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Linking inter-individual variability to endocrine disruptors: insights for epigenetic inheritance

Sarah E. Latchney[1] · Ashley M. Fields[1] · Martha Susiarjo[1]

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Abstract
Endocrine disrupting chemicals (EDCs) can induce a myriad of adverse health effects. An area of active investigation is the multi- and transgenerational inheritance of EDC-induced adverse health effects referring to the transmission of phenotypes across multiple generations via the germline. The inheritance of EDC-induced adverse health effects across multiple generations can occur independent of genetics, spurring much research into the transmission of underlying epigenetic mechanisms. Epigenetic mechanisms play important roles in the development of an organism and are responsive to environmental exposures. To date, rodent studies have demonstrated that acquired epigenetic marks, particularly DNA methylation, that are inherited following parental EDC exposure can escape embryonic epigenome reprogramming. The acquired epimutations can lead to subsequent adult-onset diseases. Increasing studies have reported inter-individual variations that occur with epigenetic inheritance. Factors that underlie differences among individuals could reveal previously unidentified mechanisms of epigenetic transmission. In this review, we give an overview of DNA methylation and posttranslational histone modification as the potential mechanisms for disease transmission, and define the requirements for multi- and transgenerational epigenetic inheritance. We subsequently evaluate rodent studies investigating how acquired changes in epigenetic marks especially DNA methylation across multiple generations can vary among individuals following parental EDC exposure. We also discuss potential sources of inter-individual variations and the challenges in identifying these variations. We conclude our review discussing the challenges in applying rodent generational studies to humans.

Introduction

The developmental origins of health and disease concept (DOHaD) postulates that early life exposures can have long-term impact for later health (Barker 1995). Originally proposed in the context of heart disease (Barker 1995), the DOHaD concept links environmental stressors with health outcomes. One pervasive environmental stressor is manmade endocrine disrupting chemicals (EDCs). EDCs are ubiquitous environmental chemicals arising from different sources, including pesticides, food constituents, and packaging industries. The World Health Organization (WHO) definition of an endocrine disruptor includes any exogenous chemical or mixture of chemicals that can modify the function(s) of the endocrine system (Solecki et al. 2017). Epidemiological studies and clinical evidence suggest that early life EDC exposure targets different organ systems, leading to a range of negative health effects. In rodents, these adverse health effects include breast, ovarian, testicular, and prostate cancers, as well as reproductive and neurodevelopmental impairments and metabolic syndromes (Supplemental Table 1).

The structure of many EDCs resembles endogenous hormones. Exposure alters the endocrine system by interacting with endogenous hormone receptors, changing the levels of circulating endogenous hormones, and interfering with the synthesis, transport, and metabolism of endogenous hormones (Solecki et al. 2017). Endocrine disruption in utero is of special concern for fetal health for several reasons. First, EDCs can interfere with the endogenous activities of hormones that promote fetal growth (Solecki et al. 2017). Second, the formation and specialization of fetal tissue are characterized by developmental time windows that are

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✉ Martha Susiarjo
martha_susiarjo@urmc.rochester.edu

[1] Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA

especially sensitive to environmental exposures. Exposure to EDCs during sensitive developmental periods can potentially re-direct the course of fetal tissue development (Rodier 1994; Bateson et al. 2004). Third, the enzymes involved in EDC metabolism and clearance are not fully developed in the fetus (Choudhary et al. 2003), contributing to their sensitivity to EDC toxicity (Chamorro-Garcia et al. 2013; Li et al. 2014a). Lastly, EDC exposure in utero can lead to adverse health effects that are not evident until adult life (Anway and Skinner 2008).

The identification of EDCs and their specific mechanism of action(s) that contribute to adult-onset disease is an area of active investigation. One proposed mechanism is epigenetic modification of developmental genes, including DNA methylation and posttranslational histone modifications (Heard and Martienssen 2014). Because embryonic and germ cell development occur simultaneously with reprogramming of the epigenome, EDC exposure during this time could disrupt the proper erasure, re-establishment, and/or maintenance of epigenetic marks (Jirtle and Skinner 2007). Accumulating evidence has further shown that EDCs can produce epigenetic modifications resulting in diseases that are transmitted to future generations not directly exposed to the EDC. As listed in Supplemental Table 1, these EDCs include the fungicide vinclozolin, pesticides (dioxin, methoxychlor, and dichlorodiphenyltrichloroethane; DDT), biocides (tributyltin; TBT), plastics (Bisphenol A; BPA and dibutyl phthalate; DBP), phthalates (bis(2-ethylhexyl) phthalate; DEHP), and EDC mixtures. Exposures to these EDCs have been shown to cause negative health effects including renal, reproductive, neurobehavioral, and immune dysfunctions (Supplemental Table 1).

