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Transgenerational inheritance and its modulation by environmental cues

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Abstract

The epigenome plays an important role in shaping phenotypes. However, whether the environment can alter an organism's phenotype across several generations through epigenetic remodeling in the germline is still a highly debated topic. In this chapter, we briefly review the mechanisms of epigenetic inheritance and their connection with germline development before highlighting specific developmental windows of susceptibility to environmental cues. We further discuss the evidence of transgenerational inheritance to a range of different environmental cues, both epidemiological in humans and experimental in rodent models. Doing so, we pinpoint the current challenges in demonstrating transgenerational inheritance to environmental cues and offer insight in how recent technological advances may help deciphering the epigenetic mechanisms at play. Together, we draw a detailed picture of how our environment can influence our epigenomes, ultimately reshaping our phenotypes, in an extended theory of inheritance.

1. Introduction

The definition and associated understanding of “epigenetics” have dramatically shifted since the 1940's coining of the term *epigenotype* by Waddington (Waddington, 2012). Nearly 60 years later, the currently most accepted definition of epigenetics was put forth by Wu and Morris (Wu & Morris, 2001). They proposed that epigenetics should be defined as: “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence.” As evidenced by the extensive correspondence associated with this proposal (Wu & Morris, 2001), the introduction of the concept of heritability in epigenetics was quite remarkable. Indeed, while epigenetic mechanisms operate on a short timescale for gene regulation, the potentially long-lasting nature of some epigenetic modifications has garnered significant attention for its ability to explain biological phenomena that unfold across several generations.

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Contrasting with the relative stability of epigenetic marks, the epigenome is remarkably malleable and sensitive to environmental influences. Thus, epigenetics is intimately tied to the research into the Developmental Origins of Health and Disease (DOHaD), which aims to uncover the mechanisms by which early life (pre- or peri-natal) exposures may condition the health of an individual over their life course (Barrett, 2017; Bianco-Miotto, Craig, Gasser, van Dijk, & Ozanne, 2017; Ong, Lin, & Holbrook, 2015). In the context of an in utero (i.e., prenatal) exposure directly altering the health outcomes of the next generation, this phenomenon would call for an intergenerational role for the epigenome. Even more remarkable is the wealth of evidence, in a variety of species, indicating that many different types of environmental cues elicit epigenetic changes and phenotypic alterations over several generations. In many instances, these health outcomes are detected in generations beyond the point at which any directly exposed cells remain. This mechanism of epigenetic inheritance is termed “transgenerational” and is the most intriguing since it implies a transfer of biological information by germ cells independently of DNA sequences, yet, acting on gene expression and functioning in parallel with sequence-based regulation. Perhaps unsurprisingly, transgenerational epigenetic inheritance (TEI) has attracted both significant research interest and controversy when applied to humans (Breton et al., 2021).

In this chapter, we explore the molecular mechanisms underlying TEI. We first review the epigenetic mechanisms of inheritance, then discuss their involvement in germ cells development. We then focus on the evidence for TEI, but also highlight the knowledge gaps that require significant research investment. We end this chapter with forward-thinking approaches that we propose to deploy to address the remaining black boxes in the field.

2. Epigenetic mechanisms of inheritance

Epigenetic mechanisms make use of reversible modifications of chromatin states that impact gene expression (Bird, 2007; Murr, 2010). Such modifications include chemical changes of the DNA itself, in particular DNA methylation, changes in histones post-translational modifications, changes in nucleosomes position and composition, non-coding RNA (ncRNA)-mediated modifications of the chromatin structure and changes in high-order chromatin structure within the nucleus (Fig. 1). Importantly, epigenetic states are heritable and ensure the maintenance and long-term stability of gene expression across cell division and during development (Almouzni & Cedar, 2016). Here, we briefly review known mechanisms of epigenetic inheritance.

In mammals, DNA methylation mainly consists in the addition of a methyl group onto cytosines (5mC) within the context of CpG dinucleotides (Bird, 2002; Goll & Bestor, 2005). Dense regions of CpGs – termed CpG islands (CGIs) – are associated with the majority of human and mouse genome promoters and their methylation constitutes a major mechanism for stable repression of gene expression (Haberle & Stark, 2018; Zhu, Wang, & Qian, 2016). The DNA methyltransferase DNMT1, recruited by the ubiquitin E3 ligase UHRF1, has been shown to reproduce DNA methylation patterns during DNA replication and repair, ensuring faithful maintenance of methylation profiles (Nishiyama et al., 2020; Sharif et al., 2007). DNMT3A and DNMT3B have long been considered as “de novo” methyltransferases for their capacity to establish new patterns of DNA methylation (Okano, Bell, Haber, & Li,

1999). However, the distinction between maintenance and de novo DNA methyltransferases has been challenged by observations of their cooperation in establishing DNA methylation patterns (Haggerty et al., 2021; Lyko, 2018).

