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Elusive inheritance: Transgenerational effects and epigenetic inheritance in human environmental disease

Suzanne N. Martos[*], **Wan-yee Tang**, and **Zhibin Wang**[*]

Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA

Abstract

Epigenetic mechanisms involving DNA methylation, histone modification, histone variants and nucleosome positioning, and noncoding RNAs regulate cell-, tissue-, and developmental stage-specific gene expression by influencing chromatin structure and modulating interactions between proteins and DNA. Epigenetic marks are mitotically inherited in somatic cells and may be altered in response to internal and external stimuli. The idea that environment-induced epigenetic changes in mammals could be inherited through the germline, independent of genetic mechanisms, has stimulated much debate. Many experimental models have been designed to interrogate the possibility of transgenerational epigenetic inheritance and provide insight into how environmental exposures influence phenotypes over multiple generations in the absence of any apparent genetic mutation. Unexpected molecular evidence has forced us to reevaluate not only our understanding of the plasticity and heritability of epigenetic factors, but of the stability of the genome as well. Recent reviews have described the difference between transgenerational and intergenerational effects; the two major epigenetic reprogramming events in the mammalian lifecycle; these two events making transgenerational epigenetic inheritance of environment-induced perturbations rare, if at all possible, in mammals; and mechanisms of transgenerational epigenetic inheritance in non-mammalian eukaryotic organisms. This paper briefly introduces these topics and mainly focuses on (1) transgenerational phenotypes and epigenetic effects in mammals, (2) environment-induced intergenerational epigenetic effects, and (3) the inherent difficulties in establishing a role for epigenetic inheritance in human environmental disease.

Keywords

Transgenerational; Epigenetic; Mammals; Environment

1. Introduction

Barker et al. postulated that organs undergo developmental programming in utero that predetermines subsequent physiological and metabolic adaptations during adult life (Barker et al., 1993; Hales and Barker, 2001). Classic examples include association between low birth weight and a greater risk of coronary heart disease, hypertension, stroke, depression,

[*]Corresponding authors. 615 N. Wolfe Street, Room E7618, Baltimore, MD 21205, USA. Tel.: +1 410 955 7840. smartos1@jhu.edu (S.N. Martos), zwang47@jhu.edu (Z. Wang).

type 2 diabetes, and osteoporosis in later life (Barker et al., 1993; Fernandez-Twinn and Ozanne, 2006; Gluckman and Hanson, 2004). The observation from the Dutch famine studies also provided a proof of this concept. The paradigm is rooted in the process of developmental plasticity (Bateson et al., 2004) that argues that most human organs, prior to full maturation, are capable of re-directing their course of development based on early life clues that forecast later-life demands. It is thought that a fetal environment can alter various organ systems, rendering the individual more susceptible to disease in later life when there is a chance to encounter a second, disease-promoting stimulus (Burdge et al., 2009). Epigenetics now underpins the developmental reprogramming by demonstrating the molecular relationship between the environment and gene expression (Jirtle and Skinner, 2007; Tang and Ho, 2007).

Within the last decade, studies have been published, suggesting the possibility of transgenerational epigenetic effects in mammals (Anway et al., 2005; Padmanabhan et al., 2013; Rassoulzadegan et al., 2006). These observations force us to question whether environment-altered epigenetic marks can be inherited over generations to contribute to human disease. We now present the possible epigenetic mechanisms underlying the developmental plasticity (adaptive epigenetic variations) and epigenetic inheritance towards exogenous environmental factors and whether these epigenetic effects persist in subsequent generations. We briefly define transgenerational, discuss how mammalian epigenetic reprogramming should prevent most epigenetic marks from persisting across multiple generations, and summarize established mechanisms of transgenerational epigenetic inheritance in non-mammalian eukaryotic organisms. Then we review mammalian models demonstrating transgenerational phenotypes and environment-induced intergenerational epigenetic effects. Finally, we discuss the difficulties in establishing a role for epigenetic inheritance in human environmental disease and why this necessitates an understanding of the molecular basis underlying transgenerational phenotypes to determine potential implications for human health.

1.1. Transgenerational versus intergenerational epigenetic effects

Many studies have described intergenerational effects as evidence for transgenerational inheritance. However, correcting the misconception that these two terms are interchangeable is necessary for progress to be made in the study of epigenetic inheritance in human disease. Clarifying these definitions will allow researchers to develop and choose appropriate model systems for examining environmental influences on intergenerational and transgenerational phenotypes and associated mechanisms. In mammals, pregnant females exposed to environmental factors, such as nutrition, hormones, toxicants, or stress can affect fetal development. Such in utero exposures can also affect developing germ cells within the fetus (Skinner, 2008). Environment-induced epigenetic changes are referred to as ***intergenerational*** when they occur in the adult female organism (F0), the first generation of offspring (F1), or the second generation of offspring (F2), because the adult, the fetus, and the primordial germ cells (PGCs) would be directly exposed to the inducing agent. Effects may be ***transgenerational*** only when observed in subsequent generations (F3 or later) in the absence of exposure to the inducing agent or environmental factor that initiated the change. Effects observed in the male germline during the second-generation offspring (F2) may be