In this review, we summarize research on EDC exposure and epigenetic modifications by focusing on inter-individual variations. For clarity, we first discuss two common epigenetic mechanisms—DNA and posttranslational histone modifications—and how acquired changes in epigenetic marks can be transmitted to future generations. Using the rodent literature, we examine sources of inter-individual variations that arise from EDC exposure and the challenges associated with conducting epigenetic inheritance studies. Lastly, we discuss the relevance of rodent epigenetic inheritance studies to humans.

DNA methylation and histone modifications as potential marks for epigenetic inheritance

The term "epigenetics" was initially used to describe multiple phenotypes that can arise from a single genotype (Waddington 1942). The definition has evolved to describe a mitotically stable and heritable phenotype that occurs without alterations of the underlying DNA sequence (Berger et al. 2009). Identifying and understanding the significance of epigenetic marks has become an area of great interest for environmental health, particularly related to in utero exposures and their impact on health and disease outcomes later in life.

There are several well-established epigenetic mechanisms. In this review, we focus on DNA methylation, as the majority of EDC-related epigenetic inheritance studies to date focus on the inheritance of acquired DNA methylation patterns. DNA methylation is functionally associated with epigenetic gene silencing, including genomic imprinting and X-chromosome inactivation (Jones 2012; Schubeler 2015). DNA methylation entails the covalent addition of a methyl group (–CH3) to the 5′ carbon of a cytosine residue, generating 5-methylcytosine (5mC). In mammals, 5mC is the predominant form of methylated DNA and is necessary for mammalian development. Other DNA methylation modifications have been reported, including the conversion of 5mC to 5-hydroxymethylcytosine (Tahiliani et al. 2009), 5-carboxylcytosine (He et al. 2011), and/or 5-formylcytosine (Ito et al. 2011). In *C. elegans* and *Drosophila*—organisms that lack or have low levels of 5mC—the N6-methyladenine form of methylated DNA has been reported (Wion and Casadesus 2006; Sun et al. 2015).

Cytosine methylation largely occurs in 5′-CpG-3′ dinucleotide—or CpG—regions. While approximately 70% of single CpG sites are hypermethylated, regions with increased CpG density such as promoter regions—known as CpG islands—are typically hypomethylated (Messerschmidt et al. 2014). With the advent of novel single-base resolution DNA methylation techniques such as whole genome bisulphite sequencing and methylC-sequencing, methylation in non-CpG regions such as CpA, CpT, and CpC (Ramsahoye et al. 2000; Patil et al. 2014) and in sites containing CHG and CHH sequences (where H = A, C, or T; Lister et al. 2009, 2011, 2013) have been identified. Although the functional relevance remains unknown, non-CpG methylation appears to regulate the expression of tissue- and cell-specific genes via mechanisms not yet characterized (Patil et al. 2014).

Although its relevance to EDC-induced epigenetic inheritance has not been well characterized, posttranslational histone modifications have been extensively studied in the context of developmental reprogramming and epigenetic inheritance (Gaydos et al. 2014; Inoue et al. 2017; Zenk et al. 2017). Histones form octameric protein complexes called nucleosomes, around which DNA coils. Histones undergo many posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. These molecular marks regulate gene expression by altering chromatin structure, thereby influencing gene activation and repression. In the context of development, histone methylation is one of the most studied modifications. For example, histone (H) lysine (K) 27 trimethylation

(me3) and H3K4me3 marks function as epigenetic regulators to repress and activate gene expression, respectively. Both H3K27me3 and H3K4me3 coexist on the promoter of genes essential for development, differentiation, and proliferation, suggesting that active and repressive histone marks exist in an inherently balanced state (Hu et al. 2013; Voigt et al. 2013; Denissov et al. 2014). These bivalent promoters include *Nanog, Oct4*, and *Sox2*, all transcription factors that dictate the developmental potential and fate of embryonic stem cells (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). The coexistence of H3K27me3 and H3K4me3 on developmental genes potentially allows embryonic stem cells to self-renew in an undifferentiated state, yet remain poised to differentiate into specific cell types in response to developmental signals (Voigt et al. 2013; Geisler and Paro 2015). The mechanism underlying the coexistence of active and repressive histone modifications is unknown. Research into the molecular crosstalk between various histone modifications may provide insights into the susceptibilities of these histone marks to environmental exposures.