In addition to changes in the DNA sequence itself, changes in nucleosome position and composition, in particular on the amino-terminal tails of histones, have also been shown to participate in epigenetic memory of phenotypes (Bannister & Kouzarides, 2011; Venkatesh & Workman, 2015). Multiple post-translational modifications (PTMs) of histone tails have been identified, including methylation, acetylation, ubiquitination, sumoylation, and this repertoire is in perpetual expansion with the discovery of crotonylation (Fang et al., 2021; Tan et al., 2011), malonylation, succinylation, and glutarylation (Sabari, Zhang, Allis, & Zhao, 2017). Histone PTMs can directly regulate chromatin compaction but can also indirectly regulate gene expression through the recruitment of chromatin-modifying enzymes, which has led to the notion of a histone code (Strahl & Allis, 2000; Tan et al., 2011). Indeed, specific genomic regions are occupied by histones displaying different PTMs and these epigenetic signatures are predictive of transcriptional states. For instance, transcriptionally-active enhancers are enriched in a subset of histone marks, including histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1) (Heintzman et al., 2007; Hon, Hawkins, & Ren, 2009). Maintenance of histone marks is regulated by the controlled recruitment of histone modifiers, principally during DNA replication (Shan, Fang, & Jia, 2021).

Non-coding RNAs regulate epigenomes by recruiting chromatin-modifying complexes (Holoach & Moazed, 2015). Regulatory ncRNAs can be classically classified according to their size, with transcripts longer than 200 nt termed long ncRNAs (lncRNAs) and smaller transcripts including microRNAs (miRNAs) or piwi-interacting RNAs (piRNAs) (Kaikkonen, Lam, & Glass, 2011). Small ncRNAs-mediated inheritance of epigenetic states is a hallmark in *Schizosaccharomyces pombe*, *Drosophila* and *Caenorhabditis elegans* (Heyt & Thakur, 2021). In mammals, piRNAs and sperm-borne ncRNAs are the most well-understood small ncRNAs in the inheritance of epigenetic states (Chen, Yan, & Duan, 2016; Yan, 2014). In addition, lncRNAs are important regulators of epigenetic states and serves as hub for the concerted recruitment of multiple types of chromatin modifiers that are relevant for epigenetic inheritance (Legoff, D’Cruz, Tevosian, & Smagulova, 2019).

Finally, the three-dimensional organization of chromatin domains within the nucleus has been shown to be an important epigenetic mechanism (Pombo & Dillon, 2015). Indeed, depending on its transcriptional competence, chromatin dynamically transits between higher-order organization forms within different subnuclear compartments (Pombo & Dillon, 2015). Technical advances in the recent years have provided tools to analyze chromatin conformation but the three-dimensional control of epigenetic inheritance still needs to be better defined (Zheng & Xie, 2019).

3. Substrates for epigenetic inheritance

Germ cells are at the origin of new organisms and ensure a continuous link across generations and species (Lesch & Page, 2012). The germ cell lineage is responsible for

the faithful propagation of genetic and epigenetic information to the next generation. Thus, for TEI to occur, the germline information needs to be altered and these changes retained throughout development. In this section, we will briefly review the development of germ cells and their epigenetic dynamics. We highlight key windows of susceptibility to environmental cues. We will further discuss how environmentally-induced epigenetic changes can be sustained during the intense epigenetic remodeling undergone by the germline.