transgenerational when induced during exposure to the adult male (F0) and his germline (F1). Importantly, this does not imply that all epigenetic effects in F3 after gestational female exposure or F2 after male exposure are necessarily epigenetic inheritance. Parental effects (Daxinger and Whitelaw, 2012; Whitelaw and Whitelaw, 2008), recapitulation (Waterland, 2014) and DNA sequence changes (Heard and Martienssen, 2014) should be excluded. For example, that seminal fluid can affect the uterine environment (Bromfield, 2014; Robertson, 2005) and impact offspring phenotype (Bromfield et al., 2014) implies that paternal effects could also influence developing PGCs (F2), independent of germline-transmitted effects. Examples of non-germline maternal effects are described later in Sections 2.2 and 2.4. Several reviews have previously described distinguishing between intergenerational and transgenerational effects in greater detail (Daxinger and Whitelaw, 2012; Heard and Martienssen, 2014; McCarrey, 2014; Schmidt, 2013; Skinner, 2013). Up to date, the majority of environmental toxicants are shown to influence somatic cells (in F0 and/or F1 germ cell) via epigenetic mechanisms and induce disease phenotypes in mammals but not transmit those epigenetic effects into F3 (mother exposed) or F2 (father exposed). Transgenerational inheritance of epigenetic changes is commonly shown in plants only. Limited studies have demonstrated that environmental toxicants are able to promote transgenerational inheritance of phenotypes and diseases states in mammals. Findings from either aspect can help us to define the exposure window to the nutritional, hormonal, or stress/toxin environments that may induce the adaptive and/or heritable epigenetic changes on the developing embryo and its germline, and cause disease phenotypes in subsequent generations.

1.2. Epigenetic reprogramming in mammals

An understanding of the resetting of epigenetic marks during development is needed to investigate the role of epigenetic inheritance in human disease. Within the mammalian life-cycle, the genome undergoes two global epigenetic reprogramming events, once in the zygote and second in the developing PGCs, reviewed in Cowley and Oakey (2012), Hackett and Surani (2013), Heard and Martienssen (2014) and McCarrey (2014). For zygote reprogramming after fertilization, the paternal genome is rapidly demethylated, and the maternal genome is passively demethylated; after implantation, genome-wide de novo methylation occurs and is completed by embryonic day 6.5 (E6.5) in mice (Smith et al., 2012). Regions of the mouse genome resistant to zygotic reprogramming include imprinted differentially methylated regions (DMRs), intracisternal A particles (IAPs), and L1Md_A retroelements, a family of long interspersed elements (LINEs) (Smith et al., 2012). Coincident with post-fertilization demethylation, the mammalian zygote undergoes a process, maternal-to-zygotic transition, during which maternal RNAs are degraded, and the embryonic genome becomes transcriptionally active. The timing of zygote genome activation varies across species (1–2-cell stage in mice and 4–8-cell stage in humans) and prior to this, pre-ovulation-accumulated maternal RNAs and proteins direct developmental processes, reviewed in Li et al. (2013) and Tadros and Lipshitz (2009).

Germline reprogramming has indeed raised the question as to how these ‘reprogrammed’ marks are being inherited. Gamete imprinting via DNA methylation machinery is reported as establishment of epigenetic patterns for future generations (Trasler, 2006). Following

post-implantation de novo methylation, cells in the epiblast are induced to become PGCs and global demethylation occurs (E6.5 to E9.5); a subset of specific sequences, including imprinted DMRs, maintain methylation until E10.5 and are then demethylated by E13.5 (Seisenberger et al., 2012). After E13.5 sex-specific de novo methylation occurs: in males, methylation of germ cells is completed by birth; in females, methylation occurs between birth and puberty (Cowley and Oakey, 2012; Smallwood et al., 2011). At E13.5, mouse PGCs are extremely hypomethylated, but site-specific resistance to demethylation occurs for some IAPs, as well as single-copy genomic regions including, some CpG islands (CGIs) and non-CGI promoters near IAPs (Seisenberger et al., 2012). A recent study identified 4730 regions, including 233 single-copy regions, that maintained greater than 40 percent CpG methylation on E13.5 in female mouse PGCs (Hackett et al., 2013; Waterland, 2014). That "two rounds of epigenetic erasure leave little chance for inheritance of epigenetic marks, whether programmed, accidental, or environmentally induced," argues against transgenerational epigenetic inheritance in mammals (Heard and Martienssen, 2014). Indeed, for transgenerational epigenetic inheritance to occur, epigenetic factors would have to either evade or otherwise be protected from global erasure/degradation during reprogramming. More studies on the underlying mechanisms of how environmental factors modulate the epigenetic modifications in germ cells are required in the future. The reversible and responsive nature of epigenetic modifications toward the environment leads us to wonder whether this reprogramming will persist or be further modified throughout the course of life of the organism.

1.3. Transgenerational epigenetic inheritance in eukaryotic organisms

Mechanisms of transgenerational epigenetic inheritance have been demonstrated mostly in plants and non-mammalian animal models. Transcriptional silencing in plants and non-mammalian animal models indicate that small RNA may initiate stable chromatin silencing that can then be inherited over generations (Heard and Martienssen, 2014). Often associated with transposable elements or repeats, RNA-Dependent DNA Methylation (RdDM) and paramutation, in plants, depend on RNA interference (RNAi), reviewed in Heard and Martienssen (2014). Plant epigenetic reprogramming and epiallele transmission are discussed in Kawashima and Berger (2014). Described as a "conversion-type phenomenon" (Coe, 1959) to explain the heritable and reversible induction of phenotype (Brink, 1956), paramutation involves one allele inducing a heritable epigenetic change in the other without DNA sequence modification (de Vanssay et al., 2012).

Paramutation in the fruit fly, *Drosophila melanogaster*, establishes transgenerational silencing of transposable elements. Silencing is mediated by Piwi-interacting RNAs (piRNAs), small RNAs that interact with Argonaute-family proteins in the Piwi clade (Stuwe et al., 2014). Transcribed from transposon sequence-enriched genomic regions, termed piRNA clusters, primary piR-NAs are then processed and incorporated into Piwi proteins to silence target sequences (Brennecke et al., 2007). In addition to recognizing established transposable elements, the piRNA pathway can recognize new transposable elements that invade the host genome. Insertion of an exogenous sequence into a piRNA cluster results in repression of similar sequences in euchromatic regions of the genome (Muerdter et al., 2012; Roche and Rio, 1998; Ronsseray et al., 2003) and novel transposons

can become integrated into piRNA clusters once an organism has been exposed (Khurana et al., 2011). Stable transgenerational epigenetic inheritance (over 50 generations) via paramutation is initiated when maternally inherited piRNAs, transmitted through oocyte cytoplasm, lead to de novo silencing of homologous regions on the paternal allele (de Vanssay et al., 2012).