DNA methylation, histone modifications, and the multi- and transgenerational phenomena

An understanding of multi- and transgenerational inheritance requires an understanding of how epigenetic marks are reset during fetal development. In mice, two rounds of genome-wide DNA methylation occur during early embryonic development (Heard and Martienssen 2014). The first round occurs following fertilization to erase and reset the gamete epigenome for pluripotency (Guo et al. 2014; Smith et al. 2014). The paternal genome is rapidly demethylated, and the maternal genome is passively demethylated. This resetting of the gamete epigenome is required for sexual reproduction, to allow the embryo to commence its cellular differentiation program with a hypomethylated genome. Along with demethylation, maternal RNAs are degraded and the embryonic genome becomes transcriptionally active, a process called maternal-to-zygotic transition (Smith et al. 2012, 2014; Guo et al. 2014). The second round of DNA methylation occurs in primordial germ cells (PGCs) and includes the erasure imprinted differentially methylated regions (DMRs) before new imprints are re-established. Erasure in PGCs also includes potentially deleterious epimutations that may lead to adult-onset diseases. The erasure and re-establishment of DNA methylation during both reprogramming windows are tightly regulated so that epimutations are not transmitted into the new generation (Heard and Martienssen 2014). At this point, full germline potency is restored in the embryo (Hajkova et al. 2002).

Despite tight regulation of epigenetic mechanisms, evidence shows that in utero exposure to EDCs including vinclozolin and BPA can produce epimutations that become "imprinted-like." These epigenetic epimutations can escape erasure, transmit through multiple generations, and lead to adult-onset diseases (Supplemental Table 1; Anway et al. 2008). The transmission of acquired epigenetic marks begins with changes in the germline during fetal gonadal sex determination (Anway et al. 2005). Acquired epigenetic marks in the germline are transmitted to the embryo and can alter the transcriptomes and epigenomes in all tissues and cell types and may give arise to adult-onset disease. Two types of generational inheritance exist: multigenerational or transgenerational. While the primary site of action in both types of inheritance is the germline, the difference depends on whether the affected generation is directly exposed to the environmental agent (Xin et al. 2015). Multigenerational inheritance occurs when parental exposures during pregnancy influence the phenotype in the developing embryo (F1) and its gametes (F2). In this case, the adult (F0), the fetus (F1), and the exposed gametes (F2) are all directly exposed to the environmental agent. In contrast, transgenerational inheritance is only observed when the phenotype is found in generations not directly exposed to the environmental agent (McCarrey 2014; Martos et al. 2015). This type of inheritance has been observed in plants (Holeski et al. 2012), *Drosophila* (Xing et al. 2007; Ruden and Lu 2008), *C. elegans* (Kaati et al. 2007; Katz et al. 2009), rodents (Anway et al. 2005), and humans (Kaati et al. 2007; Painter et al. 2008). Importantly, if the epigenetic changes reside in the female germline, the first generation to not experience direct exposure is the F3. If the epigenetic changes reside in the male germline, the first generation to not experience direct exposure is the F2. The multi- or transgenerational inheritance of acquired epigenetic marks can be transmitted through the maternal or paternal lineage. However, if the female germline is the source of exposure—as in the case for studies examining methoxychlor (Manikkam et al. 2014), DEHP (Li et al. 2014b; Pocar et al. 2017), and EDC mixtures (Nilsson et al. 2012)—it is necessary to control for physiological and behavioral effects from the gestating mother. Maternal effects can confound the transmission of epigenetic marks to the offspring (Francis et al. 1999; Weaver et al. 2004), and, as discussed in more detail later in this review, can be a source for inter-individual differences in heritable phenotypes.

