cytokine gene expression	A significant increase in IL-4, IL-5 and INF- γ gene expression were observed	MBEP, MBUP, MEHP, MOP, MINP, MIDP	Quantitative competitive RT-PCR and real-time PCR	Glue et al., 2002

(Continued)

TABLE 1 | Continued

Model	Life-cycle timing of impact of phthalate exposure	Impact of exposure	Epigenetic dysregulation associated with exposure	Associated phthalate di-esters or monoesters	Analysis method	References
Whole blood from umbilical cord at birth and children at 9 years	Fetal stage	Asthma, inflammation, restricted child growth, and poor sperm quality	Inverse association between MEP concentration and cord blood Alu repeats Inverse association between DEHP and Alu repeat methylation in children at 9 years of age	MEP, MBP, MiBP, MEHP, MEHHP, MEOHP, MECPP, MBzP, MCPP, MCOP, MCNP	Pyrosequencing	Huen et al., 2016
Whole blood from umbilical cord	Fetal stage	Genes related to androgen response, estrogen response, spermatogenesis enriched	Altered DNA methylation	DEHP	HM450K	Chen et al., 2018
Whole blood from children	Childhood	Decreased methylation of TNF-α gene promoter and childhood asthma	Detection of DNA methylation by pyrosequencing, real-time PCR	MEHP	Quantitative PCR	Wang et al., 2015
Whole blood from children	Childhood	Skinfold thickness in girls 8 to 14 years old. No direct link between phthalate exposures and adiposity measures mediated by changes in DNA methylation	Altered DNA methylation of H19 in girls	MEP,MBP, MiBP, MCPP, MBzP, MEHP, MEHHP,MEOHP, MECPP	Pyrosequencing	Bowman et al., 2019
Serum from pregnant women	During gestation	Higher levels of miR-9-5p, miR-29a-3p and miR-330-3p in sera of patients with gestational diabetes mellitus compared to non-diabetic subjects	miRNA expression	MBP, MiBP, MBzP, MEHP	Real-Time PCR	Martinez-Ibarra et al., 2019
Murine pregnancy models						
Pregnant rats	F1 generation reproductive stage	Adult testicular function	Hypermethylation in SF-1 and Sp-1 promoter regions of Leydig cells	DEHP	Real-Time PCR	Sekaran and Jagadeesan, 2015
Pregnant rats	F1 generation reproductive stage	Adult male testicular and prostate disease	Altered DNA methylation in sperm and transgenerational inheritance	DEHP	Quantitative PCR	Manikkam et al., 2013)
Pregnant rats	F1 generation reproductive stage	Genes controlling immune response affected by *in utero* DEHP exposure	DNA methylation alterations throughout epigenome of adult male adrenal glands	DEHP	Reduced-representation bisulfite sequencing	Martinez-Arguelles and Papadopoulos, 2015
Pregnant mice	F2 generation childhood	Allergic airway inflammation	Altered DNA methylation and transgenerational model	BBP	MassARRAY	Jahreis et al., 2018
Pregnant rats	F1 generation	Low and high exposure groups had higher body weight than control group	Altered DNA methylation of Srebf1 and Srebf2	DEP,DEHP, DBP, DiNP, DiBP, BBP	EZ DNA Methylation Gold Kit	Moody et al., 2019
Pregnant rats	F1 Male reproductive stage	Altered ano-genital distance, prostate weight, and testosterone levels	Non-coding miRNA	Mixture of DEHP,DEP, DBP, DiBP, BBzP, DiNP	(i) RNAs sequenced by HiSeq2500 platform (Illumina) (ii) High performance sequencing—sncRNAs (NovaSeq Sequencing System)	Scarano et al., 2019

(Continued)

TABLE 1 | Continued

Model	Life-cycle timing of impact of phthalate exposure	Impact of exposure	Epigenetic dysregulation associated with exposure	Associated phthalate di-esters or monoesters	Analysis method	References
Other models						
Follicular fluid	Female reproductive stage	Dysregulation of follicular growth, ovarian function, and fertility	EV-miRNA	DEHP,MBP, MEOHP,MEHHP, MECPP	TaqMan Open Array Human microRNA panel	Martinez-Ibarra et al., 2019
Spermatozoa	Reproductive stage	Genes associated with growth and development, and basic cellular function, and diminished blastocyst quality	Differential DNA methylation	MEHP, MEOHP, MBP, MCOCH	HM450K	Wu et al., 2017a
Placental derived extracellular vehicles circulating in maternal blood	Fetal stage	Expression of mi-518e associated with increased BBP	EV-miRNA	BBP	TaqMan Open Array Human microRNA panel	Zhong et al., 2019
Mouse liver and testes	Adult reproductive stage	DEHP causes toxicity in liver- liver is involved in steroid metabolism and is known to be a DEHP target organ.	51 DEHP-regulated genes were identified involved in-peroxisome proliferation, xenobiotic detoxification, oxidative stress response, immune function, steroid hormone metabolism, testis development, and pheromone transport	DEHP	Analysis of DEHP induced gene expression changes in liver using microarray screening of Murine Genome U74Av2 Arrays (MGU74Av2) (Affymetrix, Santa Clara, CA)	Wong and Gill, 2002

hESC, Human embryonic stem cell; EB, Embryoid Body; EV, Extracellular Vesicle.

Transgenerational studies with other EDCs like dioxin or TCDD which is a persistent environmental toxicant show that unexposed TCDD-lineage F2 offspring have defects in reproduction, skeletal muscle system and sex ratio in offspring. Moreover, the decrease in fertility and egg release in control female zebrafish is due to the unexposed, TCDD-lineage F2 male zebrafish. The ancestral TCDD exposure affects reproductive success of male zebrafish across multiple generations (De Felice et al., 2002). In a follow up study, the transgenerational effect of TCDD on zebrafish reproductive success was found to be the result of altered DNA methylation (De Felice et al., 1999). The authors performed whole genome methylation analysis of adult zebrafish exposed to sublethal levels of TCDD during the developmental period and found both DMR- and CpG-specific changes in the DNA methylation profile. The authors observed that several genes were differentially methylated in the exposed compared to the unexposed, and many of those genes were responsible for reproductive success or epigenetic modifications (De Felice et al., 1999). Thus, similar transgenerational studies are required for phthalates.

The epigenetic tags of the chromatin are of two types—(i) transient which can be removed and (ii) permanent which is heritable (Ayala-Garcia et al., 2013). The transient epigenetic tags enable the organism to adjust their gene expression status in relation to changes in their environment—in contrast permanent epigenetic tags gives rise to an epigenetic memory which modulates the cells' genetic and metabolic response to environmental changes for the rest of the organism's life (Ayala-Garcia et al., 2013). When permanent chromatin epigenetic tags occur in the stem cells, gametes—they are inherited by their progenies both at the cellular and organismal levels and are thus transgenerationally heritable (Dolinoy et al., 2007; McCarrey, 2012). Thus, the highly dynamic process of epigenetic tagging as perpetuated by the epigenetic memory is responsible for the phenotypic plasticity in an organism. Tagging occurs as a response to changes in environmental conditions at any time point in the life course (Ayala-Garcia et al., 2013). Humans are simultaneously exposed to several xenobiotics and thus the interaction of phthalates with other environmental toxins should be taken into account when studying their roles in causing diseases in the population (Benjamin et al., 2017). One major drawback is that epidemiological studies are mostly limited to developed countries (Benjamin et al., 2017). We have limited reports so far on the impact of phthalates from Asia, Africa and South America. In a 2018 study of Children's Health and Environmental Chemicals in Korea (CHECK) cohort, comprising of matched pregnant woman-fetus pairs recruited from four cities of Korea, phthalate metabolites like MiBP, MnBP, MEHP, MEHHP, and MEOHP (metabolites of DEHP), MEP,

persistent organic pollutants (POPs), heavy metals, and BPA were linked with decreased neurodevelopmental performances and behavioral scores of toddlers (Kim et al., 2018). In another Mothers and Children's Environmental Health Study composed of 460 mother–infant pairs between 2006 and 2009 revealed that prenatal exposure to phthalates is inversely associated with the Mental and Psychomotor Developmental indices (MDI and PDI, respectively) of particularly male infants, at 6 months as measured by the Korean Bayley Scales of Infant Development (Bayley, 1993; Park, 2006; Kim et al., 2018).

The European Union (EU) has adopted strict regulations around use of phthalates and other EDCs- it is an interesting natural experiment that could be used to investigate how these diseases change in Europe. The impact of phthalates varies from one population to population based on their food habits and lifestyles. The concentration of phthalate metabolites in human body widely varies based on demographics (Benjamin et al., 2017). A joint, co-ordinated and conscious effort by various nations is needed to manage the existing health issues caused by EDCs like phthalates across the globe plus the onset of new cases which will result in huge expenditures for a nation's economy in the years to come. A Steering Committee of scientists in the EU evaluated a range of health and economic costs due to EDC exposures based on epidemiological and toxicological evidences. The disease and dysfunctionality of life caused due to EDC exposures in the EU is estimated to cost hundreds of billions of Euros per year (Trasande et al., 2015). As a global population, we should adapt the 5 R's (Reduce, Reuse, Recycle, Rethink, and Restrain) for controlling the environmental exposure to phthalates and other EDCs for our future generations.

FUTURE PERSPECTIVES

An emerging view in the field of epidemiologic research is that intrauterine growth period of the fetus is a critical window of susceptibility during which environmental toxicants can affect the developmental trajectories and cause epigenetic information to be transmitted between generations (Morkve Knudsen et al., 2018). Germ cells undergo extensive epigenetic reprogramming starting from embryonic stage to mature reproductive stage and are vulnerable to environmental stressors during those reprogramming phases (Wu et al., 2015). A true transgenerational event is one in which epigenetic information is transmitted across generations through the germline, and is known to occur when a man or woman (F0) and their germ cells to the F1 generation are directly subjected to any environmental stressor and the F2 offspring is the first generation which is a true case for transgenerational epigenetic inheritance (Horsthemke, 2018; Morkve Knudsen et al., 2018).

The various mechanisms which play an important role in passing on information from one generation to another are DNA methylation, histone modification, or changes in non-coding RNA (Heard and Martienssen, 2014; Wu et al., 2015; Sales et al., 2017; Horsthemke, 2018). Intergenerational effects occur when a pregnant woman (F0) is subjected to environmental stress, and the developing fetus including the germline of the fetus may be affected leading to altered phenotype of the child (F1) and possibly the next generation (F2). Intergenerational epigenetic inheritance is the transfer of epigenetic marks from the gametes to the embryo for only one generation. The third generation (F3) is the first generation that could exhibit transgenerational epigenetic inheritance (Morkve Knudsen et al., 2018). Multigenerational exposures are exposure related events observed across multiple generations (Skinner, 2008).

Where can research improve to develop a better understanding of the biological mechanisms underpinning phthalate exposures and human disease? Environmental epidemiology needs to focus on accurate characterization of phthalate exposure by using multiple samples across time to best capture exposure status. Epigenetic studies of phthalate exposures should consider how mixtures of phthalates affect gene expression beyond the traditional classifications of low and high molecular weight.

In conclusion, future studies of phthalates and other environmental chemicals must examine potential multigenerational effects of exposures. Maternal exposures, both prior to and during pregnancy, can potentially affect the developing egg and fetus. Paternal exposures can potentially affect the sperm. More studies with model organisms, such as with zebrafish, are needed to examine the mechanisms of multigenerational inheritance of phenotypes induced by chemicals such as phthalates.

AUTHOR CONTRIBUTIONS

SD conducted the literature review, drafted, and revised the manuscript. DH provided critical review of the manuscript and revised the manuscript. DAR provided critical review of the manuscript. DMR conceived the review, provided critical review of the manuscript, and revised the manuscript.

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GLOSSARY OF ACRONYMS

Phthalates:

BBP: Benzyl butyl phthalate

BBzP: Butylbenzyl-phthalate

BzBP: Benzylbutyl phthalate

DBP: Dibutyl phthalate

DCHP: Dicyclohexyl phthalate

DEHP: Di-(2-ethylhexyl) phthalate

DEP: Diethyl phthalate

DiBP: Diisobutyl phthalate

DiDP: Di-isodecyl phthalate

DiNP: Di-isononyl phthalate

DMP: Dimethyl phthalate

DnOP: Di-n-octyl phthalate

mBP-glu: Monobutyl phthalate glucuronide

mBzP: Mono-benzyl phthalate

mBzP-glu: Monobenzyl phthalate glucuronide

MCNP: Mono (carboxy-isononyl) phthalate

MCOP: Mono (carboxy-isooctyl) phthalate

MECPP: Mono-2-ethyl-5-carboxypentyl phthalate

MEHHP: Mono-2-ethyl-5-hydroxyhexyl phthalate

MEHP: Mono-2-Ethylhexyl Phthalate

MEOHP: Mono (2-ethyl-5-oxohexyl) phthalate

MEP: Mono ethyl phthalate

MHiBP: Mono-hydroxyisobutyl phthalate

MiBP: Mono-isobutyl phthalate

MiDP: Mono-iso-decyl phthalate

MiNP: Mono-isononyl phthalate

MnBP: Mono-n-butyl phthalate

MOP: Mono-n-octyl phthalate

Others:

ADAMTS: A Disintegrin and Metalloproteinase with Thrombospondin Motifs

AGD: Anogenital distance

BPA: Bisphenol A

5caC: 5-carboxylcytosine

CERHR: Center for the Evaluation of Risks to Human Reproduction

CTD: Comparative Toxicogenomics Database

DC: Dendritic cells

DMR: Differentially methylated regions

DNMTs: DNA methyltransferases

EDCs: Endocrine disrupting chemicals

ESCs: Embryonic stem cells

E_2: Estrogen

ER: Estrogen receptor

5fC: 5-formylcytosine

GDM: Gestational diabetes mellitus

HM450K: Human Methylation 450K BeadChip

HMW: High molecular weight

huESCs: Human embryonic stem cells

5hmC: 5-hydroxymethylcytosine

IGF2: Insulin-like growth-factor 2

IL: Interleukin

LMW: Low molecular weight

LINE-1: Long interspersed nucleotide elements

5mC: 5-methylcytosine

miRNAs: microRNA

NTP: National Toxicology Program

PBMC: Peripheral blood mononuclear cells

PVC: Polyvinyl chloride

P_4: Progesterone

SREBPs: Sterol regulatory element binding proteins

TET: Ten-eleven translocation

TNF-α: Tumor necrosis factor-α

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ORIGINAL RESEARCH
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Epigenetic Vulnerability of Insulator CTCF Motifs at Parkinson's Disease-Associated Genes in Response to Neurotoxicant Rotenone

*Dana M. Freeman and Zhibin Wang**

Laboratory of Environmental Epigenomes, Department of Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, United States

CCCTC-binding factor (CTCF) is a regulatory protein that binds DNA to control spatial organization and transcription. The sequence-specific binding of CTCF is variable and is impacted by nearby epigenetic patterns. It has been demonstrated that non-coding genetic variants cluster with CTCF sites in topological associating domains and thus can affect CTCF activity on gene expression. Therefore, environmental factors that alter epigenetic patterns at CTCF binding sites may dictate the interaction of non-coding genetic variants with regulatory proteins. To test this mechanism, we treated human cell line HEK293 with rotenone for 24 h and characterized its effect on global epigenetic patterns specifically at regulatory regions of Parkinson's disease (PD) risk loci. We used RNA sequencing to examine changes in global transcription and identified over 2000 differentially expressed genes (DEGs, >1.5-fold change, FDR < 0.05). Among these DEGs, 13 were identified as PD-associated genes according to Genome-wide association studies meta-data. We focused on eight genes that have non-coding risk variants and a prominent CTCF binding site. We analyzed methylation of a total of 165 CGs surrounding CTCF binding sites and detected differential methylation (|>1%|, q < 0.05) in 45 CGs at 7 PD-associated genes. Of these 45 CGs, 47% were hypomethylated and 53% were hypermethylated. Interestingly, 5 out of the 7 genes had correlated gene upregulation with CG hypermethylation at CTCF and gene downregulation with CG hypomethylation at CTCF. We also investigated active H3K27ac surrounding the same CTCF binding sites within these seven genes. We observed a significant increase in H3K27ac in four genes (FDR < 0.05). Three genes (PARK2, GPRIN3, FER) showed increased CTCF binding in response to rotenone. Our data indicate that rotenone alters regulatory regions of PD-associated genes through changes in epigenetic patterns, and these changes impact high-order chromatin organization to increase the influence of non-coding variants on genome integrity and cellular survival.

Keywords: CTCF, Parkinson's disease, single nucleotide polymorphisms, rotenone, DNA methylation, histone modifications

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Edited by:
Yanqiang Li,
Houston Methodist Research Institute, United States

Reviewed by:
Chunyuan Jin,
New York University, United States
Lin An,
Sanofi, United States

***Correspondence:**
Zhibin Wang
zwang47@jhu.edu

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States (de Lau and Breteler, 2006). More than 800 genetic association studies have been conducted to interpret genetic contribution to PD etiology (Lill et al., 2012; Coetzee et al., 2016). Genome-wide association studies (GWAS) evaluate the association of common genetic variants to a phenotype or disease outcome. Since 2005, thousands of variants have been identified to have a significant association with a disease and more than 1600 single-nucleotide polymorphisms (SNPs) have been identified as genetic risk variants for PD (Lill et al., 2012). However, unlike rare monogenetic associations, the functional consequences of most of these variants have yet to be determined. Over 90% of all indexed SNPs including those associated with PD occur in non-coding regions of the genome (Maurano et al., 2012; Verstraeten et al., 2015). This discovery led to the hypothesis that SNPs in the human genome interact with regulatory elements to control gene expression (Wang et al., 2019). This is supported by expression of quantitative trait loci (eQTLs) defined as genetic regions that are enriched at positive GWAS sites and explain variability in the expressivity of a gene (Nica et al., 2010). Despite these advances, it remains a challenge to determine which genetic variants in a broad region of variants is the driver of gene expression changes particularly when regulatory element interactions are long-range (Do et al., 2016). SNPs cluster within enhancers and can modify PD risk. These observations of PD-associated SNPs have been described in multiple cell types (Coetzee et al., 2016). With this new evidence, studies are now focusing on interactions of regulatory elements to understand how genetic associations trigger disease biology within the brain.

CCCTC-binding factor can play a long-range cis-regulatory role that insulates genes from their surrounding signaling environment by directing chromatin looping (Phillips and Corces, 2009). Functional CTCF binding sites are required for the formation of distinct structural domains within a three-dimensional chromosomal organization (Ong and Corces, 2014; Tang et al., 2015). CTCF binding is dependent upon DNA sequence (CCGCGNGGNGGCAG) and allelic hypomethylation (Wang et al., 2012). Thus, genetic variants and epigenetic patterns within binding sites can contribute to dysfunctional CTCF allele-specific binding (Tang et al., 2015; Wang et al., 2019).

Approximately 85% of PD cases cannot be explained by genetic predisposition alone (Franco et al., 2010; Verstraeten et al., 2015; Labbé et al., 2016). Therefore, it is likely that most cases are caused by the interplay of common SNPs with environmental factors. Environmental factors can modulate the association of a genetic variant with a disease (Lee et al., 2011). For instance, exposures that impact allele-specific methylated regions in the genome can influence CTCF binding and thus influence non-coding variants' effect on genetic expression (Wang et al., 2019). GWAS association signals are complex in that they can cover a broad region of DNA with several polymorphisms, so we focused on environmentally induced epigenetic changes in CTCF binding regions nearby risk-associated genes to explore mechanisms of gene–environment interactions in PD.

In our pesticide-induced cellular model, we used rotenone, a naturally occurring insecticide and potent inhibitor of complex I in the mitochondrial electron transport chain. The primary use of rotenone today is as a piscicide to terminate invasive or noxious species of fish. Permissible application concentrations up to 250 ppb can be applied to public and recreational waters (United States Environmental Protection Agency [USEPA], 2007). Rotenone is a widely accepted PD toxicant and can robustly replicate pathology via depletion of ATP, generation of reactive oxygen species, damage of nigrostriatal tissues, and death of dopamine producing cells in the midbrain (Dawson et al., 2002; Cicchetti et al., 2009). It has also been shown to cause these types of cellular pathology in HEK293 (Orth et al., 2003; Teixeira et al., 2018). While cell line HEK293 is a human immortalized cell line derived originally from primary embryonic kidney cells, it has been found to have a genetic signature similar to neurons (Stepanenko and Dmitrenko, 2015). We chose this cell line given their well-characterized genome and ENCODE regulatory elements (ENCODE Project Consortium, 2012; Lin et al., 2014).

DNA methylation and histone acetylation are epigenetic modifications implicated in rotenone-induced neurotoxicity (Huang et al., 2019). DNA hypomethylation has been reported in response to pesticide exposure (Hou et al., 2012), and we discovered that rotenone reduces DNA methylation at DNMT1-dependent regions in the human genome (Freeman et al., 2020). Histone acetylation patterns have been more extensively studied in rotenone-induced PD due to its high correlation with gene expression and enhancer activation (Wang et al., 2008). Most studies agree that rotenone-induced neurodegeneration is associated with pathological hyperacetylation as a result of impaired homeostatic activity of HATs and HDACs (Feng et al., 2015; Park et al., 2016; Harrison et al., 2018; Wang et al., 2018; Huang et al., 2019).

In this study, we examined rotenone-induced changes in DNA methylation and histone acetylation patterns at CTCF binding sites adjacent to PD-associated genes. Eight selected genes had identified disease-risk SNPs in a non-coding region and were indexed by a meta-data analysis of over seven million human polymorphisms (Lill et al., 2012). We hypothesize that rotenone exposure modifies epigenetic patterns at CTCF binding motifs and affects its allele-specific transcription factor binding. We postulate that this mechanism could mediate the interchange between genetic variants and regulatory elements controlling transcription and genomic stability.

MATERIALS AND METHODS

Cell Culture and Treatment of Human Cell Line HEK293

All media reagents and chemicals in cell culture were purchased from Sigma (St. Louis, MO, United States). Human cell line HEK293 was grown in Dulbecco's Modified Eagle Medium

with high glucose, L-glutamine, and sodium pyruvate. Media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin–streptomycin. HEK293 cells were confirmed by ATCC. Cells were treated at approximately 70% confluency with 200 nM rotenone or DMSO vehicle control (<0.001%) for 24 h. Cell viability was measured with trypan blue (0.4%) staining, and cells were counted manually with a hemocytometer. Cells used for experiments were at least 85% viable relative to the vehicle control.

Qualitative Analysis of Total 5mC Levels

Genomic DNA was extracted from two replicates of DMSO or rotenone-treated HEK293 using 1:1:1 phenol: chloroform: isoamyl alcohol (Sigma, St. Louis, MO, United States). Global DNA methylation was first measured by dot blot analysis. Bisulfite-treated DNA (30–60 ng/μL) was denatured at 95°C for 5 min and then cooled at 4°C for 5 min in a conventional thermocycler (MyCycler; Bio-Rad; Hercules, CA, United States). DNA was spotted onto 0.45-micron nitrocellulose paper as 1- or 2-μL drops and dried for 30 min at room temperature. The membrane was UV cross-linked at 3000 Hz and incubated in anti-5methylcytosine (5mC) primary antibody overnight at 4°C (Epigentek 33D3; Farmingdale, NY, United States). The membrane was washed with TBST and incubated with a secondary antibody conjugated to HRP for 1 h at room temperature (Santa-Cruz Biotechnology anti-mouse IgG sc-2005; Dallas, TX, United States). The membrane was washed again with TBST after secondary incubation and visualized with chemiluminescence (ProSignal Femto; Prometheus; Raleigh, NC, United States). We used a serial dilution of 100% 5mC standard (Zymo Research D5012; Irvine, CA, United States) to create a standard ladder (**Supplementary Figure 1**).

HEK293 Western Blot for Global H3K27ac

HEK293 cells were collected after a 24-h treatment, and histones were extracted using the Abcam Histone Extraction kit according to kit instructions (Cambridge, United Kingdom). Histone protein concentration was measured by Qubit Protein Assay from Thermo Fisher Scientific (Waltham, MA, United States). Protein (5 μg) was loaded onto 4–15% Bio-Rad Page Gels and transferred to 0.45 μm nitrocellulose (Bio-Rad, Hercules, CA, United States). The blots were incubated with H3K27ac primary antibody (1:1000; Abcam ab4729) overnight at 4°C and anti-rabbit IgG conjugated secondary antibody (1:5000, Santa Cruz Biotechnology Sc-2357) for 1 h at room temperature. The histone protein was normalized to total histone 3 (H3; Abcam ab1791) and quantified with ImageJ software. We tested significance by comparing the ratio of H3K27ac/H3 with a two-tailed Student's paired *t*-test ($p < 0.05$).

RNA Extraction and RNA Sequencing Library Construction

Total RNA was extracted from two replicates of DMSO or rotenone-treated HEK293 using the TRIzol method (Invitrogen, Carlsbad, CA, United States). A total of 2 μg per sample was used for library construction using the TruSeq Sample Preparation kit from Illumina (San Diego, CA, United States). Poly-A-containing mRNA molecules were isolated from total RNA using oligo-dT attached magnetic beads. Isolated mRNA was then fragmented and synthesized into double-stranded cDNA according to kit instructions. Ligation of unique Illumina adapter indices was completed for each sample before bead purification. Libraries were loaded onto a 2% agarose gel, and library products between 200–800 bp were purified using the mini-Elute gel extraction kit from Qiagen (Hilden, Germany). Approximately 150 ng was sent for sequencing on a HiSeq 2000 platform with 100-bp paired-end reads.

RNA Sequencing Data Analysis

Adapter sequences were removed from the raw sequencing data, and individual libraries were converted to the fastq format. Sequencing reads were aligned to the human genome (hg19) with TopHat2 (v2.0.9) (Kim et al., 2013). For mRNA analyses, the RefSeq database (Build 37.3) was chosen as the annotation references. Read counts of annotated genes were obtained by the Python software HTSeq count (Anders et al., 2015). DEGs were defined as those with a 1.5-fold change in expression using FDR < 0.05 from the edgeR package (Robinson et al., 2010). Gene Ontology annotation was done with Gorilla online platform and visualized with Revigo and Cytoscape (Eden et al., 2009; Supek et al., 2011; Otasek et al., 2019).

RNA Sequencing Validation With Quantitative Reverse Transcription-PCR

Total RNA was extracted from an additional replicate of HEK293 treated with DMSO or rotenone using the same procedure as stated above. A total of 500 ng RNA was converted to cDNA with the PrimeScript RT reagent kit with gDNA eraser from Takara (Kusatsu, Japan). We selected 10 genes for quantitative PCR (qPCR) analysis using primers listed in **Supplementary Table 1**. All qPCR reactions were performed on a 7500 Real-Time PCR system from Applied Biosystems (Foster City, CA, United States) using the iTaq Universal SYBR Green Supermix from Bio-Rad (Hercules, CA, United States). The change in expression was normalized to the GAPDH housekeeping gene and expressed as fold change ($2^{-\Delta\Delta CT}$).

Identification and Selection of PD-Associated Genes

We identified PD-associated genes using the National Health Genomic Research Institute GWAS Catalog (Buniello et al., 2018). We searched for all associations both reported and mapped using the trait "Parkinson's disease" (EFO_0002508) which included 39 publications investigating genomic signatures of both familial and environmentally driven PD as well as Lewy body pathology and Parkinsonism in frontotemporal lobe dementia (**Supplementary File 2**). We calculated the frequency for various region types (non-coding, regulatory, coding) within the 246 known genetic variants provided by GWAS Catalog. We compared 399 reported and mapped genes to our list of DEGs. We then cross-referenced these genes with the PD gene online resource which analyzed over 800 publications and seven

million polymorphisms (Lill et al., 2012). We selected five genes that remained significant in the PD gene meta-analysis, were represented in at least two studies, and had their most significant variant in a non-coding region (**Table 2**). We also selected three additional genes from the PD gene database that were represented in our RNA sequencing data (**Table 2**). The first, *UBOX5*, was among the most significant polymorphisms identified by the meta-analysis (Lill et al., 2012; Nalls et al., 2014). The other two, *PARK2* and *CHCHD2*, have significant polymorphisms according to the PD gene database but are also reported to have autosomal mutations that contribute to familial disease cases (Lill, 2016).

Region Selection for Bisulfite and ChIP Primer Design

CCCTC-binding factor transcription factor binding was observed using the Uniform Transcription Factor Binding data found in the ENCODE Regulation super track in UCSC Genome Browser. We selected all CTCF transcription factor binding sites detected with ChIP-seq experiments from the ENCODE consortium from 2007 to 2012 (ENCODE Project Consortium, 2012). We also predicted which cytosine would overlap the binding motif using the CTCF binding prediction tool database v2.0 (Ziebarth et al., 2012). Primer design was focused on CTCF binding sites for both bisulfite sequencing and ChIP-qPCR experiments (further described below).

Bisulfite-DNA Conversion and Bisulfite-Amplicon Sequencing Library Construction

Genomic DNA was extracted from two replicates of DMSO or rotenone-treated HEK293 using phenol: chloroform: isoamyl alcohol (Sigma, St. Louis, MO, United States). A total of 200 ng DNA was bisulfite-converted using the Sigma DNA Imprint Modification kit two-step protocol. Bisulfite-converted DNA (BS-DNA) was amplified with primers for selected regions designed with MethPrimer (Li and Dahiya, 2002) (**Supplementary Table 2**). Amplified BS-DNA products were run on a 2% EtBr agarose gel and purified using the mini-Elute gel extraction kit from Qiagen (Hilden, Germany). Purified products for each sample were pooled together, and 1 ng was used for library preparation using the Illumina Nextera XT DNA Library Preparation kit. Each sample was tagged with a unique Nextera XT adapter (San Diego, CA, United States). Sequencing libraries were quality checked via Bioanalyzer and run on an Illumina MiSeq platform to generate 150-bp paired-end reads.

Bisulfite-Amplicon Sequencing Analysis for Methylation Patterns at CTCF Binding Sites

The raw fastq files were imported into the Galaxy web platform (Afgan et al., 2016). Reads with quality score <30 were filtered out, and reads with quality score >30 were trimmed with Trim Galore (Krueger, 2015). Reads were mapped to the human genome (hg19) using bwa-meth (Pedersen et al., 2014). MethylDackel was used for methylation calling, and per-cytosine contexts were merged into per-CPG metrics[1]. Duplicates and singletons identified in alignment were ignored from the methylation call. Minimum and maximum per-base depths were 1000× and 100,000×, respectively. The output was selected for methylKit format. Coverage statistics and differentially methylated regions were calculated for CG sites with methylKit installed in R (v3.5) (Akalin et al., 2012). Differentially methylated cytosines were defined as being present in both biological replicates, having a minimum absolute difference of 1% using the coverage weighted mean and having a SLIM adjusted q-value < 0.01 using the methylKit logistic regression model (Ning et al., 2011). The change in mean percent methylation (Δme) for all CpG sites within a defined region was calculated by taking the mean number of methylated versus non-methylated CpG sites from the pooled control and treated samples and using Fisher's exact test FDR < 0.05.

Chromatin Immunoprecipitation

All chemicals were purchased from Sigma unless otherwise noted (St. Louis, MO). HEK293 cells were harvested after a 24-h treatment and resuspended in fresh media at 10×10^6 cells/mL in a conical tube. Cells were fixed with 1% formaldehyde for 10 min at room temperature. Reaction was stopped with 0.2 M glycine and incubation at room temperature for 5 min. Fixed cells were centrifuged for 5 min at 300 × g and 4°C and washed with 1 mL cold PBS. Fixed cell pellet was stored at -80°C until chromatin immunoprecipitation (ChIP).

Cell pellets were resuspended at approximately 1×10^6 cells/0.1 mL with PBS + 0.5% Triton-X + 1% protease inhibitor cocktail and incubated on ice for 10 min prior to centrifugation for 5 min at 400 × g 4°C. The pellet was resuspended in TE buffer pH 8.0 with protease inhibitor and PMSF. Cells were sonicated at high intensity for 30 s on/60 s off until DNA fragments were within 200–800 bp as checked by 2% agarose gel. After sonication, samples were centrifuged for 15 min at 14,000 × g 4°C to pellet insoluble material. Sheared chromatin was transferred to RIPA buffer, and 10% of total chromatin was saved for input DNA extraction.

Chromatin immunoprecipitation was done with Dynabeads Protein A (Invitrogen, Carlsbad, CA, United States) and 4 μg of primary ChIP-grade antibody (H3K27ac Abcam ab4729; CTCF Millipore 07-729; Rabbit IgG Santa Cruz Biotechnology sc-2025). Beads were washed with lithium chloride (LiCl 0.25 M) buffer, and immunoprecipitated DNA was extracted from beads using the phenol: chloroform method. DNA was quantified using Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific, Waltham, MA, United States).

ChIP-qPCR Analysis

We selected eight genes for quantitative real-time PCR (qPCR) analysis using primers listed in **Supplementary Table 3**. Primers were designed with NCBI Primer Blast at H3K27ac peaks surrounding the predicted CTCF binding site (Ye et al., 2012). All qPCR reactions were performed on a 7500 Real-Time PCR

[1]https://github.com/dpryan79/MethylDackel

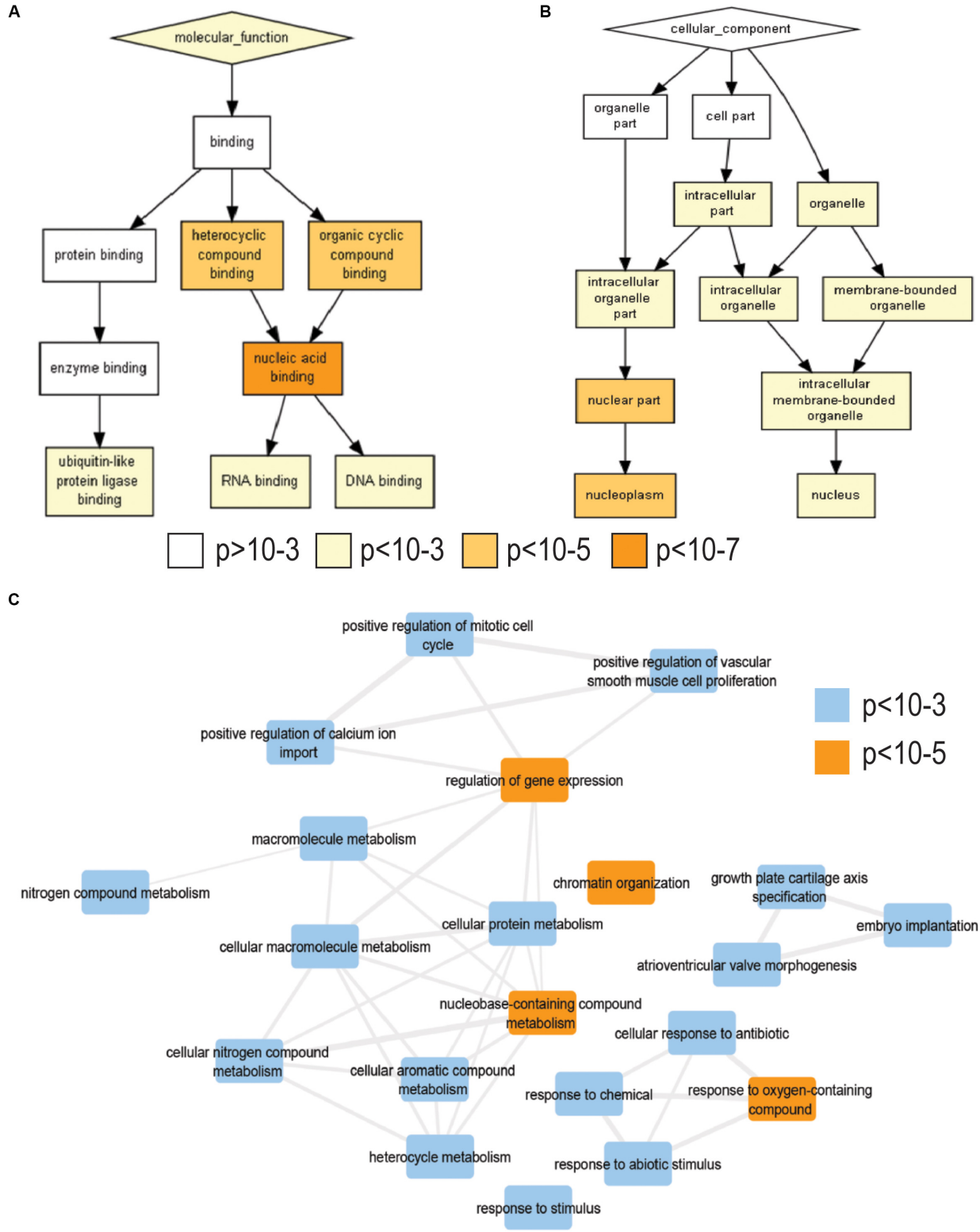

FIGURE 1 | Gene Ontology enrichment analysis of RNA-sequencing data. **(A)** Gene Ontology molecular functions enriched in our differentially expressed genes from rotenone-treated HEK293 cells. **(B)** Gene Ontology cellular component enriched. Color gradient indicates enrichment *p*-value: white, $>10^{-3}$; yellow, 10^{-3}–10^{-5}; light orange, 10^{-5}–10^{-7}; dark orange, $<10^{-7}$. Enrichment *p*-value and adjusted FDR for each term are listed in **Supplementary Tables 5, 6**. **(C)** Network analysis of significantly enriched biological processes. Blue boxes enrichment *p*-value $< 10^{-3}$ and orange boxes enrichment *p*-value $< 10^{-5}$. Rotenone treatment changes the expression of genes involved in transcription factor signaling in the nucleus.

system from Applied Biosystems (Foster City, CA, United States) using the iTaq Universal SYBR Green Supermix from Bio-Rad (Hercules, CA, United States). H3K27ac and CTCF enrichment was calculated from the Ct threshold value as a percent of the total input DNA. Rabbit IgG samples were used as a negative control (**Figures 5**, **6B**).