In the roundworm, *Caenorhabditis elegans*, dsRNA or piRNA can initiate epigenetic silencing via the RNAi/chromatin pathway, which involves RNAi factors, chromatin proteins, and histone methyltransferases (Heard and Martienssen, 2014). Epigenetic silencing at endogenous genes and transgenes can last more than 20 generations and depends on *rrf1*, an RNA-dependent RNA polymerase (Heard and Martienssen, 2014). Additional details for small RNA-mediated transgenerational inheritance in *C. elegans* are reviewed in Castel and Martienssen (2013).

Many of these established pathways and factors implicated in transgenerational epigenetic inheritance in non-mammalian models have not been thoroughly explored in mammalian models. Studies attempting to identify non-genetic inheritance in mammalian models should expand the scope of their studies to include similar potential mechanisms.

2. Transgenerational phenotypes in mammals

The discovery that vinclozolin, an anti-androgenic fungicide classified as nonmutagenic, could produce transgenerational (F1 through F4) effects on male fertility and altered DNA methylation patterns in F2 and F3 sperm (Anway et al., 2005) lead to the hypothesis that environment-induced epigenetic changes, inherited through the germline, could contribute to disease. Since then, a number of environmental chemicals have been demonstrated to induce transgenerational phenotypes. In spite of this, a clearly defined molecular basis for inheritance, presumed to be epigenetic, has yet to been established (Schmidt, 2013). The proposed mechanism is that “epimutations” induced in the male germline become “imprinted-like” allowing them to escape post-fertilization DNA methylation erasure; the altered epigenome then leads to altered transcriptomes in somatic cells resulting in adult-onset diseases (Nilsson and Skinner, 2014; Skinner et al., 2013a). However, DNA sequence changes cannot be ruled out when genes responsible for phenotypes are unknown (Heard and Martienssen, 2014). Here we review the studies demonstrating transgenerational phenotypes and associated epigenetic changes induced by environments.

2.1. Transgenerational effects of environmental exposures

In the initial study of vinclozolin (Anway et al., 2005), gestating female (F0) rats were exposed to vinclozolin between days E8 and E15, corresponding with epigenetic reprogramming of germs cells and sex determination. Following transient gestational exposure to F0 females, F1, F2, F3, and F4 adult males had significantly increased spermatogenic apoptosis, decreased sperm number, and reduced sperm motility. Similar effects on sperm were observed in offspring of vinclozolin-lineage F2 males crossed with untreated females, but not in vinclozolin-lineage F2 females crossed with untreated males, suggesting that the phenotype is transmitted through the male, but not female parent. PCR amplification of methylation-sensitive restriction enzyme digested DNA demonstrated

altered DNA methylation patterns in F2 (4 out of 8) and F3 (2 out of 5) vinclozolin-treated lineages. The transgenerational increase in spermatogenic cell apoptosis was replicated in inbred Fisher and outbred Sprague Dawley rat strains (Anway et al., 2006a). Subsequent studies found additional adult disease phenotypes after female F0 vinclozolin exposure, including prostate abnormalities in F1–F4 males (Anway and Skinner, 2008), severe anemia during pregnancy in F1–F3 females (Nilsson et al., 2008), and sexually dimorphic changes in anxiety behaviors (Skinner et al., 2008). These studies demonstrate that transgenerational manifestation of vinclozolin-induced phenotypes depends on the sex of the offspring and originate from exposure during specific developmental intervals. Additionally, transcriptome characterizations found mRNA expression of 196 genes to be consistently different in testis among the F1–F3 vinclozolin-lineage animals compared to the F1–F3 controls (Anway et al., 2008). Affected genes had roles histone modification, chromatin remodeling, and DNA methylation, including significant reductions of *Dnmt3a* in F1–F2, *Dnmt1* in F1–F3, *Dnmt3L* in F1–F3, and *Ehmt1* in F1–F3 (Anway et al., 2008). Altered expression of these epigenetic enzymes provides the possibility that epigenetic processes could be involved. Transcriptome alternations in prostate (F3 males) (Anway and Skinner, 2008), Sertoli cells (Guerrero-Bosagna et al., 2013), and hippocampus and amygdala (F3 males and females) (Skinner et al., 2008) have also been reported.