3.1 Epigenetic dynamics during germ cells development

Germ cells are specified early in the developing embryo, in the perigastrulation epiblast, around embryonic day 7.5 (E7.5) in the mouse (Magnúsdóttir & Surani, 2014; Saitou & Yamaji, 2012) (Fig. 2). The founding population of mouse primordial germ cells (mPGCs) consists of about 40 cells and arise following specification cues—WNT3 signaling and bone morphogenetic proteins (BMP4, BMP2 and BMP8b)—from the extraembryonic ectoderm and visceral endoderm. Specification results in the induction of germline transcriptional programs, regulated by the lineage-determining transcription factors PRDM14, BLIMP1/PRDM1 and TFAP2C (Tang, Kobayashi, Irie, Dietmann, & Surani, 2016). From E9.5, mPGCs start migrating through the hindgut endoderm towards the genital ridge. The colonization of the genital ridge is accompanied by cell proliferation and an intense wave of epigenetic remodeling, which creates a naïve epigenome (Tang et al., 2016). In particular, mPGCs undergo a global and extensive loss of DNA methylation to reach a level of 5% of global DNA methylation (Guibert, Forné, & Weber, 2012; Hajkova et al., 2002; Seisenberger et al., 2012) (Fig. 2). Histone reprogramming and changes in histone post-translational modifications have been proposed to be functionally coordinated to preserve genomic stability and safeguard the genome from DNA demethylation and spurious transcription (Ancelin et al., 2006; Kurimoto et al., 2015; Seki et al., 2007). Indeed, mPGCs present a progressive reduction in global levels of H3K9me2 followed by an increase in global levels of H3K27me3, an increase of open domains marked by H3K4me1 and a global decrease in H2A/H4R3me2s (Ancelin et al., 2006; Kurimoto et al., 2015; Nagano et al., 2022; Ohta et al., 2017; Seki et al., 2007). In addition, it has been recently shown that the three-dimensional organization of the chromatin within the nucleus also preserves mPGCs from pervasive transcription, with an augmented chromatin insulation mediated by CTCF (Nagano et al., 2022).

At their arrival in the developing somatic gonad, by E11.5, mPGCs are on the onset of gametogenesis, during which sex differentiation and meiosis will occur, ultimately generating the functional gametes: oocytes or spermatozoa (Larose et al., 2019). During this time, mPGCs undergo licensing, a developmental transition regulated by the RNA-binding protein DAZL, that enables the cells to acquire the competence to respond to feminizing or masculinizing cues produced by the fetal ovary or testis (Gill, Hu, Lin, & Page, 2011). Subsequently, mPGCs will progress either to spermatogenesis or oogenesis depending on the sex of the embryo and epigenetics marks will be re-established in a sex-specific manner (Fraser & Lin, 2016; Kota & Feil, 2010; Sasaki & Matsui, 2008).

Oogenesis, the process of formation of mature female gametes, begins with differentiation of female PGCs into oogonia around E13.5 (Sánchez & Smitz, 2012) (Fig. 2). Oogonia proliferate by mitosis to form a pool of primary oocytes that arrest at the prophase stage of the first meiotic division until after birth when they are assembled into primordial follicles. Upon puberty, a small subset of oocytes will resume meiosis and undergo meiotic maturation concomitant with the process of ovulation prior to fertilization. During oogenesis, epigenetic marks are distinctively re-established on the genome (Sendžikait & Kelsey, 2019; Tomizawa, Nowacka-Wozuk, & Kelsey, 2012). At the later stages of their development, after birth, oocytes undergo de novo DNA methylation (Hiura, Obata, Komiyama, Shirai, & Kono, 2006). Mature oocytes are lowly methylated (40%) and present a unique bimodal DNA methylation pattern, with gene bodies of actively-transcribed genes being mostly hypermethylated (>75%) and large-scale genomic domains being hypomethylated (<25%) termed partially-methylated domains (PMDs) (Kobayashi et al., 2012; Veselovska et al., 2015) (Fig. 2). Mechanistically, de novo DNA methylation is mainly mediated by the DNA methyltransferase DNMT3A, guided by DNMT3L (Bourc'his, Xu, Lin, Bollman, & Bestor, 2001; Kaneda et al., 2004; Lucifero et al., 2007). Regions destined for DNA methylation have been shown to present specific histone signatures (reduced H3K4me2/3 and increased H3K36me3), indicating the importance of crosstalk in epigenetic reprogramming (Stewart et al., 2015; Xu et al., 2019). Furthermore, the oocyte PMDs are also associated with non-canonical distributions of histone modifications. Indeed, H3K4me3, a mark typically enriched at active promoters was found on intergenic domains where it accumulates following the activity of the H3K4 methyltransferases MLL2 and SETD1 and correlates with genome-wide transcriptional silencing (Andreu-Vieyra et al., 2010; Dahl et al., 2016; Hanna et al., 2018; Sha et al., 2020; Yu et al., 2017). Accumulation of the repressive mark H3K27me3 has also been reported on the oocyte PMDs but its functional relevance appears less clear (Inoue, Jiang, Lu, Suzuki, & Zhang, 2017; Zheng et al., 2016). A recent study highlighted that H2AK119ub1 was pervasively distributed in oocyte PMDs and that the co-existence of H3K27me3 and H2AK119ub1 at promoters was associated with gene silencing (Mei et al., 2021), further pointing to the importance of mechanistic crosstalk in oocytes epigenetic reprogramming.