RESULTS

Rotenone-Induced Stress Alters Transcription Factor Intracellular Signaling

To characterize the changes in gene expression upon rotenone exposure, we treated HEK293 cells with rotenone 200 nM for 24 h. We then used RNA-seq analyses to identify over 2000 DEGs in response to rotenone (**Supplementary File 3**). To gain insights of impacted biological processes, we performed gene ontology enrichment analysis on 1853 of these DEGs with known HUGO (hgnc) symbol and cell description using a p-value threshold of $p < 10^{-3}$. Among enriched biological processes, we observed a significant induction of the oxidative stress response, transcription factor activity, and chromatin organization ($<10^{-3}$) (**Figure 1C**). We observed a significant enrichment of genes involved in nucleic acid binding ($<10^{-7}$) and DNA binding ($<10^{-5}$) with the nuclear cell component being most represented ($<10^{-5}$) (**Figures 1A,B** and **Supplementary Tables 5, 6**). We analyzed pathway nine enrichment of the top 200 genes with the largest change in expression using reactome pathways (Fabregat et al., 2018; **Supplementary Table 4**). Three of the top five pathways enriched in our data were major transcription factor pathways including SMAD, NOTCH, and TP53 which have implications in PD reviewed in the discussion section.

Alteration of PD-Associated Genes Stands Out Upon Rotenone Exposure

The GWAS Catalog is a public database of approximately 72,000 variant–trait associations from over 3500 publications (Buniello et al., 2018). Out of 246 PD-associated variants with genetic sequence context information in the GWAS Catalog, 220 variants (89%) were in non-coding regions (intron, intergenic, regulatory, and exon) (**Supplementary File 2**). Intronic variants constituted most of the known polymorphisms. We searched our DEGs for PD-associated genes and identified 14 genes from the GWAS Catalog (**Supplementary File 2**). Of these genes, 13 were also considered significant PD-associated genes according to meta-analysis data in the PD gene (Lill et al., 2012). We validated the RNA sequencing results for 10 of these genes and were able to validate 8 of them with qPCR analysis (R^2 = 0.96) (**Figure 2**). We selected five genes (*ITGA8*, *GPRIN3*, *FER*, *CNKSR3*, *BMP4*) and three additional genes from the PD gene meta-analysis (*UBOX5*, *PARK2*, *CHCHD2*) for further examination of epigenetic patterns (**Table 1**).

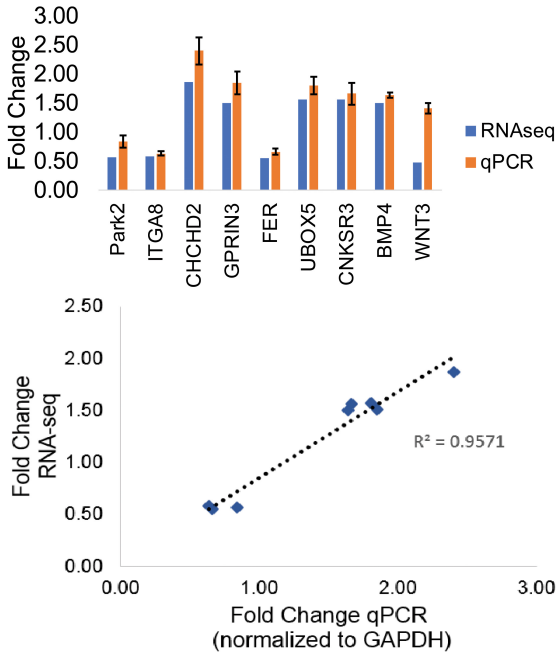

FIGURE 2 | RNA-sequencing validation of PD-associated genes. Fold-change comparison of RNA sequencing results versus qPCR results and the linear calibration curve of RNA sequencing results with qPCR results expressed as fold change in expression.

Selected Genes Contain Prominent CTCF Binding Sites in Their Regulatory Non-coding Regions

To examine any potential CTCF motifs within these selected genes, we visualized CTCF binding using experimental data from ENCODE and the CTCF binding prediction tool from the Cui Lab at the University of Tennessee (ENCODE Project Consortium, 2012; Ziebarth et al., 2012). Intriguingly, all selected genes had at least one prominent CTCF binding site in a regulatory non-coding region (**Table 1**). We designed both bisulfite primers and ChIP primers at these sites using ENCODE regulation data from the Broad Institute (ENCODE Project Consortium, 2012). The polymorphisms in these regions that were recognized by the SNP database (dbSNP[2]) were also analyzed with Regulomedb, a database that annotates SNPs with known or predicted interactions with regulatory elements in intergenic regions (Boyle et al., 2012). We determined that SNPs within selected regions at two genes, *GPRIN3* and *FER*, were present at CTCF binding sites and were active in the brain (**Table 2**). The rank of an SNP represents the number of available datasets for that polymorphism, and the score is generated based on the integrated results from available datasets. In this analysis, the polymorphism listed at each gene was present in datasets from experimental transcription factor binding, matched transcription factor position-weight matrix (PWM), and DNase footprinting. We checked the HEK293 genome using the online

[2]http://www.ncbi.nlm.nih.gov/SNP

TABLE 1 | Selected non-coding genetic variants.

HGNC	Log2 FC	FDR	Motif sequence	SNP*	Number of studies	*p*-value	Cell function
FER	−0.86	6.21E-07	AGCAGAGCA	rs13178668	13	<0.05	Tyrosine kinase activates cell surface signaling.
Park2	−0.82	3.55E-03	GTTGCCAGTAGGTGGCTCAC	rs4388272	13	<0.05	E3 ubiquitin ligase targets proteins for degradation.
ITGA8	−0.78	7.24E-06	GGAAGTCCA	rs10737104	15	2.70E-07	Transmembrane receptor activates various cell signaling.
BMP4	0.59	1.16E-02	GGAAGTGCG	rs148491084	3	<0.05	Secreted regulatory protein regulates development.
GPRIN3	0.60	3.31E-04	GGAACTGAA	rs10014765	15	6.48E-05	G-protein regulates neurite outgrowth.
CNKSR3	0.65	2.44E-05	CTCCCTCTACCTGT	rs145160741	12	<0.05	Scaffold protein signals membrane dynamics.
UBOX5	0.65	6.46E-04	CGTCCTCCAGTGGA	rs55785911	21	3.30E-10	Interacting protein signals ubiquitin proteasome pathway.
CHCHD2	0.90	2.00E-04	GGAAGAGCA	rs11978209	12	<0.05	DNAbinding protein signals oxidative stress response.

** SNP information found at PDGene Database (Lill et al., 2012)*

database[3] and did not find either variant in our cells (Lin et al., 2014). This information provides additional evidence that CTCF binding sites among common non-coding variants may be critical in disease pathogenesis.

Rotenone Modifies Epigenetic Patterns Across the Genome

We used the dot blot method to qualitatively assess changes in DNA methylation levels in cells exposed to rotenone. The total methylation level was detected with anti-5mC antibody and visualized using chemiluminescence. After 24 h, 5mC levels were strikingly reduced in genomic DNA (**Figure 3A**).

Next, we asked the extent to which rotenone exposure impacted histone acetylation. To investigate histone acetylation at active enhancers with PD-relevant SNPs, we selected H3K27ac mark (Wang et al., 2008) and examined H3K27ac levels from extracted histones using western blot. We measured a significant 1.3-fold increase in H3K27ac in rotenone-treated cells compared to the DMSO vehicle control ($p < 0.05$) (**Figure 3B**).

Rotenone Alters DNA Methylation Patterns at CTCF Binding Sites in Regulatory Regions of PD-Associated Genes

Because CTCF binding is methylation sensitive and changes in CG methylation correlate with disease risks (Wang et al., 2012), we next examined a total of 284 CG nucleotides from eight regions surrounding our selected genes. Our amplicon-sequencing results demonstrated that 233 of these nucleotides met our minimum requirement of 1000 × coverage (**Supplementary Figure 2**). We focused our analysis on 165 CG sites that met minimum coverage requirements and overlapped predicted CTCF binding motifs at seven of the selected genes. There were 45 differentially methylated CG sites, and 53% were hypermethylated (**Table 3**). Two of these CG sites, *FER* cg143 at chr5:102025097 and CHCHD2 cg217 at chr7:56174103, were significantly hypomethylated (*FER cg143*$\Delta = -4.4$) and hypermethylated (*CHCHD2 cg217*$\Delta = 1.7$) at the predicted CTCF binding sequence (**Figures 4A,B**). The DNA sequence of the CTCF motif was predicted with the CTCF prediction tool by Ziebarth et al. (2012). The different motif sequences, or PWMs, found within the enhancer of these two genes are listed in **Figures 4A,B**. The score of each PWM corresponds to the log-odds of the observed sequence being specific rather than randomly generated. Two genes, *PARK2* and *UBOX5*, were significantly hypomethylated (*PARK2*$\Delta = -1.3$) and hypermethylated (*UBOX5*$\Delta = 0.33$) across the entire CTCF binding region with $p < 0.05$ but did not remain significant after multiple hypothesis testing (FDR > 0.05) (**Figures 4C,D**). Collectively, we conclude that methylation of CTCF binding motifs was vulnerable to rotenone exposure.

[3]http://hek293genome.org/v2/

TABLE 2 | Regulomedb results for SNPs within selected regions.

Gene	chr:start	SNP ID	Rank	Score
GPRIN3	chr4:90228735	rs2116326	2a	0.96
FER	chr5:108084548	rs113728457	2a	0.92

Rotenone Alters H3K27ac at CTCF Binding Sites in Regulatory Regions of PD-Associated Genes

We used ChIP-qPCR to test whether local H3K27ac enrichment overlapped CTCF binding sites in PD-associated genes. Our ChIP-qPCR results demonstrate that four genes (*GPRIN3*, *UBOX5*, *FER*, and *BMP4*) had significantly increased H3K27ac at CTCF binding motifs with FDR < 0.05. One gene, *CNKSR3*, had reduced H3K27ac at its CTCF binding motif but was not statistically significant ($p = 0.07$; FDR = 1) (**Figure 5A**). H3K27ac enhancer activity was correlated with gene upregulation in

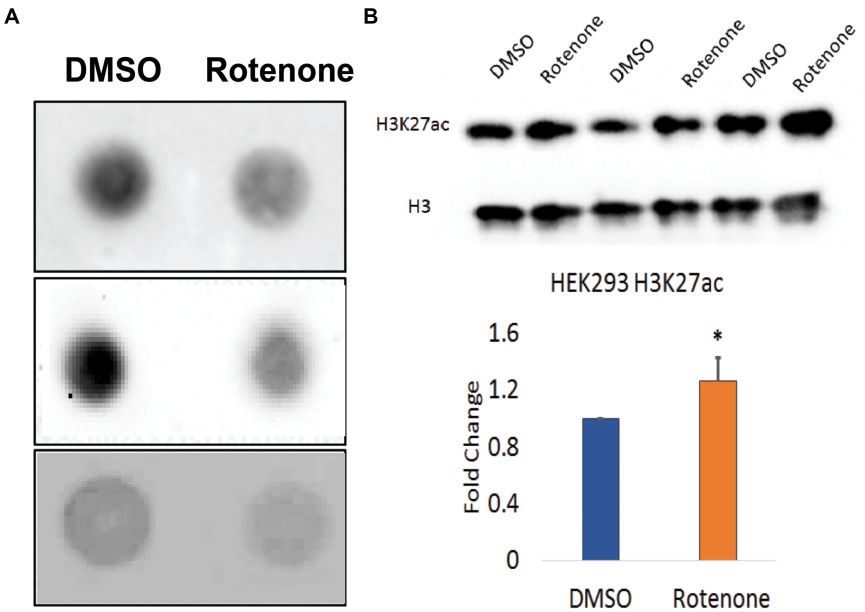

FIGURE 3 | Global epigenetic patterns in rotenone treated *HEK293* cells. **(A)** DNA methylation was visualized by dot blot method using the anti-5mC antibody. Three biological replicates are shown in this image. The standard ladder using 100% 5mC standard is shown in **Supplementary Figure 1**. **(B)** Global histone H3K27ac levels were measured from total extracted histones using Western blot. Total histone H3 was used as the loading control. Three biological replicates are shown in this image. This western blot was quantified using ImageJ software and is shown as the fold change in the amount of H3K27ac relative to the vehicle (DMSO) control. $*p < 0.05$ using a paired Student's *t*-test with the ratio of H3K27ac/H3.

three genes (*GPRIN3, UBOX5, BMP4*). *FER* was downregulated despite increased H3K27ac in its first intron but may be related to changes in DNA methylation within that region, altered posttranscriptional regulation, or limitations of targeted ChIP analysis. Interestingly, the H3K27 region amplified in qPCR overlapped at least one differentially methylated cytosine for all four significantly enhanced genes (**Table 4**). Only one of the genes without H3K27ac enrichment, *CHCHD2*, also had differentially methylated cytosines within the amplified region. These genes had both increased and decreased changes in percent methylation.

Rotenone Increases CTCF Binding at Three PD-Associated Genes

To determine whether altered DNA methylation and H3K27ac patterns would affect CTCF binding, we measured CTCF enrichment at its binding motif at seven PD-associated genetic loci. CTCF binding was increased at three genes (*PARK2, GPRIN3*, and *BMP4*) (**Figure 6A**). *BMP4* had one hypomethylated CG and increased H3K27ac within our selected region. There was an increase in CTCF binding and mRNA expression. *PARK2* had two hypomethylated CGs but no increase in H3K27ac in its CTCF binding domain. In this region, CTCF binding increased and mRNA expression decreased. *GPRIN3*, unlike the other two genes, had more hypermethylated CGs within its CTCF binding motif but the closest CG to its consensus sequence was also hypomethylated. There was increased H3K27ac enrichment at *GPRIN3* and increased CTCF binding. *GPRIN3* mRNA was significantly upregulated in response to rotenone. These data suggest that both DNA methylation and H3K27ac influence CTCF transcription factor binding and impact the expression of PD-associated genes (**Table 5**).

DISCUSSION

We selected rotenone based on its ability to model gene–environment interactions in rodents and non-mammalian models of PD (Cannon and Greenamyre, 2013; Johnson and Bobrovskaya, 2015). It is estimated that chronic exposure to concentrations of approximately 20–30 nM of rotenone is enough to cause degeneration of dopaminergic neurons in the midbrain (Greenamyre et al., 2003). While rotenone is a potent inhibitor of complex I in the electron transport chain of mitochondria, rotenone has been shown to cause neurodegeneration by mechanisms unrelated to its effect on complex I (Sherer et al., 2007; Choi et al., 2008). The transcriptome and its regulation have become a focus for understanding these mechanisms outside of the electron transport chain (Cabeza-Arvelaiz and Schiestl, 2012). We observed large-scale changes in gene expression profiles, and many of these genes were enriched in processes involved in gene regulation and chromatin organization (**Figure 1**). The pathway analysis of DEGs also revealed a large involvement in major intracellular transcription factor pathways. For instance, SMAD proteins are critical for transducing signals from the transforming growth factor (TGFβ) receptors at the plasma membrane which are essential for midbrain dopaminergic survival (Hegarty et al., 2014). Notch signaling is known to

TABLE 3 | Differentially methylated CG sites at Parkinson's disease-associated genes.

CHR_GENE	CG	*p*-value	*q*-value	Delta
chr14_54422869_54423420_BMP4	54423352	5.48E-08	5.60E-08	−3.3
chr20_3140226_3140678_UBOX5	3140420	2.59E-27	9.41E-27	2.3
chr20_3140226_3140678_UBOX5	3140429	3.46E-34	1.62E-33	3.6
chr4_90228647_90229070_GPRIN3	90228692	2.37E-07	2.24E-07	−1.1
chr4_90228647_90229070_GPRIN3	90228700	1.87E-13	3.06E-13	1.3
chr4_90228647_90229070_GPRIN3	90228702	3.42E-13	5.41E-13	1.2
chr4_90228647_90229070_GPRIN3	90228709	1.23E-33	5.49E-33	−2.5
chr4_90228647_90229070_GPRIN3	90228753	2.38E-09	2.82E-09	1.1
chr4_90228647_90229070_GPRIN3	90228761	1.33E-13	2.29E-13	1.7
chr4_90228647_90229070_GPRIN3	90228764	4.10E-07	3.73E-07	1.0
chr4_90228647_90229070_GPRIN3	90228792	2.58E-11	3.61E-11	−1.2
chr4_90228647_90229070_GPRIN3	90228822	4.03E-16	8.78E-16	1.7
chr4_90228647_90229070_GPRIN3	90228849	1.47E-36	8.03E-36	2.7
chr4_90228647_90229070_GPRIN3	90228860	3.23E-44	2.88E-43	1.8
chr5_108084418_108084954_FER	102025087	1.53E-06	1.26E-06	−1.6
chr5_108084418_108084954_FER	102025097	5.23E-76	8.56E-75	−4.4
chr5_108084418_108084954_FER	102025117	1.62E-140	1.59E-138	−4.9
chr5_108084418_108084954_FER	102025151	2.40E-14	4.62E-14	−1.3
chr5_108084418_108084954_FER	102025176	2.79E-12	4.14E-12	1.6
chr5_108084418_108084954_FER	102025225	1.05E-16	2.39E-16	1.9
chr5_108084418_108084954_FER	102025228	1.12E-19	2.98E-19	3.4
chr5_108084418_108084954_FER	102025231	1.23E-11	1.79E-11	−2.8
chr5_108084418_108084954_FER	102025234	1.56E-15	3.26E-15	1.7
chr5_108084418_108084954_FER	102025251	4.73E-03	2.52E-03	−1.8
chr5_108084418_108084954_FER	102025320	5.38E-07	4.77E-07	1.9
chr5_108084418_108084954_FER	102025330	3.76E-04	2.32E-04	1.3
chr5_108084418_108084954_FER	102025334	2.60E-04	1.67E-04	−1.7
chr5_108084418_108084954_FER	102025341	1.36E-15	2.90E-15	2.7
chr5_108084418_108084954_FER	102025408	2.37E-07	2.24E-07	2.1
chr5_108084418_108084954_FER	102025430	2.26E-03	1.28E-03	−1.4
chr6_154830537_154830958_CNKSR3	154830543	8.22E-22	2.44E-21	−2.5
chr6_154830537_154830958_CNKSR3	154830584	1.66E-24	5.63E-24	1.8
chr6_154830537_154830958_CNKSR3	154830770	2.43E-59	2.65E-58	−1.5
chr6_154830537_154830958_CNKSR3	154830810	1.02E-43	8.35E-43	1.7
chr6_154830537_154830958_CNKSR3	154830817	3.60E-14	6.66E-14	−1.1
chr6_154830537_154830958_CNKSR3	154830837	1.41E-93	6.93E-92	3.3
chr6_154830537_154830958_CNKSR3	154830863	8.54E-20	2.33E-19	−1.0
chr6_163277806_163278291_PARK2	163277841	3.89E-04	2.38E-04	−1.7
chr6_163277806_163278291_PARK2	163277943	8.27E-07	7.12E-07	−2.0
chr7_56173886_56174373_CHCHD2	56174016	1.67E-08	1.76E-08	−1.1
chr7_56173886_56174373_CHCHD2	56174033	4.52E-04	2.75E-04	−1.9
chr7_56173886_56174373_CHCHD2	56174103	1.71E-05	1.29E-05	1.7
chr7_56173886_56174373_CHCHD2	56174107	1.01E-08	1.11E-08	−1.1
chr7_56173886_56174373_CHCHD2	56174149	2.07E-04	1.37E-04	1.6
chr7_56173886_56174373_CHCHD2	56174179	3.24E-04	2.05E-04	1.2

have an important role in regulating genes involved in nervous system development and synaptic plasticity (Ables et al., 2011). Lastly, the TP53 pathway is perhaps the most well-known of the toxicant-induced signaling mechanisms to control cell cycle progression and cellular survival. It is thus a critical regulator of programmed cell death in PD and rotenone-induced neurotoxicity (Venderova and Park, 2012).

To determine if the gene expression changes discussed above were due to changes in global levels of epigenetic patterns, we performed dot blots and Western blots to examine global levels of DNA methylation (5mC) and H3K27ac (**Figures 3A,B**). DNA methylation is the best-studied epigenetic modification, and small changes in methylation at regulatory regions of the genome can have substantial effects on genome integrity during

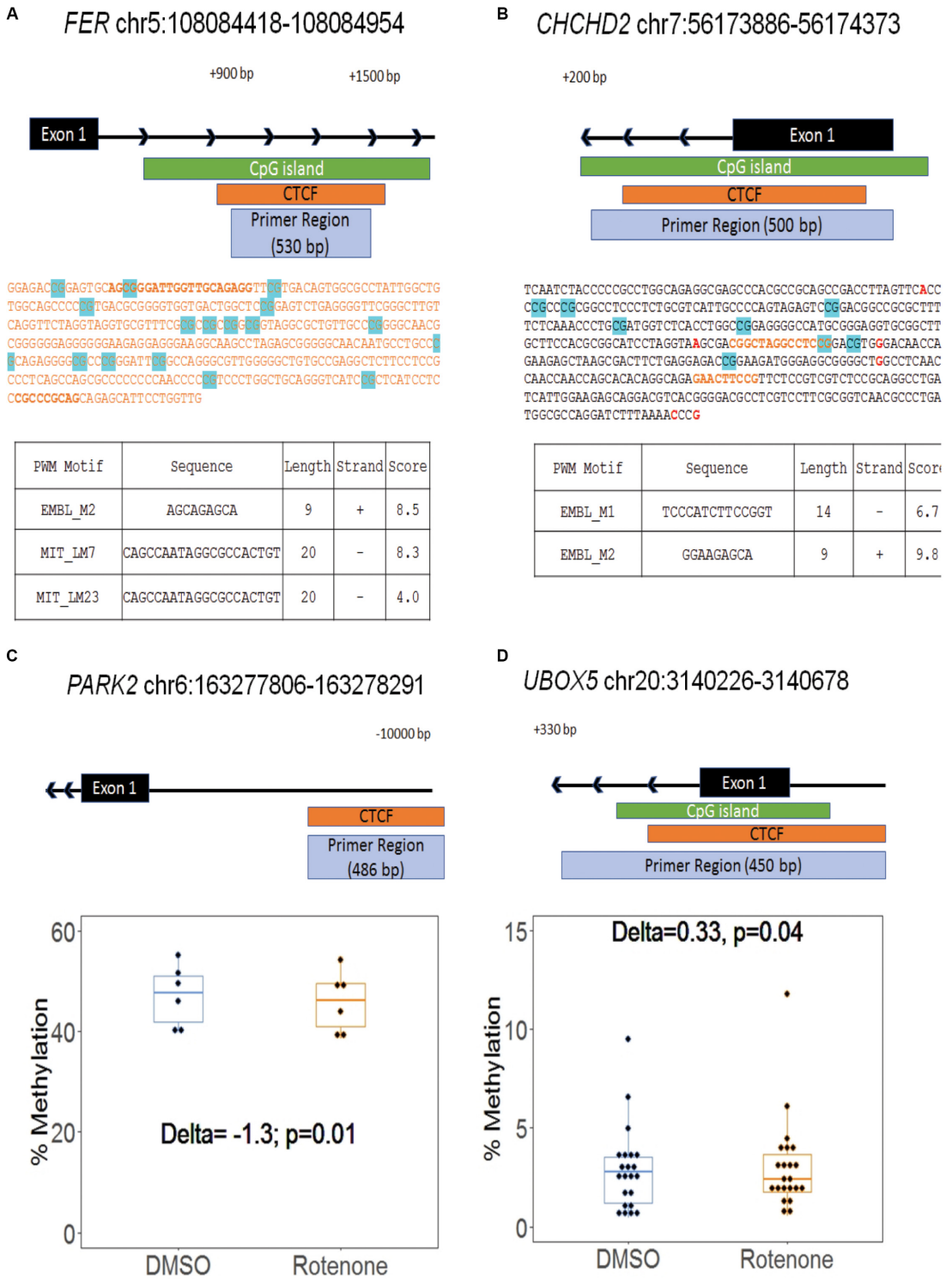

FIGURE 4 | Differential methylation within CTCF motifs at Parkinson's disease genes. **(A,B)** Two genes, *FER and CHCHD2*, had differential methylation at CG sites within their predicted CTCF binding motif. The amplified region at both genes covered the first exon and intron. The highlighted blue CG sites are all with significant differential methylation (>|1%|; *q*-value < 0.05). Red text emphasizes a common SNP. Orange text represents CTCF binding sites in the human genome identified by ENCODE. The bold orange text is the sequence motif predicted by the Cui Lab CTCF prediction tool (Ziebarth et al., 2012). The output of the CTCF binding prediction tool is listed in the table with the name of the position weight matrix motif, motif sequence, motif length, strand orientation, and the integrated output score. **(C,D)** Two genes, *PARK2* and *UBOX5*, had a significantly different methylation across the region. Each dot represents a CG within the region. Delta indicates the change in the mean CpG methylation percentage and the associated *p*-value from Fisher's exact test.

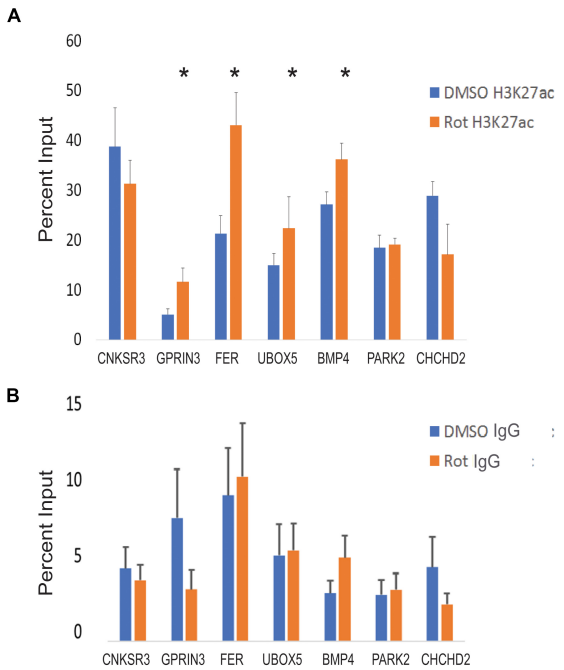

FIGURE 5 | CTCF site H3K27 enhancer activation in response to rotenone. **(A)** The local abundance of H3K27ac within CTCF binding sites at Parkinson's disease-associated genes was measured with ChIP-qPCR and expressed as the percent of total DNA input used for immunoprecipitation. **(B)** The negative control for ChIP analysis was Rabbit IgG. Significance was tested with paired Student's *t*-test using the percent input of vehicle (DMSO) vs rotenone, and *post hoc* analysis for multiple hypotheses was done using the false discovery method. *FDR < 0.05.

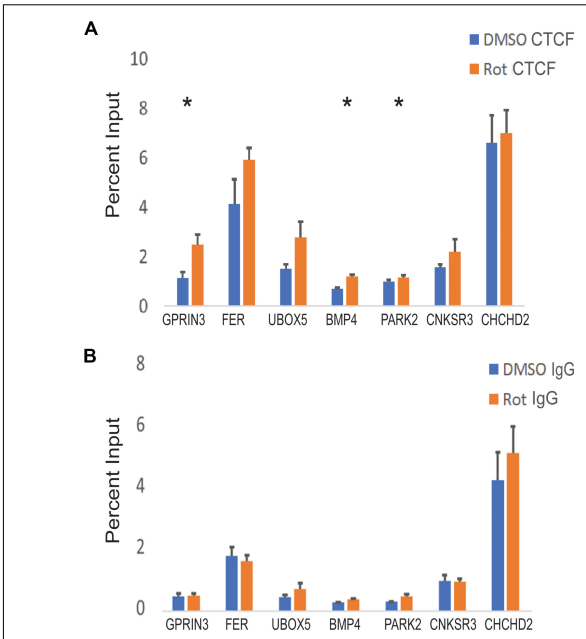

FIGURE 6 | CTCF binding at PD-associated genes in response to rotenone. **(A)** The local abundance of CTCF binding at Parkinson's disease-associated genes was measured with ChIP-qPCR and expressed as the percent of total DNA input used for immunoprecipitation. **(B)** The negative control for ChIP analysis was Rabbit IgG. Significance was tested with paired student's *t*-test using the percent input of vehicle (DMSO) vs rotenone and *post hoc* analysis for multiple hypotheses was done using the false discovery method. *FDR < 0.05.

aging (Horvath, 2013). As seen with other pesticide models, we observed a global decrease in total 5mC in response to rotenone. We also chose to look at global H3K27ac levels because it is tightly correlated with gene expression and vulnerable to environmentally driven enhancer activation (Wang et al., 2008). We observed a significant increase in H3K27ac across the genome. H3K27ac is not only an important mark to distinguish poised from active enhancers in bivalent chromatin but also a critical epigenetic modulator in post-mitotic neurons (Maze et al., 2015).

We searched the DEGs for non-coding risk variants associated with PD (fc = fold change). We discovered 13 genes with significant association to PD using GWAS meta-data. We focused on eight genes (*ITGA8* 0.6 fc, *GPRIN3* 1.5 fc, *FER* 0.6 fc, *CNKSR3* 1.6 fc, *BMP4* 1.5 fc, *UBOX5* 1.6 fc, *PARK2* 0.6 fc, and *CHCHD2* 1.9 fc) that remained significantly associated in at least two studies with their most significant variant lying in a non-coding region (PDGene; Lill et al., 2012). *UBOX5* was the most significantly associated variant according to GWAS meta-data. Furthermore, *UBOX5* was the only identified gene with its most significant non-coding variant having known interactions with regulatory elements such as CTCF (Regulomedb; Boyle et al., 2012).

UBOX5 is predicted to have a role in the ubiquitin proteasome system, a well-known PD pathway involved in protein quality control and cellular detoxification (McNaught and Jenner, 2001; McNaught et al., 2003). This pathway is involved in the function of multiple PD-associated genes most notably *PARK2* which encodes a ubiquitin ligase. Mutations in *PARK2* account for approximately 50% of familial early-onset PD, but the frequency of these mutations decreases with age (Bekris et al., 2010). These mutations generally occur at exon sequences, but other less penetrant but significantly associated polymorphisms with higher frequency in the population occur more often at intronic or regulatory sequences of the gene.

Each of the selected genes had a CTCF binding site determined by ENCODE and the CTCF prediction tool (ENCODE Project Consortium, 2012; Ziebarth et al., 2012). We used the online tool, Regulomedb, to investigate whether SNPs in these CTCF binding regions had evidence of an interaction with CTCF (**Table 2**). There was evidence of a CTCF interaction in the brain in two of the selected genes *GPRIN3* and *FER*. This rank score generated by Regulomedb is indicative of the strength of the evidence for this interaction with one being the greatest.

CCCTC-binding factor binds regions with allele-specific methylation and preferentially binds the unmethylated allele (Wang et al., 2018). The methylation status of these allele-specific methylated regions is critical to functional CTCF binding and can explain as much as 41% of its variability (Wang et al., 2012). We have previously identified allele-specific methylated regions in the human genome and verified their sensitivity to rotenone exposure (Martos et al., 2017; Freeman et al., 2020). Therefore,

TABLE 4 | Differentially methylated CG sites within H3K27ac-enriched regions.

CHR_GENE	CG	*p*-value	*q*-value	Delta
chr14_54422869_54423420_BMP4	54423352	5.48E-08	5.60E-08	−3.3
chr20_3140226_3140678_UBOX5	3140420	2.59E-27	9.41E-27	2.3
chr20_3140226_3140678_UBOX5	3140429	3.46E-34	1.62E-33	3.6
chr4_90228647_90229070_GPRIN3	90228822	4.03E-16	8.78E-16	1.7
chr4_90228647_90229070_GPRIN3	90228849	1.47E-36	8.03E-36	2.7
chr4_90228647_90229070_GPRIN3	90228860	3.23E-44	2.88E-43	1.8
chr5_108084418_108084954_FER	1.02E + 08	1.05E-16	2.39E-16	1.9
chr5_108084418_108084954_FER	1.02E + 08	1.12E-19	2.98E-19	3.4
chr5_108084418_108084954_FER	1.02E + 08	1.23E-11	1.79E-11	−2.8
chr5_108084418_108084954_FER	1.02E + 08	1.56E-15	3.26E-15	1.7
chr5_108084418_108084954_FER	1.02E + 08	4.73E-03	2.52E-03	−1.8
chr5_108084418_108084954_FER	1.02E + 08	5.38E-07	4.77E-07	1.9
chr5_108084418_108084954_FER	1.02E + 08	3.76E-04	2.32E-04	1.3
chr5_108084418_108084954_FER	1.02E + 08	2.60E-04	1.67E-04	−1.7
chr5_108084418_108084954_FER	1.02E + 08	1.36E-15	2.90E-15	2.7
chr5_108084418_108084954_FER	1.02E + 08	2.37E-07	2.24E-07	2.1
chr5_108084418_108084954_FER	1.02E + 08	2.26E-03	1.28E-03	−1.4

we hypothesized that CTCF binding sites at PD-associated genes would also be vulnerable to rotenone. Out of 165 CG sites that met minimum coverage requirements and overlapped predicted CTCF binding motifs, we detected 45 differentially methylated cytosines (**Table 3**). In two of the genes, the cytosines within the CTCF consensus sequence were differentially methylated but not in any consistent direction (*FER*-hypomethylated; *CHCHD2*-hypermethylated) (**Figures 4A,B**). We saw a similar trend in *PARK2* and *UBOX5* which had differential methylation across the whole binding region but did not change in a consistent direction (*PARK2*-hypomethylated) and (*UBOX5*-hypermethylated) (**Figures 4C,D**). Overall, there was a slight increase in hypermethylated cytosines (53%) indicating a potential decrease in CTCF binding capacity.

One of the primary functions of CTCF is to act as an insulator by blocking enhancer–promoter interactions (Phillips and Corces, 2009). CTCF is thus tightly correlated with enhancer activity, and its interaction with active enhancers topologically is much greater than with silent regions of the genome (Ren et al., 2017). Histone acetylation patterns also determine chromatin structure, and the histone mark H3K27ac is correlated with active enhancer regions (Wang et al., 2008; Creyghton et al., 2010). p300 is one of the primary histone acetyltransferase enzymes and loads the acetyl group onto the lysine tail of histone 3 at active regions. CTCF binding sites are often located next to at least one p300 binding site and interacts with p300 at the chromatin with active acetylation (Ren et al., 2017).

Histone acetylation patterns are vulnerable to environmental factors and like DNA methylation are heritable (Chinnusamy and Zhu, 2009; Dai and Wang, 2014; Zhu et al., 2018). It is likely that histone acetylation patterns also contribute to the role of genetic variants in disease pathogenesis. We tested H3K27ac levels at CTCF binding sites to determine if acetylation patterns were also sensitive to rotenone at PD-associated genes. We saw an increase in H3K27ac at four of the eight identified genes suggesting strong chromatin interactions with CTCF (FDR < 0.05) (**Figure 5A**). Notably, all four genes with H3K27ac also overlapped a differentially methylated cytosine within the CTCF binding region (**Table 4**). This suggests a cross talk mechanism with DNA methylation patterns and H3K27ac enrichment at CTCF binding sites to control chromosomal organization and SNP impacted gene expression.

We observed increased CTCF binding at three differentially expressed PD-associated genes (**Figure 6A**). *PARK2* is a well-known genetic factor in PD as described earlier. Increased CTCF binding at its upstream enhancer decreased its expression, thereby affecting its role in the ubiquitin proteasome system. *BMP4* is a gene that encodes bone morphogenetic protein 4, and it regulates neurite outgrowth and axonal transport through the activation of the TGFβ/Smad pathway which is disrupted according to RNA sequencing reactome enrichment data (**Supplementary Table 4**). Increased CTCF binding was associated with increased *BMP4* promoter expression which is essential for dopaminergic neuron differentiation and survival (**Table 5**; Hegarty et al., 2014). *GPRIN3* encodes a protein involved in microtubule dynamics and neurite outgrowth which are both impaired in rotenone-induced neurotoxicity (Cabeza-Arvelaiz and Schiestl, 2012). Interestingly, increased CTCF binding occurred at *GPRIN3* within an active transcription start site in the substantia nigra (**Table 5**;

TABLE 5 | Correlation of gene expression changes with CTCF binding at PD-associated genes.

	PARK2	*GPRIN3*	*BMP4*
RNA	↓	↑	↑
Methylation	↓	↑↓	↓
H3K27ac	–	↑	↑
CTCF	↑	↑	↑

Boyle et al., 2012). It is also associated with a fully penetrant PD mutation causing a triplication of these loci and doubling of *GPRIN3* mRNA transcripts (Devine et al., 2011). This observed increase in mRNA transcripts in a mature dopaminergic neuron differentiated from a patient was comparable to the observed 1.5-fold change increase in our rotenone-treated cells.

DATA AVAILABILITY STATEMENT

RNA sequencing data has been submitted to the Gene Expression Omnibus (GEO) repository at NCBI Databank with record number GSE147617. Bisulfite-amplicon sequencing data is publicly available on the sequence read archive (SRA) at NCBI Databank with Bioproject ID PRJNA615220. All other data used to generate this report are available upon request from the corresponding author ZW (zwang47@jhu.edu).