In contrast, a different group exposed outbred female (F0) Wistar rats to vinclozolin during gestation (E6 to E15) and reported no adverse transgenerational anti-androgenic effects in F1–F4 male offspring for two separate studies (Schneider et al., 2008, 2013). The first study reported decreased and the second reported increased spermatogenic cell apoptosis in F1–F3 male offspring after maternal F0 vinclozolin treatment. A study by an additional independent research group, using inbred Sprague–Dawley rats, reported no effects on spermatogenesis in F1 or F2 males after maternal (F0) exposure to vinclozolin (Inawaka et al., 2009). The reason is undetermined, but the discrepancy suggests the possible influence of genetic variations among strains (Heard and Martienssen, 2014; Inawaka et al., 2009; Schneider et al., 2013). Additionally, the original research group (Anway et al.) reported intraspecies differences in susceptibility to vinclozolin in mouse strains after F0 female exposure during gestation (E7 to E13). Inbred 129 mice were not sensitive to vinclozolin-induced prostate, kidney, or testis phenotypes, but apoptosis of spermatogenic cells occurred; whereas, outbred F3-generation CD-1 mice developed prostate, kidney, and testis abnormalities (Guerrero-Bosagna et al., 2012). A second group exposed inbred FVB/N mice to vinclozolin during gestation (E10 to E18) and reported changes in CpG methylation at five imprinted DMR in sperm; paternally imprinted genes decreased and maternally imprinted genes increased (Stouder and Paoloni-Giacobino, 2010). Methylation changes were observed in F1 through F3 but appeared to be returning to normal methylation levels by F3. Sperm motility was reduced in F1, but not in F2 and F3. Although not a genome-wide analysis, this suggests that CpG methylation changes at imprinted genes were not correlated with phenotype (Schneider et al., 2013). Alternatively, these results may not be comparable to the previously described studies because exposure was extended to E18, which overlaps with the androgen receptor vulnerability window (Schneider et al., 2013) and, therefore, was not limited to germ cell reprogramming and sex determination. Herein, it

suggests that the experimental designs and exposure windows determine the transgenerational effect of vinclozolin in epigenome.

Since the initial vinclozolin study, a number of endocrine-disrupting chemicals (EDCs), including 2,3,7,8-tetrachlorodibenzo [p]dioxin (TCDD) (Manikkam et al., 2012b), permethrin and N,N-diethyl-meta-toluamide (DEET) mixture (Manikkam et al., 2012c), jet fuel JP-8 (Tracey et al., 2013), plastics mixture (bisphenol-A (BPA), bis (2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP)) (Manikkam et al., 2013), dichlorodiphenyltrichloroethane (DDT) (Skinner et al., 2013b), and methoxychlor (Manikkam et al., 2014) have been assessed for phenotypes and DNA methylation changes in F3 rat sperm using similar designs as the original vinclozolin study. Transgenerational phenotypes observed in rats include prostate and kidney disease (Anway and Skinner, 2008; Anway et al., 2006b; Manikkam et al., 2013, 2012c; 2012b; 2012a; Tracey et al., 2013), mammary tumors (Anway et al., 2006b), abnormalities of the immune system (Anway et al., 2006b; Tracey et al., 2013), neurological and behavioral effects (Crews et al., 2012, 2007; Gillette et al., 2014; Skinner et al., 2008), reproductive effects (Guerrero-Bosagna et al., 2013; Nilsson et al., 2012, 2008), altered mate preference (Skinner et al., 2014), and obesity (Skinner et al., 2013b; Tracey et al., 2013), reviewed in Crews et al. (2014) and Nilsson and Skinner (2014). In addition to the paternally-transmitted phenotypes, recent studies demonstrate that some effects from DDT and methoxychlor are transmitted maternally (Manikkam et al., 2014; Skinner et al., 2013b). Independent research groups have also reported transgenerational phenotypes from exposure to EDCs or other environmental factors. Effects observed in mice from gestational (F0) exposure were altered gene expression, social interaction, and social recognition (Wolstenholme et al., 2012, 2013) from BPA; adipose depot size, increased adipocytes, and fatty livers from tributyltin (TBT) (Chamorro-García et al., 2013); spermatogonial stem cell defects from di-(2-ethylhexyl) phthalate (DEHP) (Doyle et al., 2013); and hyperactivity from nicotine (Zhu et al., 2014). Taken together, these studies suggest that vinclozolin is not unique in its ability to induce transgenerational effects and that gestational exposure can lead to a diverse array of phenotypic consequences in subsequent generations.

Epimutations have been proposed as potential evidence for transgenerational epigenetic inheritance of toxicant-induced disease phenotypes (Nilsson et al., 2012). DMR, considered to be epi-mutated if reproducible in three experiments, in gene promoters were determined in F3 male rat sperm: 48 for vinclozolin (Guerrero-Bosagna et al., 2010), 50 for dioxin, 33 for jet fuel (Manikkam et al., 2012a),197 for plastics mixture, 361 for pesticides mixture, 39 for DDT, and 37 for methoxychlor (Manikkam et al., 2014). The locations of the epimutations appear to be unique to the chemicals as there is little overlap between most pair-wise comparisons and no overlap among all chemicals; plastics mixture and pesticides mixture had the most overlap, with 109 epimutations in common (Manikkam et al., 2014). Sperm from F3 vinclozolin-lineage CD-1 mice had 66 promoters with 68 differentially methylated regions (Guerrero-Bosagna et al., 2012). These results indicate that the effect a toxicant has on the epigenome exhibits some chemical specificity.

The window of susceptibly for induction of epimutations in the rat, E7/8 to E14/15, is equivalent to E6/7 to E12/13 in the mouse, which coincides with global demethylation in

PGCs and hypomethylation, but excludes the remethylation phase (Section 1.2) (McCarrey, 2014). Differentially expressed genes and epimutations were determined in germ cells from male F3 vinclozolin-lineage rats on E13 (PGCs) and E16 (prospermatogonia) (Skinner et al., 2013a). There were 592 differentially expressed genes between vinclozolin-lineage germ cells and controls at E13 and 148 at E16, with only 25 at both E13 and E16 (Skinner et al., 2013a). E13 PGCs had 24 epimutations and E16 prospermatogonia had 13 epimutations, with only one gene promoter in common; neither E13 nor E16 cells had epimutations in common with those previously identified in male F3 vinclozolin-lineage mature sperm. This finding does not support specific CpG methylation-based epi-mutation(s) being programmed in PGCs and directly transmitted from sperm to offspring for the inheritance of vinclozolin phenotypes. Unfortunately, there are disadvantages in using a rat model to study potential epigenetic mechanisms involved in the trans-generational inheritance of the observed disease phenotypes. Extrapolating from findings in mice (Section 1.2), we assume that reprogramming-resistant regions could exist in the rat genome as well. Determining whether rat epimutations would be resistant to post-fertilization reprogramming is hindered in that much less is known about the rat genome and epigenome, compared to the mouse and human genomes. For example, genome-wide, base-resolution DNA methylation profiling across different stages of embryonic and germ cell development have not yet been published for the rat. Furthermore, genomic regions resistant to germline erasure in the mouse are often associated with transposable elements or repeats and likely display interspecies differences in position and regulation, which prevents direct extrapolation of resistant loci from the mouse genome to the rat genome (Sections 1.2, 2.4 and 4.3).