Spermatogenesis, the process of formation of mature male germ cells, begins with differentiation of male PGCs into prospermatogonia/spermatogonial stem cells (SSCs) around E13.5 (Fayomi & Orwig, 2018; Griswold, 2016) (Fig. 2). Prospermatogonia arrest at a mitotic G1/G0 phase until puberty during which mitosis and spermatogenesis resumes. Spermatogenesis takes about 34.5 days to complete in the mouse and is classically divided in three major phases: mitotic divisions of prospermatogonia into spermatogonia, two sets of meiotic divisions of spermatogonia to generate the haploid spermatids and spermiogenesis during which spermatids are transformed into spermatozoa (Fig. 2). Contrary to oocytes, spermatozoa DNA is highly methylated (>80%) and de novo DNA methylation is initiated before birth in the prospermatogonia between E15.5 and E18.5 (Ben Maamar, Beck, Nilsson, McCarrey, & Skinner, 2022; Kubo et al., 2015) (Fig. 2). DNA methylation in spermatozoa is evenly distributed, with the exception of the presence of PMDs in intergenic regions (Kubo et al., 2015; Shirane, Miura, Ito, & Lorincz, 2020). Mechanistically, DNMT3A guided by DNMT3L have also been shown to be important for spermatozoa

DNA methylation (Bourc'his et al., 2001; Kato et al., 2007; Kobayashi et al., 2013). As for oocytes, DNA methylation and histone post-translation modifications are functionally linked, with domains destined for DNA methylation marked by H3K36me2 and H3K27me3 (Nagano et al., 2022; Shirane et al., 2020). Importantly, spermatogenesis is characterized by histone-to-protamine transition. While spermatogonia DNA is initially wrapped around canonical histones, these are progressively replaced first by testis-specific histone variants then by protamines during spermiogenesis (Bao & Bedford, 2016). Protamines are small arginine-rich nuclear proteins that allow strong DNA binding and tight packaging into the compact spermatozoa head. About 1% of the mature mouse spermatozoa genome retains canonical histones that present post-translational modifications transmitted to the early zygote and persisting in the early embryo (Brykczynska et al., 2010; Hammoud et al., 2009), suggesting a role for these canonical histones in epigenetic inheritance (Siklenka et al., 2015; Van Der Heijden et al., 2008). Finally, spermatozoa also encode a large proportion of small and long ncRNAs, which roles in sperm epigenetic remodeling remain to be determined (Jodar, 2019), but that have nevertheless been linked to epigenetic inheritance in the offspring (Chen, Yan, & Duan, 2016).

Following fertilization, another extensive epigenetic remodeling occurs asymmetrically to remove parental epigenetic marks in each parental genomes before fusion (Okada & Yamaguchi, 2017). Soon after the entry of the spermatozoon in the oocyte, the paternal genome is demethylated via Tet3-mediated oxidation and protamines are exchanged for the maternally-provided histones that eventually acquire post-translational modifications (Okada & Yamaguchi, 2017). In contrast, the maternal genome remains largely protected from demethylation through the H3K9me2-mediated recruitment of Dppa3/PGC7/STELLA (Nakamura et al., 2012). Certain maternal histone marks, such as H3K36me3, are erased prior zygotic genome activation (ZGA), whereas other marks such as the non-canonically-distributed H3K4me3 are redistributed to canonical promoters upon ZGA (Xia & Xie, 2020). This has led some authors to categorize histone remodeling in the early embryo based on functions: epigenetic marks that are required for gametogenesis, for transcriptional control both in the gametes and in the embryo or “passenger” marks that appear dispensable for the gametes but that are nevertheless maintained and could play a role later in embryogenesis (Xia & Xie, 2020). Of note, although early epigenetic reprogramming erases much of the parental epigenomes, certain loci called imprinting control regions (ICRs) conserve epigenetic features that are parentally-inherited and maintained in the offspring (Tucci et al., 2019). Understanding the mechanisms of epigenetic memory on ICRs is relevant for deciphering the mechanisms of epigenetic inheritance of environmental cues. ZGA marks a key step in early development during which the zygote acquires its transcriptional independency and establishes its own epigenome. As the embryo develops towards the blastocyst stage, active histone marks (H3K4me3 and H3K27ac) are established on promoters and enhancers, respectively, whereas DNA methylation and repressive histone marks are not restored until implantation, which could be functionally relevant for totipotency (Xia & Xie, 2020).