Although DNA methylation has been a major focus of most multiple generational studies related to EDCs, increasing data have shown that histone modifications can also be inherited across generations and serve as additional epigenetic inheritance marks. In particular, H3K27me3 has been found in *Drosophila* (Ciabrelli et al. 2017; Zenk et al. 2017), *C. elegans* (Gaydos et al. 2014), *Xenopus* (Akkers et al.

2009), mouse, and human (Hammoud et al. 2009, 2014; Brykczynska et al. 2010; Erkek et al. 2013) germ cells at developmental genes, and is transmitted to future generations. In *Drosophila*, H3K27me3 localizes in the oocytes and remains present in the maternal pronucleus upon fertilization. The prezygotic enrichment of H3K27me3 in the *Drosophila* maternal germline is thought to protect against excessive accumulation of active histone marks that can trigger inappropriate gene activation and premature cellular differentiation (Zenk et al. 2017). Another *Drosophila* study showed that stable epigenetic mutations containing varying degrees of the H3K27me3 mark were transmitted to the F2 progeny, providing evidence that H3K27me3 can contribute to epigenetically inheritable variations in phenotypes (Ciabrelli et al. 2017). In *C. elegans*, H3K27me3 is transmitted to new embryos by both sperm and oocytes, providing additional evidence that H3K27me can contribute to epigenetically inheritable phenotypes during development and across generations (Gaydos et al. 2014). In mice, H3K27me3 is enriched in the female germline and regulates genomic imprinting of genes (e.g., *Sfmbt2, Gab1, Slc38a4*, and *Phf17*) in the developing embryo (Inoue et al. 2017). These studies suggest that some histone modifications are transmitted from the germline into future generations and have the potentials to serve as additional marks for epigenetic inheritance. More studies are needed to characterize how environmental exposure affects the inheritance of these additional marks and the consequences on developmental programming of the new embryo.

Sources of inter-individual variability in multi- and transgenerational EDC exposures

Transgenerational inheritance of DNA methylation resulting from vinclozolin exposure was first reported in 2005 (Anway et al. 2005). An increasing number of studies have linked specific EDCs with DNA methylation inheritance across multiple generations (Supplemental Table 1). However, the presence of inter-individual variations that occur with epigenetic inheritance and their potential sources is less frequently discussed. In this review, we identify potential sources of inter-individual variation for the EDC studies listed in Supplemental Table 1, which is also summarized in Table 1.

Study design

Vinclozolin was the first endocrine disruptor used to study the transgenerational actions of EDCs on DNA methylation and adult-onset disease (Anway et al. 2005). Results from this study, however, were not without discrepancies, as vinclozolin did not produce transgenerational phenotypes in studies performed by independent labs (Schneider et al. 2008, 2013; Inawaka et al. 2009; Iqbal et al. 2015). The lack of reproducibility was likely due to embryonic exposure periods (Schneider et al. 2008, 2013; Iqbal et al.

Table 1 Sources of inter-individual variations

Sources	Examples	Comments
Study design	Exposure periods Route of administration Dose Sensitivities of rodent strains	Consideration for environmentally relevant exposure periods, administration routes, dose, and genetic strains for optimal applicability to the human condition
Epivariations	Naturally occurring variations in unexposed control animals	Epivariations may exert greater influence on methylation patterns than EDCs May present itself as low-frequency disease states May result from organism's attempt to correct for methylation errors
Tissue heterogeneity	Cell fate decisions Cell differentiation Cell type distribution Critical period of cellular reprogramming	DNA methylation states can be a major determinant of tissue heterogeneity Small changes in DNA methylation states in the cell population driving the diseased phenotype can be significant
Genomic features	Copy number variations Small nucleotide polymorphisms Partially methylated domains Strain (inbred, outbred, transgenic) Non-mendelian inheritance	Some modes of genetic inheritance can appear as epigenetic
Maternal effects	Intrauterine position Genotype ratio Sex ratio Hormone milieu Litter size Maternal physiology and behavior Parent-of-origin phenotypes	Maternal physiology and behavior and in utero environment can significantly impact the toxicodynamic profile of EDCs and influence its inheritance across generations

2015), administration routes (Schneider et al. 2008), and genetic strains of mouse (Iqbal et al. 2015) and rat (Inawaka et al. 2009).