Overall, attributing these transgenerational effects on disease susceptibility to non-genetic mechanisms is supported by differential DNA methylation observed in sperm of the F3 generation following gestational exposure of F0 females to nonmutagenic toxicants compared to unexposed controls. Perturbations to epigenetic regulatory enzymes suggest that these phenotypes involve epigenetic processes. Currently, there is not enough detailed molecular evidence to demonstrate the transgenerational maintenance of toxicant-induced epigenetic status (i.e. an epigenetic mark altered by exposure that is present in the offspring and inherited from the parental germline (Waterland, 2014)) or to conclusively rule out confounders, such as DNA sequence changes, parental effects, or recapitulation. For these reasons, the specific mechanisms of heritability for these toxicant-induced trans-generational effects need to be elucidated. Research is also needed to clarify how the epimutations affect gene regulation and disease etiology (Nilsson and Skinner, 2014).

2.2. Mutation-induced epigenetic instability and transgenerational phenotypes

A recent mouse study demonstrated the importance of accounting for parental effects in experimental models for exploring mechanisms of transgenerational inheritance in mammals. In the study, they found that the disruption of folate metabolism caused by a mutation in the methionine synthase reductase (*Mtrr*) gene of F0 females or males caused epigenetic perturbations and lead to growth defects and congenital malformations (Padmanabhan et al., 2013). Effects persisted through generations F2–F4 of wild-type offspring derived from wild-type mothers (F1). F0 metabolic deficiencies could potentially alter reproductive phenotypes in F1 wild-type mothers (e.g., affect F1 uterine development).

If the F1 (wild-type) maternal environment were responsible for the observed adverse developmental outcomes and epigenetic perturbations, then F3 germ cells developing in F2 fetuses would be considered exposed to the altered maternal environment (F1 phenotype) caused by the *Mtrr* mutation in the F0 generation. To separate potential maternal effects from those directly caused by F0 grandparental *Mtrr*-deficiency on F2 gametes, embryo transfer experiments were conducted on grandprogeny (F2). Results from these experiments implicated maternal uterine environment in growth defects and maternal grandparental *Mtrr*-deficiency in transgenerational congenital malformations. Affected wild-type grandprogeny (F2) derived from *Mtrr*-deficient F0 males or females were determined to have epigenetic instability, indicated by alterations in DNA methylation at imprinted DMRs in placentas; whereas, phenotypically normal wild-type litters (F1) from *Mtrr*-deficient F0 males did not display epigenetic instability. Taken together this suggests some phenotypic effects initiated by maternal grandparental *Mtrr* deficiency are transmitted through the germ cells and are, at least in part, mediated by epigenetic mechanisms.

2.3. RNA-mediated paramutation-like effects

In addition to toxicant- and mutation-induced transgenerational effects, RNA-mediated, paramutation-like effects have been described in mice. In this example, wild-type offspring derived from a parent (male or female) heterozygous for an engineered, mutated allele, Kit^{tm1Alf}, displayed the mutant phenotype, white feet and tail tips (Rassoulzadegan et al., 2006). The phenotype was still partially visible in F2 but disappeared gradually in subsequent generations. The inheritance of the phenotype, despite the absence of genetic inheritance of the Kit^{tm1Alf} allele, suggests the possibility of epigenetic inheritance. Microinjection of RNA isolated from Kit^{tm1Alf} heterozygote sperm or brain, miR-221, or miR-222 into fertilized eggs was sufficient to induce the phenotype (Rassoulzadegan et al., 2006), which indicates RNA-mediated effects. The transgenerational inheritance does not occur in other *Kit* null alleles without a transgene insertion (Daxinger and Whitelaw, 2012), which raises the question of whether similar phenomena would arise naturally. The same group demonstrated that the injection of other microRNAs into fertilized eggs results in the transgenerational inheritance of additional phenotypes but did not lead to a stable increase in microRNAs to pass to the next generation (Grandjean et al., 2009; Wagner et al., 2008). This observation suggests that the transmission to subsequent generations is initiated, but not maintained, by the same RNA molecules. Furthermore, establishment of the paramutant-like phenotypes requires *Dnmt2*, responsible for methylation of cytosine in RNA (Kiani et al., 2013). While initiated by the transfer of RNA from sperm to offspring, the molecular basis for how the observed phenotypes are inherited throughout generations has not been established. Importantly, these studies establish that RNA-mediated epigenetic mechanisms might play a similar role in mammalian transgenerational inheritance, as they do in plants and non-mammalian animal models.

2.4. Transgenes and metastable epialleles

The non-mendelian inheritance of transcription activity at transgenes is presumed to be epigenetic because inbred littermates have variable transcription (Daxinger and Whitelaw, 2012). Variable expression and non-mendelian inheritance patterns of endogenous genes at metastable epialleles, defined as “allele[s] at which the epigenetic state can switch and

establishment is a probabilistic event" (Rakyan et al., 2002), are often associated with transposable elements and are thought to result from incomplete reprogramming of epigenetic marks (Rakyan et al., 2001).