AUTHOR CONTRIBUTIONS

DF designed and performed experiments with supervision from ZW. Both authors analyzed and interpreted data and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

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Demethylation of the NRF2 Promoter Protects Against Carcinogenesis Induced by Nano-SiO_2

Dan Lou[1,2†], Xiaoyi Wei[3†], Ping Xiao[1], Qian Huo[1], Xinyu Hong[1], Jingqiu Sun[1], Yi Shuai[4*] and Gonghua Tao[1*]*

[1] Shanghai Municipal Center for Disease Control & Prevention, Shanghai, China, [2] Department of Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, United States, [3] Department of Food Science, Shanghai Business School, Shanghai, China, [4] Syngenta (China) Investment Company Limited, Shanghai, China

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***Correspondence:**
Dan Lou
dlou1@jhu.edu
Yi Shuai
yi.shuai@syngenta.com
Gonghua Tao
taogonghua@scdc.sh.cn

† These authors have contributed equally to this work and share first authorship

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Nano silicon dioxide (Nano-SiO_2) has been widely used in industries such as the field of biomedical engineering. Despite the existing evidence that Nano-SiO_2 exposure could induce oxidative stress and inflammatory responses in multiple organ systems, the carcinogenicity of Nano-SiO_2 exposure has rarely been investigated. Thus in this study, two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as *in vitro* models to investigate the carcinogenicity of Nano-SiO_2. Our results revealed that Nano-SiO_2 induces a malignant cellular transformation in human bronchial epithelial cells according to the soft agar colony formation assay. The carcinogenesis induced by Nano-SiO_2 was also confirmed in nude mice. By using immunofluorescence assay and high-performance capillary electrophoresis (HPCE), we observed a genome-wide DNA hypomethylation induced by Nano-SiO_2. Besides the reduced enzyme activity of total DNMTs upon Nano-SiO_2 treatment, altered expression of DNMTs and methyl-CpG binding proteins were observed. Besides, we found that the expression of NRF2 was activated by demethylation of CpG islands within the NRF2 promoter region and the overexpression of NRF2 could alleviate the carcinogenesis induced by Nano-SiO_2. Taken together, our results suggested that Nano-SiO_2 induces malignant cellular transformation with a global DNA hypomethylation, and the demethylation of NRF2 promoter activates the expression of NRF2, which plays an important role in protecting against the carcinogenesis induced by Nano-SiO_2.

Keywords: Nano-SiO_2, malignant transformation, carcinogenesis, DNA methylation, NRF2

INTRODUCTION

Nano silicon dioxide (Silica nanoparticles, Nano-SiO_2), due to its special characteristics of large surface area and optical transparency, has been widely used in various fields including biomedical imaging, drug delivery, cosmetics, and electronics industry (Vivero-Escoto et al., 2012; Sweeney et al., 2016; Mohajerani et al., 2019). Meanwhile, its widespread applications raise potential health risks to humans through occupational and environmental exposure. Recent studies have demonstrated that Nano-SiO_2 exposure in a short term could induce oxidative stress, mitochondria dysfunction, and inflammatory responses in multiple organ systems

(Song et al., 2009; Napierska et al., 2010; Fruijtier-Polloth, 2012; Lu et al., 2013), among which respiratory system is the primary site that exposed to the airborne Nano-SiO_2 particles (Song et al., 2009; Yu et al., 2015).

Long-lasting nanomaterials in certain tissues have been reported to cause chronic adverse effects such as carcinogenesis due to their special physicochemical properties (Hirose et al., 2011), and nano-sized particles might be more carcinogenic than micron-sized particles concerning long-term exposure (Gebel, 2012). Inhalation of TiO_2 nanoparticles was shown to increase the risk of lung cancers in rats (Bermudez et al., 2004) and carbon nanotube exposure through inhalation is carcinogenic to the lungs of male and female rats (Kasai et al., 2016). Although SiO_2 has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) in 1997 (Iarc., 1997), there is limited data on carcinogenicity following chronic exposure to Nano-SiO_2. Nano-SiO_2 can be localized to the nucleus thereby affecting nuclear integrity and causing DNA damage (Chen and von Mikecz, 2005; Wang et al., 2007).

Alterations in DNA methylation, a typical epigenetic modification, have been reported in cells exposed to Nano-SiO_2. A global genomic hypomethylation induced by Nano-SiO_2 was observed in HaCaT cells and the CpGs in the promoter of PARP1 gene were demethylated, resulting in DNA repair dysfunction (Gong et al., 2010, 2012b). Abnormal DNA methylation occurs frequently in the process of carcinogenesis (Miousse et al., 2018; Dreval et al., 2019), through devastating genome stability and activating aberrant transcription (Gama-Sosa et al., 1983). Decreased global DNA methylation and increased expression of DNA methyltransferases (DNMTs) were demonstrated in the early stage of Nano-SiO_2-induced malignant cellular transformation (Seidel et al., 2017), indicating that DNA methylation is involved in the Nano-SiO_2-induced carcinogenesis.

Nuclear factor erythroid-2-related factor 2 (NRF2) is a key transcription factor involved in cellular responses to stresses induced by electrophiles, oxidants, and chemicals (Motohashi and Yamamoto, 2004). In addition to stress responses, NRF2 plays a contradictory role in cancers (Wu et al., 2019). Loss of NRF2 ultimately leads to malignant cellular transformation in the prostate gland of murine models (Frohlich et al., 2008). NRF2 dysfunction in mice accelerates the acetylhydrolase-induced hepatocarcinogenesis (Marhenke et al., 2008). On the contrary, accumulating evidence indicated that increased activation of NRF2 frequently occurs in multiple types of tumors, which promotes cancer cell growth and metastasis formation (Liu et al., 2016; Zhang et al., 2016; Lu et al., 2017). The hypomethylation of CpG islands within the NRF2 promoter region was reported in colorectal cancer (Kang et al., 2014; Zhao et al., 2015). Decreased NRF2 expression due to hypermethylation of CpG islands within the NRF2 promoter region was found to be associated with prostate cancer (Yu et al., 2010; Khor et al., 2014).

In the present study, we demonstrated that Nano-SiO_2 induces a malignant cellular transformation and a global DNA hypomethylation in human bronchial epithelial cells. Intriguingly, we found that demethylation of CpG islands within the NRF2 promoter region increases the NRF2 gene expression, which inhibits the carcinogenesis induced by Nano-SiO_2.

MATERIALS AND METHODS

Chemicals and Reagents

The 15 nm Nano-SiO_2 particles were purchased from Wan Jing New Material Co., Ltd. (Hangzhou, Zhejiang, China) and were characterized as previously described (Gong et al., 2012a). Nano-SiO_2 samples were sonicated to distribute in the solution as evenly as possible. Dosing solutions were prepared by dissolving the calculated amount of Nano-SiO_2 in the cell culture medium, following the approved standard operating procedures for handling toxic agents. All other reagents were obtained from commercial sources and were of the highest available grade.

Cell Culture

Human bronchial epithelial cell lines (16HBE and BEAS-2B) and adenocarcinomic human alveolar basal epithelial cell line (A549) were purchased from the Cell Bank in Chinese Academy of Sciences Cell Bank in Shanghai. Frozen cells were thawed and expanded in MEM medium supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 U/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C and passaged at about 80% confluence.

Nano-SiO_2 Treatment

Human bronchial epithelial cell lines were treated with Nano-SiO_2 for several passages and harvested at certain time points as shown in **Figure 2A**. In detail, 16HBE and BEAS-2B cells were grown in complete MEM medium until they reached a confluence of about 70–80%. Cells were then treated with Nano-SiO_2 (10.0 μg/ml for 16HBE cells, 40.0 μg/ml for BEAS-2B cells) for 24 h and changed into the complete MEM medium until they are ready for subculture, these cells treated with Nano-SiO_2 for one passage are referred to P1. Accordingly, cells treated as above for n passages are named as P(n). Cells cultured in complete MEM medium for the same passages without any Nano-SiO_2 treatment are taken as the negative control. A549, an epithelial cell line derived from human lung carcinoma, was used as a positive control.

Cell Viability Measurement by MTT Assay

Cell viability was determined by MTT assay. 16HBE and BEAS-2B cells growing at the exponential phase were seeded in 96-well plates with a density of 5×10^4cells/ml. After treated with various dosages of Nano-SiO_2 (0, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 μg/ml for 16HBE, 0, 1.0, 5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 μg/ml for BEAS-2B) for 24 h, 50 μl MTT solution was added to each well and incubated for another 4 h. After adding 150 μl DMSO, the absorbance of each well was measured at 490 nm using a spectrophotometer. Each treatment group has at least three replicates. Cell viability was obtained as a percentage of the value of viable cells in the control groups.

Anchorage-Independent Cell Growth Measured by Soft Agar Colony Formation Assay

Anchorage-independent growth of Nano-SiO_2-treated human bronchial epithelial cells (16HBE and BEAS-2B) was measured by soft agar colony formation assay. Cells treated with Nano-SiO_2 for various passages (P0, P8, P16, and P32 of 16HBE cells, P0, P15, P30, and P45 of BEAS-2B cells) were trypsinized. Cells were resuspended at a density of 1×10^4/ml in MEM medium with 0.3% agar and plated over 3 ml of a solidified complete MEM culture medium containing 0.5% agar. Cells were then incubated in a humidified atmosphere with 5% CO_2 at 37°C for 3 weeks. The colonies were photographed and counted by using ImageJ software.

Tumorigenicity in Nude Mice

The tumorigenicity of the Nano-SiO_2-treated human bronchial epithelial cells (16HBE and BEAS-2B) was determined in nude mice. Balb/c-nu male nude mice (4 weeks old) purchased from the Laboratory Animal Center in Shanghai, were housed in ventilated microisolators with sterile food and water. After one week of acclimation, mice were randomly divided into eight treatment groups, with three mice in each group. Specifically, four groups of mice were injected with different passages of 16HBE cells (P0, P8, P16, and P32), another four groups of mice were injected with different passages of BEAS-2B cells (P0, P15, P30, and P45). Nude mice injected with cells cultured in complete MEM medium for the same passages without any Nano-SiO2 treatment were taken as the negative control. Cells were harvested by trypsinization, washed, and resuspended in PBS. Cell suspensions were then injected (0.2 ml) subcutaneously to mice at a density of 1×10^7 cells/ml. Tumor growth was monitored for 90 consecutive days after injection by measuring the major diameter of the tumor externally with a slide caliper. Mice were sacrificed by cervical dislocation if the major diameter of its tumor reaches about 2 cm. Mice were kept for more than 90 days after injection. The tumor sizes were measured at the fifth week after injection and the incidence and latency period of tumorigenesis were recorded. The incidence was presented as the number of mice with a tumor at the site of injection versus the total number of mice. The latency period is defined as the time required for a tumor to be able to visually detect.

Quantitative Reverse Transcription-PCR (qRT-PCR) Assay

Total RNAs from three cell lines (16HBE, BEAS-2B, and A549) were isolated and reverse transcribed into cDNAs by using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio Inc., Japan). Four genes related to the NRF2 signaling pathway were selected for qRT-PCR assay. Primers are listed in **Supplementary Table S1**. All qPCR reactions were performed on an Applied Biosystems™ StepOne™ Real-time PCR system using iTaq™ Universal SYBR Green Supermix, with three technical replicates. The amplification procedure was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. Relative quantification of target genes was performed using the $\Delta\Delta$Ct method with GAPDH as a reference gene.

Western Blotting Assay

Cells were washed with ice-cold PBS and lysed on ice with a protease inhibitor cocktail. Protein concentrations were measured by BCA method. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were probed with NRF2 (1:500), HO1 (1:1000), SOD1 (1:1000), GST (1:1000), DNMT1 (1:500), DNMT3a (1:500), DNMT3b (1:500), MeCP2 (1:500), MBD2 (1:500), GAPDH (1:10000) antibodies (Santa Cruz Biotechnology, Inc., Texas, United States) at 4°C overnight. The bands were visualized after incubation with a chemiluminescent substrate. Quantification of the band density was determined by densitometric analysis.

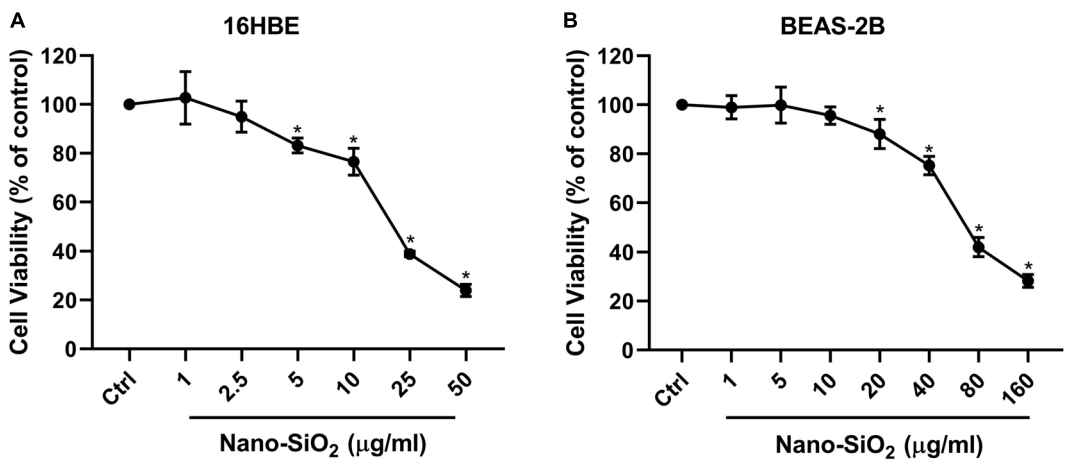

FIGURE 1 | Cell viability of two types of human bronchial epithelial cells **(A:** 16HBE cell line, **B:** BEAS-2B cell line) upon treatment with Nano-SiO_2 for 24 h. Values were mean ± SD ($n = 3$); *$p < 0.05$, control versus Nano-SiO_2 treatment.

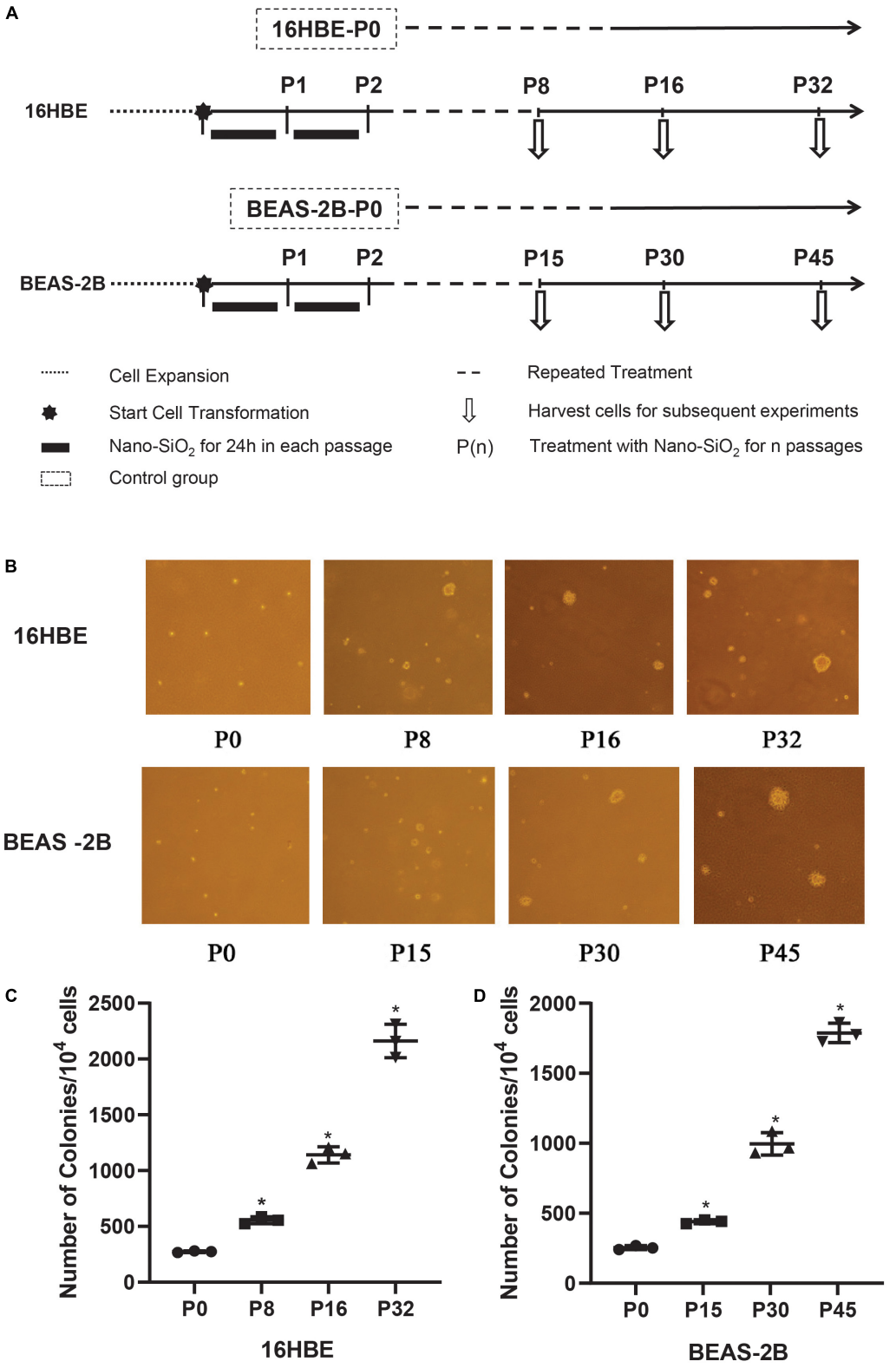

FIGURE 2 | The effects of Nano-SiO_2 on soft agar colony formation of 16HBE and BEAS-2B cells. **(A)** The experimental protocol indicates the Nano-SiO_2 treatment timeline of 16HBE and BEAS-2B cells. **(B)** Representative colonies on soft agar plates. Cells treated with Nano-SiO_2 (10 μg/ml for 16HBE cells, and 40 μg/ml for BEAS-2B cells) or without Nano-SiO_2 (16HBE-P0, BEAS-2B-P0) were grown on soft agar plates after indicated passages. **(C,D)** Dot plots showing the number of colonies developed from 16HBE and BEAS-2B cells. The data are shown as mean ± SD ($n = 3$); $^*p < 0.05$, control (P0) vs. Nano-SiO_2 treatment.

5-mC Content Measurement by Immunofluorescence Assay

Cells were fixed with 4% paraformaldehyde for 15 min and then fixed with cold formaldehyde for 5 min, washed with PBST, and then incubated with hydrochloric acid at 37°C for 30 min. After blocked with 3% PBST-BSA for 30 min, samples were incubated with anti-5-mC antibody (1:1000) at 37°C for 1 h and incubated with FITC-conjugated secondary antibody (1:400) for 30 min. Finally, coverslips were then incubated with DAPI for double staining and then mounted on glass slides by using Fluo-Antifading medium II. The fluorescence intensity was detected by using an inverted fluorescent microscope.

Measurement of DNMT Enzymes Activity

Cells treated with Nano-SiO_2 for different passages (P0, P8, P16, and P32 of 16HBE cells, P0, P15, P30, and P45 of BEAS-2B cells) and A549 cells were harvested by trypsinization. Nuclear was extracted and total DNMT enzymes activity was determined by EpiQuik^{TM} DNA Methyltransferase Activity/Inhibition Assay Kit (EpiGentek, United States) according to the procedures provided by the manufacturers.

Qualitative Analysis of DNA Methylation by MSP

Genomic DNA was extracted and treated with bisulfite as previously described (Gong et al., 2012a). After sodium bisulfite conversion, PCR assay was conducted to analyze the DNA methylation level in specific *NRF2* gene loci quantitatively. Primer sets M1 and M2 were designed and synthesized to amplify the methylated DNA, primer sets U1 and U2 were designed and synthesized to amplify the unmethylated DNA. PCR products were separated by electrophoresis at 100 V for 40 min on 2.2% agarose gels. Primers were listed in **Supplementary Table S1**.

Cell Transfection

The cells (16HBE and BEAS-2B) were separately transfected with NRF2 shRNA(h) lentiviral particles (sc-37030-V) or plasmid pEGFP-NRF2 to construct NRF-2 knockdown (KD) or overexpression (OE) cell lines, according to the manufacturer's instructions. For the knockdown of NRF-2, NRF2 shRNA(h) lentiviral particles (shRNA-NRF2) and control shRNA Lentiviral Particles (shRNA-Ctrl) were transfected into cells. After transfected for 24 h, the transfection culture medium was replaced with complete culture medium and incubated for 72 h. Then, 10 μg/ml puromycin dihydrochloride was used to select and achieve stable knockdown cell lines. For the overexpression of NRF-2, pEGFP containing NRF-2 gene sequence (pEGFP-NRF2) or empty pEGFP (pEGFP) were transfected into cells. Full length cDNAs of NRF-2 were amplified through RT-PCR using specific forward 5′-CACCATGGGAATGGACTTGGAGCTGCC-3′ and reverse 5′-CTAGTTTTTCTTAACATCTGGCTTCTTAC-3′ primers. After transfected for 24 h, cells were selected by G418. Selected cells were harvested for transfection efficiency confirmation and subsequent experiments (**Supplementary Figure S1**).

Statistical Analysis

Data were represented by the means ± SD of at least three independent experiments. Statistical significances among experimental groups were evaluated by ANOVA followed by the Tukey *post hoc* test performed with the GraphPad Prism (version 8.0.2; San Diego, CA, United States).

RESULTS

Nano-SiO_2 Reduces Cell Viability of Human Bronchial Epithelial Cells

Since the respiratory system is the primary site that exposed to the airborne Nano-SiO_2 particles (Song et al., 2009; Yu et al., 2015), two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as our *in vitro* models. To investigate the cytotoxicity of Nano-SiO_2, we determined the effects of Nano-SiO_2 on the viability of human bronchial epithelial cells by MTT assay. The survival rate of cells treated with Nano-SiO_2 was expressed as the percentage of that of cells in the control group without nano-SiO_2 treatment. For 16HBE cells, no significant change in cell viability was observed when treated with 1.0 or 2.5 μg/ml Nano-SiO_2 for 24 h. After exposure to 10.0 μg/ml Nano-SiO_2 for 24 h, cell viability was reduced to 77.04% of the control group ($p < 0.05$) (**Figure 1A**). For BEAS-2B cells, no significant change in cell viability was observed when treated with 1.0, 5.0, or 10.0 μg/ml Nano-SiO_2 for 24 h (**Figure 1B**). Cell viability was significantly decreased when treated with Nano-SiO_2 no less than 20.0 μg/ml. At a concentration of 40.0 μg/ml Nano-SiO_2, cell viability was reduced to 75.18% of the control group after 24 h ($p < 0.05$) (**Figure 1B**).

With the increase of Nano-SiO_2 concentration, the reduction of cell viability in both cell lines became more severe, showing a dose-dependent manner. Based on the cell viability results, 10 μg/ml and 40 μg/ml Nano-SiO_2 were used as the treatment

TABLE 1 | Tumorigenicity in nude mice of 16HBE and BEAS-2B cells treated with Nano-SiO_2.

Cell lines	Treatment group	Tumors formed at the injection site		
		Incidence[a]	Latency period[b] (weeks)	Tumor size (mm)[c] Diameter mean (range)
16HBE	P0	0/3	NA	NA
	P8	3/3	5	2 (2–3)
	P16	3/3	3	4 (3–6)
	P32	3/3	1	7 (6–9)
BEAS-2B	P0	0/3	NA	NA
	P15	3/3	5	3 (2–4)
	P30	3/3	3	4 (4–6)
	P45	3/3	2	8 (6–9)

[a] *The incidence was presented as the number of mice with a tumor at the site of injection versus the total number of mice.* [b] *The latency period is the time required for a tumor to be able to visually detect. No tumor regression was ever observed.* [c] *The tumor sizes were measured at the 5th week after injection.*

dosage in subsequent experiments for 16HBE and BEAS-2B cells, respectively.

Nano-SiO_2 Induces Malignant Transformation of Human Bronchial Epithelial Cells

To confirm the malignant cellular transformation induced by Nano-SiO_2, a soft agar colony formation assay was performed to evaluate the ability of Nano-SiO_2-treated cells to grow independently on a solid surface (anchorage-independent growth). The number of colonies grown from 16HBE cells cultured in complete MEM medium (control) for 8, 16, and 32 passages without Nano-SiO_2 exposure was first compared, and no obvious difference was observed (data not shown). Similarly, no obvious difference in the numbers of the colony was observed among the 15th, 30th, and 45th passage of BEAS-2B cells in the

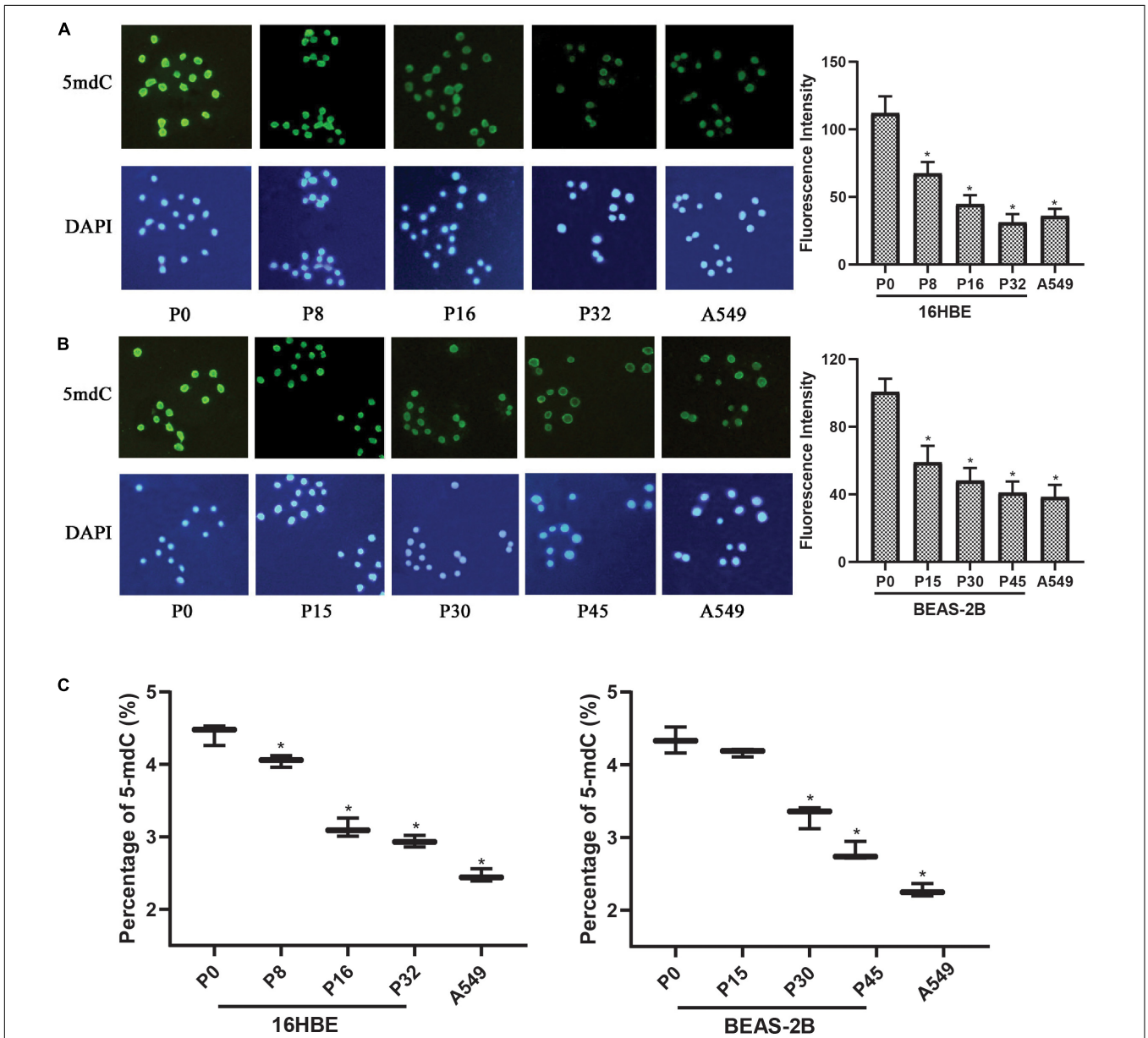

FIGURE 3 | Nano-SiO_2 exposure induces global DNA hypomethylation in human bronchial epithelial cells. 16HBE cells were treated with 10 μg/ml Nano-SiO_2 for 8, 16, or 32 passages and BEAS-2B cells were treated with 40 μg/ml Nano-SiO_2 for 15, 30, or 45 passages. **(A,B)** Immunofluorescence assay was performed to detect 5-mC levels in 16HBE and BEAS-2B cells. 5-mC was visualized by immunostaining with the antibodies against 5-mC (green) and nuclei were visualized by staining with DAPI (blue). Quantification of relative fluorescence intensity was shown on the right. **(C)** The levels of 5-mdC normalized to that of cytosine were shown in percentage. The data are presented as mean ± SD ($n = 3$). *$p < 0.05$, compared with the control group (P0). A549, an epithelial cell line derived from human lung carcinoma, was used as a positive control.

control group (data not shown). Hereafter, when compared with treatment groups, the control group was named as 16HBE-P0 or BEAS-2B-P0.

We observed that with the prolonged exposure to Nano-SiO_2, both 16HBE and BEAS-2B cells developed a significant amount of colonies in the soft agar (**Figure 2B**). Specifically, after treated with 10 μg/ml Nano-SiO_2 for 8 passages, ten thousand 16HBE cells developed 555 colonies in the soft agar, which is approximately twice that from 16HBE-P0 cells which are not exposed to Nano-SiO_2. As the Nano-SiO_2 exposure continued for 32 passages, ten thousand 16HBE cells developed 2161 colonies, which is eight times as many as those growing from 16HBE-P0 cells (**Figure 2C**).

Similarly, after treated with 40 μg/ml Nano-SiO_2 for 15 passages, ten thousand BEAS-2B cells developed 440 colonies, which is approximately twice as many as the colonies growing from BEAS-2B-P0 cells. As the Nano-SiO_2 exposure continued for 45 passages, ten thousand BEAS-2B cells developed 1788 colonies, which is seven times as many as the colonies growing from BEAS-2B-P0 cells (**Figure 2D**). The results indicated that Nano-SiO_2 could induce malignant cell transformation in human bronchial epithelial cells and promote tumorigenesis *in vitro*.

Tumorigenicity of Nano-SiO_2-Transformed Cells in Nude Mice

To assess the *in vivo* carcinogenicity of Nano-SiO_2-treated cells, twenty-four nude mice received subcutaneous injections of human bronchial epithelial cells with various treatments (three mice in each group). The features of the tumor formed at the site of cell injection are summarized in **Table 1**. We did not observe any tumor when 16HBE or BEAS-2B cells were not exposed to Nano-SiO_2 (P0). In contrast, both 16HBE and BEAS-2B cells treated with Nano-SiO_2 formed tumors in nude mice within five weeks. The latent period of tumorigenesis induced by cells treated with Nano-SiO_2 for a longer period was generally shorter than cells treated for a shorter period. Also, the size of tumors growing in nude mice correlated with the duration of Nano-SiO_2 treatment. Specifically, at the fifth week after injection, P8-, P16-, and P32- 16HBE cells formed tumors with a mean diameter of 2, 4, and 7 mm, respectively. Similarly, at the fifth week after injection, P15-, P30-, and P45-BEAS-2B cells formed tumors with a mean diameter of 3, 4, and 8 mm, respectively. Cells treated with Nano-SiO_2 for a longer time tend to induce larger tumors in nude mice. These results indicated that human bronchial epithelial cells could be malignantly transformed by Nano-SiO_2 exposure and acquire tumorigenicity *in vivo*.

Nano-SiO_2 Induces Global DNA Hypomethylation in Human Bronchial Epithelial Cells

Since global changes in DNA methylation are a hallmark of carcinogenesis, the levels of genome-wide DNA methylation in Nano-SiO_2 treated cells were determined. Immunofluorescence assay was first performed to estimate the relative intensity of 5-mC in human bronchial epithelial cells (16HBE and BEAS-2B cells) and A549 cells (human lung carcinoma cell line, as a positive control).

A significant decrease in the fluorescence intensity of 5-mC was observed in both 16HBE and BEAS-2B cells treated with Nano-SiO_2. For 16HBE cells, the mean fluorescence intensity of 5-mC was reduced significantly by 39.85%, 60.14%, and 72.18% in P8, P16, and P32 cells, respectively, when compared with the control group (16HBE-P0) (**Figure 3A**). For BEAS-2B cells, the mean fluorescence intensity of 5-mC was reduced significantly by 41.40%, 52.18%, and 59.23% in P15, P30, and P45 cells, respectively, when compared with the control group (BEAS-2B-P0) (**Figure 3B**).

High-performance capillary electrophoresis (HPCE) assays were performed to further investigate the levels of genome DNA methylation in cells treated with Nano-SiO_2. We found that the levels of 5-mdC were reduced in cells exposed to Nano-SiO_2 in both 16HBE and BEAS-2B cells when compared with the non-exposed cells in P0, the control group (**Figure 3C**). Besides, the extent of 5-mdC reduction correlated with the duration of Nano-SiO_2 exposure (**Figure 3C**). These results suggested that Nano-SiO_2 exposure induces genome-wide DNA hypomethylation of human bronchial epithelial cells, and the decrease of DNA methylation is aggravated with prolonged Nano-SiO_2 exposure.

Influences of Nano-SiO_2 on the Expression of DNA Methylation-Associated Proteins

To characterize the global DNA hypomethylation in cells treated with Nano-SiO_2, the total activity of DNA methyltransferases (DNMTs) catalyzing DNA methylation was investigated. We found that the total activity of DNMTs in both cell lines (16HBE and BEAS-2B) was gradually inhibited with the prolonged treatment with Nano-SiO_2 (**Figures 4A,B**). We further determined the levels of DNMTs in cells treated with Nano-SiO_2. According to the results of western blot analysis, we found that the levels of DNMT1 are increased while DNMT3a protein expression is decreased in cells treated with Nano-SiO_2 (**Figures 4C,D**). Further investigation on levels of methyl CpG binding proteins (MeCP-2 and MBD2) revealed that Nano-SiO_2 exposure significantly increases MBD2 protein expression in both 16HBE and BEAS-2B cells. No significant change was observed in levels of MeCP-2 in both cell lines after Nano-SiO_2 treatment (**Figures 4C,D**).

Nano-SiO_2 Inhibits the CpG Methylation at the Promoter Region of NRF2 Gene

The transcription factor NRF2 involved in stress response plays a role in cancer development. Expression of the NRF2 gene is known to be influenced by the status of methylation of CpG islands in the promoter region in cancer cells (Kang et al., 2014; Zhao et al., 2015). Therefore, we asked whether DNA methylation

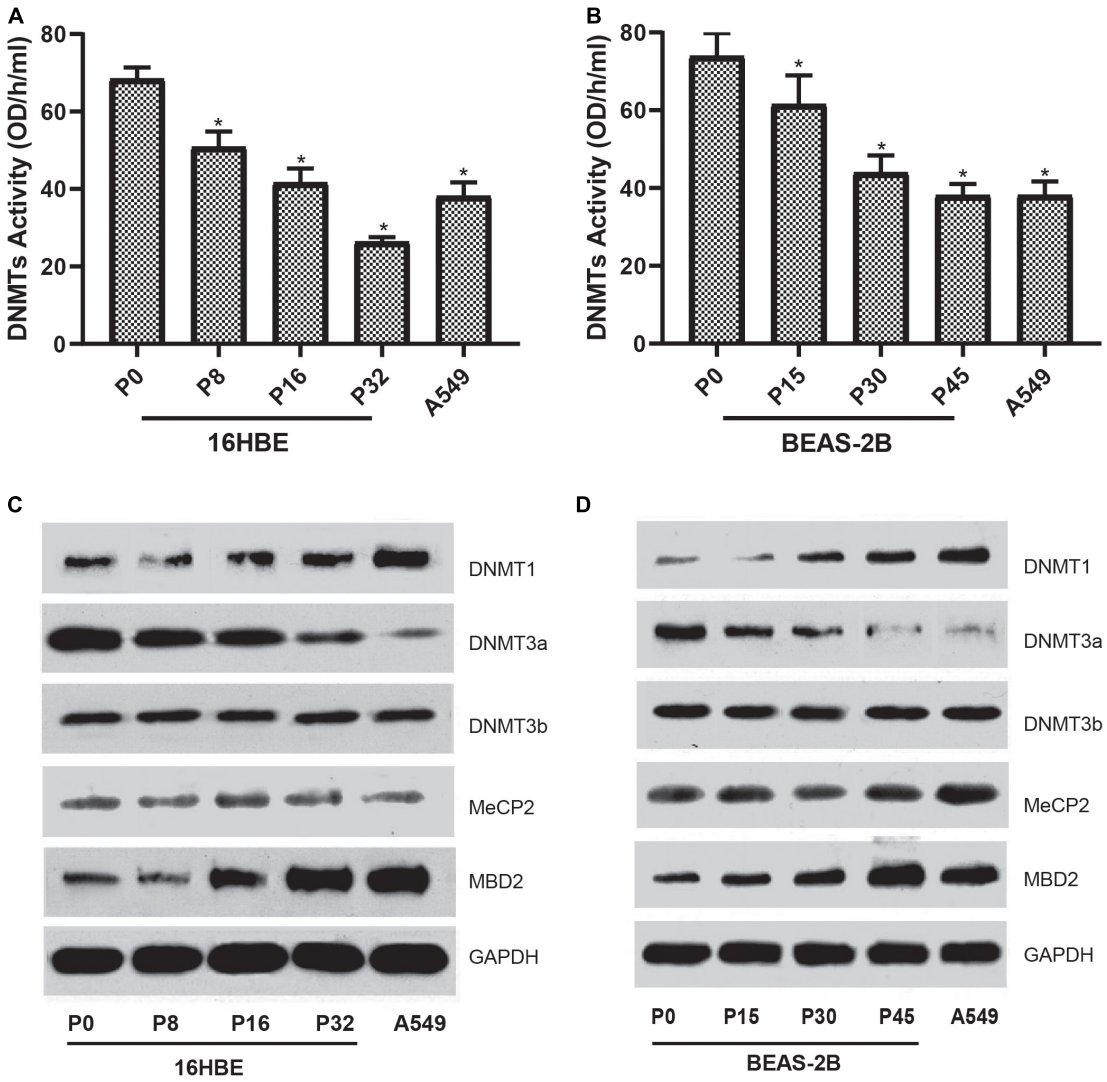

FIGURE 4 | Effects of Nano-SiO_2 on the expression of DNA methylation-associated proteins in human bronchial epithelial cells. DNMTs activity of 16HBE cells **(A)** and BEAS-2B cells **(B)** treated with Nano-SiO_2 for various durations were determined. The data are shown as mean ± SD (n = 3). *$p < 0.05$, compared with the control group (P0). A549, an epithelial cell line derived from human lung carcinoma, was used as a positive control. Levels of DNMT-1, DNMT-3a, DNMT-3b, MeCP-2, and MBD-2 in 16HBE cells **(C)** and BEAS-2B cells **(D)** treated with Nano-SiO2 for different passages were evaluated using western blot. GAPDH was used as the loading control.

of the promoter region of the NRF2 gene is influenced by Nano-SiO_2 exposure. We conducted a methylation-specific PCR (MSP) to assess the methylation status of CpG sites at the NRF2 promoter region. As illustrated in **Figure 5A**, two sets of primers (**Supplementary Table S2**) were designed to detect the methylation status in different CpG islands (region A and region B). The methylation level at region A of 16HBE cells treated with Nano-SiO_2 for 16 (P16) and 32 (P32) passages were decreased when compared with the untreated group (16HBE-P0). Similarly, the methylation level at region A of BEAS-2B cells treated with Nano-SiO_2 for 15, 30, and 45 passages was also reduced when compared with the untreated group (BEAS-2B-P0). However, no obvious changes were found at region B in 16HBE or BEAS-2B cells after Nano-SiO_2 treatment (**Figures 5B,C**).