Notably, the vinclozolin dose linked to the transgenerational effects was 100 mg/kg/day. The dose is approximately 80,000-fold higher than its reference dose from rat toxicity studies (Alyea et al. 2014), and higher than doses that induce multigenerational reproductive malformations in mice (i.e., 3.125 mg/kg/day) and the observed LOAEL (i.e., 4.1 mg/kg/day) in rat reproductive toxicity (Gray et al. 1999). More importantly, estimated human exposure to vinclozolin ranges from 34 to 78 ng/kg/day (Alyea et al. 2014). It remains unclear if exposure to environmentally and physiologically relevant doses of vinclozolin (Alyea et al. 2014) produces transgenerational effects, and future studies should focus on doses that are more relevant for humans.

Another limitation is the lack of continuous exposure assessment following EDC administration. This is critical as biomonitoring studies determine internal circulating and excreted levels of the EDC of interest. Collection and analysis of biomonitoring data allows for translating the findings from animal studies to health risks for humans. Without biomonitoring of EDC levels, as is the case for many EDC-related transgenerational studies, it is unknown if the doses administered in rodent studies resulted in physiologically relevant exposure levels for humans. For example, the levels of conjugated and unconjugated BPA detected in human blood and urine samples are found in the nanogram per milliliter (ng/mL) range (Vandenberg et al. 2010). Given this low-dose range, it is difficult to determine the physiological relevance of doses tested in animal transgenerational studies in the absence of biomonitoring studies. Jointly, variations in study design, the use of supraphysiological doses, and the lack of exposure level assessments underscore the need for future studies that investigate dose–response relationships using relevant routes of exposures at doses within environmentally relevant doses to validate contributions of epigenetic inheritance in diseases.

Epivariations

Epivariations are naturally occurring variations in epigenetic marks in unexposed control animals. While epivariations have been documented in several non-EDC studies (Bock et al. 2008; Irizarry et al. 2009; Carone et al. 2010; Ficz et al. 2011; McCullough et al. 2016), they have not been frequently reported in the EDC literature. The exception was one study which reported that the lack of transgenerational effects of vinclozolin exposure was partially attributed to high variability of spermatogenic cell apoptosis in unexposed control rats (Schneider et al. 2008). In some cases, naturally occurring epivariations could exert a greater influence on methylation patterns than environmental exposures (Carone et al. 2010; Shea et al. 2015) and present itself as low-frequency disease states (Schneider et al. 2013). Epivariations could also result from attempts to correct for errors in DNA methylation (Iqbal et al. 2015), although this hypothesis remains to be tested. Additional studies examining the contribution of naturally occurring epivariations to inter-individual variability may unveil additional multi- and transgenerational effects that otherwise would be overlooked.

Tissue heterogeneity

Evidence from immunofluorescence (Ficz et al. 2013), locus-specific (Smallwood et al. 2014), and single-cell DNA methylome (Shea et al. 2015; Gravina et al. 2016) studies suggest that DNA methylation is a major determinant of tissue heterogeneity. Interestingly, the frequency of methylation variability reported in Gravina et al. (2016) was more than three orders of magnitude higher than the frequency of somatic DNA mutations reported in Busuttil et al. (2007). This suggests that slight changes in methylation patterns may have profound consequences on tissue heterogeneity and individual phenotypes. As such, variations in tissue heterogeneity may be reflected by variations in DNA methylation levels. Close scrutiny of the studies listed in Supplemental Table 1 demonstrates that variations in methylation levels observed between EDC-exposed and unexposed rodents are small in magnitude, typically between 1 and 10%, but are statistically significant (Stouder and Paoloni-Giacobino 2010; Li et al. 2014a; Susiarjo et al. 2015). Depending on which cell population has altered DNA methylation states, these small differences can produce profound effects on phenotype (Lappalainen and Greally 2017). For example, if DNA methylation changes by 10% with EDC exposure—but the change occurs in the cell population driving the diseased phenotype—then small variations in methylation patterns can significantly increase disease risk (Lappalainen and Greally 2017). Correlating changes in DNA methylation levels with alterations in the distribution of cells expressing these alleles could predict disease risk and variations among individuals (Houseman et al. 2012). This strategy was by used by Houseman et al. (2012) in which DNA methylation patterns were used as a surrogate for cell type distribution and was validated in a subsequent study in which Jaffe and Irizarry (2014) demonstrated that the cellular composition in peripheral blood samples explained the majority of age-related variability of DNA methylation states. Therefore, identifying cellular types distribution would benefit studies that observe increased frequency and severity of a disease with age (Anway and Skinner 2008) or studies that observed increased (Skinner et al. 2015b) or decreased (Anway et al. 2008; Stouder and Paoloni-Giacobino 2010, 2011) transgenerational effects with each successive generation.