For example, the agouti locus (*A*) controls pigmentation of the mouse, the coat color phenotype of A^{vy}/a heterozygotes ranges from yellow to various degrees of yellow background with black mottling to pseudoagouti (i.e. resembles wild-type agouti pattern) (Dickies, 1962). Coat color correlates with other varied phenotypes observed in genotypically similar littermates, including obesity and susceptibility to tumor formation (Wolff, 1978). Furthermore, the maternal and grandmaternal, but not the paternal, phenotype affects the distribution of phenotypes among offspring derived from A^{vy}/a heterozygotes crossed with *a/a* homozygotes (Morgan et al., 1999; Wolff, 1978), suggesting that phenotypic variation is mediated by maternal factors. The A^{vy} mutation, caused by an IAP element insertion 100 kb the transcription start site for the wild-type coding sequence, drives ubiquitous, constitutive transcription of a gene that has tissue- and stage-specific expression in wild-type mice (Bultman et al., 1992; Duhl et al., 1994; Waterland and Jirtle, 2003). Varying degrees of epigenetic silencing, indicated by hypermethylation at the IAP of the A^{vy} locus, suppresses transcription of agouti gene to generate mottled and pseudoagouti phenotypes (Morgan et al., 1999).

Physiologic and metabolic differences among mothers were thought to explain dependence of offsprings' phenotype on maternal phenotype (Wolff, 1978, 1971). Embryo transfer experiments (to separate maternal environment from germline effects) indicate that phenotype distribution results from inheritance, not maternal environment (Morgan et al., 1999). Interestingly, characterization of CpG methylation in gametes, zygotes, and blastocysts demonstrated that the A^{vy} allele undergoes dynamic post-fertilization DNA methylation reprogramming consistent with established genome-wide patterns (Blewitt et al., 2006). That is paternal A^{vy} allele was dramatically reduced (from 73% to 15%) within 9–11 h after fertilization, indicating active demethylation; maternal A^{vy} allele was not rapidly demethylated (69% methylation in zygote), but was completely unmethylated by the blastocyst stage (Blewitt et al., 2006). Thus, CpG methylation at the A^{vy} allele is not likely the epigenetic mark responsible for the transgenerational phenotypes (Daxinger and Whitelaw, 2012), because it is erased within the mammalian lifecycle. In A^{iapy} mice, which are similar to A^{vy} mice in that DNA methylation at an IAP insertion inversely correlates with agouti expression and determines the distribution of offspring phenotypes (Michaud et al., 1994), low expression of maternal oocyte-specific *Dnmt1* isoform shifted offspring coat distribution toward yellow (Gaudet et al., 2004). This result indicates that the pre-implantation control of agouti expression contributes to adult phenotype (Gaudet et al., 2004); however, whether the A^{iapy} epiallele escapes post-fertilization reprogramming or whether the A^{vy}/a phenotypes are similarly dependent on oocyte-specific *Dnmt1* has not been reported.

3. Environment-induced intergenerational epigenetic effects

Currently, studies showing transgenerational epigenetic inheritance towards environmental stimuli are limited. Nonetheless, a number of human and animal studies have demonstrated

that toxicant exposure and nutritional status during pregnancy can affect epigenetic marks in developing offspring, which provides a molecular explanation for increased disease susceptibility from fetal exposures (Bailey and Fry, 2014; Hilakivi-Clarke, 2014; O'Hagan and Tang, 2014; Soubry et al., 2014; Waterland, 2014). The Agouti viable yellow (A^{vy}) mouse model is, perhaps, the most well characterized animal model demonstrating a role for environmental factors in phenotypic variation through alteration of DNA methylation. Methyl supplementation of female (F0) diet during gestation shifted the distribution of offspring (F1) (Wolff et al., 1998) and grand offspring (F2) (Cropley et al., 2006) phenotypes toward pseudoagouti when the A^{vy} allele is inherited from F0 males to F1 females to F2 offspring, indicating intergenerational effects from F0 gestational exposure. Gestational methyl supplementation-induced phenotypic changes correlated with the degree of CpG methylation at the IAP insertion site (Waterland and Jirtle, 2003). Similarly, maternal methyl supplementation increased CpG methylation of the $Axin^{Fu}$ metastable epiallele and shifted the phenotype distribution of offspring (Waterland et al., 2006). Environment-mediated epigenetic regulation of the mutant allele(s) provides a molecular basis for the phenotypic variation observed among these isogenic mice.

Other environmental exposures during gestation have also been shown to affect offspring phenotype and CpG methylation of the A^{vy} epiallele. Genistein, a soy phytoestrogen, induced hypermethylation and shifted offspring distribution toward pseudoagouti (Dolinoy et al., 2006). Bisphenol A (BPA), an estrogenic chemical used to manufacture plastics and resins, induced hypomethylation and shifted offspring distribution toward yellow, but methyl donor or genistein supplemented maternal diets were able to counter the effects of BPA on coat color distribution (Dolinoy et al., 2007). However, methyl donor-induced hypermethylation, indicated by coat color, was not inherited transgenerationally through the female germline (Waterland et al., 2007).

Animal models involving dietary manipulation have demonstrated the importance of gestational nutrition in determining offspring disease susceptibility. In utero exposure to high-fat diet in mice is shown to induce paternal obesity with increase in adiposity and initiate intergenerational transmission of obesity and insulin resistance in two generations of offspring accompanied with changes in sperm microRNA content and germ cell methylation status (Fullston et al., 2013). In contrast, caloric restriction during pregnancy alters DNA methylation in the sperm of F1 offspring mice at regions resistant to zygotic reprogramming. Although these changes do not persist in F2 tissues, the results suggests in utero nutritional exposures during critical windows of germ cell development can impact the male germline methylome that may influence the risk of metabolic disease in the offspring (Radford et al., 2014). Nonetheless, another group demonstrated no changes in male germline DNA methylation in a dietary protein restriction model (Carone et al., 2010). Overall, it is clear that both toxicants and nutrients can induce epigenetic changes with intergenerational phenotypic consequences. Certainly, in animal models, the exposure window to the environmental stimuli is critical for the establishment of intergenerational epigenetic changes.