Expression of the NRF2 Gene Is Upregulated in Cells Exposed to Nano-SiO_2

We further investigated the expression of NRF2 gene using qRT-PCR for mRNA and western blot analysis for protein in cells treated with Nano-SiO_2. Our results showed that Nano-SiO_2 exposure significantly increases the mRNA and protein expression of the NRF2 gene in both cell lines (**Figures 6A–D**), which is consistent with the hypomethylation of global DNA

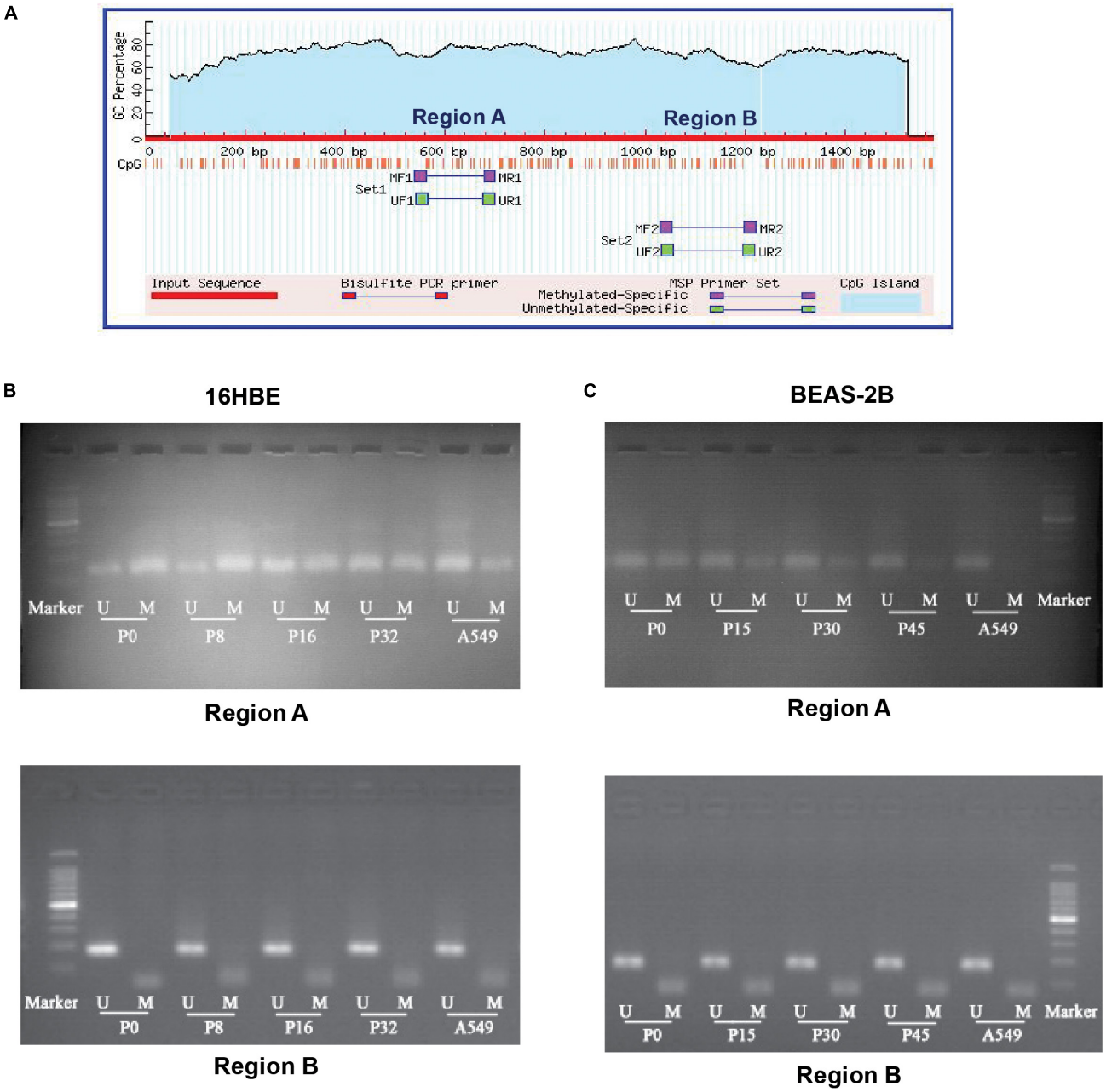

FIGURE 5 | MSP analysis of DNA methylation of the promoter of the *NRF2* gene. **(A)** Schematic graph showing CpG islands in the *NRF2* promoter region and binding sites for MSP PCR primers. **(B,C)** Representative electrophoresis results of PCR products of CpG islands in the *NRF2* promoter region using primers illustrated in **(A)**. M: methylated alleles. U: unmethylated alleles. A549 was used as a positive control.

induced by Nano-SiO_2 (**Figures 3A,C**). Furthermore, we also determined the expression levels of several gene targets (HO-1, SOD1, and GST) of the transcription factor NRF2 upon Nano-SiO_2 exposure. Analysis using qPCR and western blot showed that the mRNA and protein levels of HO-1, SOD1, and GST were also significantly increased by Nano-SiO_2 treatment. The mRNA and protein expression of these anti-oxidative genes regulated by NRF2 largely correlated with the duration of Nano-SiO_2 exposure (**Figure 5**). These results revealed that the NRF2 gene and its target genes were up-regulated by Nano-SiO_2 exposure.

NRF2 Inhibits the Carcinogenesis of Human Bronchial Epithelial Cells Induced by Nano-SiO_2

Since the expression of the NRF2 gene is up-regulated by Nano-SiO_2, we further investigated the role of NRF2 in carcinogenesis

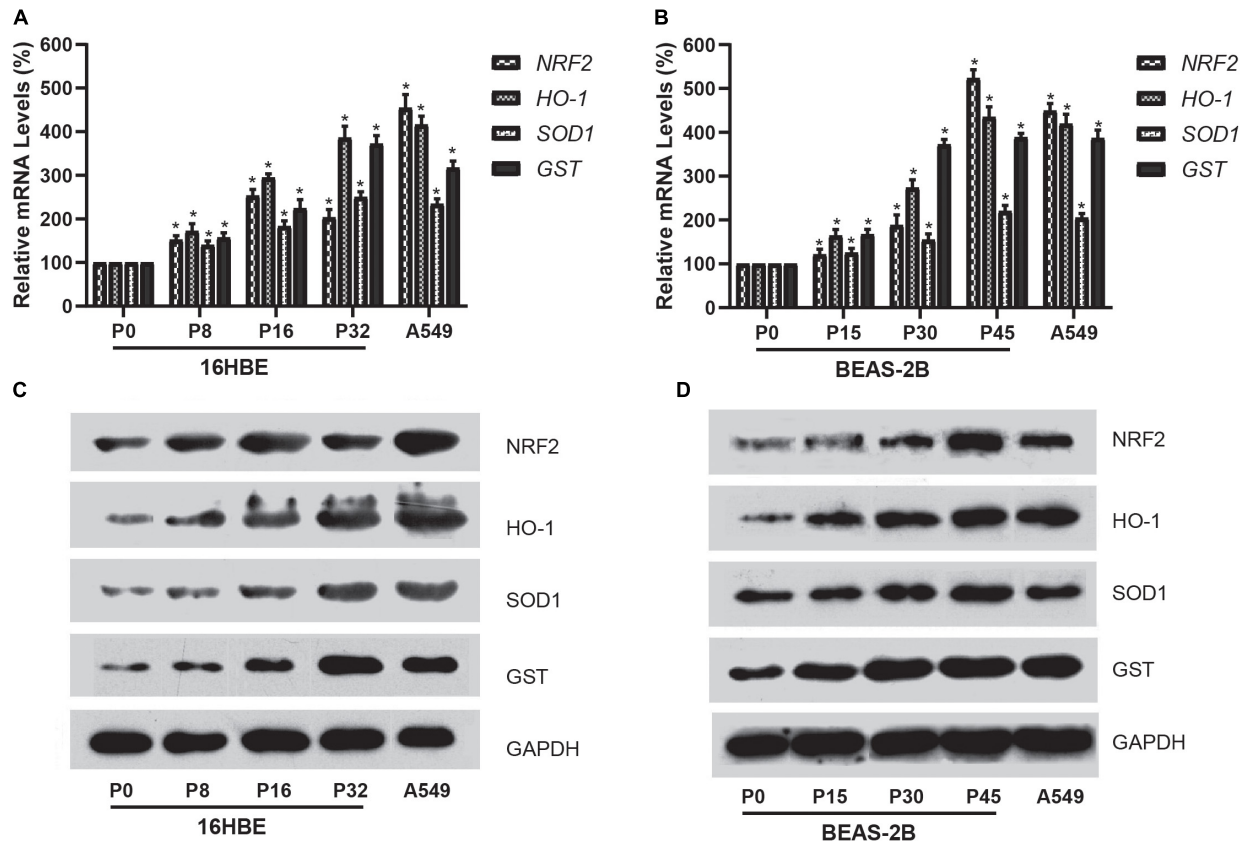

FIGURE 6 | Effects of Nano-SiO_2 on the expression of *NRF2* and its target genes in human bronchial epithelial cells. **(A,B)** mRNA expression of NRF2, HO-1, SOD1, and GST genes in 16HBE or BEAS-2B cells were determined by qRT-PCR. **(C,D)** Protein expression levels of NRF2, HO-1, SOD1, and GST in 16HBE or BEAS-2B cells were evaluated by western blotting analysis. GAPDH was used as the internal control for normalization. The data are presented as mean ± SD ($n = 3$). $^*p < 0.05$, compared with the untreated control group (P0). A549 was used as a positive control.

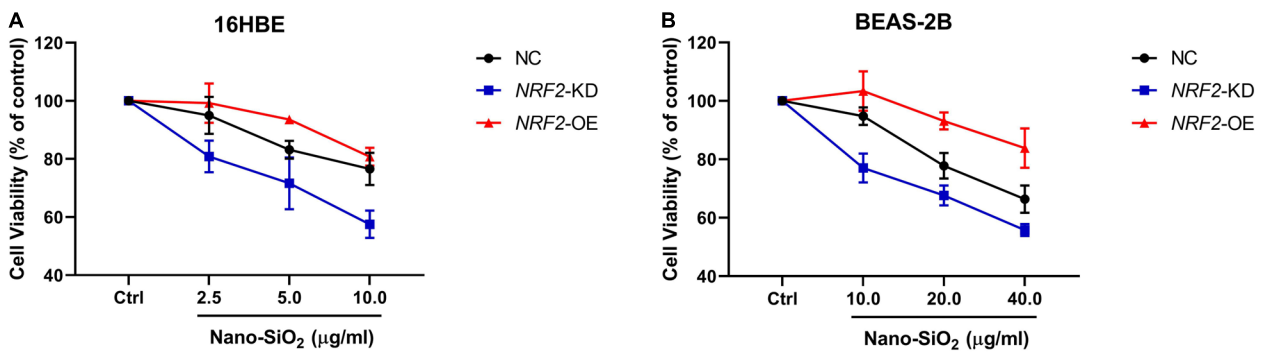

FIGURE 7 | The NRF2 protein protects against the cytotoxicity of Nano-SiO_2 on human bronchial epithelial cells. Cells **(A)** 16HBE, **(B)** BEAS-2B) were treated with Nano-SiO_2 particles for 24 h. Values were mean ± SD ($n = 3$). NC: cells (16HBE or BEAS-2B) transfected with negative control; NRF2-KD: knockdown of the NRF2 gene; NRF2-OE: NRF2 overexpression.

induced by Nano-SiO_2 by knockdown or overexpression of the NRF2 gene. Cells were transfected with vector harboring the NRF2 gene or the NRF2 gene shRNA to achieve overexpression (NRF2-OE) or knockdown (NRF2-KD) of the NRF2 gene and levels of NRF2 protein were determined using western blot (**Supplementary Figures S1A–D**). Cell viability assay showed that neither knockdown nor overexpression of the NRF2 gene introduces cytotoxicity to 16HBE and BEAS-2B cells (**Supplementary Figures S1E,F**). Thereafter, the effects of NRF2-OE and NRF2-KD on the viability of cells treated with Nano-SiO_2 were determined using the MTT method. We observed that NRF2-OE increases the viability of both 16HBE and BEAS-2B

cells after treated with Nano-SiO_2, whereas NRF2-KD tends to reduce the cell viability upon Nano-SiO_2 treatment (**Figure 7**). These results suggest that the NRF2 attenuates the cytotoxicity induced by Nano-SiO_2 exposure. To investigate the role of NRF2 protein in the tumorigenicity induced by Nano-SiO_2, the tumorigenicity of cells with NRF2-OE or NRF2-KD exposed to Nano-SiO_2 were determined both *in vitro* and *in vivo*. The 16HBE and BEAS-2B cells with NRF2-OE or NRF2-KD were treated with Nano-SiO_2 (10.0 μg/ml for 16HBE cells, 40.0 μg/ml for BEAS-2B cells) for various passages and followed with soft agar colony formation assay and nude mice injection. With the prolonged Nano-SiO_2 exposure, both NRF2-OE and NRF2-KD cells (16HBE and BEAS-2B) developed a significant amount of colonies in the soft agar (**Tables 2**, **3**). Interestingly, we observed that cells with NRF2-OE formed a lower number of colonies than cells of the control group, while NRF2-KD was able to increase the formation of colonies on soft agar. Consistent with the *in vitro* assay, both 16HBE (P8, P16, P32) and BEAS-2B (P15, P30, P45) cells with NRF2-OE formed tumors of a smaller size than the control group, while cells with NRF2-KD tend to increase the average tumor size (**Figure 8**). Taken together, results from both the *in vitro* and *in vivo* assay suggest that the NRF2 protein plays an inhibitory role in the tumorigenesis induced by Nano-SiO_2 exposure.

DISCUSSION

In this study, two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as the *in vitro* models to investigate the carcinogenicity of Nano-SiO_2. We observed the dose-dependent cytotoxic effects of Nano-SiO_2 on the human bronchial epithelial cells. Also, we showed the tumorigenicity of Nano-SiO_2 in human bronchial epithelial cells.

TABLE 2 | Summary of colony formation in soft agar of 16HBE cells treated with Nano-SiO_2.

Treatment Group	Cell number	Number of Colonies		
		16HBE	16HBE-ShRNA-NRF2	16HBE-pEGFP-NRF2
P0	1×10^4	273 ± 8	284 ± 12	267 ± 10
P8	1×10^4	555 ± 32*	730 ± 30*#	498 ± 13*#
P16	1×10^4	1141 ± 73*	1758 ± 108*#	846 ± 47*#
P32	1×10^4	2161 ± 150*	2247 ± 64*#	1486 ± 43*#

*$p < 0.05$, compared with normal cells without Nano-SiO_2 exposure; #$p < 0.05$, compared with normal cells treated with Nano-SiO_2 for the same passages.

TABLE 3 | Summary of colony formation in soft agar of BEAS-2B cells treated with Nano-SiO_2.

Treatment Group	Cell numbers	Number of Colonies		
		BEAS-2B	BEAS-2B-ShRNA-NRF2	BEAS-2B-pEGFP-NRF2
P0	1×10^4	254 ± 14	260 ± 8	246 ± 7
P15	1×10^4	440 ± 14*	844 ± 53*#	324 ± 19*#
P30	1×10^4	996 ± 80*	1476 ± 51*#	705 ± 72*#
P45	1×10^4	1788 ± 69*	1915 ± 35*#	1155 ± 79*#

*$p < 0.05$, compared with normal cells without Nano-SiO_2 exposure; #$p < 0.05$, compared with normal cells treated with Nano-SiO_2 for the same passages.

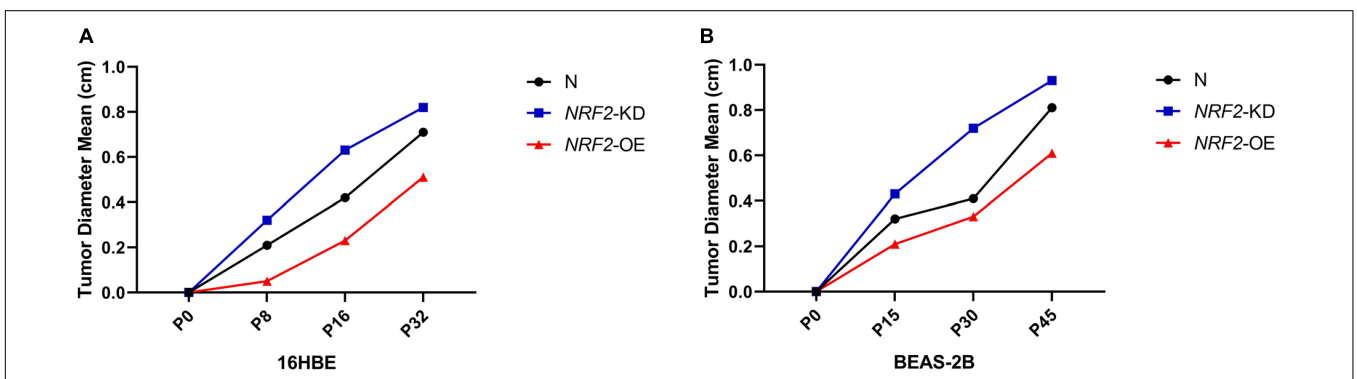

FIGURE 8 | NRF2 expression affects the tumorigenicity of Nano-SiO_2-transformed cells in nude mice. **(A,B)** The major diameter of the tumor formed at the subcutaneous injection site in nude mice. Tumor sizes were measured precisely 5 weeks after injection. The data are presented as a mean of tumor diameter. N: 16HBE or BEAS-2B cells without transfection; NRF2-KD: knockdown of NRF2; NRF2-OE: overexpression of NRF2.

As a hallmark of carcinogenesis, alterations in DNA methylation occur frequently in a wide range of cancers (Miousse et al., 2018; Dreval et al., 2019). Our results demonstrated a global genomic hypomethylation in cells exposed to the Nano-SiO_2, which is consistent with the reduction of the total enzyme activity of DNA methyltransferases. Although the total activity of DNMTs is reduced, we found that the expression of DNMT1 protein is elevated and the expression of DNMT3a protein is downregulated. The DNMT1 is responsible for the methylation of tumor suppressor genes (Jair et al., 2006; Ting et al., 2006), the increased expression of which might induce hypermethylation of the tumor suppressor genes, thereby inhibiting the expression of tumor suppressors and promoting carcinogenesis induced by Nano-SiO_2. The DNMT3a protein is known for the *de novo* CpG methylation independent of replication, which is consistent with the global hypomethylation induced by Nano-SiO_2. Loss of DNMT3a has been reported to be associated with leukemia pathogenesis and poor prognosis (Shivarov et al., 2013). Moreover, MBD2 and MeCP2 that can bind to and mediate the repression of methylated tumor suppressor genes (Klose and Bird, 2006; Lopez-Serra et al., 2008; Mian et al., 2011), were also induced by the Nano-SiO_2 exposure.

In addition to the global DNA hypomethylation, we observed a decreased methylation of CpG islands within the NRF2 promoter region in human bronchial epithelial cells exposed to Nano-SiO_2. The NRF2 protein is a key transcription factor involved in cellular defensive mechanisms against stresses induced by electrophiles, oxidants, and chemicals (Motohashi and Yamamoto, 2004). Under the basal condition, NRF2 is localized in the cytoplasm by binding to its cytosolic repressor Kelch-like ECH-associated protein 1 (KEAP1) (Motohashi and Yamamoto, 2004). In response to stress, NRF2 dissociates from KEAP1 and translocates to the nucleus, where it activates the transcription of its target genes to maintain cellular homeostasis (Panieri and Saso, 2019). Short-term exposure to Nano-SiO_2 can increase the expression of the NRF2 gene (Liu et al., 2017), which is consistent with our results that the NRF2 gene expression is up-regulated in both mRNA and protein levels in cells exposed to Nano-SiO_2 exposure. Moreover, our findings revealed that levels of HO1, SOD1, and GST, which are encoded by antioxidative gene targets of the NRF2 protein (Itoh et al., 1997), are increased upon Nano-SiO_2 exposure. These results suggest that NRF2 promotes the stress responses of cells treated with Nano-SiO_2.

Activation of the NRF2 protein is suggested to suppress carcinogenesis, especially in its early stage (Wu et al., 2019). Loss of NRF2 disrupts the antioxidant axis resulting in increased oxidative stress, ultimately leading to DNA damage and the initiation of malignant cellular transformation (Frohlich et al., 2008). In the tumor microenvironment, the tumor suppressor gene BRCA1 activates NRF2 (Gorrini et al., 2013), whereas the oncogene *Fyn* mediates nuclear export and induces degradation of NRF2 (Niture et al., 2014). NRF2-deficient mice are more susceptible to carcinogens and develop severer tumors in the urinary bladder and liver (Iida et al., 2007; Kitamura et al., 2007).

Collectively, our results showed that NRF2 plays an important role in protecting against the carcinogenesis induced by Nano-SiO_2 exposure. Moreover, hypomethylation at the promoter region of the NRF2 gene contributed to the alterations of NRF2 upon Nano-SiO_2 exposure. It should, however, be noted that histone modifications are also important for NRF2 gene transcription and it cannot be excluded that the alterations of NRF2 at both transcription and protein levels are caused by a more intrinsic mechanism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai Municipal Center for Disease Prevention and Control.

AUTHOR CONTRIBUTIONS

DL, GT, and YS jointly conceived this project and supervised the experiments. DL and XW designed the research. DL, XW, PX, QH, JS, and XH performed the experiments and analyzed the experimental results. DL, XW, YS, and GT prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00818/full#supplementary-material

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Conflict of Interest: YS is employed by the Syngenta (China) Investment Company Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

BRIEF RESEARCH REPORT
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Tissue- and Sex-Specific DNA Methylation Changes in Mice Perinatally Exposed to Lead (Pb)

Kai Wang[1], Siyu Liu[1], Laurie K. Svoboda[2], Christine A. Rygiel[2], Kari Neier[2], Tamara R. Jones[2], Justin A. Colacino[2], Dana C. Dolinoy[2] and Maureen A. Sartor[1,3]**

[1] Department of Computational Medicine and Bioinformatics, School of Medicine, University of Michigan, Ann Arbor, MI, United States, [2] Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI, United States, [3] Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, MI, United States

Lead (Pb) is a well-known toxicant that interferes with the development of a child's nervous and metabolic systems and increases the risk of developing diseases later in life. Although studies have investigated epigenetic effects associated with Pb exposure, knowledge of genome-wide changes with *in vivo* low dose perinatal Pb exposure in multiple tissues is limited. Within the Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium, we utilized a mouse model to investigate tissue- and sex-specific DNA methylation. Dams were assigned to control or Pb-acetate water, respectively. Exposures started 2 weeks prior to mating and continued until weaning at post-natal day 21 (PND21). Liver and blood were collected from PND21 mice, and the DNA methylome was assessed using enhanced reduced representation bisulfite sequencing (ERRBS). We identified ∼1000 perinatal Pb exposure related differentially methylated cytosines (DMCs) for each tissue- and sex-specific comparison, and hundreds of tissue- and sex-specific differentially methylated regions (DMRs). Several mouse imprinted genes were differentially methylated across both tissues in males and females. Overall, our findings demonstrate that perinatal Pb exposure can induce tissue- and sex-specific DNA methylation changes and provide information for future Pb studies in humans.

Keywords: DNA methylation, lead exposure, ERRBS, tissue, sex

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Naoko Hattori, National Cancer Center Research Institute (Japan), Japan

****Correspondence:***
Dana C. Dolinoy
ddolinoy@umich.edu
Maureen A. Sartor
sartorma@umich.edu

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INTRODUCTION

DNA methylation is a chemical modification of DNA that can affect gene expression without changing the underlying sequence (Robertson, 2005). During embryonic development of mammals, DNA methylation from parents is for the most part erased and re-established in gametogenesis and early embryogenesis (McSwiggin and O'Doherty, 2018). DNA methyltransferases (DNMTs) are required for establishment and maintenance of DNA methylation (Castillo-Aguilera et al., 2017). Another family of enzymes, ten-eleven translocation (TET), is involved in the demethylation process in mammals (Kohli and Zhang, 2013). High levels of DNA methylation in gene promoter regions are associated with repression of transcriptional activity (Weber et al., 2007), and DNA methylation changes are associated with many diseases, including cancer, atherosclerosis, and Alzheimer's disease (Zawia et al., 2009; Bergman and Cedar, 2013).

A variety of environmental exposures, including air pollution, tobacco smoke, and toxicants, including metals, are associated with altered DNA methylation levels (Baccarelli et al., 2009; Fragou et al., 2011). Reprogramed DNA methylation levels during development can be maintained through cell division and influence disease status in later life. Therefore, it is important to investigate the DNA methylation changes resulting from early life exposures (Ideta-Otsuka et al., 2017).

Lead (Pb) is a well-known toxicant that affects almost all human organs and systems (Muller et al., 2018). In the United States, approximately 400,000 deaths every year have been attributed to Pb exposure (Lanphear et al., 2018). Even low blood Pb levels (<5 μg/dL) are associated with increased risk of certain diseases such as hypertension, atherosclerosis, and left-ventricular hypertrophy. Children with Pb exposure are at increased risk of developing non-communicable chronic diseases in later life (Lanphear et al., 2018), and children with blood Pb levels <7.5 μg/dL demonstrate intellectual deficits (Lanphear et al., 2019). Recent studies show that Pb can influence DNA methylation, which can further impair cognitive development and result in behavioral problems (Min et al., 2017). Besides the effects of Pb on the nervous system, Pb can also accumulate in and cause oxidative damage to many human tissues, including heart, liver, kidney, and reproductive organs (Patra et al., 2001; Long et al., 2016). At the cellular level, Pb enhances the peroxidation of membrane lipids, affects membrane proteins and ultimately damages the molecular functions (Sandhir and Gill, 1995). In mouse brain, Pb induced DNA methylation changes are correlated with altered mRNA expression (Sánchez-Martín et al., 2015). In addition to coding genes, DNA methylation status of murine IAP transposons can be influenced by Pb exposure (Montrose et al., 2017). Although studies exist on the epigenetic effects of Pb exposure, research on environmentally relevant perinatal Pb exposure in multiple tissues within the same cohort are limited.

To understand how environmentally relevant perinatal Pb exposure affects genome-wide DNA methylation in different tissues, we performed studies as part of the Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium (Wang et al., 2018). In this study, a mouse model of human-relevant environmental exposure to Pb was used to investigate tissue- and sex-specific DNA methylation. Enhanced reduced representation bisulfite sequencing (ERRBS) (Garrett-Bakelman et al., 2015) was used to measure DNA methylation changes in liver (target tissue) and blood (surrogate tissue) collected from post-natal day 21 (PND21) mice with and without perinatal Pb exposure.

MATERIALS AND METHODS

Animal Exposure, DNA Extraction, and ERRBS

In this study, experiments were performed in wild-type non-agouti *a/a* mice from a colony of viable yellow agouti (A^{vy}) mice maintained over 230 generations resulting in mice genetically invariant that are 93% identical to C57BL/6J (Waterland and Jirtle, 2003; Weinhouse et al., 2014). Exposure to Pb was carried out by adding Pb-acetate to drinking water, which was provided to mice *ad libitum*. Pb-acetate mixed with distilled water with a Pb concentration of 32 ppm was used for exposure. This concentration results in human relevant maternal exposure levels in the 16–60 μg/dL range, as previously described (Faulk et al., 2013). Dams were randomly assigned to control and exposure groups. The exposure started 2 weeks prior to mating and continued until weaning at post-natal day 21 (PND21). Tissues (i.e., blood and liver) were collected from 7 males and 7 females for each group, 1–2 males and 1–2 females per litter. Blood was collected through cardiac puncture immediately following CO2 euthanasia and the left lobe of the liver was dissected and flash frozen in liquid nitrogen and then stored at −80°C. AllPrep DNA/RNA/miRNA Universal Kit (Qiagen #80224) was used for DNA extractions. Enhanced reduced representation bisulfite sequencing was performed at the University of Michigan Epigenomics and Advanced Genomics Cores (Garrett-Bakelman et al., 2015). More details about animal exposure, tissue collection, DNA extraction, and ERRBS can be found in Svoboda et al. (2019). Procedures of this study were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

Data Processing and DMC/DMR Analysis

For quality control of ERRBS data, FastQC (v0.11.3) was used to assess the quality of all sequenced samples (Andrews, 2010). TrimGalore (v0.4.5) was applied to trim adapter and low quality bases (Krueger, 2015). After trimming, reads shorter than 20 bp were removed from further analysis. Bismark (v0.19.0) was used for mapping and methylation calling (Krueger and Andrews, 2011) with Genome Reference Consortium Mouse Build 38 (mm10) as the reference genome. Bowtie2 (v2.3.4) was used as backend alignment software, and all alignments were performed with default parameters, i.e., 0 mismatches with multi-seed length of 20 bp (Langmead and Salzberg, 2012). The unmethylated lambda phage DNA was used to calculate the bisulfite conversion rates. For methylation calls, CpG sites with less than five reads covered were discarded from further analysis. The wCorr R package (version 1.9.1) was used to calculate the weighted genome-wide pair-wise sample correlations of CpG methylation, with the read counts at each CpG site as the weights. The methylSig R package (version 0.5.2) was used to detect differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) (Park et al., 2014). A window size of 50 bp was applied for discovering DMRs. The differential methylation test was performed by using the *methylSigDSS* function. Cytosines or windows with sufficient coverage in at least four samples per treatment were used for DMC and DMR detection, respectively. Adjusted *p*-values were obtained using FDR (Benjamini and Hochberg, 1995). FDR less/equal than 0.15 and absolute difference in methylation larger/equal than 10% were used to obtain the final DMCs and DMRs. Significant DMCs/DMRs were annotated with the annotatr Bioconductor package (version 1.8.0) (Cavalcante and Sartor, 2017). The *annotate_region* function was used to generate different genomic annotations, including CpG islands, CpG shores, CpG shelves,

CpG intervals, promoters, exons, introns, 5′UTRs, 3′UTRs, enhancers, and 1–5 kb upstream of TSSs. Proportion tests were used to identify overrepresented annotation results.

Pathway Analysis

The *chipenrich* R Bioconductor package (version 2.6.1) was used to evaluate biological pathways enriched with significant DMRs (Welch et al., 2014). Four analyses were performed stratified by tissue and sex (i.e., male blood, male liver, female blood, and female liver). Locus definition *nearest_tss* (the region spanning the midpoints between the TSSs of adjacent genes) was used to discover enriched Gene Ontology (GO) terms. All three ontologies (i.e., Biological Process, Cellular Component, and Molecular Function) were used. An FDR < 0.05 cutoff was used for selecting significantly enriched GO terms.

Annotating DMR Genes With Mouse Imprinted Genes and the CTD Database

To further interpret our results, we compared DMRs to mouse imprinted genes and the Comparative Toxicogenomics Database (CTD). Mouse imprinted genes were collected from Williamson et al. (2013) and Tucci et al. (2019). After removing redundancy, 303 genes were used for comparison. The genome annotation GTF file (NCBI *Mus musculus* Annotation Release 108) was used to obtain mouse non-imprinted genes (49,846 genes) and calculate the proportion of non-imprinted genes relate to DMRs. Imprinted genes annotated to DMRs were firstly identified for each tissue and sex, and then the overlaps determined. For CTD, all mouse genes with Pb-acetate exposure were downloaded from CTD. Genes with interaction of Pb-acetate on the mRNA level with changes in expression or methylation were extracted. Similar to above, genes annotated to DMRs from each tissue and sex were firstly compared to the CTD genes. We then combined these results to determine which genes were identified across tissues and sexes.

RESULTS

Differential DNA Methylation (DMCs and DMRs) With Perinatal Pb Exposure

To investigate the effects of perinatal Pb exposure on DNA methylation, we performed ERRBS on liver and blood DNA from offspring mice on post-natal day 21 (PND21). The numbers of total examined cytosines/regions were consistent across tissues and sexes (see **Figure 1** and **Supplementary Table S1**). From our ERRBS data, for both tissues, about 5% of all CpG sites across the mouse genome were tested to identify statistically significant DMCs and DMRs. Genome-wide sample correlations of CpG methylation indicates the samples were more highly correlated within groups (see **Supplementary Figure S1**). The average bisulfite conversion rate and read depth of all covered CpG sites from our ERRBS samples were 99.9% and 93.2, respectively (see **Supplementary Table S2**). About 1000 significant DMCs and hundreds of significant DMRs were detected by comparing Pb treated and control mice from each sex and tissue combination. The methylation changes of most cytosines/regions were 10 to 30%, with some as high as 67% (see **Supplementary Figure S2** for distribution). Most changes were tissue and sex specific. From the results, we found male blood contained the most DMCs/DMRs compared to the others, with the majority being hypo-methylation. In other sex-tissue combinations, similar numbers of hypo- and hyper-DMCs/DMRs were identified. Among the DMRs, 1 hypo-DMR and 2 hyper-DMRs were identified in both male tissues. However, no DMRs were discovered in both female tissues. In liver, 3 hypo-DMRs and 1 hyper-DMR were found across sexes, while in blood, 3 hypo-DMRs were found for both sexes (**Figure 1E**). A similar number of overlapping DMCs were identified (see **Supplementary Figure S3** for DMC Venn diagram).

The significant hyper- and hypo-DMRs were annotated to examine their genomic locations relative to all tested CpG sites and regions (**Figure 1**). The genomic locations of these DMRs revealed a similar pattern across tissues and sex. From the proportion test, most of the CpG sites/regions are significantly enriched in different genomic regions including CpG islands, 5′UTR, promoter region, intron, etc. (**Supplementary Table S3**). Although hundreds of genes are related to DMRs, only a small number of common genes are identified across tissues and sexes (see **Supplementary Figure S4**). 38 and 29 genes were identified across tissues in male and female, respectively. Five genes were detected in both tissues and in both sexes, i.e., *Prdm16*, *Hjurp*, *Cdh23*, *Bc1*, and *Arid1b*. Associated pie charts show the percentage of the genes related to hyper-, hypo-, or mixed (i.e., hyper and hypo) direction DMRs. From these pie charts, only a small number of genes are associated with mixed direction DMRs (see detail in **Supplementary Table S4**).

Pathway Analysis of Tissue- and Sex-Specific DMRs

To further understand the biological pathways altered by perinatal Pb exposure, we investigated the enrichment of cellular pathways with DMRs. Significant DMRs from each tissue- and sex-specific analysis were used for enrichment testing, and FDR < 0.05 was used to select significantly enriched GO terms. Before removing redundant identified GO terms, three common GO terms *metanephros morphogenesis*, *sensory perception of chemical stimulus*, and *metanephric nephron morphogenesis* were discovered in liver across sexes. In the non-redundant results, female liver had the most enriched terms, while male blood only had one GO term (GO:0003724) (**Figure 2**). To further investigate the DMRs behind these enriched GO terms, we found most GO terms are enriched by mixed DMRs (i.e., hyper and hypo-methylated) (see **Supplementary Table S5**). However, in female blood, there is one GO term (GO:1903205) is enriched only by hyper-DMRs. In female liver, there is an enriched GO term (GO:0045737) that only related to hypo-DMRs. In addition, four GO terms (GO:0043457; GO:0072170; GO:1901532; GO:0003338) are enriched only by hypo-DMRs in male liver.