In utero environment

In animals with litters, intrauterine position can affect the genotype ratios, sex ratios, behavior, and the amount of endogenous sex hormones, leading to variations among individuals (Crews and Gore 2014). A female rodent that develops between two females in utero, for example, produces litters with female-biased sex ratio, whereas a female that developed between two males in utero produces a litter with a male-biased sex ratio. This is because the intrauterine position can determine fetal hormone levels, which can be transferred from one fetus to another (Howdeshell et al. 1999). In one study, female rats prenatally exposed to BPA developed precocious onset of puberty. When the intrauterine position of the fetus was considered, however, the effect was restricted to individuals having the highest background levels of endogenous estrogen during in utero development (Howdeshell et al. 1999). Therefore, consideration for intrauterine position would be insightful for studies in which the EDC was administered during a critical period of sexual differentiation versus throughout the entire gestation period (see Supplemental Table 1 for exposure periods). Intrauterine position and its influence on endogenous hormones can also influence the responsiveness and sensitivity to EDCs depending on sex (Skinner et al. 2008a, b, 2015a; Gillette et al. 2014) as well as sexual selection (Crews et al. 2007). Thus, intrauterine position—including in unexposed litters—can influence sex ratios, genotype ratios, litter size, and stress responses. Because humans are typically not multiparous, however, the relevance of intrauterine position remains to be determined. In its place, consideration of the maternal global hormonal milieu as a function of age, environment, exposure level, and the presence of certain health conditions that affect the hormonal environment makes human studies even more complex.

Challenges to studying epigenetic multi- and transgenerational inheritance

While epigenetics can confer stable, heritable, functional changes in gene expression, challenges exist when studying the transmission of epigenetic marks to future generations. In this section, we discuss the challenges that are commonly associated with EDC-induced epigenetic inheritance. These challenges are also summarized in Table 2.

Soma-to-germline transmission

The first notable challenge relates to the established dogma that hereditary information flows only from germline to soma (Lim and Brunet 2013). In this theory, only the germline can transmit genetic information across generations and germline mutations are required and necessary for multi- and transgenerational inheritance (Lim and Brunet 2013). However, it is possible that environmental exposure can cause epigenetic modifications in the germline either directly or indirectly through the soma. Supporting this, there is recent evidence for RNA transfer from somatic cells to germ cells (Cossetti et al. 2014; Chen et al. 2016). For example, injection of human melanoma cells stably expressing enhanced green fluorescent protein (EGFP) revealed the presence of EGFP RNA in epididymal spermatozoa, near circulating exosomes (Cossetti et al. 2014), suggesting that soma RNA can be transferred to the germline via extracellular vesicles. Interestingly, transgenerational vinclozolin exposure can dysregulate microRNAs in PGCs (Brieno-Enriquez et al. 2015), alluding the prospect that non-coding RNAs could be a candidate mechanism for soma-to-germline transmission. Moreover, endogenous hormones—of which endocrine disruptors resemble and interact with—are proposed to play a role in the soma-to-germline transmission of epigenetic marks, although direct evidence in animals are lacking (Sharma 2013). Mechanisms of soma-to-germline epigenetic transfer in EDC generational studies remain largely unexplored but are necessary to conclusively

Table 2 Challenges to studying multi- and transgenerational epigenetic inheritance

Challenges	Comments
Unidentified mechanisms of epigenetic erasure	Identifying novel imprinted DMRs, IAPs, transposable repeat elements, and "escapee" genes may reveal novel epigenetic erasure mechanisms
Soma-to-germline transmission	Non-coding RNAs may serve as a candidate mechanism Potential role of endogenous hormones in influencing soma-to-germline transmission
Genetic influences	Genomic features may be mistaken for an epigenetic mechanism Need to unify epigenetic and genetic heritability mechanisms
Statistical analysis	Clear definition of the experimental unit Consideration of sample sizes

demonstrate that epigenetic marks in gametes can be replicated, escape erasure, and be transmitted across multiple generations.