4. Establishing a role for epigenetic inheritance in human environmental disease

4.1. Transgenerational human disease

Evidence of transgenerational epigenetic inheritance in plant and animal models suggests that it may be a ubiquitous phenomenon in eukaryotic organisms and could plausibly occur in mammals. Evidence from mammalian animal studies indicates that epigenetic instability resulting from deficiency in folate metabolism, initiated by a genetic mechanism, can cause transgenerational outcomes and that environmental and dietary factors can affect DNA methylation intergenerationally in mammals. Several human cohorts like famine and DES exposure demonstrated the intergenerational effect of nutrients and EDCs on disease phenotypes (Bygren et al., 2014; Klip et al., 2002; Veenendaal et al., 2013, 2012). However, whether environmental perturbations induce epigenetic changes and these changes can be transgenerationally inherited through epigenetic mechanisms to influence human disease susceptibility remains unknown. Furthermore, distinguishing between epigenetic inheritance and epigenetic bio-markers remains challenging, especially in humans. Few epidemiological studies that demonstrate intergenerational and possibly transgenerational associations have assessed the following conditions: paternal line food supply, maternal line famine and prenatal nutrition, smoking, and paternal line betel-quid chewing, recently reviewed in Pembrey et al. (2014).

4.2. Limitations of human epidemiology studies

That we have evidence for transgenerational epigenetic inheritance in animal models with relatively short lifespans and generation times, such as *D. melanogaster* and *C. elegans* is not surprising. In addition to the time required for multi- or transgenerational studies in humans, limited sample availability makes it difficult to study dynamic genome–epigenome interactions (Section 4.4). While often sufficient for identification of stable biomarkers, human samples do not necessarily provide adequate mechanistic insight needed to establish epigenetic inheritance and rule out genetic inheritance, parental effects, and recapitulation. Furthermore, human samples are often limited to those that are minimally invasive, such as blood, urine, saliva, skin, and post-mortem samples, which are inadequate for studying the complex epigenetic dynamics that occur during development (Section 1.2). Finally, the inability to completely control for other environmental exposures makes it difficult to identify relevant epigenetic and genetic changes for a particular factor and rule out confounding effects from other environmental influences.

4.3. Limitations of interspecies extrapolation of phenotypes

Animal models can be used to study molecular mechanisms in organisms over multiple lifecycles and throughout development. It is tempting to extrapolate phenotypic consequences of environment-induced epigenetic changes (e.g., adverse outcomes from toxicant exposure) observed in animal models to humans. While similar epigenetic mechanisms likely apply to humans, perturbations to these mechanisms may not necessarily affect the same endogenous gene(s) or pathway(s) as in animal models. For example, estimates suggest that mice and humans have 30 percent of imprinted genes in common

(Skaar et al., 2012) and IAP elements, responsible for the lability of the agouti epiallele (Sections 2.4 and 3), are not found within the human genome. Therefore, direct interspecies extrapolation of phenotypes identified in rodent models may be insufficient for explaining particular human traits or disease susceptibility. However, other families of transposable elements within the human genome could impart similar characteristics to endogenous human genes. A mechanistic understanding of interspecies genomic and epigenomic differences would be particularly important in extrapolating effects observed in rodents involving imprinted genes, mouse IAPs, and other transposable elements to humans.

One study demonstrated interspecies differences in the regulation of retrotransposons at transcription start sites (TSSs) in mouse and human genomes using multiple cell and tissue types for each species (Faulkner et al., 2009). The comparison used cap analysis gene expression (CAGE) followed by high-throughput sequencing, which provides detailed information on activity at TSSs by capturing and sequencing the first 20 nucleotides from the 5′ end of full-length cDNAs. They found that approximately 18.1 percent of mouse and 31.4 percent of human TSSs were located within repetitive elements, but only 5.2 percent of mouse and 2.8 percent of human TSSs with more than 100 CAGE tags were from retrotransposons. This finding indicates that although abundant throughout mammalian genomes, TSSs of retrotransposons tend have lower transcription than non-repeat TSSs. Furthermore, transcription from repetitive element-associated TSSs differed between cell and tissue types. Interestingly, transcription of retro-transposons was poorly conserved between mice and humans, even in comparable tissue types. Although whether this reflects species-specific epigenetic regulation of transposable elements or differences between human and mice retrotransposon families was not determined. Overall, this genome-wide characterization of transcription at repetitive elements in both mouse and human cells/tissues suggests that regulation of retrotransposons in mammalian genomes displays species specificity and that variation also occurs within a species among different developmental stages and tissue types. These differences might need to be taken into account to extrapolate from rodent models to human diseases.

The dependence on transposable elements for both metastable epialleles and inherited paramutations suggests that transposable element-associated genes that escape DNA methylation erasure in both zygotes (Borgel et al., 2010; Smallwood et al., 2011) and PGCs (Guibert et al., 2012; Hackett et al., 2013) could be candidate regions to explore for heritable epialleles in mammals. With bioinformatic approaches and bisulfite sequencing, a recent study identified retrotransposon-derived candidate epialleles in the mouse genome (Ekram et al., 2012). Identification of similar candidate regions within the rat and human genomes would be useful for understanding interspecies similarities and differences in genomic regions that have the potential to provide insight into potential transgenerational epigenetic inheritance in mammals.