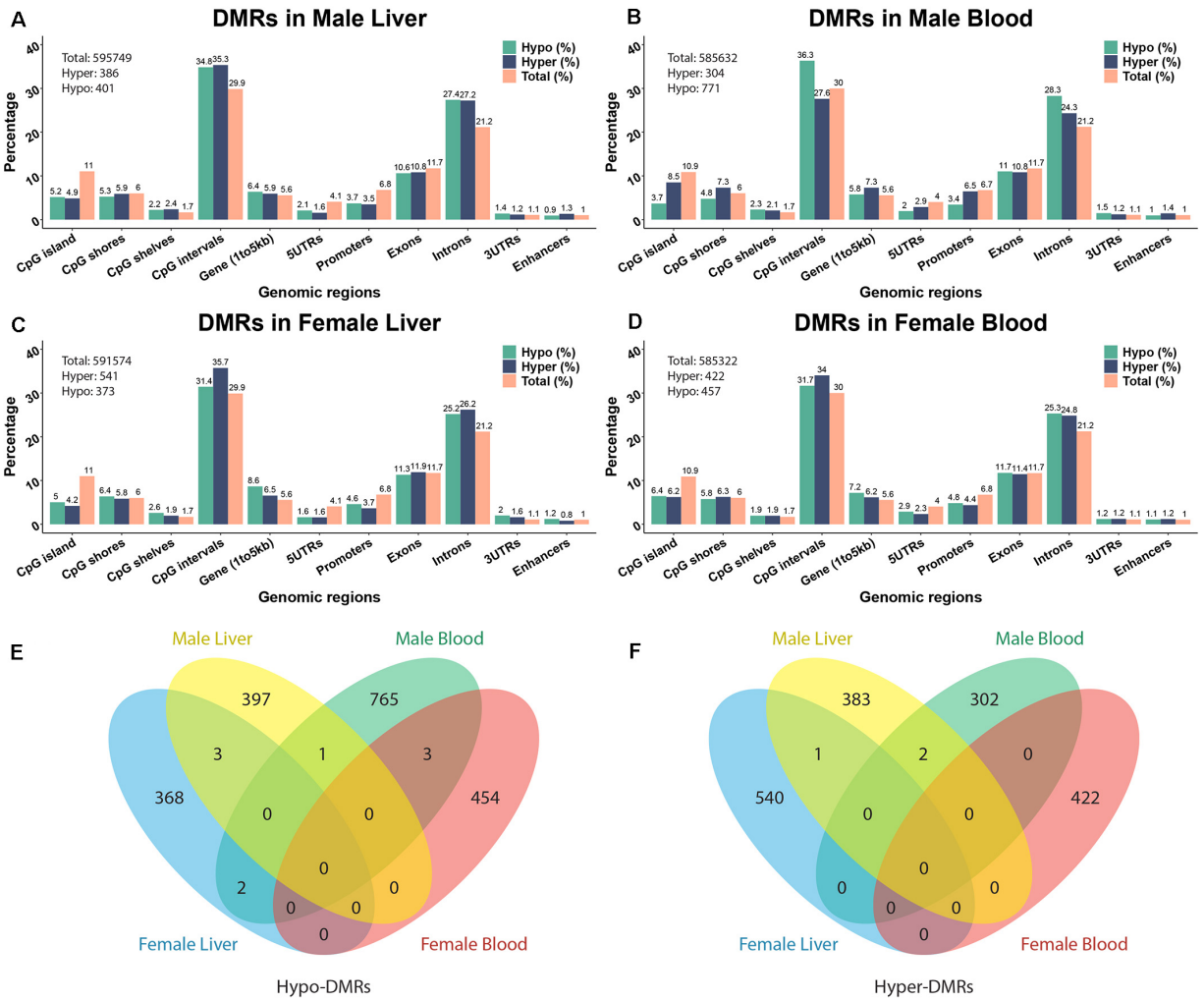

FIGURE 1 | The annotation of Pb-associated differentially methylated regions (DMRs) and related genes. **(A–D)** depict DMR annotations of male liver, male blood, female liver, and female blood. **(E,F)** Venn diagram showing the overlapping of hypo- and hyper-DMRs from different tissues in two sexes. The DNA methylation changes are expressed as lead vs control.

Several Mouse Imprinted Genes Contain DMRs

The effects of perinatal Pb exposure on imprinted genes in liver and blood were investigated. In total, 1879 genes were related to DMRs from our results (**Supplementary Figure S4**). Overall, 303 mouse imprinted genes were used for this comparison, with 36 genes (11.88%) being associated with significant DMRs (**Table 1**). This is compared to 3.69% of all other genes (1843 of 49,846 total genes) associated with significant DMRs. Within these 36 genes, *Arid1b* contained DMRs in both liver and blood across both sexes. Besides *Arid1b*, no imprinted genes with DMRs were detected in liver across sexes. In blood, the imprinted gene *Trappc9* had DMRs in both sexes. In female liver and blood, *Trappc9* and *Smoc2* both had DMRs. In male liver and blood, besides *Arid1b*, *Pde10a* contained DMRs across tissues (see **Supplementary Table S6** for details).

Pb-Associated Genes in the Comparative Toxicogenomics Database Contain DMRs

The Comparative Toxicogenomics Database (CTD) (Mattingly et al., 2006) is a curated resource that annotated known mouse Pb exposure-related target genes. Genes with expression changes and methylation changes due to Pb exposure were collected from the CTD (data updated March 2020), and after removing redundancy, 1190 genes were identified. Of these 1190 genes, only one gene contained a DNA methylation change in an exon region according to the CTD. Therefore, we focused on genes with Pb exposure related expression changes in the CTD. In total, 119 genes with at least one DMR from our results were annotated in CTD with changes at the transcription level due to Pb exposure. These 119 genes are therefore likely to be dysregulated at least partially due to DNA methylation changes in response to Pb. In female tissues, 39 and 36 genes with DMRs from blood and liver

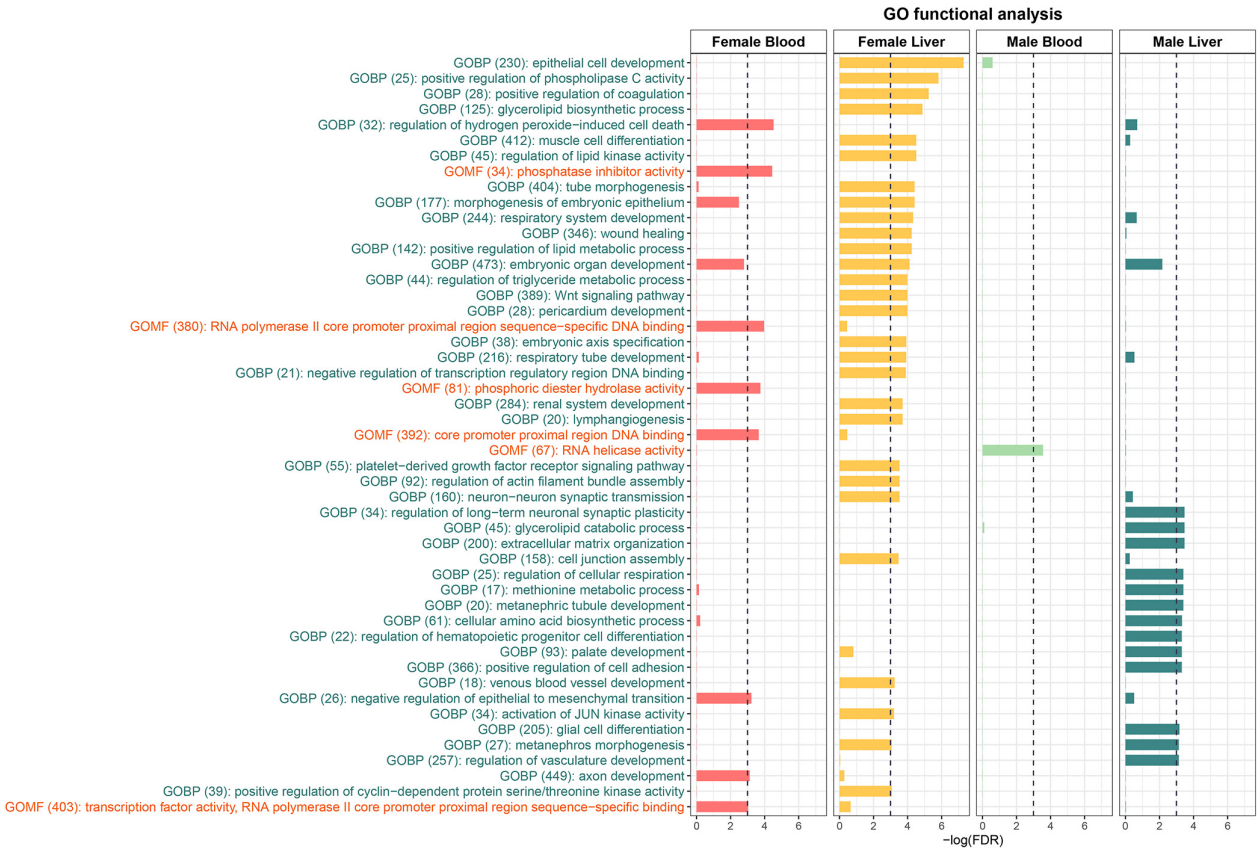

FIGURE 2 | The Enriched GO terms identified from the DMRs. GOBP stands for Gene Ontology Biological Process and GOMF stands for Gene Ontology Molecular Function. No Gene Ontology Cellular Component terms were significantly enriched.

were Pb-associated, respectively. *Art3*, *Bri3*, *Ephb1*, and *Ldlrad4* were the four genes found across female tissues. In male tissues, 39 genes were detected from blood and 18 genes were detected from liver. *Prkca* was the only gene detected across male tissues (**Figure 3**). In blood, 9 and 8 genes were involved in nucleic acid-templated transcription in female and male, respectively. In male liver, there were six genes in this biological process. Gene list and associated DMRs can be found in **Supplementary Table S7**.

DISCUSSION

Pb is a common environmental exposure that is related to many health problems. In this study, we specifically investigated the effect of perinatal Pb exposure on DNA methylation in two tissues and both sexes using an established mouse model of perinatal environmental exposures. Because regulated DNA methylation targets are usually clustered into certain regions, and not single CpG sites, we focused on analysing DMRs to explore the biological effects of the Pb-induced methylation changes (Gaspar and Hart, 2017). In the two female tissues as well as in the male liver, similar numbers of hyper- and hypo-DMRs were identified. In male blood, however, we observed many more hypo-methylated than hyper-methylated regions. The sequencing depth of male blood samples did not show any bias compared to other tissues, suggesting that the result is not due to a technical effect of coverage level. However, it is possible that the trend toward hypomethylation is due to an unknown technical effect. If biologically meaningful, the biological mechanism underlying this observation is currently unclear. A potential explanation is that perinatal Pb exposure can modulate the expression of *Dnmt1* and reduce the activity of DNMT1 (Wu et al., 2008; Schneider et al., 2013; Ordemann and Austin, 2016), the maintenance DNA methyltransferase (Sobolewski et al., 2018); thus, sex- and tissue-specific alterations in *Dnmt1* expression may contribute to the high level of hypo-methylation in male blood. Future mechanistic studies are necessary to answer this question.

Previous studies revealed that DNA methylation in different genomic contexts relates to unique biological functions. The DNA methylation status of non-CpG island sites are more dynamic and tissue-specific than CpG islands (Jones, 2012). After annotating DMRs to genic regions, we found five genes - *Prdm16*, *Hjurp*, *Cdh23*, *Bc1*, and *Arid1b* – that were detected with DMRs across a combination of tissues and sex. Among these genes, four of them displayed DMRs mostly within intronic regions with mixed methylation directions (i.e., hyper and hypo). *Hjurp* showed inconsistent methylation direction within each

TABLE 1 | Imprinted genes with differentially methylated regions (DMRs).

Gene symbol	Male liver	Male blood	Female liver	Female blood
Adam23		●		
Adamts2	●			
Ampd3			●	
Arid1b	●	●	●	●
Axl	●			
Ccdc40		●		
Cdkn1c				●
Dact2	●			
Ddc	●			
Epas1			●	
Etv6	●			
Gab1		●		
Gnas			●	
H13			●	
Htra3	●			
Igf2r				●
Kcnk9				●
Mest		●		
Nespas			●	
Park2		●	●	
Pde10a	●	●		
Pde4d		●		
Plagl1		●		
Rian			●	
Sfmbt2		●		
Sh3gl3	●			
Slc38a2				●
Slc38a4		●		
Smoc2			●	●
Thbs2		●		
Trappc9		●	●	●
Usp29	●			
Wt1			●	
Xlr3b			●	
Zdbf2				●
Zfp64	●			

The colors of dots indicate the types of DMRs that associated with the genes. Blue represents hyper-DMRs, green represents hypo-DMRs, and black represents mixed-type (hyper and hypo) DMRs.

tissue (hypo- in blood and hyper- in liver) across sex on an exon/3′UTR region. The gene products of *Hjurp* have been identified as an important factor of DNA binding that promotes cell mitosis and chromosomal segregation (Barnhart-Dailey et al., 2017). This gene has been linked to many cancers including liver cancer, breast cancer, lung cancer, and glioma (Chen et al., 2018). Previous work suggests that methylation in exons can affect the alternative splicing events of the gene (Shayevitch et al., 2018), so perinatal Pb exposure may affect the function of *Hjurp* on a transcriptional regulation level through alternative splicing caused by DNA methylation changes.

In liver, *Prdx6b* was identified with a hyper-methylation change on an exon region in male and hypo-methylation in female. *Prdx6b* is an antioxidant gene involved in redox regulation of the cell. It has been reported that an elevated blood Pb concentration is associated with an increased risk of non-alcoholic fatty liver disease (Zhai et al., 2017). Mice with highly expressed *Prdx6b* in liver showed more protection from lipid accumulation after a high-fat diet (Lee et al., 2019).

In blood, we found the promoter region of *Chchd2* is hypo-methylated in both sexes. This gene is associated with Parkinson's disease in humans, which is a long-term degenerative disorder of the central nervous system (Funayama et al., 2015). Our results show that perinatal Pb exposure can affect the methylation status of the promoter region of this gene in a surrogate tissue at a young age (PND21) in mouse. The biological consequences of the altered DNA methylation resulting from perinatal Pb exposure need to be further examined.

To further understand the biological pathways associated with DNA methylation changes caused by perinatal Pb exposure, we identified GO terms enriched with identified DMRs. In female liver, GO terms *triglyceride metabolic process*, *lipid metabolic process*, and *regulation of lipid kinase activity* were significantly enriched. Similar GO terms *methionine metabolic process* and *glycerolipid catabolic process* were enriched in male liver. Previous studies show Pb exposure can damage the cell membranes through lipid peroxidation in liver (Sandhir and Gill, 1995). As a target tissue of Pb exposure, liver plays an important role in fat metabolism. From this study, the results suggest the perinatal Pb exposure can affect metabolic pathways through the alteration of DNA methylation. In female blood, *regulation of hydrogen peroxide-induced cell death* and *phosphatase inhibitor activity* were the most highly enriched. However, in male liver, the same GO terms are not significantly enriched. In blood, only a handful of GO terms were enriched, but it also shows more enriched GO terms in females than males. These together indicate that there are sex differences in the effects of perinatal Pb exposure.

Genomic imprinting is an epigenetic phenomenon in which the mono-allelic expression of certain genes occurs in a parent-of-origin specific manner. This phenomenon is caused by differential epigenetic modifications that are established in the germline and maintained through mitotic cell division in next generation (Tucci et al., 2019). Genomic imprinting is critical to normal development, and defects in imprinting are associated with neurodevelopmental and metabolic diseases (Bartolomei and Ferguson-Smith, 2011). Recent studies demonstrate that genomic imprinting is sensitive to environmental exposures as well as maternal nutrition and metabolic status (Monk et al., 2019). Imprinted genes are typically controlled by imprinting control regions (ICRs) and identification of ICRs is currently an active research area. Existing studies indicate that the ICR can vary by developmental stage and tissue. For example, the expression of *Gnas* in the mouse blood can be affected by DNA methylation changes that are not located in the previous identified imprinting control region (Kochmanski et al., 2018; Svoboda et al., 2019). The predefined ICR of this gene is within exon 1A of the *Nespas* gene, however, the DMR detected from our data did not overlap with this ICR. It is plausible that the DMRs located on non-ICRs may still be important for regulation of gene expression.

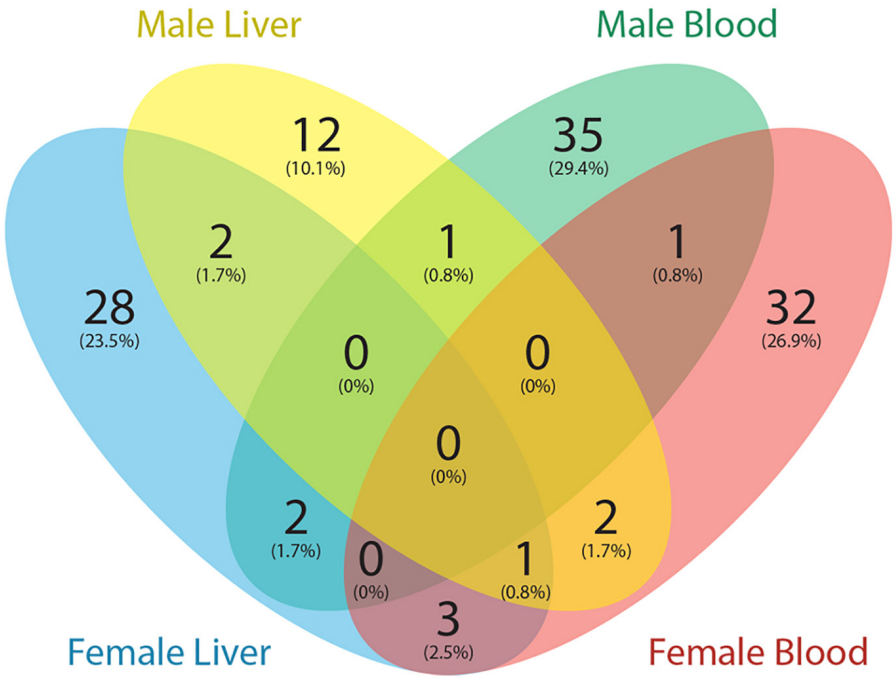

FIGURE 3 | Venn diagram showing the number of genes with DMRs overlapped with Pb exposure related genes from the Comparative Toxicogenomics Database (CTD).

From our results, one imprinted gene, i.e., *Arid1b*, was differentially methylated across both sexes and tissues. The product of this gene is a component of chromatin remodeling complex and may also play a role in cell cycle activation (Li et al., 2010; Santen et al., 2012). This gene contained DMRs in intronic regions in all four comparisons, thus the regulation of this gene through DNA methylation is worth further examination. In females, *Smoc2* was annotated to hyper-DMRs and hypo-DMRs in blood and liver, respectively. This gene is related to calcium ion binding and glycosaminoglycan binding (Peeters et al., 2016). In our results, the 3′UTR of this gene was covered by hyper-DMRs and hypo-DMRs in blood and liver, respectively. Since the 3′UTR is a putative functional DNA methylation site that can affect the expression of genes (Choi et al., 2009; McGuire et al., 2019), further studies are needed to address the potential functional consequences of altered methylation of this gene in female tissues. Overall, the percentage of imprinted genes with DMRs (11.88%) is higher than other genes with DMRs (3.69%). This suggests that perinatal exposures might differentially affect the methylation status of imprinted genes and further impact the normal developmental process.

To further investigate our results, we compared genes with DMRs from this study to those linked to Pb exposure in the Comparative Toxicogenomics database (CTD). We downloaded all mouse Pb exposure related target genes from the CTD to perform the analysis, and found a small portion (∼10%) of genes from the CTD were identified with DMRs in this study. Since the CTD dataset mainly represents genes that have an expression level change with Pb exposure, and our study was focused on DNA methylation changes, the limited overlap between these two datasets is not surprising. Among these genes, *Prkca* is the only gene identified in both male tissues (hypomethylation of 3′UTR in blood and intronic hypermethylation in liver). The product of this gene, protein kinase C (PKC), is strongly activated by Pb, causing dysregulation of neurotransmitter release and second-messenger systems (Tarrago and Brown, 2017). Our results indicate that this gene can be epigenetically affected by Pb exposure in blood and liver. In the CTD database, expression of *Prkca* was affected by Pb exposure at both the mRNA and protein levels. Thus, the methylation changes of this gene may contribute to the observed expression changes.

Since ERRBS was used to examine the DNA methylation changes and only about 10% of total CpG sites in mouse genome were covered by solid reads, this study cannot provide the whole genome-wide DNA methylation changes caused by perinatal Pb exposure. However, tissue and sex specific DMRs were still identified from our data. Future studies should focus on finding other potential perinatal Pb exposure related methylation changes, discovering the longitudinal biological consequences of the perinatal Pb exposure, and properly applying the findings to human environmental epigenetics studies.

DATA AVAILABILITY STATEMENT

All ERRBS data is publicly available at Gene Expression Omnibus (GEO) under accession ID GSE150670.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Michigan Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

MS, DD, LS, and JC conceived of the study. CR, TJ, and KN performed the tissue collection and sample preparation for ERRBS. KW and SL carried out the data analysis. KW wrote the manuscript and all authors edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00840/full#supplementary-material

FIGURE S1 | The genome-wide weighted sample correlations.

FIGURE S2 | The distribution of DNA methylation changes of differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs).

FIGURE S3 | The overlap among differentially methylated cytosines (DMCs) among sexes and tissues.

FIGURE S4 | Venn diagram showing the number of DMR related genes detected from different tissues in two sexes along with associated pie graph depicting the percentage of methylation directions of each gene set. The DNA methylation changes are expressed as lead vs control.

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The Involvement of Metals in Alzheimer's Disease Through Epigenetic Mechanisms

*Menghua Cai, Xiangjin Zhang, Wei He and Jianmin Zhang**

State Key Laboratory of Medical Molecular Biology, Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China

Alzheimer's disease (AD) is the most frequent cause of dementia among neurodegenerative diseases. Two factors were hypothesized to be involved in the pathogenesis of AD, namely beta-amyloid cascade and tauopathy. At present, accumulating evidence suggest that epigenetics may be the missing linkage between genes and environment factors, providing possible clues to understand the etiology of the development of AD. In this article, we focus on DNA methylation and histone modification involved in AD and the environment factor of heavy metals' contribution to AD, especially epigenetic mechanisms. If we can integrate information together, and that may find new potential targets for the treatment.

Keywords: Alzheimer's disease, DNA methylation, histone modifications, metals, demenita

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Sanofi, United States
Shuo Zhang,
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United States

****Correspondence:***
Jianmin Zhang
jzhang42@163.com

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INTRODUCTION

Neurodegenerative disorders are characterized by the progressive accumulation of misfolded proteins, which trigger damage of synapses, disturb network of pathway, facilitate death of specific neuronal populations, and finally initiate diseases. Several factors were hypothesized to be associated with the etiology of those diseases, including genetic and environmental factors. Alzheimer's disease (AD) is the most common neurodegenerative disease, and the hallmarks of AD pathology are an accumulation of Aβ to form amyloid-plaques and aggregation of phosphorylated tau to constitute neurofibrillary tangles (NFTs). Aβ is viewed as the core stone and trigger of diseases, which induces the dysfunction of synapses, loss of neurons, and ultimately dementia, with the existence of Aβ plaques and NTFs (Morris et al., 2014). Hyperphosphorylation changed the conformation of tau, which was believed to play a role in synaptic plasticity and facilitated its misfolding in pathological process (Zhang et al., 2016). Beside, apolipoprotein E (*ApoE*) gene shows strong association with risk for AD, for ApoE combined directly with Aβ to promote its aggregation and that facilitated tau phosphorylation inducing NFTs (Brecht et al., 2004).

Epigenetics is the study of heritable and reversible changes in gene expression, including DNA methylation, multi-modification of histones, and microRNA (Collotta et al., 2013), which occur without a change in the DNA sequence. This article reviewed DNA methylation and histone modifications to exhibit latest understanding about the role epigenetics plays in AD.

DNA Methylation

The first report epigenetic changes in AD found hypomethylation of *amyloid precursor protein* (*APP*) from an AD patient (West et al., 1995). In a pair of monozygotic twins, levels of DNA methylation significantly decreased in temporal neocortex neuronal nuclei of the AD twin (Mastroeni et al., 2009). Besides, DNA methyltransferase (DNMT) decreased in entorhinal cortex layer II of AD patients (Mastroeni et al., 2010). In a recent research, the patient group showed 25% reduction of DNA methylation levels in mitochondrial DNA D-loop region (Stoccoro et al., 2017), suggesting the underlying role of mitochondrial DNA methylation in AD. Hypomethylation of *BRCA1* was observed in AD patients, and this result was in consistent with the higher expression of its mRNA (Mano et al., 2017). Through comparing brains of mouse models and AD patients, hyper-methylation of three genes namely TBXA2R, SPTBN4, and SORBS3 resulted in silence of these genes in AD process (Sanchez-Mut et al., 2013).

Histone Modification

Comparing the temporal cortex and hippocampus, the twin with AD showed a significantly higher level of H3K9me3, a sign of gene silence, and H3S10 phosphorylation, a regulator of chromatin structure (Wang et al., 2013). The brains from AD patients showed hyper-acetylation in histone H3 and H4 (Narayan et al., 2015). Histone deacetylation catalyzed by histone deacetylase (HDAC) results in a condensed state of chromatin and consequent transcriptional repression. HDAC2 increased in AD-related neurotoxic insults *in vitro*, two mouse models and patients with AD, which decreased the histone acetylation of genes related to memory and inhibited their expression (Graff et al., 2012). Tau interacts with HDAC6 to decrease its activity. Through this way, tau promoted the acetylation of related genes (Perez et al., 2009). As a feedback and compensation, the expression of HDAC6 was significantly increased. In a mouse model of AD, decreased HDAC6 facilitated the recovery of learning and memory through disturbing mitochondrial trafficking dysfunction caused by Aβ (Govindarajan et al., 2013). Importantly, the AD mouse model treatment with valproic acid (VPA), one of widely used HDAC inhibitors in clinical research, has shown exciting results. VPA significantly decreased Aβ production by inhibiting γ-secretase cleavage of APP and alleviated the memory deficits of the AD mice (Qing et al., 2008).

Roles of Metals in AD

Plumbum

Plumbum facilitated the concentrations of free radicals, which leaded to the death of neurons. Pb exposure stimulated the serine/threonine phosphatases to impair memory formation (Rahman et al., 2011). Pb exposure leaded to the DNA methylation changes in the whole blood cells (Hanna et al., 2012). Early exposure of Pb increased Aβ product in old age. While in aged monkeys exposed to Pb as infants, the expression of APP and BACE1 elevated, and the activity of DNMT decreased (Wu et al., 2008). In rodents exposed lead, the expression of APP increased 20 months later, implying that lead exposure showed a life-long risk of AD (Basha et al., 2005).

In mice model of AD exposure to Pb, the levels of DNMT1, H3K9ac, and H3K4me2 decreased, the level of H3K27me3 increased, while the concentration of DNMT3a did not change (Eid et al., 2016). Besides, Pb exposure altered the production of tau (Dash et al., 2016). In mice expressing human APP, Pb stimulated the production of Aβ (Gu et al., 2011). Pb also disturbed the clearance of Aβ plaques by suppressing the activity of neprilysin (Huang et al., 2011). In primates with early exposure of Pb, their brains showed overexpression of APP and Aβ through hypo-methylation of related genes when aging. Yegambaram also reported that early exposure of Pb leaded to overexpression of APP, BACE1, and PS1, one of their regulators (Yegambaram et al., 2015). Both of them suggest that early exposure of Pb played a role in the development of AD when aging.

Arsenic

S-adenosyl-methionine (SAM) is essential for methylation of inorganic arsenic to detoxication, and it is also the metyl-donor required by DNA methyltransferases. So, it is reasonable to speculate that arsenic exposure leads to hypo-methylation of DNA and facilitates tumor-related gene expression (Zhao et al., 1997). Insufficiency of SAM leaded to hypomethylation of *PS1* and *BACE* genes. This hypomethylation increased the expression of PS1 and BACE, which facilitated the production of Aβ (Fuso et al., 2005). Besides, arsenic inhibited the expression of the DNA methyltransferase genes, DNMT1 and DNMT3a (Reichard et al., 2007). Sodium arsenite exposure inhibited HDAC p300 for attenuating H3K27ac at enhancers in mouse embryonic fibroblast cells (Zhu et al., 2018). Su reported a dose-response relationship between the environmental concentration of total arsenic in topsoils and the prevalence and mortality of AD in European countries (Yegambaram et al., 2015).

Environmental toxin arsenite induced a remarked increase in the phosphorylation of several sits in tau, including Thr-181, Ser-202, Thr-205, Thr-231, Ser-262, Ser-356, Ser-396, and Ser-404, which was in coincidence with results from AD (Giasson et al., 2002). Gong argued that arsenic stimulated the generation of free radicals, which leaded to oxidative stress and neuronal death (Gong and O'Bryant, 2010). When mothers were exposed to arsenic during pregnancy, their children showed a higher activation of inflammation-related pathways involved in the development of AD (Fry et al., 2007).

Aluminum

Aluminum has been reported to induce neurofibrillary degeneration in neurons of higher mammals in 1970s (Crapper et al., 1973). McLachlan reported a dose-effect association between the risk of AD and residual aluminum in municipal drinking water. The estimated relative risk of AD for residents with drinking water containing more than 100 ug/L of Al was 1.7 (McLachlan et al., 1996). Walton (2014) reported that long term intake of Al was an etiology of AD. A 15-year follow-up implemented by Rondeau et al. (2009) also showed a significant association between a high daily intake of aluminum and increased risk of dementia. Al could selectively interact with Aβ to facilitate the formation of fibrillar aggregation, while copper, iron, or zinc could not (Bolognin et al., 2011).

In transgenic mice overexpressed human APP (Tg2576), dietary Al stimulated the expression and aggregation of Aβ through increasing oxidative stress (Pratico et al., 2002). In embryo rat hippocampal neurons, high concentration of Al facilitated the production of ROS induced by Fe (Xie et al., 1996). Al facilitated the degradation from APP to the aggregation of Aβ (Kawahara et al., 1994). Besides, the structure of non-Aβ component of AD amyloid was changed by the induction of Al to resist degradation and form plaque (Paik et al., 1997).

CONCLUSION

No mutation in genes has been definitely associated with neurodegenerative diseases, suggesting that, besides risk factors of gene, environmental exposure also is involved in the etiology of AD, and those two factors may be abridged through epigenetic alterations. Recently, an integrated multi-omics analyses identified molecular pathways associated with AD and revealed the H3 modifications H3K27ac and H3K9ac as potential epigenetic drivers linked to transcription and chromatin and disease pathways in AD (Nativio et al., 2020). These findings provide mechanistic insights on AD for aiming epigenetic regulation of therapeutic strategy. We should get more enlightenment from it and explore the relationship between AD and epigenetics. On this basis, we will further study the effective diagnosis, treatment, and prevention methods of AD, and develop new intervention measures for AD from the field of epigenetics.

AUTHOR CONTRIBUTIONS

MC wrote the manuscript. XZ helped to edit the manuscript. WH and JZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Review: *In vitro* Cell Platform for Understanding Developmental Toxicity

Junkai Xie[1], *Kyle Wettschurack*[1] *and Chongli Yuan*[1,2]*

[1] *Davidson School of Chemical Engineering, Purdue University, West Lafayette, IN, United States,* [2] *Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN, United States*

Developmental toxicity and its affiliation to long-term health, particularly neurodegenerative disease (ND) has attracted significant attentions in recent years. There is, however, a significant gap in current models to track longitudinal changes arising from developmental toxicity. The advent of induced pluripotent stem cell (iPSC) derived neuronal culture has allowed for more complex and functionally active *in vitro* neuronal models. Coupled with recent progress in the detection of ND biomarkers, we are equipped with promising new tools to understand neurotoxicity arising from developmental exposure. This review provides a brief overview of current progress in neuronal culture derived from iPSC and in ND markers.

Keywords: IPSC, epigenetics, neurodegenerative disease, developmental exposure, organoid

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Shuo Zhang,
University of Pennsylvania, United States
Lu Li,
Sun Yat-sen University, China

***Correspondence:**
Chongli Yuan
cyuan@purdue.edu

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INTRODUCTION

Developmental exposure to environmental chemicals, such as heavy metal [e.g., lead (Bellinger et al., 1987), manganese (Lynam et al., 1999), mercury (Falluel-Morel et al., 2007)] and organic chemicals (Thiruchelvam et al., 2002) (e.g., pesticides, herbicide, and industrial surfactants) has been associated with various neurodevelopmental and neurodegenerative diseases (NDs) in the past decades and attracted significant attention in the scientific area. These chemicals have pervaded nearly all parts of our environment, including the food chain (Watras et al., 1998), drinking water (Remoundaki et al., 2016), and atmosphere (Lynam et al., 1999) due to decades of industrial and commercial use. Chronic exposure to low-doses of environmental chemicals does not necessarily illicit immediate response in the exposed population but can have significant bearings on shaping the health of individuals later in life (Lee et al., 2003; Wang et al., 2015). Among different exposure windows, the developmental window has been considered the most sensitive to chemical exposure, which has led to the establishment of the Developmental Origins of Health and Disease (DOHaD) hypothesis. The DOHaD hypothesis postulates that the developmental and growth window presents a critical period of time where exposure to certain environmental chemicals can impose significant consequences on an individual's short and long-term health (Barker, 2007). The attempts to elucidate toxicity arising from developmental exposure, however, have been very challenging because of the latent time between exposure and disease on-set; as well as the lack of established biomarkers that "record" past exposure events and trigger disease on-set later in life.

Longitudinal epidemiology studies have been commonly used to establish the connection between developmental exposure and disease onset later in life. For example, developmental

exposure to lead (Pb) can result in an increased risk of developing learning disabilities in exposed children (Needleman et al., 1979) and NDs such as Alzheimer's disease later in life as suggested in rodent models (Eid et al., 2016). Exposure to organic pesticides such as the herbicide paraquat and fungicide maneb during the developmental stage can cause a decrease of striatal dopamine production and impairment of locomotor activity which aligns with Parkinson's disease phenotype (Thiruchelvam et al., 2002). Unfortunately, the majority of studies provide limited mechanistic insights into how toxicity affects cells. Various animal models have also been adopted, including rodent (Filipov et al., 2007; Eid et al., 2016), fish (Weber et al., 2013; Wirbisky et al., 2016), and monkey (Lasky et al., 2005) models. These have had success in assessing survival toxicity (Gad, 2014) and damage to reproductive tissues (Song et al., 2014) but have had only limited success for assessing neurotoxicity because of the vast difference between human and non-primate brains. For example, rodent models are extremely popular for the study of cancer (Ding et al., 2011) and psychiatric (Majdak et al., 2016) diseases; however, it has become increasingly clear that rodent models do not necessarily recapitulate human brain function as accurately as previously believed and thus may not be an ideal surrogate system for studying neurotoxicity arising from environmental exposures (Hodge et al., 2019). Although rodent and human brains share a conserved structure, RNA-seq studies have revealed that the expression of ion channels, neurotransmitter synthetase, and neurotransmitter receptors varies greatly between species (Hodge et al., 2019). Uncertainty in the accuracy of animal models for studying brain-related diseases has led to the popularization of human stem cells, including embryonic (ESC) and induced pluripotent stem cells (iPSCs) as an *in vitro* system for assessing neurotoxicity. In contrast to ESC, which must be collected from blastocysts, iPSCs are derived from fibroblasts of human patients. They are easier to collect; account for genetic variations among human patients and thus are becoming a preferred cell model in studying neurotoxicity. iPSCs have been differentiated into various lineages to partially mimic human organs since their debut in 2006 (Takahashi and Yamanaka, 2006). Of particular interest in assessing environmental exposure, iPSCs can be differentiated into neural stem cells (NSCs) (D'Aiuto et al., 2014) and later glutaminergic (Anderson et al., 2015), dopaminergic (Suzuki et al., 2017), and GABAergic (Lin et al., 2015) neurons that mimic part of the brain functions. Here, we will review the recent progress in using human iPSCs as a cell culture model to study neurotoxicity arising from developmental exposure.

TOWARDS RECAPTULATING (PART OF) BRAIN COMPLEXITY IN HUMAN CELL CULTURE

The brain is a complex organ and thus understandably difficult to reconstruct *in vitro* given its composition, structural, and functional complexity. However, significant progress has been made in recent years enabling partial recapitulation of a human brain in a culture dish.

Composition Complexity

The human brain consists of various cell types, including neurons (Lake et al., 2016), astrocytes (Lewis et al., 2010), and microglia (Hickey and Kimura, 1988). To address the challenge in composition complexity, various protocols have been developed for incorporating multiple cell types via co-culture systems [i.e., neuron- astrocyte (Odawara et al., 2014; Kuijlaars et al., 2016) and neuron- microglia (Haenseler et al., 2017)] differentiated from iPSC and neural progenitor cells (NPCs). **Figure 1** illustrates some commonly used co-culturing techniques that can be used to reconstruct brain-like tissues on a dish. These co-culture systems facilitate the reconstruction of complex neural systems resembling neural development (Kuijlaars et al., 2016) or mimicking inflammatory responses that are provoked in NDs such as Alzheimer's and Parkinson's disease (McGeer et al., 1988; Sapp et al., 2001). It is worth noting that not all cells found in the brain are from the same stem cell lineage. For example, microglial cells in the brain result from the differentiation of mesodermal progenitors (Murabe and Sano, 1982), rather than the neuroectoderm, as is the case with neurons (Jiang et al., 2003). The combination of multiple types of cells *in vitro* has arisen as a critical tool in the study of brain development, particularly in the study of NDs, where the interaction between multiple cell types is crucial for disease pathology (Di Malta et al., 2012; Heneka et al., 2013).

Structure Complexity

Significant advances in cell culturing approaches have occurred in recent years that enabled the transition from 2D to 3D cell culture to reconstruct the architecture of the brain. Compared to 3D culture, 2D cell cultures are typically easier to perform and more compatible with imaging-based analysis, including neurite morphology analysis and live-cell tracing (Shin et al., 2018). 3D culture, however, takes advantage of the self-assembly that iPSCs undergo when grown in a 3D environment (Lancaster et al., 2013). This self-assembly causes iPSCs to form distinct cell types native to specific areas of the brain, such as the hippocampus, ventral forebrain, and cerebral cortex (Lancaster et al., 2017; Quadrato et al., 2017). Using this method, researchers can create brain organoids that contain various discrete but interdependent brain regions (Lancaster et al., 2013). This structure complexity, particularly the development and interaction of different brain regions, can produce organoids with active neural networks capable of firing in response to stimuli (Giandomenico et al., 2019). 3D organoids provide an ideal testing platform for drug discovery (Phan et al., 2019), modeling neurological diseases (Lancaster et al., 2013), and assessing environmental toxin exposure (Forsythe et al., 2018). While early brain organoids showed significant heterogeneity (Camp et al., 2015), brain organoids generated via more recent protocols have improved reproducibility (Velasco et al., 2019; Nickels et al., 2020) but still require further optimization.