The developmental model for germ cells detailed above corresponds to the one of the mouse and is mostly conserved in mammals. The resolution of the epigenetic remodeling occurring during the germline development in humans has been challenged by technical

and ethical constraints. However the development of in vitro germ cell models and low-input epigenetic interrogation techniques has enabled to demonstrate that, in general, DNA methylation patterns are relatively conserved between mouse and humans (Hanna, Demond, & Kelsey, 2018). This further supports the use of mouse as a model organism for epigenetic studies in the germline.

3.2 Windows of susceptibility

Germ cell development is uniquely regulated by waves of epigenetic erasures and re-establishment. In particular, two major events of epigenetic reprogramming occur during mammalian development: in PGCs, serving to erase established patterns and restore pluripotency potential, and in the early zygote, serving to erase the gametic epigenomes and regain totipotency. For environmentally-induced changes in the epigenome to be passed across generations, they need to be maintained through these epigenetic events. In particular, three temporal windows of susceptibility to environmental cues can be theoretically defined during: (1) gestation, (2) post-natal development of gametes and (3) around conception (Fig. 2). Here we will further detail these temporal windows of susceptibility and the proposed mechanisms of epigenetic inheritance to environmental cues in the germline. We focus on such mechanisms that directly impact germ cells epigenomes, however, change in somatic cells, particularly in gonads, can indirectly alter the epigenome of germ cells (Bline, Le Goff, & Allard, 2020).

During gestation, maternal environmental exposure could affect PGCs' epigenome remodeling. DNA methylation, the most studied epigenetic mark, is globally reduced to the lowest-reported levels in mammalian epigenomes and would, therefore, not be a likely medium for transgenerational inheritance. However, certain loci corresponding to transposable elements have been identified as methylation escapees. Indeed, young long-terminal repeat (LTR) retrotransposons, known as endogenous retroviruses, resist programmed global demethylation during PGC reprogramming. For instance, the intracisternal A-particle (IAP) in the mouse and Alu and HERVK elements in humans have been shown to display higher DNA methylation than the rest of the epigenome (Gkoutela et al., 2015; Guo et al., 2015; Ohta et al., 2017; Seisenberger et al., 2012). Retrotransposon-associated inheritance of epialleles is a well-described phenomenon, particularly in the context of the extensively-studied agouti viable yellow (A^{VY}) epiallele. In agouti mice, an IAP retrotransposon element is inserted upstream of the *agouti* gene (Duhl, Vrieling, Miller, Wolff, & Barsh, 1994). Changes in the IAP LTR methylation status drive phenotypical changes, with unmethylated LTRs promoting ubiquitous expression of *agouti* (Duhl et al., 1994). These A^{VY} mice present a phenotype of yellow fur, obesity, glucose intolerance and increased susceptibility to tumors development (Duhl et al., 1994). Hence, epigenetic changes in endogenous retroviruses can drive inheritance of phenotypes in mammals, however, if the endovirome can serve as hotspots for epigenetic memory in the context transgenerational inheritance remains to be determined (Blake & Watson, 2016; Friedli & Trono, 2015; van Otterdijk & Michels, 2016; Zeng et al., 2022). To do so, the epigenetic mechanisms responsible for endogenous retroviruses silencing during normal development need to be better understood before analyzing the effect of epigenetic enzymes recruitment upon altered environmental conditions.

The sex-specific reestablishment of epigenetic marks on gametes occurs in part after birth and during puberty, with differences between oocytes and spermatozoa. Hence, epigenetic changes in gametes postnatally, following exposures to environmental cues, has been proposed as a mechanism of transgenerational inheritance. Multiple studies have, in fact, reported the existence of aberrant differentially-methylated regions (DMRs) in paternal epigenomes in response to environmental cues (see Section 3). However, it is unlikely that phenotypic changes in the offspring are mediated solely by DNA methylation, considering the importance of epigenetic crosstalk in spermatogenesis. Rather, it is proposed that cumulative effects of dysregulation epigenetic patterns in the earlier development are sustained and exacerbated in transcriptional regulatory networks (Kremsky & Corces, 2020). In this sense, changes in sperm small ncRNAs, that has garnered much attention recently as a medium for epigenetic inheritance, could also participate in this global change (Chen, Yan, & Duan, 2016). Compared to the breadth of data on paternal-mediated epigenetic inheritance, much less is known on maternal-mediated inheritance, mainly due to the paucity of biological material for epigenetic studies. The advent of new techniques for chromatin interrogation and for modeling oocytes development in vitro will bring to light mechanisms of maternal-mediated inheritance in response to environmental cues (see Section 4).