Genetic influences

Methylation states can be influenced by genotype (Kerkel et al. 2008; Zhang et al. 2009; Shoemaker et al. 2010), representing a significant confounding variable when studying epigenetic inheritance. One genetic feature is the recognition that the transmission fidelity of epigenetic marks is prone to errors (Laird et al. 2004; Modder et al. 2004). Functional sequences such as promoter regions and CpG islands are the least variable, while non-functional sequences such as repeat elements and introns are prone to errors and have higher variance between cells (Shea et al. 2015; Gravina et al. 2016). The instability of non-functional sequences could lead to copy number variations (CNVs; Guerrero-Bosagna et al. 2010; Skinner et al. 2015b) and variations in CpG density (Stouder and Paoloni-Giacobino 2011; Manikkam et al. 2012), genetic effects that, in turn, can influence the degree of methylation and complicate data interpretation. The presence of partially methylated domains (PMDs) is another genomic feature that can modify the degree of DNA methylation and subsequent gene expression (Lister et al. 2009; Schroeder et al. 2013). However, PMDs were not analyzed in any of the studies listed in Supplemental Table 1, likely because PMDs lie in genomic sites of fewer gene bodies and fewer CpG islands (Schroeder et al. 2013), regions that are not targeted in low-coverage DNA methylation assays.

The use of inbred versus outbred rodent lines also presents its own challenges when attempting to unify epigenetic and genetic mechanisms across generations. Outbred rats exposed to vinclozolin in utero exhibited decreased male fertility over three to four generations of offspring and was transmitted through male germline (Anway et al. 2005, 2006). However, this was not observed in one study using inbred rats (Inawaka et al. 2009). In mice, vinclozolin produced a more consistent transgenerational action in the outbred CD-1 mouse strain, but not in the inbred 129 mouse strain, a phenomenon known as inbreeding depression (Guerrero-Bosagna et al. 2012). Therefore, caution should be used when comparing studies using outbred and inbred lines as inbreeding may reduce the frequency or ability of an EDC to promote epigenetic transgenerational inherited phenotypes. The use of transgenic mouse lines also seems to have an impact as vinclozolin exposure in one study using transgenic mice did not produce persistent transcriptional and methylation effects into the F2 or F3 germline (Iqbal et al. 2015). Collectively, these studies allude to possible strain-dependent resilience and susceptibility to EDC-induced epigenetic phenotypes, warranting future studies that use multiple strains to identify genetic and epigenetic vulnerabilities to EDC exposure.

While a detailed discussion of additional genetic influences on transgenerational epigenetic mechanisms is outside the scope of this review—as they have not been investigated in detail in the context of EDC exposure—this topic has been comprehensively reviewed elsewhere (Lim and Brunet 2013; Heard and Martienssen 2014). For example, it is possible that transgenic animal models are not completely isogenic (Lim and Brunet 2013), leading to non-Mendelian epigenetic inheritance due to meiotic defects (Rosenberg et al. 2009; Rechavi et al. 2011). Behavioral (Francis et al. 1999; Weaver et al. 2004), microbiotic (Rosenberg et al. 2009; Tremaroli and Backhed 2012; Theodorou 2013), and metabolic effects (Lim and Brunet 2013; Heard and Martienssen 2014) can also contribute to genetic inheritance that otherwise may appear epigenetic. The presence of these genomic features highlights the need to draw clear distinctions between epigenetic- and genetic-based modifications to accurately interpret and conclude transgenerational inheritance studies.

Individual versus litter effects

Consideration for individual vs. litter effects and the respective statistical analyses used is critical when studying multi- and transgenerational inheritance. In terms of statistical analysis, the individual litter in the F1 generation should be considered as the experimental unit. For F2 and/or later generations, however, individual germ cells or animals must be considered as the experimental unit. In the case of F2 and/or later generations, identifying inter-individual variations can be difficult if it is not clear if individual germ cells or animals from the same litter were pooled for analysis. As such, technology that enables the analysis of small numbers of germ cells and cells of the pre-implantation and early post-implantation embryo are useful tools. Recent developments include advanced methylome and transcriptome technologies to analyze germ cells at the single-cell level (Smallwood et al. 2014) and novel techniques to measure slight changes in CpG methylation levels in pooled germ cells (Aiba et al. 2017).