Functional Connectivity

Although iPSCs can differentiate into neurons and express the mature neuronal markers such as MAP2 and NeuN, they

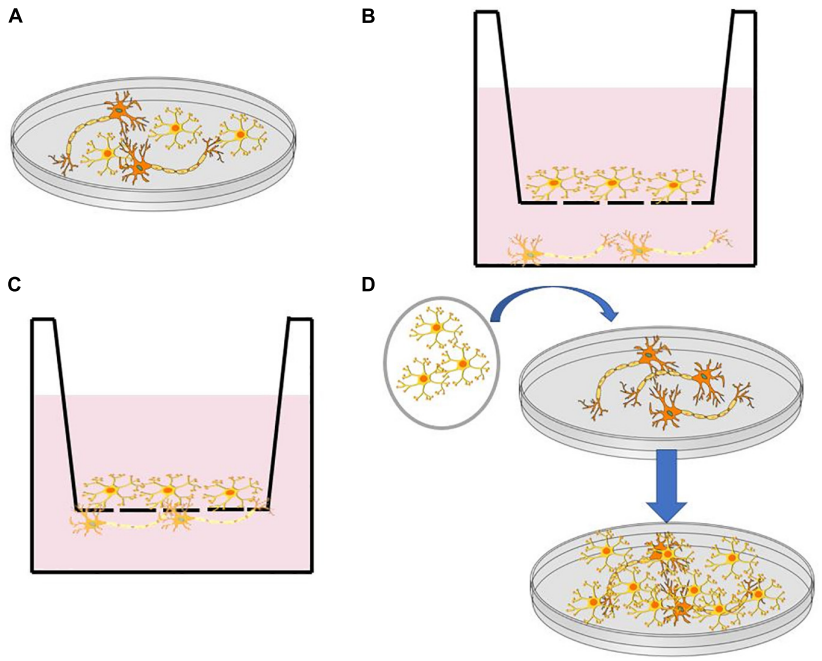

FIGURE 1 | (A) Direct co-culture in a dish. **(B)** Indirect co-culture using a transwell insert with a semipermeable membrane. **(C)** Direct co-culture through a semipermeable membrane in a transwell dish. **(D)** Transfer of cell monolayer to established culture through a membrane such as PVDF.

do not necessarily exhibit the correct electrophysiological properties, hampering their uses in assessing neural circuitry activities. Researchers have developed simplified protocols for differentiating neuronal networks that are electro-physiologically active in 2D (Gunhanlar et al., 2018) and 3D (Paşca et al., 2015; Birey et al., 2017). These protocols typically include cAMP in their culture to facilitate the establishment of neuronal connectivity (Kang et al., 2017; Gunhanlar et al., 2018). With the initial claim of success, the prevalence, composition, properties, and relevance of these established neuronal connections remain to be tested by time. The occurrence of *in vivo*-like complex neuronal activity and functional circuitry remains elusive and more in-depth analysis will be crucial for unraveling the connectivity in cultures, particularly organoids, to understand the impact of genetic and environmental chemical perturbations on human synaptogenesis, neuronal activity, and network function.

MIMICKING DEVELOPMENTAL EXPOSURE USING STEM CELL MODELS

Stem cells can undergo differentiation and maturation in a similar manner compared to animal models, providing multiple assessment windows for studying neurotoxicity. Human brains contain many unique features that define cognition (Lui et al., 2011). On average, 86.1 billion neurons can be found in the brain and spinal cord of an adult male and 16.34 billion of these are located in the cerebral cortex (Azevedo et al., 2009; Herculano-Houzel et al., 2016). 80% of the neurons in the cerebral cortex are thought to be excitatory glutaminergic neurons which were differentiated in the ventricular zone and sub-ventricular zone of the cortical wall during prenatal development from 50 postconceptional days (pcd) to 24 postconceptional weeks (pcw) at a rate of ∼3.86 million neurons per hour (Workman et al., 2013). Neurogenesis occurs in the spinal cord and brain stem beginning at 32 pcd followed by cerebral cortex (O'Rahilly, 2006). Most of this neuron generation is completed before birth. Neocortical interneurons and inhibitory GABAergic neurons are two of the few neuron types that are known to undergo differentiation after birth (Sanai et al., 2011; Radonjić et al., 2014). Although most neurons have completed differentiation before birth, the neuronal network is yet to be established. Recent studies have revealed that synaptogenesis (Peter, 1979; Kang et al., 2011), myelination (Miller et al., 2012), and synaptic pruning (Petanjek et al., 2011) are still ongoing until 20 years of age. **Figure 2** summarizes the neuronal developmental processes at different time windows in the human brain matched to various stages of stem cell differentiation. Specifically, the procedure of generating mature neurons from NPCs can be divided into two sequential steps, namely, differentiation followed by maturation. During the differentiation step, retinoic acid or bone morphogenetic protein is used to mediate neuronal differentiation signaling pathways (Cazillis et al., 2006). After that, brain-derived neurotrophic factor (BDNF) and dibutyryl cyclic adenosine monophosphate (db-cAMP) are added to facilitate neuron maturation and promote synaptogenesis (Kang et al., 2017; Gunhanlar et al., 2018). The initial differentiation stage is completed after 8 days but it can take up to 6–8 weeks for neurons

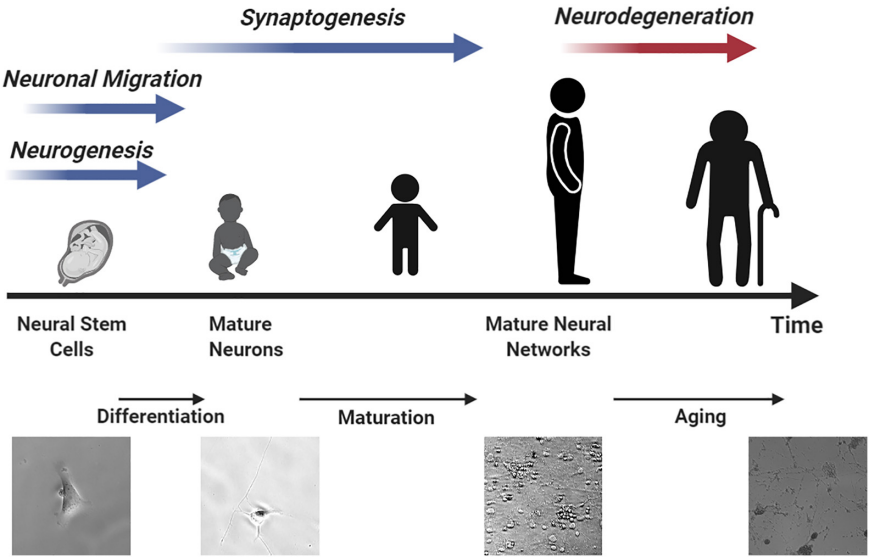

FIGURE 2 | An illustration of neurodevelopment processes matched to different stem cell differentiation time windows resembling human brain development.

to mature and develop proper electrophysiological functions (Gunhanlar et al., 2018).

ASSESS NEUROTOXICITY USING CELL CULTUR MODEL

Compared to animal models, cell culture presents several advantages for assessing neurotoxicity, including homogeneity in cell identity, compatibility with long-term monitoring, and controllable dosing. Several disadvantages remain, most notably the inability to perform behavior and aging related assessments. We will thus focus on summarizing molecular markers that have been previously established to facilitate the assessment of neurotoxicity.

Disease Biomarkers

Cell viability and proliferation are conventionally used as the starting point for assessing neurotoxicity. Although insightful for acute toxicity, limited knowledge can be gained regarding the chronic effects of neurotoxicity arising from environmental exposures. Early-stage disease biomarkers with established correlations of disease on-set thus play a critical role in assessing neurotoxicity. Several good reviews exist summarizing prevalent early-stage biomarkers that have been used to assess ND risks (Molinuevo et al., 2018; Ehrenberg et al., 2020). For example, Aβ42/Aβ40 ratio (Doecke et al., 2020) and phosphorylated tau p-Thr181 tau (Thijssen et al., 2020) and p-Thr205 tau (Barthélemy et al., 2020)) can be used to assist the diagnosis and prognosis of AD. α-synuclein and α-synuclein phosphorylation can be used in PD for similar purposes (Wang et al., 2012; Lin et al., 2017). These biomarkers can be visualized in cells exposed to environmental chemicals using either immunofluorescence or immunohistology methods, or quantified via ELISA, providing a robust approach to connect environmental exposure with neurodevelopmental and NDs.

Neuronal Activity

Recent expansion of toolsets to conduct electrophysiology measurements have further expanded our capability to probe neural circuit activities related to environmental exposure. Synaptic and ion channel activities, membrane potential, and action potential can be conventionally recorded using patch-clamp and multi-electrode array (MEA). These approaches have been primarily used to record changes in neural circuits of animal brain slices (Cholanian et al., 2017), cultured primary cells (Cannady et al., 2017; Dunn et al., 2018), and cell cultures (James et al., 2017) responding to various environmental chemical exposures. Neurons derived from iPSCs can establish electro-physiologically mature neuronal networks that closely resemble mature primary neurons (Gunhanlar et al., 2018). Recently, patch-clamp and MEA have been applied to cortical neurons derived from iPSCs of AD patient and healthy control; and demonstrated that AD neuronal cultures exhibit increased spontaneous firing, slow oscillatory events, and hypersynchronous circuit activity (Ghatak et al., 2020). Furthermore, various imaging probes, including calcium indicators, i.e., Fura-2 (Tsien et al., 1982; Grynkiewicz et al., 1985) and GCaMP (Chen et al., 2013; Yang et al., 2018); membrane potential probes, i.e., ASAP2 (Yang et al., 2016; Chamberland et al., 2017) and Voltron (Abdelfattah et al., 2019); and neurotransmitter probes, i.e., iGluSnFR (Marvin et al., 2013) and dLight (Patriarchi et al., 2018) can be introduced to neuronal cultures to monitor neural circuit activity *in situ*. These measurements collectively provide a viable approach to characterize abnormal circuitry activity that are known to be altered in various neurodevelopmental and neurodegenerative conditions. For example, AD neuronal cultures show oscillatory

events and hypersynchronous network activity compared to their wild-type isogenic controls (Ghatak et al., 2020). Furthermore, neurons derived from iPSCs of Autism Spectrum Disorders (ASD) have significantly altered glutamate neurotransmitter release and reduced spontaneous firing rate (Russo et al., 2018).

Epigenetic Changes

Epigenetic modifications account for inheritable changes in chromatin that are not accounted for by DNA mutations. Epigenetic modifications have been increasingly recognized as viable markers for various neurological diseases and are being actively researched. Among different epigenetic modifications, DNA methylation, such as cytosine methylation (5mC), hydroxymethylation (5hmC), and histone acetylation have attracted the most significant attention. Mutations in DNA and histone methyltransferase have been identified as risk factors in autism (Satterstrom et al., 2020). In addition, abnormal alterations in H3K9ac and H3K27ac were identified as the major distinctions between aging and AD brains (Nativio et al., 2020). Furthermore, CpH hypomethylation is accelerated in AD brains compared to normal aging ones (Li et al., 2019). Abnormal DNA methylation of ASCC1 and SLC7A11 has also been linked to PD (Vallerga et al., 2020). Similar methylation features were also revealed in iPSC models reprogrammed from patients iPSCs (Fetahu et al., 2019). Interestingly, environmental exposure of neuronal cells can also lead to major changes in DNA methylation, histone methylation, and acetylation. This suggests that epigenetic mechanisms may serve as a potential bridging marker between environmental exposure and neurological conditions (Pavanello et al., 2009; Luo et al., 2014; Wirbisky-Hershberger et al., 2017; Lin et al., 2020). The detection of epigenetic changes by protein-based fluorescent probes allows for real-time analysis of the changes to the global epigenome levels. So far protein probes have been developed for binding and visualization of both DNA methylation and histone modifications (Hendrich and Bird, 1998; Lungu et al., 2017; Sanchez et al., 2017; Sanchez et al., 2019). Further development of nanobody fragments have provided an additional domain that allows for high specificity and affinity for selected epigenetic targets (Hattori et al., 2016; Jullien et al., 2016). Accurate understanding and quantification of epigenetic changes that proceed chemically induced disease development could provide a system that can detect neurological conditions at an earlier time point.

CONCLUSION

In closing, patient derived iPSCs offer a promising platform to assess neurotoxicity given its ability to partially recapitulate the complexity and functionality of *in vivo* neurological structures. The advent and exploration of both 2D and 3D iPSC systems provide great promise for the examination of environmental exposure effects on adolescent brains. In addition, these cell assemblies provide a platform that more closely matches the developmental timeline and disease phenotypes of a human brain in comparison to rodent models. The application of environmental chemicals to iPSC is in its early stages, but provides a clear opportunity to understand the mechanisms that affect the human brain when exposed to heavy metals and pesticides. The use of phenotypic assays that quantify ND risk factors, neuron activity, and epigenetic changes can be used to elucidate the molecular mechanisms that are perturbed by environmental exposure. With the application of the numerous live-cell compatible techniques to human iPSCs, we are at an optimal position to understand the biological response to chronic toxin exposure and how it informs long-term disease development.

AUTHOR CONTRIBUTIONS

All authors have contributed in planning and writing the review.

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frontiers in Genetics

MINI REVIEW
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Epigenetic Alteration Shaped by the Environmental Chemical Bisphenol A

Tengfei Qin[1†], Xiaoping Zhang[2†], Ting Guo[1], Ting Yang[3], Yahui Gao[1], Wei Hao[1,4] and XiangFen Xiao[5*]*

[1]Henan Collaborative Innovation Center of Modern Biological Breeding, Henan Institute of Sciences and Technology, Xinxiang, China, [2]School of Medical Science, Chifeng University, Chifeng, China, [3]Department of Nephrology, Affiliated Hospital of Beihua University, Jilin City, China, [4]School of Life Sciences, The Chinese University of Hong Kong, Shatin, China, [5]Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

Bisphenol A (BPA) is extensively used in plastic products and epoxy resins. The epigenetic response to the environmental chemical BPA was involved in multiple dysfunctional categories, such as cancer, the reproductive system, metabolism, pubertal development, peripheral arterial disease, infant and childhood growth, and neurodevelopment outcomes. In this mini-review, we described the recent progress of the epigenetic effects of the environmental chemical BPA, including DNA methylation, histone methylation, and toxic epigenomics. Notably, the histone modification changes under BPA exposure are summarized in this review. DNA methylation accompanied by transcriptional changes in key genes affected by BPA exposure is related to various processes, including neural development, cancer pathways, and generational transmission. In addition, BPA could also affect histone modifications in many species, such as humans, rats, and zebrafish. Finally, we reviewed recent studies of the toxico-epigenomics approach to reveal the epigenetic effect of BPA exposure genome-wide.

Keywords: bisphenol A, epigenetic alteration, DNA methylation, histone modifications, toxico-epigenomics

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Dan Lou,
Heidelberg University, Germany
Soterios A. Kyrtopoulos,
National Hellenic Research Foundation, Greece
Min Huang,
Ningxia Medical University, China

***Correspondence:**
XiangFen Xiao
xxf0614@163.com
Wei Hao
haowei9111@gmail.com

[†]These authors have contributed equally to this work

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INTRODUCTION

Bisphenol A (BPA) is one of the most extensively used chemical components in the plastics and epoxy resins of large consumer products, including water bottles, baby bottles and storage containers, pitchers, tableware, and other storage goods (Kamrin, 2004). Almost everyone can be exposed to BPA every day from the environment, especially from food intake. BPA has been detected in multiple biological samples, including urine, blood, saliva, and even breast milk (Vandenberg et al., 2010).

Accumulating evidence shows that BPA, an endocrine-disrupting chemical (EDC), interferes with hormonal signaling pathways, which is acutely toxic to living organisms (Diamanti-Kandarakis et al., 2009). In recent years, many adverse effects on the reproductive system have been found to be associated with BPA exposure in both animals and humans (Shankar et al., 2012; Adeyi and Babalola, 2019).

Epidemiological studies discovered that cancer, reproductive system, metabolism, pubertal development, peripheral arterial disease, infant and childhood growth, and neurodevelopment outcomes were associated with BPA exposure (Matuszczak et al., 2019). For example, urinary BPA concentrations were reported to be associated with serum reproductive hormones.

Compared with unexposed BPA workers, exposed workers were more likely to suffer from male sexual dysfunction (Braun and Hauser, 2011). Urinary BPA levels were significantly correlated with peripheral arterial disease based on the analysis of representative samples (Shankar et al., 2012).

Bisphenol A exposure can induce epigenetic misregulation, especially early persistent exposure, which can affect the development of offspring. Epigenetic changes, including modifications in DNA, RNA, and histones combined with noncoding RNA (ncRNA) and miRNA-associated gene silencing, are currently thought to initiate and sustain transcriptional regulation without alteration of DNA sequencing (Weinhold, 2006). Compared to genetic alterations, epigenetic regulation is more dynamic in response to environmental changes and environmental exposures. The major DNA modification is DNA methylation of 5-mC in cytosine in both symmetric and asymmetric contexts (CG, CHG, and CHH). Oxidation of methylated cytosine (5-mC), methylation of adenine (A), and other modifications are also regarded as significant epigenetic regulators (Kumar et al., 2018). Histone modification is a covalent posttranslational modification (PTM) of histones. Histone modification of acetylation and methylation on histone H3 could regulate chromatin structure and transcription (Bannister and Kouzarides, 2011). Epigenetic modifications are tightly controlled during human development, especially in the very early stages from fertilization to birth. ncRNAs are a group of functional RNA molecules transcribed from DNA but not translated into proteins (Wei et al., 2017). ncRNAs have been found to be involved in various processes, such as histone modification, DNA methylation targeting, and gene silencing. Numerous miRNAs have been shown to play significant roles in virtually every cellular process and are required for animal development, cell differentiation, and homeostasis.

To conduct our review on the most recent studies about the epigenetic effects of BPA, we used a combination of the key words "bisphenol A" AND "epigenetic" OR "DNA methylation" OR "histone OR "miRNA" to search the PubMed database from 2012 to August 2020. In total, we found approximately 200 papers and manually checked each article. We then classified them into three categories: DNA methylation, histone modification, and both DNA methylation and histone methylation. In addition, we further selected studies with high-throughput sequencing data, such as toxico-epigenomics study cases. Because a comprehensive review has only published the miRNA changes induced by BPA exposure, we will not review the miRNA and ncRNA parts in this review (Sabry et al., 2019; Farahani et al., 2020).

New and ongoing studies are continuously revealing the role of epigenetics in human disorders and fatal diseases linked to environmental exposure, including endocrine disruptors (BPA, DDT, phthalates; Dutta et al., 2020); tobacco smoke (Wu et al., 2019); diesel exhaust particles, such as PM2.5 and PM10; heavy metals, such as arsenic, nickel, and lead (Wang et al., 2020); and other indoor and outdoor pollutants (Mileva et al., 2014; Zhu et al., 2018; Freeman et al., 2020). A systematic review summarized that BPA has many effects, such as hormonal alterations, alteration of male reproductive organs, and epigenetic changes involving the male reproductive system (Cariati et al., 2020). In this review, we summarized the recent discoveries of BPA-mediated epigenetic alterations in DNA methylation and histone modification. In addition, we also reviewed toxico-epigenomics studies on different kinds of animal models.

BPA AND DNA METHYLATION

Bisphenol A could affect DNA methylation as an endocrine-disrupting chemical. In **Table 1**, we summarized the genes that have been reported to be methylated or demethylated under BPA treatment since 2012, while another review reported BPA-affected genes epigenetically until 2012 (Singh and Li, 2012).

Bisphenol A was extensively found to be involved in neurodevelopment by affecting DNA methylation. Kundakovic et al. (2013) found that *in utero* BPA exposure induced sex-specific DNA methylation changes in *ERα* [estrogen receptor 1 (*Esr1*)] in the brain. Furthermore, they detected enduring DNA methylation changes in brain-derived neurotrophic factor (*BDNF*), a specific gene related to nervous system development, that were induced by parental BPA exposure (Kundakovic et al., 2015). These results indicated that BPA exposure-induced DNA methylation changes in specific genes may underlie long-lasting effects on brain function, behavior, and neurodevelopment. Another group of scientists found that, by binding to tyrosine kinase B (TrkB), BDNF induces TrkB autophosphorylation, which activates the RAS-MAPK pathway, and finally, CREB (cAMP responsive element binding protein) is activated at the serine site. By increasing the expression of *BDNF* and the antiapoptotic gene *Bcl-2*, CREB could increase the survival of nerve cells, synaptic plasticity, and neurogenesis. A BPA or ethinyl estradiol (EE) consortium study revealed that exposure to BPA or EE may lead to *BDNF* gene expression. Exposure may also lead to changes in DNA methylation in the hippocampus and hypothalamus of adult rats. The changes in *BDNF* in the hippocampus exhibited some connections with the performance of female mice in the Barnes maze (Cheong et al., 2018). The *Grin2b* gene is important for the regulation of neural morphology, learning, and memory. Genetic polymorphisms in Grin2b have been demonstrated to be involved in neurodevelopmental diseases and disorders. Alavian-Ghavanini et al. (2018) found that prenatal BPA exposure is connected with epigenetic changes in *Grin2b* in female rats and humans. In addition, the global levels of 5-mC and 5-hmC were significantly increased after BPA treatment in human neuroblastoma cells (SH-SY5Y; Senyildiz et al., 2017).

Cancer cell proliferation has been reported to be mediated by DNA methylation induced by BPA exposure. Recently, Li et al. (2020) revealed the pivotal role of TET dioxygenases and DNA hydroxymethylation in the bisphenol-stimulated proliferation of breast cancer cells. They found that BPA or its replacement BPS increased the promoter methylation of TET2, leading to an inhibition of TET2 expression and DNA hydroxymethylation. A decrease in DNA hydroxymethylation induces increased proliferation of ER-positive breast cancer

TABLE 1 | DNA hypomethylation and hypermethylation induced by bisphenol A (BPA).

Genes	Epigenetic alterations	Treatment methods	Target tissue	References
ERα	DNA hypomethylation	BPA given to female mice during pregnancy	Brain of F1 offspring mice	Kundakovic et al. (2013)
BDNF	DNA hypomethylation	Pregnant mice were orally treated with BPA	Blood of female mice and hippocampus of F1 mice	Kundakovic et al. (2015)
Igf2	DNA hypermethylation	Pregnant F0 SD rats were orally treated with BPA	Sperm of adult F1 male rats and male F2 pancreatic beta-cells	Mao et al. (2015)
	DNA hypermethylation	Early-life exposure to BPA and/or variable diet	paired mouse tail tissue	Kochmanski et al. (2017)
Stat3	DNA hypermethylation	Liver tissue exposed to BPA	Fetal liver of mice and humans	Weinhouse et al. (2015)
MEST	DNA hypermethylation	Prenatal BPA potential exposure	Human cord blood and visceral fat tissue of the offspring of BPA-exposed dams	Junge et al. (2018)
Grin2b	DNA hypomethylation	Prenatal BPA potential exposure	Hippocampus of female rats and humans	Alavian-Ghavanini et al. (2018)
TET2	DNA hydroxymethylation	MCF-7 cells treated with BPA or bisphenol S (BPS)	Breast cancer cells	Li et al. (2020)
Scgb2a1	DNA hypomethylation	BPA oral route of exposure	Adult rat prostate	Wong et al. (2015)

cells. Secretoglobins are a family of small secreted proteins and are thought to be involved in many processes, such as inflammation, tissue repair, and tumorigenesis. Wong et al. (2015) used a rat model for the developmental reprogramming of susceptibility to prostate carcinogenesis. Using RNA-seq, they found that the gene expression of *Scgb2a1* was significantly upregulated in the prostate of adult rats neonatally exposed to BPA. DNA methylation analysis of the *Scgb2a1* promoter revealed significant CpG island hypomethylation upstream of the TSS of *Scgb2a1* in the reprogrammed prostate. Their results indicate that BPA exposure could reprogram *Scgb2a1* expression in the adult prostate epigenetically during the development of the prostate (Wong et al., 2015).

In another study, the DREAM (digital restriction enzyme analysis of methylation) method was also applied in the zebrafish embryo for the study of DNA methylation modifications. Site-specific methylation in the promoter of the vasa gene was more responsive in the three examined genes, indicating that the vasa gene could be a potential marker for BPA exposure in zebrafish (Bouwmeester et al., 2016). Signal transducer and activator of transcription 3 (Stat3) is an essential transcription factor and plays roles in immune signal translocation. From the mouse fetal liver, DNA methylation profiles within STAT3 changed following the BPA level in human fetal liver samples, indicating that STAT3 could serve as a translationally relevant candidate biomarker (Weinhouse et al., 2015).

Transposable elements accounted for 45 and 37.5% of the human genome and mouse genome, respectively. Upon BPA exposure, Faulk et al. (2016) found remarkable DNA methylation changes in transposons in the human liver (1,251 individual transposon loci), while they did not detect significantly detectable differential DNA methylation in mice (only 19 loci were identified).

Recent studies also showed that BPA exposure epigenetically affected imprinted genes. Insulin-like growth factor-2 (Igf2) is a critical imprinted gene (Morison and Reeve, 1998) that is mainly regulated by differentially methylated regions (DMRs; Portela and Esteller, 2010). Kochmanski et al. (2017) found that BPA exposure in the diet showed no significant effect on the methylation drift at *LINE-1*, *IAP*, *H19*, or *Esr1* but presented a marginally significant effect on age-related methylation at *Igf2*. Mao et al. (2015) revealed that the hypermethylation of Igf2 in islets was involved in generational transmission of glucose intolerance and pancreatic beta-cell impairment in F2 offspring induced by F1 early life BPA exposure. These DNA methylation changes in germ cells may facilitate generational transmission. However, in one study using mouse models, van Esterik et al. (2015) concluded that although different metabolic phenotypes in female offspring after maternal BPA exposure were observed, changes in DNA methylation could not explain the abnormal metabolic phenotypes. BPA can disrupt human placental epigenetic modifications, including genomic imprinting, DNA methylation, and the expression of ncRNAs (Strakovsky and Schantz, 2018). Furthermore, Kochmanski et al. (2018) profiled genome-wide 5-hmC levels and discovered that perinatal BPA exposure induced persistent 5-hmC markers at multiple imprinted loci in mouse blood during development.

These recent discoveries indicate that BPA exposure could affect DNA methylation and hydroxymethylation at multiple loci related to the neural system, cancer cell proliferation, and imprinted genes and could further affect the expression levels and their functions in the cell.

BPA AND HISTONE MODIFICATIONS

Apart from DNA methylation, histone modifications are another type of epigenetic marker for transcriptional regulation. Sometimes changes in histone modifications may accompany DNA methylation. Here, we reviewed the recent findings of histone modification changes under BPA treatment.

For example, BPA could alter the histone H3K4me3, histone acetylation, and RNA polymerase II (RNAP II) recruitment to the long noncoding RNA (lncRNA) HOTAIR in human breast cancer cells (MCF7) and in the mammary glands of rats (Bhan et al., 2014a). As another example, enhancer of Zeste homolog 2 (EZH2), a histone methyltransferase specific to H3K27, was found to

be transcriptionally induced by the estrogenic endocrine disruptor BPA in MCF7 cells and the mammary glands of ovariectomized (OVX) rats (Bhan et al., 2014b).

Furthermore, HOXC6 and HOXB9 are homeobox-containing genes related to mammary gland development that are overexpressed in various cancers. BPA treatment induced the expression of HOXC6 and HOXB9 in human breast cancer cells (MCF7) and the mammary glands of ovariectomized (OVX) rats. Luciferase assays indicated that estrogen-response elements (EREs) located in the HOXB9/HOXC6 promoter are irreplaceable for BPA-induced expression. Estrogen receptors (ERs) and other ER coregulators can bind to the HOXB9/HOXC6 promoter in the presence of BPA, resulting in increased chromatin H3K4me3, histone acetylation, and gene activation. It is possible that the increase in H3K4me3, histone acetylation, and recruitment of RNAP II at the HOXC6/HOXB9 promoters was caused by exposure to BPA (Hussain et al., 2015; Deb et al., 2016). In another study, BPA induced a significant decrease in H3K9ac and H3K9me3 levels overall in SH-SY5Y cells (Senyildiz et al., 2017).

BPA exposure could affect porcine oocyte maturation. Wang et al. (2016) found that the oocyte maturation rate was significantly reduced with 250 μM BPA treatment *in vitro*. The intensity of H3K4me2 and the 5-mC levels were decreased after BPA treatment, suggesting that disturbed epigenetic modification might inhibit the meiotic progression of oocytes. Using *Chironomus riparius* as an animal model, Lee et al. (2018) observed some potential interactions between altered H3K36 and metabolites caused by BPA exposure.

Exposure to BPA could impair primordial germ cell migration. *cxcr4b* and *sdf1a* are two key genes involved in PGC migration. Lombo et al. (2019) found that cxcr4b and sdf1a were highly dysregulated in zebrafish embryos exposed to BPA. Within the genital ridge, no significant changes were determined in germ or somatic cells. However, the males exhibited a reduced H3K9ac level in sperm with embryonic BPA exposure. In another study using male zebrafish, a high dose of BPA affected spermatocyte function. The mRNA level of epigenetic remodeling enzymes in testes was misregulated. BPA also triggered the activity of histone acetyltransferase (HAT), resulting in increased levels of H3K9ac, H3K14ac, and H4K12ac (Gonzalez-Rojo et al., 2019).

In summary, BPA exposure could affect histone modification in humans, rats, porcines, *C. riparius*, and zebrafish accompanied by impairment of primordial germ cell migration.

BPA AND THE TOXICO-EPIGENOMICS

Toxicogenomics utilizes comprehensive gene expression data to profile gene expression features that strongly correlate with genetic toxicity (Suter et al., 2004; Gomase and Tagore, 2008). Indeed, with the development of high-throughput sequencing technology, apart from gene expression, DNA methylation, histone modification, or even RNA modification could be detected at the genome-wide scale to reveal the epigenetic response to toxins and environmental pollutants. Toxico-epigenomics emerged from the combination of epigenomics technology and classical toxicology. Using the toxico-epigenomics approach, we can study epigenetic alterations at specific loci under environmental exposure and the role of the epigenome as a possible mediator of the exposure effect (Chung and Herceg, 2020).

Faulk et al. (2015) compared the DNA methylation levels of three groups with different BPA concentrations. They found that BPA levels were positively and negatively associated with methylation in CpG islands and CpG shores, respectively. They also detected hypermethylated regions at the SNORD115 loci.

Using a mouse model, Weinhouse et al. (2014) revealed the dose-dependent incidence of hepatic tumors in adult mice following perinatal exposure to BPA. While the mechanism of the effect is unclear, they performed epigenome-wide DNA methylation profiles using DNA methylation arrays of adult mouse livers to reveal the methylation changes associated with perinatal BPA exposure and disease in mice with and without liver tumors. They proposed that the Jak/Stat and Mapk signaling pathways would be altered in comparisons of cancer and normal tissues. On the other hand, these Jak/Stat and Mapk pathway-related genes were differentially methylated between different doses of BPA (Weinhouse et al., 2016).

Multiple studies have found that BPA exposure could change the epigenetics of the offspring of exposed parents using low-throughput methods. Aiba et al. (2018) found that there was no significant difference in methylation levels in any CpG site in the control and low-dose BPA-treated groups. This suggested that the effect of low-dose BPA exposure on hippocampal DNA methylation levels at the fetal stage is extremely small.

Jadhav et al. (2017) employed genome-wide methyl-binding domain sequencing of the breasts of 100-day-old rats exposed prepubertally to BPA. They found that altered DNA methylation under BPA exposure can effectively provide predictive value for poor survival in TCGA breast cancer patients.

Bisphenol A has been thought to be related to obesity and diabetes. Using a mouse model, Anderson et al. (2013) revealed that in adult female mice, maternal dietary BPA exposure altered metabolic phenotypes, such as lowering body fat and hormone homeostasis. To elucidate the epigenetic mechanisms related to the metabolic changes, they applied the MBD-enriched DNA fragment and hybridized it to the DNA methylation array. Through bioinformatic analysis, they found that the differentially methylated genes were enriched in metabolic pathways. Furthermore, four candidate genes (*Jak-2*, *Rxr*, *Rfxap*, and *Tmem238*) were selected to assess DNA methylation as a mediating factor that connects perinatal BPA exposure to metabolic phenotypes. DNA methylation status at the four genes was used in mediational regression analysis and was recognized as a mediator in the mechanistic pathway of developmental BPA exposure (Anderson et al., 2017).

BPA exposure has been reported to change behavior in fish models. One study showed that alternating light stimulation with BPA could obviously decrease the zebrafish swimming speed. Olsvik et al. (2019) applied WGBS analysis to fish exposed to BPA and found that *dnmt1* and *cbs* may be affected

by BPA. They did not detect a significant effect on global DNA methylation but found that, in 4,873 genes, there were 20,474 differentially methylated (DM) sites. Some of the DM sites occurred in two protocadherin γ-subfamily genes related to axon targeting. Their data revealed that low-dose BPA exposure could affect the methylation patterns of genes associated with the nervous system and finally affect swimming speed.

In summary, most of the time, BPA exposure could be involved in changes in DNA methylation at the whole genome level in rat, mouse, and fish models. The changes in DNA methylation locus sites were enriched in cancer, metabolism, and nervous system pathways.

CONCLUSIONS AND PERSPECTIVES

As one of the most widely used chemical components, BPA was reported to influence the dysfunction of different aspects. Although multiple animal studies and epidemiological studies have shown that BPA could be involved in various diseases, the mechanisms of how BPA affects the disease are still not clear. Epigenetic changes, including DNA methylation, histone modification, and ncRNA, have been demonstrated to be related to disease development under environmental exposure. We reviewed the recent progress of epigenetic changes under BPA exposure. BPA exposure could affect the DNA methylation of multiple genes that could contribute to brain development, cancer progression, and important signaling pathways. On the other hand, histone modifications also change significantly at some loci upon BPA exposure.

High-throughput sequencing data from epigenetic profiling of toxic exposure enables us to study the epigenetic mechanism of the toxicological effect of environmental exposure. Recently, epigenomics approaches, including WGBS, ChIP-seq, and ATAC-seq, have been employed to explore the epigenetic regulation of environmental exposure. However, the majority of the studies were focused on DNA methylation changes under BPA exposure. Recently, more systematic data from the exposure were published for the community to explore. For example, the National Institute of Environmental Health Sciences (NIEHS) launched Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET) to study the transcriptional and epigenetic regulation of environmental toxins, including arsenite, BPA, DEHP, Pb, and TBT (Wang et al., 2018). With the development of new technologies, the cost of high-throughput sequencing will decrease significantly. We will see an increasing number of studies on the effects of BPA, which will help us to better understand the epigenetic changes related to diseases induced by BPA. Additionally, further investigations in this field will facilitate our understanding of the pathogenesis of these diseases, thus helping in the treatment of diseases associated with BPA exposure.

AUTHOR CONTRIBUTIONS

XX and WH contributed equally to the design and coordination of the study. TQ, XZ, and XX collected the data; with the help from the TG and TY, YG, XX, WH, TQ, and XZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Function of the Metals in Regulating Epigenetics During Parkinson's Disease

Xiangzhen Wei[1], *Menghua Cai*[2] *and Lifang Jin*[3]*

[1] Department of Biological Sciences, Xi'an Jiaotong-Liverpool University, Suzhou, China, [2] Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College, Beijing, China, [3] Department of Biological Sciences, Shaoxing University, Shaoxing, China

Parkinson's means Parkinson's disease, a chronic degenerative disease of central nervous system. The main area which is affected by this disease is motor system. Since it firstly founded by James Parkinson in his 1817 publication, nowadays, people still have lots of questions about this disease. This review mainly summarizes the epigenetics of Parkinson's. DNA methylation is one of the epigenetic mechanisms of Parkinson's. During the development of disease, global hypomethylation, and hypermethylation happen in different areas of patients. Another epigenetic mechanism is histone modification. People believe that some metals can induce Parkinson's disease by modulating epigenetic mechanisms. This review summarizes the relationships between different metals and Parkinson's disease. However, the specific roles of most metals in epigenetics are still unknown, which need further research.

Keywords: Parkinson's disease, epigenetic, metal, DNA methylation, synucleinopathy

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Yanqiang Li,
Boston Children's Hospital and Harvard Medical School, United States

Reviewed by:
Chongming Jiang,
Baylor College of Medicine, United States
Dana Freeman,
National Institute of Neurological Disorders and Stroke (NINDS), United States

***Correspondence:**
Lifang Jin
lifangj@usx.edu.cn

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disease (ND) with movement disorder (de Lau and Breteler, 2006), which is characterized by the progressive and substantial loss of dopaminergic neurons in substantia nigra. The hallmark of PD is the accumulation of cytoplasmic proteins, especially α-synuclein, a major competent of Lewy Bodies (LB). Moreover, PD patients show symptoms of resting tremor, slow movement, muscular rigidity, and postural instability (Nataraj and Rajput, 2005). Point mutations in α-synuclein are known to cause familial PD, accounting for about 5% of PD patients (Chang and Fox, 2016). Idiopathic PD, which accounts for about 90–95%, usually refers to a syndrome characterized by late-onset Parkinsonism (Ceravolo et al., 2009). In this review, we discuss the roles of α-synuclein in PD, especially its synthetic effect. Then we discuss the epigenetics of Parkinson's, mainly about DNA methylation. Histone modification also plays an important role in PD. However, the direct evidence between histone modification and metals is lacking. At last, we summarize the roles of different metals in Parkinson's.

Synucleinopathy

Intriguingly, α-synuclein was first found in the brains of AD patients (Uéda et al., 1993), and then was viewed as the main part of Lewy bodies in PD (Irizarry et al., 1998). Deas et al. (2016) reported that oligomeric α-synuclein suppressed the production of glutathione and showed the toxicity of neurons, while fibrillar forms could not. In a mice model expressing human α-synuclein, a progressive memory loss and motive dysfunction was observed (Fernagut and Chesselet, 2004).

There are several explanations for α-synuclein neurotoxicity. All of them lead to the activation of the apoptosis pathway and neuron death ultimately (Scarlet et al., 2015). In PC12 cell lines, the overexpression of the A53T mutant α-synuclein resulted in the death of 40% of neurons through the following mechanisms:

(1) Inducing the releasing of mitochondrial cytochrome C, leading to the breakdown of the respiratory chain, leakage of ROS, and mitochondrial dysfunction. In human neuroblastoma cells, overexpression of α-synuclein resulted in elevated ROS and inhibition of the respiratory chain (Parihar et al., 2009).
(2) Increasing endoplasmic reticulum (ER) stress (Smith et al., 2005). In SHSY cells, overexpression of α-synuclein also stimulated the release of mitochondrial cytochrome C (Parihar et al., 2008). In a mouse model of α-synuclein overexpression, endoplasmic reticulum stress, a mechanism triggered by misfolding proteins, showed a synthetic effect with α-synuclein in the onset of PD (Colla et al., 2012a,b).
(3) α-synuclein can form a pore in membranes, leading to the disturbance of calcium metabolism and cell death. Through single-channel electrophysiology, pores formed by α-synuclein were observed directly in the lipid membranes of cells (Schmidt et al., 2012).
(4) In a dopaminergic-like cell line, α-synuclein induced the formation of leak channels reminiscent (Feng et al., 2010). In the cell line model, α-synuclein facilitated the formation of a pore in the membrane, disturbing ion homeostasis, and leading to neuron death (Danzer et al., 2007).
(5) Furthermore, α-synuclein disturbed neurotransmitter release by inhibiting the trafficking and recycling of synaptic vesicles (Wang et al., 2014).