Finally, the periconceptual environment could affect early embryo reprogramming and constitutes another window of susceptibility for TEI. In human, this specific period has been much studied for assisted reproductive technologies (ART), with accumulating evidence suggesting that ART may influence developmental trajectories in the offspring (Fleming et al., 2018). Indeed, despite advances in in vitro culture of embryos, developmental conditions remain sub-optimal (Watkins, Lucas, & Fleming, 2010), indicating the importance of the maternal reproductive tract for proper development and its sensitivity to environmental cues. In hypofertile conditions, it remains unclear what can be attributed to the periconceptual environment or to the parental gametes themselves. However, studies in mouse models have shown how variations in culture media components impact the developmental potential of the embryos (Watkins et al., 2010). In fact, the vast majority of data emerging from periconceptual sensitivity to the environment has been made in the context of maternal dietary environment (Fleming et al., 2018). In humans, maternal undernutrition was shown to be associated with subsequent adult cardiometabolic and neurological dysfunction in the offspring (Velazquez, Fleming, & Watkins, 2019). Mechanistically, metabolic dysfunction are linked with epigenetic mechanisms since several metabolites act as cofactors for epigenetic reactions (Boon, Silveira, & Mostoslavsky, 2020; Li, Egervari, Wang, Berger, & Lu, 2018). However, more studies are needed to define how the mammalian embryo responds to its immediate environment and whether epigenetic memory of periconceptual events are maintained throughout adulthood (Novakovic et al., 2019). This is further complexified by the existence of multiple simultaneous mechanisms of inheritance to environmental cues and confounding factors, particularly in social animals.

4. Evidence for environmentally-induced epigenetic inheritance in mammals

Exposure to environmental cues have the capacity to alter epigenomes and phenotypes. However, TEI postulates that an epigenetic memory of exposure is transmitted to the offspring through the germline without continuous exposure. For maternal inheritance, true transgenerational effects are detectable only at the third generation (F3). Indeed, for exposure in pregnant females (P0), the embryo (F1) is also exposed, as well as its fetal germ cells (F2). Thus, effects without continuous exposures do not occur before the F3 generation (Fig. 3). For paternal inheritance, the F2 is the first generation wherein transgenerational effects can be examined (Fig. 3). While TEI is well accepted in plants (Hauser, Aufsatz, Jonak, & Luschnig, 2011) and invertebrate animals, such as *Caenorhabditis elegans* (Baugh & Day, 2020), the existence or significance of TEI in mammals has been much disputed considering the extensive germline epigenetic reprogramming known to occur. In humans, in particular, evidence for transgenerational epigenetic inheritance to environmental cues is limited (Horsthemke, 2018; Svanes et al., 2021). This is because human studies tend to suffer from heterogeneous populations, reproducibility, recall- and selection- bias among other confounding factors (Marczylo, Jacobs, & Gant, 2016). Despite these conceptual challenges, several studies have provided evidence for at least intergenerational inheritance. Here, we compiled existing evidence of environmentally-induced transgenerational epigenetic inheritance in rodents in response to a range of environmental cues from manufactured chemicals, metals, diet to drugs (Table 1). For each environmental cue, we present the available data in humans and highlight the need for a better understanding of the molecular mechanisms of transgenerational versus intergenerational epigenetic inheritance. Of note, we focus here on environmentally-induced TEI and we do not discuss the epigenetic inheritance of behavioral phenotypes that has been covered elsewhere (Bale, 2014; Jawaid & Mansuy, 2019).

4.1 Manufactured chemicals

The first demonstration of transgenerational inheritance of an environmentally-induced phenotype in rodents was made nearly 20 years ago from studying the agricultural fungicide vinclozolin (Anway et al., 2005). Transient gestational exposure in female rats resulted in impaired sperm motility and reduced fertility in the male offspring up to the F4 (Anway et al., 2005; Anway, Memon, et al., 2006). Female descendants also developed transgenerational phenotypes up to the F4, including tumors and kidney disease (Anway, Leathers, & Skinner, 2006). Mechanistically, a methylated DNA immunoprecipitation followed by a promoter tiling microarray (MeDIP-chip) showed the presence of differentially-demethylated regions (DMRs) in F3 spermatozoa (Guerrero-Bosagna et al., 2010, 2012). However, the stability of the generated epimutations, or their relationship with known genetic abnormalities induced by vinclozolin were not assessed. Nevertheless, a following study showed that gestational vinclozolin exposure significantly altered F3 sperm-borne small ncRNAs that correlated with the DMRs and the vinclozolin transgenerational phenotypes (Schuster, Skinner, & Yan, 2016), suggesting a concerted vinclozolin-induced epigenetic transgenerational inheritance phenomenon.