Applicability of rodent multi- and transgenerational studies in humans

Studying multi- and transgenerational epigenetic inheritance in humans is more complex than rodent models. As defined in this review, a condition for multi- and transgenerational epigenetic inheritance is that the epigenetic mark and the associated phenotype are maintained for at least three generations in a gestating female and for at least two generations

if inheritance is via the male germline. The cost and subject burden required to conduct longitudinal generational studies in humans for at least three to four generations limit such studies to be done. As an example, although several human cohorts investigating famine and DES exposure have demonstrated intergenerational effect of nutrients and EDCs, respectively, on disease (Klip et al. 2002; Veenendaal et al. 2012, 2013), the transgenerational inheritance of these exposures remains to be determined.

Recently, the transmission of DNA methylation patterns was investigated for three generations in one study (Gertz et al. 2011) and for four generations in another study (Tang et al. 2016). However, the transmission of the DNA methylation profiles was genetic, and not epigenetic, in both studies. Gertz et al. (2011) reported that most of the variations observed in DNA methylation patterns were due to genetic differences, and that the genetic influence outweighed the effects of DNA methylation levels (Gertz et al. 2011). In Tang et al. (2016) allelic symmetry of DNA methylation patterns was widespread at non-imprinted loci and regulated by *cis*-activating genetic variants. These methylation patterns were also shared between somatic and germ cells from the same individual (Tang et al. 2016), emphasizing genetic-dependent transmission of DNA methylation profiles. Subsequent twin studies also revealed within-pair epigenetic variability in methylation and gene expression analyses at birth (Ollikainen et al. 2010; Gordon et al. 2011, 2012). The methylation and gene expression differences within monozygotic twins were smaller than those observed for dizygotic twins (Ollikainen et al. 2010; Gordon et al. 2012), but, again, were genetic in nature. Therefore, no conclusive evidence exists to definitively indicate that transgenerational effects observed in humans are explained by acquired epigenetic mechanisms inherited from one generation to the next. Thus, it remains to be confirmed that environmental stressors lead to transgenerational inheritance of epigenetic phenotypes in humans.

Fundamental epigenetic differences between humans and rodents also make it difficult to directly link rodent multigenerational epigenetics to humans. For example, human embryos have a unique methylation landscape compared to rodents (Court et al. 2014; Guo et al. 2014). Many transcriptional epigenetic marks are also substantially divergent between mice and humans (Tang et al. 2015). It also remains unclear if the epigenetic reprogramming events observed in mice also occur in humans. Human studies are also characterized by greater degrees of individual variations, unforeseen differences, and ethical restrictions that are vastly divergent from the controlled environment used for rodent studies. In particular, the increased heterozygosity of humans makes it difficult to distinguish between genetic and epigenetic contributions to inter-individual variabilities, of which no rodent models are available to account for. While epidemiological studies include demographic factors such as age, sex, race, ethnicity, and lifestyle factors, epivariations in humans may only be applied to truly genetically identical twins with a range of heritable phenotypes. To address the increased heterozygosity in humans, the use of non-traditional mouse strains with more complex genetic makeup, such as the Collaborative Cross inbred mouse strains (Churchill et al. 2004), could better mimic the genomic heterozygosity in humans and better predict the inter-individual variability from generational EDC exposures.

Perspective

The epigenome can act as a biosensor for EDC exposure and influence the outcome of and confer risks to adult-onset diseases following parental exposure to EDCs. Adding to this, this comprehensive review suggests that multi- and transgenerational epigenetic inheritance should be considered as a revolutionary and modern component of the DOHaD hypothesis. However, the small—but significant—epigenetic variations that exist among individuals in multi- and transgenerational epigenetic studies should not be overlooked. Sources of inter-individual differences identified herein include principles of experimental study designs, naturally occurring epivariations, tissue heterogeneity, genomic features, and maternal/in utero effects (Table 1). A solid understanding of these sources of inter-individual variations may shed light onto the previously unidentified mechanisms of epigenetic transmission. Additional research into these sources and mechanisms of inter-individual variability will also elucidate how EDC exposure leads to negative health effects and adult-onset disease, aid in establishing new risk assessment paradigms, and identify amendable factors that can be used to reduce the negative effects of endocrine disruptors.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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