EPIGENETICS

DNA Methylation

DNA methylation is the earliest characterized chromatin modifications. Since the majority of methylation occurs in CpG motifs, which are enriched in promoters. The methylation of CpG dinucleotides at the 5′ position on the pyrimidine ring, to form 5-methylcytosine (5-mC), can disrupt the cell's transcriptional machinery by blocking the binding of transcription factors and attracting methyl-binding proteins that initiate chromatin compaction and bring about gene silencing (Lunnon and Mill, 2013). So methylation results in gene silencing while de-methylation causes gene activation.

Lumine et al. (2010) reported global hypomethylation in substantia nigra in PD patients. Moreover, intron 1 of α-*synuclein* showed hypo-methylation, leading to the over-expression of α-synuclein (Jowaed et al., 2010). On the other hand, L-dopa stimulated the hypermethylation of intron 1 of α-synuclein to suppress its expression. This might be a new explanation for the positive effects of L-dopa in PD patients (Schmitt et al., 2015). PD patients demonstrated a lower level of DNA methylation in *SNCA* and *PARK2* genes compared with controls (Eryilmaz et al., 2017).

METALS

Previous researches found that there are relationships between metals and PD. On the one hand, metals, especially heavy metals are usually regarded as neurotoxins,because they can cause neuronal death by oxidative stress. For example, both iron and copper can induce oxidative stress and cause damage to neurocyte. People have a relatively clear understanding of the pathophysiology of different metals. On the other hand, recently scientists found metals can regulate epigenetics during PD. Understanding the roles of metals in epigenetics of PD might help people find a cure for PD. However, there is a lack of relevant research. The following summarizes the relevant contents about metals (**Table 1**).

Lead (Plumbum, Pd)

Neurological damage caused by lead was found in 2006 by Monnet-Tschudi et al., they found that Lead exposure causes severe swelling and loss of neurons in the central nervous system and peripheral nervous system (Monnet-Tschudi et al., 2006).

In a case-control study of 121 PD patients and 414 controls, there was a dose-effect relationship between occupational exposure of lead and the risk of PD (Coon et al., 2006). In a case-control study of 330 PD patients (216 men, 114 women) and 308 controls (172 men, 136 women), there was a dose-effect relationship between bone lead and the risk of PD (Weisskopf et al., 2010). Wright et al. (2010) also reported an association between lead exposure and LINE1 hypomethylation. Li et al. (2013) also reported an inverse association between lead exposure and LINE1 promoter hypermethylation in a case-control study.

Mercury (Hg)

Mercury is also a neurotoxin that can damage neurons (Azevedo et al., 2012). In a case-control study of 54 idiopathic PD patients and 95 controls, there was a dose-effect association between the risk of PD and blood mercury (Ngim and Devathasan, 1989).

Mercury exposure led to DNA methylation changes in whole blood cells (Hanna et al., 2012). In SH-SY5Y cells, mercury also disturbed the clearance of Aβ plaques by suppressing the activity of neprilysin (Miguel et al., 2015). Mercury showed neural toxicity since APOE4 owned a weak combination with mercury (Mutter et al., 2004). Olivieri et al. (2010) reported that mercury stimulated the expression of Aβ and phosphorylation of tau. In PC12 cells, mercury promoted the expression of Aβ and inhibited clearance at the same time (Song and Choi, 2013).

Copper (Cu)

In a case-control study of 144 patients with idiopathic PD and 464 controls, individuals with more than two decades of copper exposure showed a significantly higher association with risk of PD ($OR = 2.49$, 95% $CI = 1.06, 5.89$) (Gorell et al., 1997). There was a decreased copper concentration in substantia nigra of PD patients (Dexter et al., 1991). Cu and α-synuclein showed the synthetic effects in the inhibition of protein degradation pathways, especially the Ubiquitin Proteasome System (UPS) (Anandhan et al., 2015). To facilitate the accumulation of Aβ through inhibiting its transport,

TABLE 1 | Epigenetic and toxic effects of different metals in Parkinson's.

	Epigenetic	Principal target of metal-induced toxicity	Pathophysiology
Lead (Pb)	Cause LINE1 promoter hypermethylation	LINE1/nervous system	Oxidative stress, mitochondria dysfunction, Ca^{2+} homeostasis disruption (Chen et al., 2016a)
Mercury (Hg)	Tau phosphorylation	Tau protein/mitochondria	Loss of dopamine receptors, tubulin degeneration, axon degeneration and glutathione depletion, higher amyloid-β level (which promotes α-synuclein aggregation) (Bjorklund et al., 2018)
Copper (Cu)	Oxidative stress mechanisms, alpha-synuclein oligomerization and Lewy body formation, as well as GABA-A and NMDA receptor neurotransmission modulation	Cytochrome/mitochondria/ brain	Increased generation of ROS, DNA, and mitochondrial dysfunction
Manganese (Mn)		Globus pallidus in the basal ganglia	Impairment of dopaminergic, glutamatergic, and GABAergic transmission, as well as mitochondrial dysfunction, oxidative stress and marked neuroinflammation (Cicero et al., 2017)
Aluminum (Al)	Al facilitates the formation of alpha-synuclein fibril by activating monoamine oxidase B	Monoamine oxidase B	Calcineurin β protects brain after injury by activating the unfolded protein response. Neurobiology of disease (Chen et al., 2016b)
Iron (Fe)	Up-regulation of divalent metal transporter 1 (DMT1) (Zhang et al., 2009)	Glutamate receptors (Lau and Tymianski, 2010), Cu protein, Ceruloplasmin (Cp) (Jiang et al., 2015)	Iron stimulates the formation of intracellular aggregates of α-synuclein and promotes oxidative damage.
Zinc (Zn)	Accumulation of alpha-synuclein	Autophagy-lysosomal pathway (Cicero et al., 2017)	Tsunemi and Krainc (2014) reported that the loss of PARK9 leads to the dyshomeostasis intracellular zinc levels, which contributes to lysosomal dysfunction then leading to the accumulation of alpha-synuclein.

Cu facilitates Aβ accumulation by inhibiting clearance and stimulating production (Singh et al., 2013).

Manganese (Mn)

Manganese has manganese toxicity through impairing motor function and damaging substantia nigra and other basal ganglia nuclei by amplifying the risk of PD (Aschner and Nass, 2006). The pathology of Manganese induced Parkinson's disease is different from other idiopathic forms. In a case-control study of 144 patients with idiopathic PD and 464 controls, individuals with more than two decades of manganese exposure showed a significantly higher association with PD ($OR = 10.61$, 95% $CI = 1.06, 105.83$) (Gorell et al., 1997). Wang et al. found a dose-effect relationship between exposure to manganese through inhalation and symptoms from extrapyramidal system dysfunction (Wang et al., 1989). Mn (II) inhibited the functions of mitochondria, which lead to the death of neurons due to energy insufficiency (Gunter et al., 2010). Manganese was associated with DNA methylation. Researchers have found a new method to identify the risk resulting from toxic metal exposure by measuring the level of DNA methylation. There is an important relationship between DNA methylation aging biomarkers and the concentration of some metals. For example, if the concentration of Mn in urine increase by 1 ng/mL, PhenoAge will increase by 9.93 years (Nwanaji-Enwerem et al., 2020).

Aluminum (Al)

In a case-control study of 200 PD patients and 200 controls, there were significantly higher levels of aluminum in the substantia nigra of PD patients than controls (Altschuler, 1999). Results from Uversky offered one explanation for the cause-effect association between Al and PD. Al activated monoamine oxidase B, an enzyme that facilitated the formation of alpha-synuclein fibril in PD (Uversky et al., 2001).

Iron (Fe)

In a case-control study of 892 participants, the concentration of iron in toenails showed a positive association with the level of LINE-1 methylation (Tajuddin et al., 2013). A test of total iron concentration in the substantia nigra of 17 parkinsonian and 29 control samples showed that the substantia nigra of PD patients contained a higher level of iron (Wypijewska et al., 2010). There was an elevated total iron level and decreased ferritin content in the substantia nigra of PD patients (Dexter et al., 1991). Iron deficiency inhibited the translation of a-synuclein mRNA (Febbraro et al., 2012). Murine treated with a high concentration of iron showed symptoms of PD when aging (Kaur et al., 2007). However, another study that explored the relationship between iron in diet and risk of PD indicates that dietary iron intake does not increase the risk of PD (Cheng et al., 2015). The different results of the two experiments may result from the amount of iron and different research models. Furthermore, iron has other functions that may also induce PD. Firstly, iron has alpha-synuclein toxicity. Secondly, iron can induce a Fenton-Haber-Weiss reaction, finally causing oxidative stress (Hellman and Gitlin, 2002; Caudle et al., 2012). Both of these two functions can cause damage to brain cells and damage mitochondria, but

the specific link between them and Parkinson's is unknown, and there is still a need for further research.

Zinc (Zn)

In a case-control study of 423 PD patients and 205 controls, patients showed significantly more exposure to zinc (95% CI, 1.51–90.90) (Pals et al., 2003). Zinc induced a significant decrease of Aβ solubility and an increase of its ability to resist tryptic cleavage at the secretase site (Bush et al., 1994). Zinc facilitated neuro-filament phosphorylation in the absence of the p70 S6 kinase in N2a cells (Bjorkdahl et al., 2005). There was an elevated zinc concentration in the substantia nigra of PD patients (Dexter et al., 1991).

Cerium (Ce)

Cerium is an interesting metal that has been the subject of great research interest in recent years. Cerium was proved to have a negative effect on DNA methylation, in other words, Cerium is likely to induce PD. However, another compound of Cerium, cerium oxide nanoparticles (CeO2 NPs) shown positive effects and could cure some neurodegenerative diseases including PD. In a study researching the relationship between concentrations of Cerium and DNA methylation in blood, samples were collected from people who lived around an e-waste disassembling factory in China. In this study, there was a negative correlation between the Ce concentration of pre-workers and global DNA methylation (5-mc) in Pearson correlation and multiple linear regression analysis, $r = -0.51$, $p = 0.01$. Therefore, the concentration of Ce in blood was significantly negatively correlated with global DNA methylation. DNA hypomethylation usually relates to chromosome instability and increased mutation events by affecting the intergenomic and intron regions of DNA, especially repeat sequences and transposable elements. This suggests that Ce plays a key role in DNA methylation reduction. Taking into account that many researchers have also shown that PD is regulated by DNA methylation, it can be concluded that Cerium may increase the risk of PD by DNA methylation reduction (Li et al., 2020). However, the limitation of this research is the lack of experimental models to validate findings in Chinese workers. In other research, scientists found that CeO2 NPs can reduce α-synuclein induced toxicity in a yeast model based on the heterologous expression of the human α-synuclein. To be specific, CeO2 NPs can suppress α-syn-induced mitochondrial dysfunction, reduce the production of reactive oxygen species (ROS) in yeast cells and absorb α-synuclein directly on its surface (Ruotolo et al., 2020).

CONCLUSION

Several intriguing points should be mentioned about the roles of metals in PD development because they play both pathological and protective effects. According to previous research, some metals play both negative and positive roles in PD development, such as cerium and copper. To be specific, excessive Cu can induce the generation of ROS, causing DNA and mitochondrial dysfunction. However, Cu plays a protective role in PD patients who are Cu-deficient. Cerium was also proven to have a negative effect on DNA methylation while cerium oxide nanoparticles are used to cure PD.

Another interesting point relates to the different valence states of metals that have different toxicities. For example, divalent Fe is important in PD development because it can induce neuronal death by oxidative stress. But it can become non-toxic if divalent Fe is oxidized to trivalent Fe by ceruloplasmin and hephestin. Researchers have validated this in a double knockout mouse lacking both cap and hephestin. Considering that many metals induce Parkinson's disease by oxidative stress to cause neuronal death, we believe further research could focus on the translation of metal valence, and this might be a new method of curing PD.

AUTHOR CONTRIBUTIONS

XW drafted manuscript. MC edited and revised manuscript. LJ approved final version of manuscript. All authors contributed to the article and approved the submitted version.

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Multigenerational Epigenetic Regulation of Allergic Diseases: Utilizing an Experimental Dust Mite-Induced Asthma Model

Jairus C. Pulczinski[1†], Yan Shang[1†‡], Tyna Dao[1†], Nathachit Limjunyawong[1†], Qinying Sun[1†], Wayne Mitzner[1], Robert YS Cheng[2] and Wan-yee Tang[1,3]*

[1] Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States, [2] Center for Cancer Research, National Cancer Institute, Frederick, MD, United States, [3] Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA, United States

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Qiang Zhang,
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***Correspondence:**
Wan-yee Tang
wanyeetang@pitt.edu;
wat23@pitt.edu

[†]These authors have contributed equally to this work and share first authorship

[‡]Present address:
Yan Shang,
Department of Respiratory Medicine, Changhai Hospital, Shanghai, China

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Environmental exposures have been linked to increased asthma risk, particularly during pregnancy and in early life. Here we use a mouse model of allergic lung disease to examine the effects of pre- and perinatal house dust mite (HDM) allergen exposure on offspring phenotypic and transcriptional outcomes in three generations. We show that maternal HDM exposure (F0) acts synergistically with adult HDM exposure, leading to enhanced airway hyperresponsiveness (AHR) and lung inflammation when compared to mice exposed solely in adulthood. Additionally, a subset of F1 males were not challenged in adulthood, and used to generate F2 progeny, which was then used to generate F3 progeny. Upon adult challenge to HDM, F2, and F3 males generated from the maternal HDM (F0) exposure lineage displayed increased airway reactivity and inflammation when compared to mice exposed solely in adulthood. These findings indicate that maternal allergen exposure is capable of enhancing either susceptibly to or severity of allergic airway disease. To examine the role of epigenetic inheritance of asthma susceptibility induced by maternal HDM exposure, we utilized a genome-wide MeDIP-seq and hMeDIP-seq analysis to identify genes differentially methylated (DMG) and hydroxymethylated (DHG), and their association with the enhanced AHR. In addition, we validated the relationship between DNA methylation and mRNA expression of the DMGs and DHGs in the male sub-generations (F1-F3). We found the expression of *Kchn1*, *Nron,* and *Spag17* to be differentially hydroxymethylated and upregulated in the F1 exposed to HDM both in early life and in adulthood when compared to F1 mice exposed solely in adulthood. *Kcnh1* remained upregulated in the F2 and F3 from the maternal HDM (F0) exposure lineage, when compared to F1 mice exposed solely in adulthood. In summary, we demonstrated that maternal HDM exposure in early life can alter the gene expression and phenotype of offspring upon adult HDM exposure, resulting in more severe disease. These effects persist at least two generations past the initial insult, transmitted along the paternal line.

Keywords: asthma, epigenetic inheritance, allergen, DNA methylation, DNA hydroxymethylation

INTRODUCTION

Asthma is a common chronic disease, afflicting some 330 million individuals globally and resulting in substantial morbidity and mortality, particularly among children (Dharmage et al., 2019). Asthma rates have increased in the last few decades and it is estimated that global asthma cases will surpass 400 million in 2025 (Dharmage et al., 2019). In part due to this increase, considerable efforts have been undertaken to understand asthma risk factors and underlying mechanisms. As maternal asthma confers a greater risk to the offspring than paternal asthma, it is thought that offspring may be vulnerable to maternally mediated exposures in the pre- and perinatal period (Lim et al., 2010). Research has shown that a broad range of maternal exposures, in humans and in animal models, can increase offspring asthma susceptibility, including particulate matter, maternal stress, smoking, and allergens (Celedón et al., 2002; Lim et al., 2010; Van De Loo et al., 2016; Lee et al., 2018; Dharmage et al., 2019). DNA methylation has been proposed as one of the underlying mechanisms, given the associations between *in utero* exposures to asthma risk factors, including air pollution and cigarette smoke, and changes in global and gene specific DNA methylation of offspring (Perera et al., 2009; Herbstman et al., 2012; Lee et al., 2017). In many cases, these perturbations are in the regulatory regions of immune related genes, including *IFNG* and *FOXP3*, thus providing biological plausibility (Tang et al., 2012; Hew et al., 2015).

As our understanding of maternal exposures and their epigenetic ramifications grows stronger, new research has emerged indicating that certain exposures may have the capacity to result in multigenerational inheritance of allergic disease (Mørkve Knudsen et al., 2018). Multigenerational inheritance is a phenomenon where exposure to an insult or stimulus results in a phenotypic effect in the subsequent generation. This phenomenon can be further subdivided into intergenerational and transgenerational effects. Intergenerational effects are those seen when the offspring is directly exposed to an insult, including as gametes. In mammals, this includes the F1 generation after exposure to the F0, as the sperm or ovum of the F0 is also exposed to the insult. In the case of prenatal exposure, this can also include the F2 generation, as the germline cells of female F1 offspring are also present during the insult. This contrasts with transgenerational inheritance, in which the F3 generation, or F2 from *in utero* exposed males, display phenotypic variation due to exposure in the F0 (Mørkve Knudsen et al., 2018).

Multigenerational exposure is challenging to identify epidemiologically due to the long lifespan of humans and the ubiquitous nature of many exposures. The strongest human data on multigenerational exposure and allergic disease risk comes from cohort studies examining grandparent smoking on offspring allergic disease risk, in part because cigarette smoke exposure *in utero* is one of the strongest risk factors for asthma development. In several cohorts, grandmaternal, but not grandpaternal smoking was significantly associated with grandchild asthma risk. This was independent of maternal smoking, however, maternal smoking magnified the risk (Mørkve Knudsen et al., 2018). A separate study found that paternal grandparent smoking was associated with increased asthma risk in grandchildren. Several other studies have examined the risk of preconception smoking on asthma risk, and also found that paternal smoking prior to conception increased the F1 offspring risk of asthma and reduced offspring lung function (Mørkve Knudsen et al., 2018). Taken together, these studies offer the strongest case that asthma risk is in part influenced via multigenerational exposure. The synergistic relationship between grandmaternal and maternal exposure is particularly relevant to public health, as many of our exposures, like air pollution, cigarette smoke, endocrine disrupters, and maternal stress, are not limited to an older generation, but are persistent and ubiquitous. If such exposures are capable of multigenerational inheritance of airway hyperresponsiveness (AHR), there may be a synergistic effect due to exposure in the F0, F1, and F2.

Given the diverse stimuli capable of producing multigenerational inheritance of exposure, and work in our lab and others indicating that multigenerational epigenetic programming may be contributing to asthma risk, we sought to investigate this phenomenon from several approaches. Our lab utilized a previously validated model of mouse allergic airway disease (Shang et al., 2013; Cheng et al., 2014), in which mice are sensitized to house dust mite (HDM) allergen. HDM is an environmentally relevant and ubiquitous indoor allergen, which many asthmatics are sensitized to Gaffin and Phipatanakul (2009). The administration of HDM elicits a strong immune response, characterized by T-helper cell 2 (TH2) skewing of the immune system, and increased AHR, similar to the TH2 high atopic asthma endotype (Kuruvilla et al., 2019). Our goal was to identify the contribution of maternal HDM exposure on offspring allergic airway disease risk and severity, and test our hypothesis that the inheritance of lung phenotypic changes is modulated by epigenetic reprogramming. We exposed F0 dams to HDM or saline during gestation and used the F1 males to generate F2 and F3 male progeny. A subset of each generation (F1, F2, and F3) were further challenged by HDM or saline, and subjected for the measurement of lung function, lung inflammation, and gene transcription/regulation (DNA methylation, DNA hydroxymethylation and mRNA expression). Here, we show that early life HDM exposure is capable of inducing multigenerational inheritance of lung phenotypes characterized by increased airway hyperresponsiveness, inflammation, and persistent changes in DNA methylation and gene expression up to at least the F3 generation.

Animal Husbandry

All of the experimental procedures used were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD, United States) (MO17H187). Male and female C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, United States). Mice were allowed to acclimate to housing at JHU for 2 weeks prior to the commencement of the experiment. All mice were maintained at 22°C, and 12-hour light and 12-hour dark cycle. The mice were housed in polysulfone-ventilated cages (Technoplast, Exton, PA, United States), and provided with Harlan Teklad Global

18% Protein Extruded Rodent Diet 2018SX and drinking water *ad libitum*.

MATERIALS AND METHODS

House dust mite extract (*D. Pteronyssinus*) was purchased from Greer (Lenoir, NC) in a lyophilized form. The level of HDM endotoxin was reduced using the EndoTrap HD assay (Hyglos GmbH, Germany) according to the protocol. Endotoxin level was measured using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Rockford, IL, United States). HDM extract was then diluted with phosphate buffered saline (PBS) to 2 mg/mL of total protein as measured by the Pierce BCA Protein Assay (Thermo Scientific Rockford, IL, United States).

Early Life and Adult Exposures to HDM

This model of experimental allergic airway disease has been previously validated in our and W. Mitzner's lab (Shang et al., 2013; Cheng et al., 2014; Wagner et al., 2015). Female nulliparous mice (F0) were sensitized to HDM (100 μg of protein) or an equivalent volume of PBS via intraperitoneal injection (i.p.) 2 weeks prior to timed mating, and exposed to HDM (100 μg of protein) or an equivalent volume of PBS via intratracheal instillation (i.t.) three times a week during pregnancy and lactation, starting at embryonic day E0.5 and continuing for 3 weeks post parturition. Instillation was assisted by the use of an isoflurane vaporizer and an induction chamber. Offspring were sex separated at 4 weeks of age and allowed to mature under the same housing conditions as mentioned in the Animal Husbandry section. Once the F1 male offspring reached 6 weeks of age, half of them were sensitized to HDM or saline (i.p) at day (d)0 and challenged with HDM or saline (i.t.) on d14, 18, and 21 before AHR measurement at d23.

Generation of Sub-Generations

Male F1 mice were paired with female naïve C57Bl/6J mice at 6 weeks to produce the F2 progeny. F3 were bred in the same manner. We summarized the breeding scheme in **Figure 1**. The male progenies from F2 and F3 were subject to acute HDM exposure when they reached 6-week old, via the same methods described for F1 male.

Measurement of Airway Hyperresponsiveness (AHR)

Mice were administered ketamine/xylazine (25 mg/ml ketamine, 5 mg/ml xylazine) via intraperitoneal (i.p.) injection and once sedated and non-responsive to toe pinch they were placed on a heating pad. Mice were then intubated with a 20 g blunt cannula via tracheostomy. Lung phenotypic measurements, including airway resistance, were then assessed via flexiVent (SCIREQ, Montreal, Canada). Mice were maintained on the ventilator and paralyzed with succinylcholine (20 mg/ml) followed by methacholine challenge. Mice were exposed to increasing doses (0, 1, 3, 10, 30 mg/ml) of aerosolized methacholine chloride (Mch) (Sigma-Aldrich, St. Louis, MO, United States). The AHR was assessed by the change in pulmonary resistance (cmH2O.s/mL) as compared to the baseline using flexiVent (SCIREQ, Montreal, Canada) (Ewart et al., 1995).

Collection of BALF and Tissue

After measurement on the flexiVent, mice were removed from ventilation, and quickly exsanguinated. Bronchoalveolar lavage (BAL) fluid was then collected by flushing the lungs with 1 mL of cold PBS with complete, Mini Protease Inhibitor Cocktail (Roche, San Luis Obispo, CA, United States). BAL was processed the same day, total cell count was conducted and slides were prepared using Cytospin. Organs were then dissected and flash frozen in liquid nitrogen and stored at −80°C until analysis.

Hydroxymethylated and Methylated DNA Immunoprecipitation Coupled With Next Generation Sequencing (hMeDIP- and MeDIP- seq)

DNA was isolated from lung tissues using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Germantown, MD, United States), according to manufacturer's instructions. Sample DNA concentrations were quantified with the Quant–iT^{TM} Picogreen dsDNA assay kit (Invitrogen, CA, United States). A second quality check was done on Agilent 2100 HS DNA chip after DNAs were fragmented. 1 μg of DNA from each sample was fragmented on Covaris S2 and had an average length of 200 bp. Hydroxymethylated and methylated DNA was captured using the hMethylCap and MethylCap kit (Diagenode, NJ, United States) respectively. DNA was eluted from the protein complex. A total of 250 ng fragmented DNAs were amplified, ligated with linkers, and subjected to flow cells using the HiSeq2000 platform according to the manufacturer's NGS protocol (Illumina, CA, United States). About 20 million reads obtained by a single-run of massive parallel sequencing with 51 bp paired-end reads were found. Bioinformatics analysis on the raw sequencing data was performed with use of CLC Genomic Workbench 6.0.3 (CLC Bios, MA, United States) and followed manufacturer's standard data import protocol. Reads (peaks) were mapped to the Mouse Reference Genome Build NCBI38/mm10. Significant peaks by threshold profiles at a height equivalent to an estimated false discovery rate (FDR) 0.001 were identified. Differentially methylated regions and differential hydroxymethylated regions between HDM-exposed and saline-exposed mice were annotated to their chromosomal loci and Gene Ontology (GO) categories. The next generation sequencing (NGS) data generated in this project is deposited to the public (GEO accession number GSE169355).

Real-Time Reverse-Transcriptase-PCR (RTPCR)

Total RNA (1 μg) was isolated from lung tissues using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Germantown, MD, United States), according to manufacturer's instructions and reverse transcribed with iScript Reverse Transcriptase (BIO-RAD, Hercules, CA, United States). mRNA levels of the genes were quantified by TaqMan-based or SYBR Green-based real-time PCR. Primers were listed in **Table 7**. The $2^{-\Delta\Delta Ct}$ method

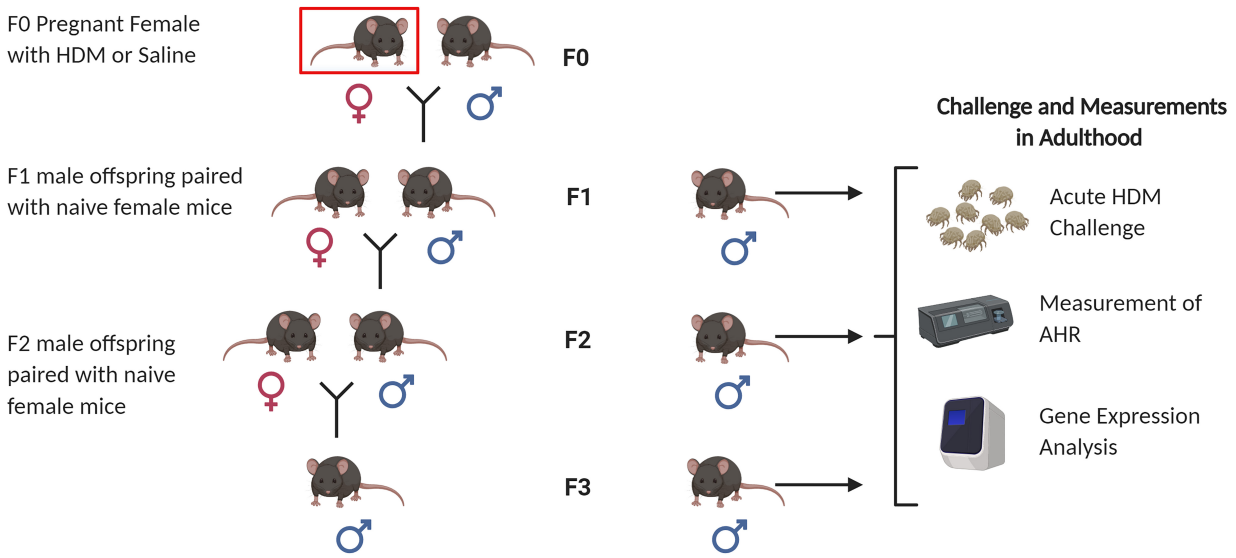

FIGURE 1 | Animal breeding scheme for generation of F1, F2, and F3 progenies. Nulliparous C57BL/6J female mice (F0) were sensitized to house dust mite (indicated by red box) at 8 weeks of age, and then paired with naïve C57BL/6J male mice to generate the F1 progeny. F1 males were paired with naïve C57BL/6J female mice to generate F2 progeny. F2 males were paired with naïve C57BL/6J female mice to generate F3 progeny. A subset of 6 week-males from each generation (F1, F2, and F3) were selected for adult HDM challenge followed by airway hyperresponsiveness (AHR) measurements, and gene expression analysis. Figure created with BioRender.com.

was used to calculate the relative expression level of transcripts normalized to *Rpl19* (Shang et al., 2013).

Statistical Analysis

Data on airway responsiveness, cell counts and relative gene expression were expressed as the mean- ± standard error of mean (SEM) with six mice per treatment group. Technical triplicates were performed in each assay. P values derived from Ordinary One-Way ANOVA with Tukey's test to correct for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ in comparison with control indicates results were statistically significant. All data were analyzed and plotted with Prism8 (GraphPad Prism version 8.4.3 for Windows, GraphPad Software, La Jolla, CA, United States)[1].

RESULTS

Effect of Maternal HDM Exposure on Lung Pathophysiology of the F1 Progenies

F1 progenies were examined for allergy induced AHR when they reached 6-week old (**Figure 2A**). In response to adult HDM challenges, F1 progenies from dams exposed to saline or HDM (F0-Saline_F1-HDM or F0-HDM_F1-HDM) had significantly increased ($P < 0.01$) airway resistance during methacholine challenge (**Figure 2B**), as well as significantly increased cellularity in the bronchoalveolar lavage fluid (BALF), when compared to their counterparts who did not received HDM challenges in their adulthood (F0-Saline_F1-Saline or F0-HDM_F1-Saline) (**Figure 2C**). These changes were accompanied by increased expression or a trend in induction of cyclin D1 (*Ccnd1*), proliferating cell nuclear antigen (*Pcna*) and calcium/calmodulin dependent protein kinase II delta (*Camk2dI*) (**Table 1**). These changes suggest that the adult acute exposure to HDM alters lung cell functions (including cell growth, cell proliferation and cell contraction) which may contribute to increased airway reactivity and airway inflammation. When we assessed the effect of maternal only HDM exposure on F1 progenies (F0-Saline_F1-Saline vs. F0-HDM_F1-Saline), we did not observe a statistically significant difference in airway reactivity, airway inflammation, or expression of AHR phenotypic genes. In the absence of maternal HDM exposure, F1 progenies (F0-Saline_F1-HDM) did not show increased collagen synthesis; collagen, type I, alpha 1 chain and Collagen, type III, alpha 1 chain (*Col1a1* and *Col3a*); in response to the adult HDM challenges. However, F1 progenies administrated to maternal HDM exposure (F0-HDM_F1-HDM) displayed significant upregulation of *Col1a* and a trend of induction of *Col3a* upon the exposure to HDM in their adulthood. In addition, there was a further HDM-induction of *Col3a* by 80% as compared to those from saline-exposed mom (vs. F0-Saline_F1-HDM). This change was associated with the further induction of AHR and production of immune cells by 70%. These results indicate maternal exposure to HDM alone did not alter the lung function of the offspring, but may promote the AHR in offspring who received the HDM challenge in their later life.

[1]www.graphpad.com

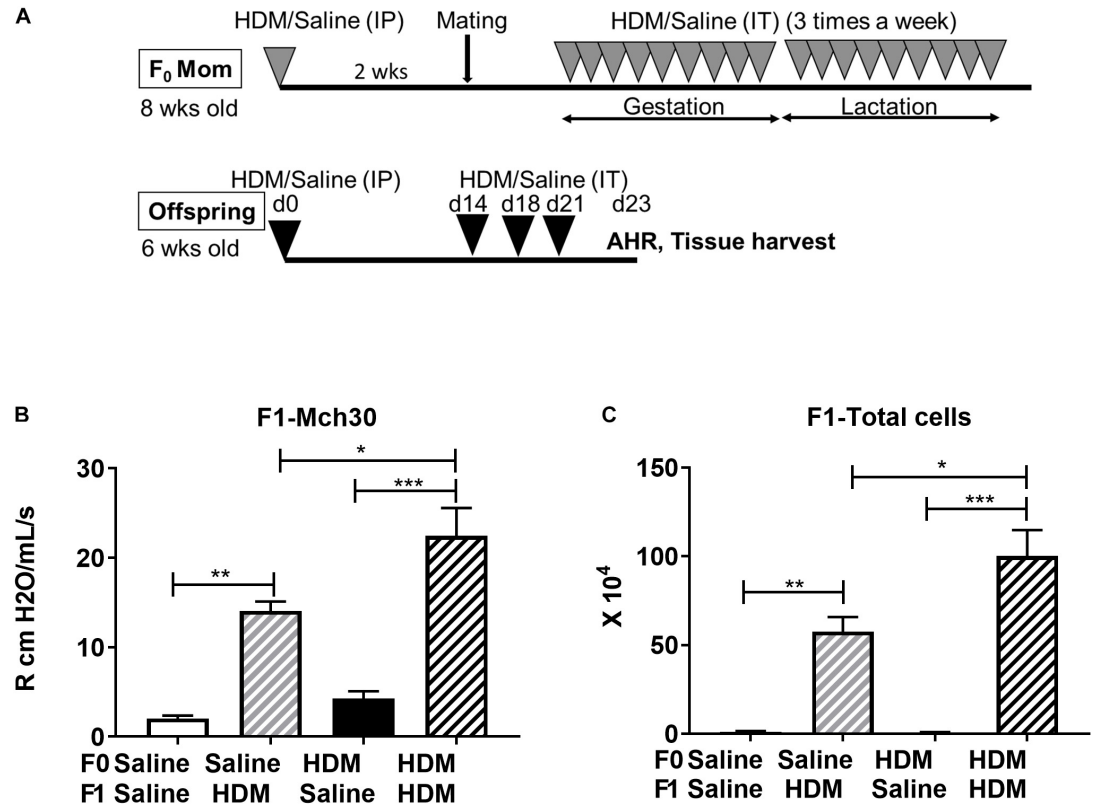

FIGURE 2 | Effect of maternal exposure to HDM on sub-generation. **(A)** Experimental protocol for acute HDM exposure-induced AHR in a mouse model. Nulliparous C57BL/6J female mice (F0), 8-week old, were sensitized with 100 μg HDM or saline (as control) intraperitoneally (i.p.) 2 weeks before conception. Subsequently, these mice were mated with naive C57BL/6J males and then challenged with 100 μg HDM or saline intratracheally (i.t.) three times a week throughout gestation and lactation (total of 6 weeks). F1 offspring were sex separated at weaning. A subset of F1 males were unexposed and mated to naïve, nulliparous C57BL/6J female mice to produce F2 progeny, additionally, a subset of F2 males were left unexposed and mated to naïve, nulliparous C57BL/6J female mice used to produce F3 progeny. Additionally, a subset of F1, F2, and F3 males were subject to acute HDM exposures to examine multigenerational effects of HDM in mice. At 6 weeks, these offspring were sensitized with 100 μg HDM or saline (as control) intraperitoneally (i.p.) on Day (d)0. On d14, mice were challenged with 100 μg HDM or saline intratracheally (i.t.) 3× a week (d14, d18, and d21). On d 23, after airway responsiveness in response to methacholine (Mch) was assessed, lung tissue, blood serum, and bronchoalveolar lavage (BAL) fluid were collected. **(B)** Mice were administered methacholine (Mch), a bronchoconstrictive agent, at increasing doses (0.1, 0.3, 1, 3, 10, and 30 mg/ml) by a 10-second aerosol inhalation. Resistance at 30 mg/ml of Mch is shown. HDM-challenged mice exhibited increased AHR compared to those exposed to PBS. This was further enhanced by maternal HDM exposure. **(C)** Total cell count was performed after staining of BAL cells with Turks solution. HDM-challenged mice exhibited increased BAL cell number compared to those exposed to saline. This was further enhanced by maternal HDM exposure. Mean with SEM shown. *P* values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Maternal Exposures to HDM Reprogram F1 Progenies' Lung Function, Through Epigenetic Modifications of Gene Transcription

We sought to test the hypothesis that maternal exposures to HDM increase offspring susceptibility to AHR by modulating lung gene expression via DNA methylation, given the fact that early-life epigenetic changes may modify lung cell function and later-life response. We previously reported that acute HDM challenges increased global 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) levels in mouse lung tissues (Shang et al., 2013). Herein, we applied MeDIP-seq and hMeDIP-seq to identify differential DNA methylation and hydroxymethylation changes in F1 progenies. 107 candidates and 118 candidates were shown differentially methylated and hydroxymethylated, respectively, when F1 progenies that received both maternal and adult exposure were compared with those that received adult HDM exposure only (F0-Saline_F1-HDM vs. F0-HDM_F1-HDM). Among these candidates, 26 genes were found in common (**Table 2**). Next, we examined if these differential epigenetic changes contribute to the changes in gene expression and increased AHR susceptibility in F1 progenies who received HDM exposures during gestation and lactation, and adulthood (**Table 3** and **Supplementary Table 1**). Among these 26 genes, changes in mRNA level of erythroid differentiation regulator 1 (*Erdr1*, $p < 0.001$), potassium voltage-gated channel, subfamily H member 1 (*Kcnh1*, $p < 0.001$), non-protein coding RNA repressor of NFAT (*Nron*, $p = 0.058$) and sperm associated antigen 17 (*Spag17*, $p < 0.001$) in lung of HDM-exposed mice were concordant with the change in DNA methylation status, as well as the further induction of airway

TABLE 1 | mRNA levels of AHR phenotypic genes in F1 progenies.