Since these pioneering observations, exposure to multiple other manufactured chemicals, belonging to the large class of endocrine-disrupting chemicals (EDCs), have been shown to be inherited transgenerationally in rodents. However, the epigenetic mechanisms at play have not always been identified. Despite this, the question of EDCs-mediated epigenetic remodeling in the germline and phenotypic inheritance across generations needs to be urgently addressed. Indeed, EDCs are ubiquitous in our environment and because they directly interfere with the endocrine hormonal system, EDCs have been linked with multiple disorders in humans, including pregnancy complications, genital malformations, and cancer (Xin, Susiarjo, & Bartolomei, 2015).

Diethylstilbestrol (DES), a nonsteroidal estrogen, was initially prescribed in the 1950s with the goal of reducing miscarriage (Fénichel, Brucker-Davis, & Chevalier, 2015; Titus et al., 2019). The medication was taken off market in the 1970s after discovering cases of vaginal clear cell adenocarcinoma and breast cancer in the offspring born to women who were gestationally exposed to DES (Fénichel et al., 2015). The cohort of children resulting from DES-exposed women were also found to develop genital tract malformations, including hypospadias and cryptorchidism in males and uterine abnormalities in females (Fénichel et al., 2015). Additionally, both sexes were more likely to develop non-reproductive diseases including diabetes, hypercholesterolemia, hypertension and osteoporosis (Fénichel et al., 2015). Beyond intergenerational effects from mother to offspring, several studies have confirmed that DES has transgenerational effects up to the third generation (Fénichel et al., 2015; Titus et al., 2019). In particular, a study demonstrated that women from the third generation following DES gestational exposure developed higher rates of menstrual irregularity, preterm delivery and vaginal epithelial changes compared to the offspring of unexposed women (Titus et al., 2019). In addition, men from the third generation following DES gestational exposure had a higher likelihood of congenital anomalies, including hypospadias and heart defects (Fénichel et al., 2015). Without any chromosomal or genetic alterations, these offspring of DES-exposed women provide evidence for transmission of epigenetic inheritance across generations. Although the molecular mechanisms of transgenerational inheritance remain unclear, DES exposure may epigenetically alter estrogen receptors and impact the role of sex steroids in differentiating the genital tract during an important fetal exposure window (Fénichel et al., 2015; Titus et al., 2019).

Dioxin/TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is a by-product of industrial combustion processes and gestational exposure of this EDC has been shown to be transgenerationally inherited up to the F3, with an observed phenotype of reduced female fertility (Bruner-Tran & Osteen, 2011). Further studies in rats showed a similar phenotype in the F3 and that the F3 sperm epigenome was altered, with the identification of several DMRs (Manikkam et al., 2012). Again, the functional relevance of these DMRs, and their conservation across rodent models, need to be addressed to properly establish the mechanisms of TEI in response to dioxin. Dioxin exposure in humans is widespread and occurs primarily via food ingestion or contact with contaminated soil, air, and water (Travis & Hattemer-Frey, 1991). Studies in an Italian human cohort, following the explosion of a trichlorophenol manufacturing plant and the release of up to 30 kg of TCDD, showed that men whose mothers were exposed in utero to dioxin had impaired semen quality (reduction in spermatozoa concentration, count and quality) (Mocarelli et al., 2011). In a Russian

cohort, whole genome bisulfite sequencing (WGBS) showed the presence of spermatozoa DMRs associated with serum TCDD concentrations (Pilsner et al., 2018). Together, these studies show that dioxin provokes spermatozoa epimutations and intergenerational phenotypes in humans, although impact across a larger number of generations has not been studied.