4.4. Genome–epigenome dynamics

DNA methylation exhibits some sequence context specificity, indicating that genetic variation among individuals could result in DNA methylation differences (Schübeler, 2012). At the same time, maintaining genome integrity requires suppression of retro-transposition

activity, accomplished via epigenetic regulation (Ishiuchi and Torres-Padilla, 2014). This observation has lead others to propose that environmental factors could result in primary, secondary, or tertiary epimutations, reviewed in McCarrey (2014). Briefly, primary epimutations would be initiated by an epigenetic change that is then inherited via epigenetic mechanisms with no changes to DNA sequence (Whitelaw and Whitelaw, 2008). Secondary epimutations would be initiated by a genetic change that causes an epigenetic change that is subsequently inherited by either genetic or epigenetic mechanisms (Whitelaw and Whitelaw, 2008). Tertiary epimutations would be initiated by an epigenetic change that causes a genetic change(s) (McCarrey, 2012). The apparent reciprocal dependence between the epigenome and genome suggests a dynamic interaction, which makes it difficult to establish whether epigenetic differences are the initial cause or consequence of DNA sequence variation, especially when the only available molecular evidence is static and temporality must be inferred.

5. Potential implications for human environmental health and disease

Without conclusive mechanistic evidence either for or against transgenerational epigenetic inheritance of environment-induced epigenetic changes in mammals, assessing the human health implications is somewhat speculative. Assuming (1) that trans-generational epigenetic inheritance occurs in mammals and we have yet to find a mechanism, (2) that because of their common use in biomedical research we would most likely establish such a mechanism in rodent models, and (3) that environment-induced perturbations to these epigenetic factors could also be inherited, we would still need to extrapolate those mechanisms to human diseases. Molecular mechanisms in the most general sense should be applicable to humans. If, for example, a transposon silencing-based mechanism were to be implicated in any transgenerational disease phenotypes observed in rodent models (Section 2.1), it would be possible for a similar silencing mechanism to occur in humans. However, interspecies differences in families of transposable elements, their insertions sites, and their influences on gene regulation (Levin and Moran, 2011) suggest that similarly regulated genomic features may not affect the same genes in humans as in animal models. Fortunately, the increasing wealth of genomic and epigenomic data should allow us to extrapolate molecular mechanisms from rodent models to identify similar regions of the human genome based on characteristic genomic and epi-genomic features. After we identify distinctive features of trans-generational inheritance in the rodent models, our next step would be to uncover details of analogous or homologous mechanisms in humans, and then to identify potential genes affected, and finally, to connect changes in expression of these genes to phenotypic (e.g., disease) consequences.

Alternatively, the observed environment-induced transgenerational phenotypes could be epigenetic manifestations of heritable genetic mutations or transmitted by other non-genetic mechanisms. Given the rarity of transgenerationally-inherited, environment-induced epigenetic changes in plants, in which mechanisms of transgenerational epigenetic inheritance have been established (Heard and Martienssen, 2014), combined with the rarity of epi-alleles reported in mammals, other scenarios could explain many examples of transgenerational phenotypes observed in mammals. A recent study assessing DNA methylation of 614 individuals from 117 families consisting of monozygotic or dizygotic

twins and their parents found that similarity in DNA methylation between relatives could be accounted for largely by genetic heritability of DNA sequence variants (McRae et al., 2014). Under a genetic inheritance scenario, we would still need detailed mechanistic information to determine affected genes and understand possible human phenotypes, especially if the DNA sequence variations were found to occur in noncoding areas of mammalian genomes.

6. Conclusions

Environmental conditions, such as toxicant exposure and metabolic deficiencies, can influence phenotypes and change epigenetic marks over multiple generations. However, whether these altered epigenetic marks are evidence of transgenerational epigenetic inheritance of environmental effects remains unknown. The demonstrated mechanisms for transgenerational epigenetic inheritance in other eukaryotic organisms and transgenerational epigenetic silencing of transposable elements in the mammalian genome suggests that it would be plausible for similar processes to exist in mammals. Then, it is not difficult to imagine that environmental conditions could alter these epigenetic processes. Although the two epigenetic reprogramming events within the mammalian lifecycle would make the transmission of epigenetic marks across multiple generations unlikely, some repetitive elements and single-copy loci resist germline erasure and some repetitive elements and imprinted loci resist early embryonic erasure. However, the rarity of epialleles and their association with silencing transposable elements in mammals leaves the possibility that toxicant or metabolic-induced epigenetic instability could give rise to de novo insertion mutations resulting from reactivation of transposable elements in offspring that were presumed to be isogenic. The mammalian lifecycle further complicates distinguishing between germline transmission and environmental influences, especially parental, as subsequent generations are never truly independent of their predecessors in natural, as opposed to experimental, mammalian systems.

Many transgenerational phenotypes appear to be mediated, if not inherited, by epigenetic processes. As such, distinguishing among epigenetic, genetic, and metabolic contributions to human environmental diseases will continue to be active areas of research and debate. Demonstrating whether transgenerational epigenetic inheritance occurs in mammals will require detailed genetic, epigenetic, transcriptomic, and metabolic profiling of well-controlled animal models with well-documented pedigrees. Novel cell-based and *in vitro* approaches for studying dynamic molecular processes will be needed to identify epigenetic factors involved and rule out possible confounding from non-germline parental and grandparental influences on offspring, such as those introduced by behavioral, metabolic, or microbiotic factors. Regardless of whether transgenerational phenotypes are transmitted via epigenetic or other mechanisms, extrapolating findings to understand the role that environmental perturbations play in human disease susceptibility necessitates a quantitative and integrated understanding of the interactions among the environment and the genome, epigenome, transcriptome, and metabolome at different stages of human development.

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