	A		B			C			D			
	F0-Saline_F1 Saline		F0-Saline_F1 HDM			F0-HDM_F1-Saline			F0-HDM_ F1 = HDM			
Gene	Mean	SEM	Mean	SEM	*p* value (B vs. A)	Mean	SEM	*p* value (C vs. A)	Mean	SEM	*p* value (D vs. C)	*p* value (D vs. B)
Ccnd1	1.838	0.708	26.169	4.708	**0.039**	1.339	0.206	0.899	28.630	4.786	0.072	0.981
Pcna	0.353	0.040	4.483	0.949	0.065	1.896	0.592	0.279	5.225	0.509	**0.045**	0.897
Col1a	0.285	0.092	0.486	0.121	0.587	0.346	0.036	0.919	0.666	0.027	**0.009**	0.545
Col3a	0.638	0.178	1.087	0.127	0.310	1.071	0.251	0.563	2.003	0.187	0.136	0.056
Muc5b	1.168	0.118	1.053	0.108	0.885	1.156	0.120	1.000	1.276	0.175	0.937	0.719
Sma	0.347	0.043	0.443	0.103	0.824	0.422	0.166	0.967	0.806	0.114	0.363	0.212
Camk2d	3.537	0.309	7.137	0.544	**0.011**	3.387	0.137	0.966	8.666	0.979	0.077	0.587
Mylk	1.146	0.187	1.230	0.043	0.967	0.989	0.160	0.914	1.164	0.094	0.787	0.913

mRNA levels measured via RTPCR. The $2\wedge(-\Delta\Delta C_t)$ method was used to calculate the relative expression level of transcripts normalized to Rpl19. P values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. Significant P values are in bold.

reactivity (F0-Saline_F1-HDM vs. F0-HDM_F1-HDM). Mice exposed to HDM during gestation and lactation showed a further increase in their gene expression (*Kchn1*, *Nron*, and *Spag17)* when they were challenged by HDM in adulthood. In contrast, maternal exposure to HDM reversed the effect of adult HDM exposure on induction of *Erdr1*. Another three gene candidates (dpy-19 like C-mannosyltransferase 1, *Dpy19l1*; leucyl-tRNA synthetase, *Lars;* and 2′-5′ oligoadenylate synthetase-like 2, *Oasl2*) were overexpressed in progenies that received adult HDM challenge, regardless of their maternal exposures (F0-Saline_F1-HDM and F0-HDM_F1-HDM). However, there was no additional induction of gene expression in mice exposed to HDM both *in utero* and in adulthood. On the other hand, we did not observe any significant change in mRNA levels of these 26 gene candidates in mice only exposed to HDM during gestation and lactation. Results suggest that the adult exposure to HDM may induce epigenetic alterations and/or advance the epigenetic effect of early-life HDM exposures on gene-specific expression, and its associated lung phenotypes in later life.

To examine if these gene-specific epigenetic changes were accompanied with changes in epigenetic modification enzymes in response to HDM (Cheng et al., 2014), we measured the expression of these enzymes (**Table 4**). F1 progenies who solely received exposure to HDM in adulthood (F0-Saline_F1-HDM and F0-HDM_F1-HDM) showed a reduction in DNA methyltransferase 3b (*Dnmt3b*) and methyl CpG binding protein 2 (*Mecp2*), when compared to their counterparts not exposed to HDM in adulthood. This change was not found in mice only exposed to HDM during gestation and lactation. There was no additional reduction in *Dnmt3b* and *Mecp2* expression in F1 progenies who received both maternal and adult challenges to HDM. F1 progenies who solely received exposure to HDM in gestation and lactation (F0-HDM_F1-Saline) showed decreased expression of DNA methyltransferase 1 (*Dnmt1*) and DNA methyltransferase 3a (*Dnmt3a*) by 2.4 and 14-fold, respectively, when compared to those from saline-exposed dams. However, F1 progenies that received both maternal HDM exposure and adult HDM exposure did not show further changes in expression of these enzymes, which function in DNA methylation. Next, we examined the effect of HDM on the expression of enzymes facilitating DNA hydroxymethylation; tet methylcytosine dioxygenase (Tet) 1 (*Tet1*) and *Tet2*. A 3-fold overexpression of *Tet1*, but no change in *Tet2*, was found in F1 progenies that received adult HDM challenges. Results indicate that the overexpression of identified gene candidates (**Table 3**) may correlate to the changes in these epigenetic modification enzymes, although further study is required to demonstrate how these epigenetic modification enzymes alter the gene transcription.

Multigenerational Effect of F0 HDM Exposure on Lung Pathophysiology of the F2 and F3 Progenies

We next examined the same parameters of allergic-induced airway reactivity and inflammation measured in the F1 progenies, in the F2 and F3 progenies. We found that the effect of maternal exposure to HDM on inheritance of the allergic responses carried over. In response to adult exposure to HDM, F2, and F3 progenies from either saline- or HDM-exposed F0 (F0-Saline_F2/3-HDM and F0-HDM_F2/3-HDM) showed increased airway reactivity (increased lung resistance in response to methacholine) and airway inflammation (induction of total number of immune cells) (**Figure 3**). These changes were accompanied by a significant induction of *Ccnd1*, *Pcna*, *Col3a*, and *Camk2d* (**Table 5**). These findings were quite similar to those observed in the in the F1 progenies. Like in the F1, without the challenge of HDM in the adulthood, there was no significant change in airway reactivity and airway inflammation in F2 and F3 progenies from grand-maternal (F0) exposure to HDM (F0-HDM_F2-Saline and F0-HDM_F3-Saline) (**Figure 3**) although these progenies showed increased expression of *Col1a* and *Camk2d*, when compared to their counterparts from saline-exposed dams (F0) (**Table 5**). With the addition of acute HDM exposure during adulthood, F2 and F3 progenies from grand-maternal (F0) exposure to HDM (F0-HDM_F2-HDM and F0-HDM_F3-HDM) showed a further induction of allergic response to methacholine challenge

TABLE 2 | List of differential hydroxymethylated and methylated genes associated with maternal HDM exposures and increased AHR.

Gene Symbol	Gene Name	GO function	Chromosome
Acox2	Acyl-Coenzyme A oxidase 2, branched chain	Signaling receptor binding and oxidoreductase activity, acting on the CH-CH group of donors	14
Aida	Axin interactor, dorsalization associated	protein domain specific binding	1
Asmt	Acetylserotonin O-methyltransferase	Protein homodimerization activity and methyltransferase activity	X
Cdr1	Cerebellar degeneration related antigen 1	Protein binding	X
Col23a1	Collagen, type XXIII, alpha 1	Extracellular matrix structural constituent, protein binding	11
Dpcd	Deleted in primary ciliary dyskinesia	Protein binding	19
Dpy19l1	dpy-19-like 1	Transferase activity, transferring glycosyl groups and mannosyltransferase activity	9
Eef2	Eukaryotic translation elongation factor 2	Protein kinase binding	10
Erdr1	Erythroid differentiation regulator 1	Not available	Y
Foxi1	Forkhead box I1	DNA-binding transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding	11
Kcnh1	Potassium voltage-gated channel, subfamily H (eag-related), member 1	Signal transducer activity and ion channel activity	1
Kdm6a	Lysine (K)-specific demethylase 6A	Protein binding and dioxygenase activity	X
Lars2	Leucyl-tRNA synthetase, mitochondrial	Binding and aminoacyl-tRNA editing activity	9
Luzp2	Leucine zipper protein 2	Extracellular region	7
Mrs2	MRS2 magnesium transporter	Magnesium ion transmembrane transporter activity	13
Muc19	Mucin 19	Extracellular region, Golgi lumen, plasma membrane	15
Nox3	NADPH oxidase 3	Oxidoreductase activity and superoxide-generating NADPH oxidase activity	17
Nron	Non-protein coding RNA, repressor of NFAT	Not available	2
Oasl2	2′-5′ oligoadenylate synthetase-like 2	RNA binding and transferase activity	5
Ranbp3l	RAN binding protein 3-like	Contributes to GTPase activator activity, Ran GTPase binding, SMAD binding	15
Rnu6	U6 small nuclear RNA	Not available	17
Smpdl3a	Sphingomyelin phosphodiesterase, acid-like 3A	Hydrolase activity and sphingomyelin phosphodiesterase activity	10
Spag17	Sperm associated antigen 17	Extracellular region, cytoplasm, cytoskeleton, microtubule, cilium	3
Tmem125	Transmembrane protein 125	Membrane, integral component of membrane	4
Ugt8a	UDP galactosyltransferase 8A	Carbohydrate binding and glucuronosyltransferase activity	3
Xcr1	Chemokine (C motif) receptor 1	G protein-coupled receptor activity and chemokine receptor activity	9

Hydroxymethylated and methylated DNA was captured using the hMethylCap and MethylCap kit. Significant peaks by threshold profiles at a height equivalent to an estimated false discovery rate (FDR) 0.001 were identified. Differentially methylated and hydroxymethylated regions between HDM-exposed and saline-exposed mice were annotated to their chromosomal loci and Gene Ontology (GO) categories.

TABLE 3 | mRNA levels of differential hydroxymethylated and methylated genes in F1 progenies. mRNA levels measured via RTPCR.

	A		B			C			D			
	F0 Saline F1 Saline		F0 Saline F1 HDM			F0 HDM F1 Saline			F0 HDM F1 HDM			
Gene	Mean	SEM	Mean	SEM	*p* value (B vs. A)	Mean	SEM	*p* value (C vs. A)	Mean	SEM	*p* value (D vs. C)	*p* value (D vs. B)
Dpy19l1	1.125	0.387	57.229	8.803	**<0.001**	1.205	0.339	>0.9999	41.083	8.178	**<0.001**	0.299
Erdr1	1.235	0.282	57.112	4.660	**<0.001**	0.282	0.054	1.000	15.180	2.583	0.432	**<0.001**
Kcnh1	0.938	0.087	263.672	24.479	**<0.001**	4.918	1.222	0.978	501.429	7.045	**<0.001**	**<0.001**
Lars2	0.175	0.043	32.978	7.785	**0.003**	0.435	0.033	>0.9999	34.603	7.404	**0.004**	0.998
Nron	0.455	0.388	24.942	3.301	**0.042**	0.297	0.143	>0.9999	48.291	3.409	**<0.001**	0.058
Oasl2	0.461	0.052	33.065	3.284	**0.003**	0.459	0.015	>0.9999	50.182	3.798	**<0.001**	0.249
Spag17	0.633	0.007	182.749	27.169	**<0.001**	2.478	0.345	0.998	323.945	42.425	**<0.001**	**<0.001**

The $2\wedge(-\Delta\Delta C_t)$ method was used to calculate the relative expression level of transcripts normalized to Rpl19. P values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. Significant P values are in bold.

TABLE 4 | mRNA levels of epigenetic modification enzymes in F1 progenies. mRNA levels measured via RTPCR.

	A		B			C			D			
	F0 Saline F1 Saline		F0 Saline F1 HDM			F0 HDM F1 Saline			F0 HDM F1 HDM			
Gene	Mean	SEM	Mean	SEM	*p* value (B vs. A)	Mean	SEM	*p* value (C vs. A)	Mean	SEM	*p* value (D vs. C)	*p* value (D vs. B)
Dnmt1	1.518	0.595	0.013	0.000	**0.003**	0.445	0.117	0.073	0.004	0.002	0.736	>0.9999
Dnmt3a	3.671	0.503	0.182	0.023	**<0.001**	0.234	0.061	**<0.001**	0.002	0.001	0.949	0.970
Dnmt3b	2.975	0.381	0.108	0.032	**<0.001**	2.019	0.389	0.131	0.014	0.005	**<0.001**	0.996
Mecp2	2.412	0.248	0.906	0.163	**0.002**	1.518	0.066	0.174	0.119	0.003	**0.010**	0.219
Tet1	0.668	0.029	3.910	0.647	**<0.001**	0.825	0.165	0.983	4.450	0.184	**<0.001**	0.542
Tet2	1.233	0.358	1.504	0.341	0.907	1.165	0.262	0.999	1.081	0.361	0.997	0.720

The $2\wedge(-\Delta\Delta C_t)$ method was used to calculate the relative expression level of transcripts normalized to Rpl19. P values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. Significant P values are in bold.

and airway inflammation, when compared to those from saline-exposed dams (F0) (**Figure 3**). This change was accompanied by an increased expression of *Col3a* in F2 progenies (p = 0.006) and a trend of induction of *Col3a* in F3 progenies (**Table 5**). The increased expression of *Col3a* potentially indicates the increased airway reactivity and inflammation via increased collagen expression. Taken together, these results suggest the multigenerational effect of the early-life exposure to HDM on offspring asthma risk, through alteration of the lung phenotypic responses.

Multigenerational Inheritance of Epigenetic Reprograming in F2 and F3 Progenies

The inheritance of allergic-induced lung phenotypic responses in the F2 and F3, suggests that the airway reactivity and the increased cellularity phenotype, as well as the expression of markers of AHR are capable of multigenerational transmission. We examined if this could be explained by the epigenetic reprogramming in F2 and F3 progenies from grandmaternal (F0) HDM exposure by assessing any differential expression of genes identified by NGS (**Table 6** and **Supplementary Tables 1, 2**). We showed that F1 progenies from dams (F0) exposed to HDM showed a further induction in expression of *Kcnh1*, *Nron* and *Spag17*. In the F2 progenies, the same induction of *Kcnh1* and *Spag17* (but not *Nron*) was seen (**Table 6** and **Supplementary Table 2**). *Kcnh1* was the only candidate gene that showed further induction in gene expression in the F3 progenies from grandmaternal (F0) HDM exposure. Our results suggest that the inheritance of the allergic phenotype could be induced by the epigenetic reprogramming of gene-specific transcription. However, these changes were not observed in F2 and F3 progenies who were not challenged by HDM in adulthood. It indicates early-life exposure to HDM did not induce a dramatic increase in allergic responses seen in the sub-generations, but may induce epigenetic programing, which may prime the offspring's lung genome to be more susceptible upon a second-hit of the exposure, leading to the development of a pronounced increased allergic responses and AHR.

DISCUSSION

In the present study, we observed that HDM exposure in adulthood results in the previously observed allergic airway

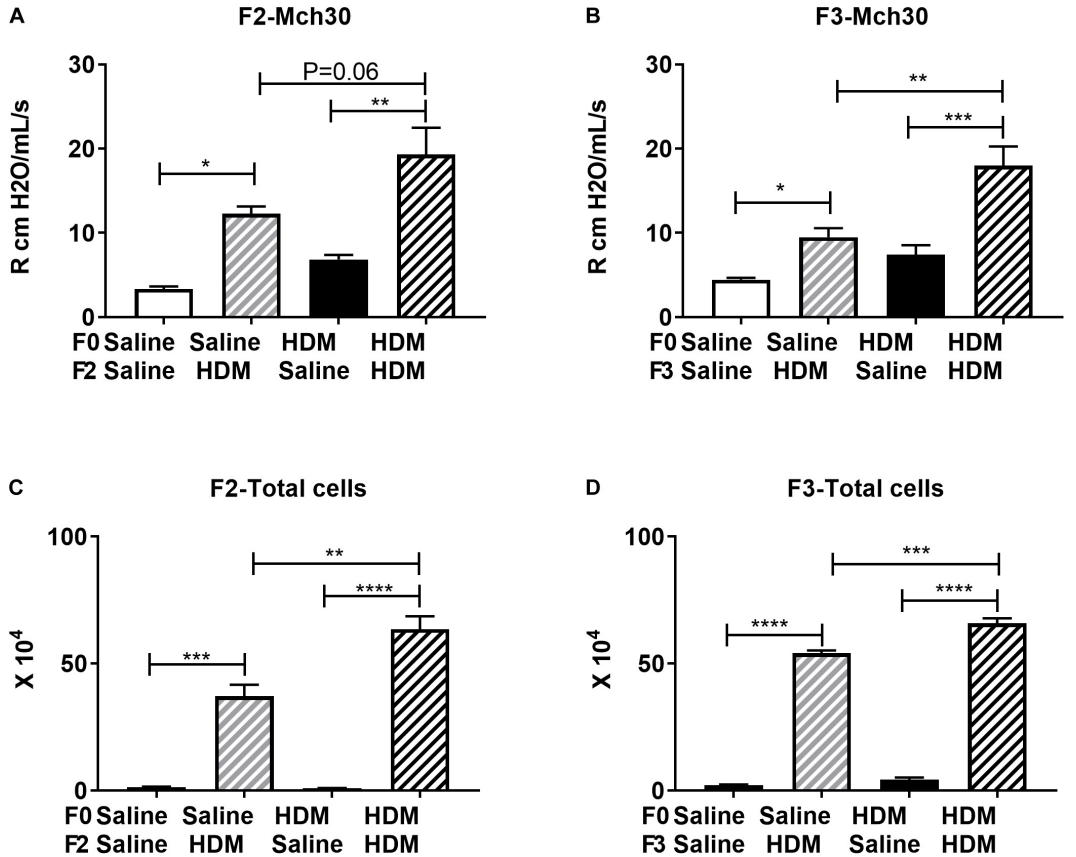

FIGURE 3 | F0 HDM exposure enhances severity to HDM induced airway hyperresponsiveness and cell count in BAL in F2 and F3. **(A)** F2 Resistance at 30 mg/ml of Mch is shown. F2 progeny from HDM-challenged mice exhibited increased AHR compared to those exposed to PBS. This was further enhanced by maternal HDM exposure. **(B)** F3 Resistance at 30 mg/ml of Mch is shown. F3 progeny from HDM-challenged mice exhibited increased AHR compared to those exposed to PBS. This was further enhanced by maternal HDM exposure. **(C)** F2 progeny from HDM-challenged mice exhibited increased BAL cell number compared to those exposed to saline. This was further enhanced by maternal HDM exposure. **(D)** F2 progeny from HDM-challenged mice exhibited increased BAL cell number compared to those exposed to PBS. This was further enhanced by maternal HDM exposure. Mean with SEM shown. *P* values derived from Ordinary One-Way ANOVA with multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

disease phenotype (Shang et al., 2013; Cheng et al., 2014), characterized by increased AHR and increased cell number in BALF. This was accompanied by the increased expression of genes governing cell proliferation (*Ccnd1*) and force-generating capacity (*Camk2d*). Unlike our previous study, we did not observe any significant change in mRNA level of *Muc5b*, *Sma*, and *Mylk* in progenies with adult exposure to HDM. We suggest the acute exposure in the present study may partially explain this, as expression of these genes was observed in a chronic HDM exposure model that was accompanied by significant airway remodeling. Strikingly, maternal and adult exposures to HDM resulted in a synergistic effect in F1 progenies (F0-HDM_ F1-HDM) when compared to adult only exposure (F0-Saline_F1-HDM), characterized by increased AHR, airway inflammation, and collagen synthesis (increased expression of *Col3a*). These changes were accompanied by changes in gene-specific methylation and hydroxymethylation as revealed by MeDIP-seq and hMeDIP-seq analysis.

To investigate the role of maternal exposure on the synergistic phenotype, we compared the progenies that received both maternal and adult exposure to HDM (F0-HDM_F1-HDM) to those who solely received adult exposure to HDM (F0-Saline_F1-HDM). We focused on the genes which showed changes in both methylation and hydroxymethylation at their promoters. As increased hydroxymethylation and decreased methylation at the promoter, and vice versa, is known to contribute to the regulation of gene transcription, we measured the mRNA levels of these candidates to validate if these epigenetic changes contribute to changes in gene expression. Maternal and adult exposure to HDM resulted in a synergistic effect on the induction of *Kcnh1*, *Nron*, and *Spag17*, when compared to that of adult only exposure (F0-HDM F1-HDM vs. F0-Saline F1-HDM). Conversely, the induction of *Erdr1* expression by adult HDM exposure was reduced in progenies with maternal (F0) exposure to HDM. Besides *Erdr1*, *Kcnh1*, *Nron,* and *Spag17*, we did observe significant changes in DNA methylation and hydroxymethylation of other 22 gene candidates that associated with the increased

TABLE 5 | mRNA levels of AHR phenotypic genes in F2 and F3 progenies. mRNA levels measured via RTPCR.

F2 Generation												
	A		B			C			D			
	F0 Saline F1 Saline		F0 Saline F1 HDM			F0 HDM F1 Saline			F0 HDM F1 HDM			
Gene	Mean	SEM	Mean	SEM	*p* value (B vs. A)	Mean	SEM	*p* value (C vs. A)	Mean	SEM	*p* value (D vs. C)	*p* value (D vs. B)
Ccnd1	1.679	0.509	14.755	2.471	0.076	1.069	0.118	0.692	17.372	5.062	0.200	0.962
Pcna	0.219	0.013	4.630	1.245	0.171	0.485	0.234	0.710	3.132	0.212	**0.004**	0.686
Col1a	0.749	0.121	1.336	0.204	0.230	0.138	0.008	0.090	1.427	0.080	**0.009**	0.972
Col3a	0.416	0.120	1.478	0.114	**0.011**	0.676	0.092	0.427	3.143	0.163	**0.002**	**0.006**
Muc5b	1.304	0.322	1.030	0.055	0.836	1.330	0.117	1.000	1.247	0.345	0.995	0.917
Sma	1.031	0.328	1.486	0.323	0.765	1.175	0.182	0.978	1.645	0.169	0.357	0.968
Camk2d	3.424	0.059	5.152	0.421	0.128	2.208	0.091	**0.003**	5.381	0.737	0.118	0.992
Mylk	1.332	0.081	1.343	0.051	1.000	0.974	0.130	0.253	1.074	0.149	0.953	0.472
F3 Generation												
	A		B			C			D			
	F0 Saline F1 Saline		F0 Saline F1 HDM			F0 HDM F1 Saline			F0 HDM F1 HDM			
Gene	Mean	SEM	Mean	SEM	*p* value (B vs. A)	Mean	SEM	*p* value (C vs. A)	Mean	SEM	*p* value (D vs. C)	*p* value (D vs. B)
Ccnd1	5.201	0.937	32.478	7.568	0.072	3.886	0.880	0.742	29.012	3.948	**0.020**	0.975
Pcna	0.682	0.231	4.419	0.338	**<0.001**	0.871	0.135	0.892	4.092	0.646	**0.041**	0.967
Col1a	1.958	0.305	3.952	0.909	0.278	3.254	0.183	**0.038**	3.303	0.561	1.000	0.926
Col3a	0.383	0.103	1.630	0.229	**0.012**	0.367	0.082	0.999	1.948	0.085	**<0.001**	0.599
Muc5b	2.788	0.307	4.797	0.825	0.220	3.079	0.422	0.941	4.572	0.692	0.356	0.997
Sma	1.116	0.165	2.130	0.333	0.121	0.974	0.134	0.907	1.905	0.265	0.099	0.949
Camk2d	3.379	0.592	6.465	0.792	0.061	2.232	0.140	0.349	3.979	0.417	0.061	0.114
Mylk	1.410	0.091	3.461	0.586	0.078	1.509	0.080	0.844	2.934	0.308	**0.049**	0.855

The $2\wedge(-\Delta\Delta C_t)$ method was used to calculate the relative expression level of transcripts normalized to Rpl19. P values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. Significant P values are in bold.

AHR. However, we did not find concordance between the induction of gene expression and methylation changes of these 22 genes. This could be explained by mechanisms other than DNA base modification, underlying the transcription of genes, although we observed acute exposure to HDM induced alteration in expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Mecp2*, and *Tet1*. Nevertheless, the methylation marks induced by HDM exposure may perhaps be used as the indicator for exposure, but not the phenotypic changes (gene expression and lung functions) if these marks are validated in other cohorts exposed to HDM.

Kcnh1, in particular, is of interest, as it has been linked to asthma-related outcomes. Recently, a study investigating environmental tobacco smoke (ETS) and lung function identified an interaction between a SNP in *KCNH1* and reduced FEV1 after ETS exposure (de Jong et al., 2017). Additionally, a study investigating season of birth with allergic outcomes identified a CpG site upstream of *KCNH1* as the mediator between spring birth dates and high serum IgE levels (Lockett et al., 2016). *KCHN1* encodes for a potassium voltage gated channel subunit that is important for smooth muscle contraction, thus it is possible that the increased expression of *KCNH1* is indicative a change in smooth muscle contractile machinery in the airways. *SPAG17* has been linked to the immune and lung related outcomes, as it has been shown to be associated with asthma susceptibility (Torgerson et al., 2012). *Spag17* is crucial for the proper function of the axoneme which forms the core of motile cilia, which are found in respiratory tract. Deletions or mutations of *Spag17* lead to immotile cilia in the nose and trachea and result in reduced mucociliary clearance, eventually leading to respiratory distress (Teves et al., 2013). We found a large increase (100-fold) in the expression of *Spag17* due to HDM exposure, which may be linked to a structural change in the airways. *Nron* is a long non-coding RNA (lncRNA) that is capable of modulating T cells at the transcriptional and protein level, as well as forming complexes with Nuclear factor of activated T cells (NFAT), and preventing NFAT translocation to the nucleus after the reception of cytokines (Xie and Liu, 2015). Given the immunological underpinnings of allergic disease and the increase in cell number in BALF, the large increase in *Nron* expression may reflect the expansion of T cell populations in the lung in response to HDM. Finally, *Erdr1* is a pleiotropic cytokine involved in hemoglobin synthesis, that has been implicated in several other disease states.

TABLE 6 | mRNA levels of differential hydroxymethylated and methylated genes in F2 and F3 progenies.

	F2										
	A		**B**			**C**			**D**			
	F0 Saline F1 Saline		**F0 Saline F1 HDM**			**F0 HDM F1 Saline**			**F0 HDM F1 HDM**			
Gene	**Mean**	**SEM**	**Mean**	**SEM**	***p* value (B vs. A)**	**Mean**	**SEM**	***p* value (C vs. A)**	**Mean**	**SEM**	***p* value (D vs. C)**	***p* value (D vs. B)**
Kcnh1	3.516	0.833	257.744	49.664	**<0.001**	3.588	0.133	>0.9999	374.755	112.999	**<0.001**	**<0.001**
Spag17	1.533	0.202	170.063	42.397	**<0.001**	1.457	0.317	>0.9999	121.977	3.923	**<0.001**	0.058
	F3											
	A		**B**			**C**			**D**			
	F0 Saline F1 Saline		**F0 Saline F1 HDM**			**F0 HDM F1 Saline**			**F0 HDM F1 HDM**			
Gene	**Mean**	**SEM**	**Mean**	**SEM**	***p* value (B vs. A)**	**Mean**	**SEM**	***p* value (C vs. A)**	**Mean**	**SEM**	***p* value (D vs. C)**	***p* value (D vs. B)**
Kcnh1	5.106	0.661	366.325	51.904	**<0.001**	2.137	0.598	0.997	471.418	77.886	**<0.001**	**<0.001**
Spag17	3.589	0.370	138.982	32.860	**<0.001**	1.844	0.182	0.999	160.806	26.801	**<0.001**	0.464

mRNA levels measured via RTPCR. The $2\wedge(-\Delta\Delta C_t)$ method was used to calculate the relative expression level of transcripts normalized to Rpl19. P values derived from Ordinary One-Way ANOVA with Tukey's test for multiple comparisons. Significant P values are in bold.

In the intestine, *Erdr1* has been shown to promote Wnt signaling, leading to epithelial growth and improved wound closure after injury to the mucosa (Abo et al., 2020). Intriguingly, *Erdr1* has been shown to be upregulated under a variety of cell stress conditions and has been shown to be negatively correlated with IL-18 signaling (Dörmer et al., 2004; Kim et al., 2016). Future work can investigate the epigenetic regulation of these genes as well as quantify and localize the expression of their products. In addition, we showed that maternal exposure to HDM induced a further induction of *Col3a* in progenies that received adult exposure to HDM. We reported that hydroxymethylation of *COL3A* promoter was associated with the increased gene expression in human ASM cells from asthmatics (Yeung et al., 2020). Taken together, maternal exposure to HDM induces gene-specific epigenetic changes in offspring in genes associated with immune response, asthma severity, and lung function. These changes may promote the observed lung phenotypic changes and result in increased susceptibility to allergic AHR.

In direct comparison to the adult only exposure, we were intrigued to see that maternal exposure to HDM (F0-HDM_F1-Saline) did not produce an overt phenotype in F1 offspring when compared to F1 (F0- Saline_F1-Saline). This was despite work in our lab and in others showing that maternal exposures to HDM, nicotine, and air pollution, and phthalates can alter the lung function in the F1 and beyond (Rehan et al., 2013; Gregory et al., 2017; Jahreis et al., 2018). Additionally, there was no change in the expression of the identified gene candidates, when compared to control (F0-HDM_F1-Saline vs. F0-Saline_F1-Saline). However, we did find that maternal HDM exposure led to a decrease in expression of *Dnmt3a*. Based on these observations alone, one might think that the maternal exposure, or perhaps its timing or duration, buffers the offspring from the deleterious effects of HDM. However, we show that maternal and adult exposure to HDM in F1 progenies (F0-HDM_F1-HDM), resulted in a synergistic effect, characterized by increased AHR and expression of *Col3a*, *Kcnh1*, *Nron*, and *Spag17*, when compared to adult only exposure. We speculated that the early-life exposure to HDM in F1 (throughout gestation and lactation) may have served as a time of early immunological sensitization. Upon exposure to HDM as an adult this may have produced the significantly enhanced response we observed. Alternatively, this early-life exposure could have altered any number of pathways, including those related to metabolism, lung function, or immune response, predisposing the F1 to enhanced disease upon adult challenge to HDM. This phenomenon is akin to the "two-hit model," in which the primary exposure predisposes the offspring to a phenotype, but a second exposure or stressor is required to initiate the phenotype (Crews et al., 2012).

We next showed that maternal exposure to house allergen can leave an epigenetic mark on subsequent descendants, resulting in an asthma-like disease transmitted along the paternal side. This effect occurred despite the fact that allergen exposure was limited to the F0 (intratracheal) and/or the F1 (during gestation and lactation). The F2 generation was generated from the paternal lineage (F1 males not exposed as adults) and naïve female mice, the offspring were never exposed to HDM before the adult, acute exposure to HDM. To our surprise, F2 progenies exposed to HDM in adulthood (F0-Saline_F2-HDM), displayed a similar phenotype as their F1 parent, characterized by significantly increased airway reactivity and increased cellularity in BALF. Furthermore, these changes were accompanied by changes in gene expression in the lung, including upregulation of *Col3a*, potentially indicating structural alterations, as well as *Kcnh1* and *Spag17* both of which were upregulated in the F1. Thus, our data

TABLE 7 | Sequence of primers for qPCR.

Gene Name	Primer Sequence-Forward	Primer Sequence-Reverse
Acox2	TCCAGAAGGCTTGCACCATT	TTTGCCTCTGGGTCACTAGG
Aida	CGCCGACTTCGACTCTTGG	TTTGCCTATGGTTTTCTTTTGTTCT
Asmt	CGCCATCTACAGGTCGGAG	GGTCGCAGATGACCCTGAAG
Camk2d	TaqMan Probe # mm0049926	
Ccnd1	ACCTGGGCAGCCCCAACAAC	GGAGGCAGTCCGGGTCACACT
Col1a	TTGGGTCCCTCGACTCCTAC	TGACTGTCCCACGTAAGCAC
Col23a	ACGGGAGAAGTTGGATGGAG	ATCTCGTCCTGATTGCCCTG
Col3a	GAGGGCCATAGCTGAACTGA	TGCAGAGTTAACAACAGTCAGC
Dpcd	CGAGCTCATCAAAGAAACCCA	CATCCTTCGGGTAGGGGAGA
Dpy19l1	GCCAGCTGGTACCGGATTTA	CCCAATCCCTCACAGCTCTC
Eef2	GTGGGGAGACCGGTACTTTG	AGAAGGTGCGGGGAAGTTTT
Erdr1	TTAGCCGCAGCTATGGTTTCT	TTCCATTCACGCCCACAGAG
Foxi1	ATGAGGACGACCCAGGCAAAG	TTCCTGCGAAAGTTTCCGTTG
Kcnh1	CTGACCCCAAACTTATCCGCA	CTGATGCCCTCATCCACGTTC
Kdm6a	AAACGCACCCACTCTACCTC	CCTTTGTGAAGCCCCTGAGT
Lars2	GGGTTTGGACCCAGAAAAGGA	GAAGACCCTTCTCTGTAAGCTGTG
Luzp2	TCAGCACCAGACAGGACTAT	TCTCTGGATGTCTTTGTCAGC
Mlck	TaqMan Probe # mm00653039	
Mrs	ACGGCAAAAGTCTCTCTGAGTT	TCAAAGACATGTGGGTCGCT
Muc19	TTGATGACCCAAGCAACCCA	TTGCTTTGGGCAGTCCTGAA
Muc5b	GAAACTGGAGCTGGGCTCTG	ATGGAGTCACTATACACTCTCTGA
Nox3	ACGGAGGAGGTCGCATCATT	GCCTGCCATTCAGCATAGTG
Nron	CACGGGTGCTGGATGACATA	ATTTAAGGGAGAGCTGGCGG
Oasl2	GAGACCGGCCCATCATCCT	CTACAGTCGTGCAACAGACCT
Pcna	CTGGGACGTCAGCTCGGGCG	TTGGACATGCTGGTGAGGTTCACG
Ranbp31	GTAGGCAGGAGGTGCGATAC	ACTCCTCGAAAAGCATGCCA
Rpl19	GGTGACCTGGATGAGAAGGA	TTCAGCTTGTGGATGTGCTC
Sma	CTTCTCCAGGGAGGAAGAGG	ACTACTGCCGAGCGTGAGAT
Smpd13a	AGCTGTGGGGCAGTTTTGG	CACACACCTTGGTACGGTCA
Spag17	CACCAACTGCGAGGACAGTA	GTAGCACCTGGTATGACCCC
Tmem125	CCTGTGTGAGAGGTGAGGGTA	TGTCAGGGTTCAGAGGGTGT
Ugt8a	TACAGGCAAAAGGCATGGGG	CCTCTGCCGATAACTGGGAT
Xcr1	GAGTCAGATGCTCTCAGTATCCCT	GGACAATGGTAGAGATGGTGGAA

indicate that early-life exposure to HDM is sufficient to transmit an asthma-like phenotype to the subsequent generation via the paternal line, and that this phenotype is accompanied by overt changes in epigenetic alterations. Similar to the F1 progenies that received saline in adulthood (F0-HDM_F1-Saline), the F2 progenies challenged with saline (F0-HDM_F2-Saline) did not display a discernable change in airway mechanics and airway inflammation. These findings support our 2-hit hypothesis, in which the maternal-only HDM exposure was not sufficient to drive a phenotype, yet resulted in altered epigenetic changes in the lung, perhaps increasing susceptibility to HDM in the event of a second insult.

Next, to examine the durability of this asthma-like phenotype, we looked at the subsequent generation, the F3, bred from F2 males (the F1 founders of this lineage only experienced HDM exposure *in utero*/early life when administered to F0) and naïve females, similarly F2 founders of this line were never exposed as adults. Here, we observed the extension of the phenotype observed in the F1 and F2. F3 progenies from paternal lineage with early-life HDM exposure showed significantly increased airway reactivity and airway inflammation, after adult, acute exposure to HDM. Like the previous generation (F2), they also displayed increased expression of *Col3a*, potentially indicative of structural changes in the airway. Additionally, they displayed increased expression of *Kcnh1*, but not *Spag17*. These findings are particularly striking as they point to multigenerational mechanisms of exposure leading to a durable genotype and phenotype. The persistence of this effect and the observed changes in expression of epigenetic modification enzymes certainly point to the epigenetic mechanisms as a possible mediator of this multigenerational effect. Furthermore, much like the F1, and F2 before them, the F3 from paternal lineage with solely maternal HDM exposure (F0-HDM_F3-Saline) showed no discernable change in AHR phenotypes and epigenetic alterations. The lack of an overt phenotype from maternal only exposure, but the clear indication of a synergistic effect upon adult exposure may hint at a role of maternal HDM exposure in priming the offspring to respond to a second insult.

In summary, HDM exposure is capable of producing multigenerational inheritance of AHR phenotype, as well as a range of transcriptional and epigenetic changes, along the paternal line, in the presence of a second insult. The second insult, acute HDM challenge, was sufficient to drive allergic airway disease pathogenesis in the F2 and F3 progenies, and resulted in a more severe disease phenotype than in adult only exposure (F0 Saline). These findings are similar to previous research in which mice exposed to diesel exhaust particles at F0 have offspring that display greater susceptibly to ovalbumin induced allergic airway disease. This increased susceptibility continued into the F2 and F3 generations, with reduced susceptibly in F3. Additionally, this susceptibility was associated with DNA methylation changes in dendritic cells, with distinct methylation patterns identified in F1, F2, and F3 (Gregory et al., 2017). Similar to our model, a second insult, in this case ovalbumin, elicited greater effects in indirectly exposed offspring. Given the similarities observed between the two models, it will be worth examining if other models of sensitization and challenge, or the use of other allergens, like cockroach or feline allergen, can produce similar multigenerational effects. Additionally, the susceptibility of these progeny to other challenges, like particulate matter or lipopolysaccharide, is worth investigating. In this study, we focused on the effect of HDM on epigenetic regulation of lung methylome because we and others reported that HDM, which is commonly found in household, induces several features of human asthma when administered to mice. Examination of shared epigenetic or transcriptomic features obtained from different exposure models could provide insight into shared underlying mechanisms of multigenerational inheritance. Our findings just scratch the surface of this phenomenon, as our understanding of the mechanisms of transgenerational inheritance are in their infancy. This does, however, make a strong case for the contribution of maternal exposures to offspring disease outcomes, including allergic airway disease. Given that we observed transgenerational effects along the paternal line, examination of the sperm, particularly miRNAs and DNA methylation and hydroxymethylation profiles are warranted. Additionally, the contribution of the maternal line to the AHR phenotype needs to be further investigated. Looking forward, identification of cell- and tissue-specific epigenetic mechanisms (such as Dnmt-dependent DNA methylation and/or Tet1-dependent DNA hydroxymethylation) responsible for the increase in expression of *Col3a*, *Kcnh1*, *Nron*, and *Spag17,* should provide a deeper insight into the molecular underpinnings of this effect.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE 169355.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Johns Hopkins University.

AUTHOR CONTRIBUTIONS

RC, YS, WM, and W-yT designed the study. NL, TD, and YS performed the lung physiological study. YS and TD performed the gene expression and methylation study with advice of W-yT and JP analyzed and interpreted the results and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.624561/full#supplementary-material

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