The plastic monomer bisphenol A (BPA) is another widespread EDC that is readily measured in humans following exposure to food and beverage containers (Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007). BPA directly impacts human metabolic and reproductive organs (Rochester, 2013). However, epidemiological studies tend to support that, although BPA can cause fetal death and growth impairment during infancy at high dose, the risks for low-dose exposure are negligible (Ranjit, Siefert, & Padmanabhan, 2010). Contrarily to the evident paucity of research in humans, several studies in rodents have addressed the consequences of in-utero exposure to BPA across generations. BPA exposure has been associated with transgenerationally-acquired phenotypes of reduced fertility and adult onset disease, correlating with spermatozoa DMRs serving as BPA epimutations (Manikkam et al., 2013; Ziv-Gal et al., 2015). In a recent study focused on paternal inheritance of BPA exposure in mice, P0 males were exposed during the entire period of sexual maturity/puberty and phenotypes were analyzed in subsequent generations (Saidur Rahman et al., 2020). Both F2 and F3 males presented reduced fertility and a global increase in DNA methylation in spermatozoa. This study highlights that EDCs provoke transgenerational phenotypes that are both maternally and paternally inherited. Considering the different role of DNA methylation in oogenesis and spermatogenesis, further studies will need to focus on a better understanding of EDCs-mediated epigenetic remodeling in germ cells from P0 exposed parents.

In this regard, epigenetic crosstalk between histone PTMs and DNA methylation might be relevant in ensuring transgenerational inheritance to EDCs. For instance, gestational exposure to the herbicide atrazine was associated with reduced spermatozoa number in male F3 due to altered spermiogenesis (Hao et al., 2016). This phenotype was associated with a transgenerational global decrease in H3K4me3 level in the F3 (Hao et al., 2016). Occupancy of H3K4me3 was studied by chromatin immunoprecipitation followed by sequencing (ChIP-seq) and integration with transcriptomic data demonstrated that the epigenetic status of promoters of genes associated with germ cell differentiation were transgenerationally altered. Additionally, differential expression of lncRNAs in the F3 was also observed (Hao et al., 2016), further pointing to more potential mechanistic cross-talk in transgenerational inheritance of atrazine.

4.2 Metals

Toxic metals are ubiquitous in our environment and exposure has been associated with serious adverse health outcomes in human populations, such as cancer, cardiovascular diseases, and neurological disorders (Ray, Yosim, & Fry, 2014). With accumulating data showing that environmental toxic metals can impact epigenomes (Ray et al., 2014), their transgenerational inheritance has started to be studied in more depth.

Arsenic is a ubiquitous naturally-occurring toxic substance in the environment (Chung, Do, & Hong, 2014). Arsenic compounds dissolve readily in water; thus, consumption of contaminated water is a global route of exposure (Chung et al., 2014). Arsenic exposure is associated with a constellation of negative health effects impacting all major organs and organ systems and is a major carcinogen (Argos et al., 2010; Smith et al., 1992). For instance, in a Mexican human cohort, elevated arsenic levels in water correlated with hypermethylation in tumor-suppressor genes, pointing towards an epigenetic component in arsenic carcinogenic capacity (Smeester et al., 2011). Transgenerational phenotypes in response to arsenic exposure have been reported in *Caenorhabditis elegans* (Yu & Liao, 2016), and in zebrafish (Hernández-Sánchez, Valles, & Bardullas, 2022). In humans, a study in a Bengali cohort demonstrated that arsenic exposure in early pregnancy altered the cord blood methylome of the offspring, with more pronounced effects in male than in female newborns (Broberg et al., 2014). The identified DNA methylation changes were associated with cancer-related genes, which would suggest a sex specificity in arsenic carcinogenic capacity (Broberg et al., 2014). In agreement with epidemiological studies in humans, intergenerational effects of parental arsenic exposure have been reported in the mouse (Gong et al., 2021; Yin et al., 2021) and in the rat (Nava-Rivera et al., 2021). The first study on transgenerational inheritance of arsenic exposure in the mouse identified that pre-conceptional paternal exposure was associated with reduced adiposity in the F2 and increased adiposity in the F3 (Gong et al., 2021). Epigenetic mechanisms were not assessed but their adaptative changes were proposed to be responsible for the opposed phenotypes between the F2 and the F3 generations (Gong et al., 2021). More recently, another study in a mouse model of maternal gestational exposure to arsenic showed that the male F3 had impaired fertility associated with a decrease in the methylation levels in two specific imprinted genes (Yin et al., 2021). Finally, a study of pre-conceptional exposure to arsenic in both male and female rats showed impaired reproductive functions in the F2/F3 that were associated with a global increase in DNA methylation in gonads (Nava-Rivera et al., 2021). Thus, while there is evidence of phenotypic transgenerational inheritance of arsenic exposure, the role of DNA methylation in this phenomenon is unclear. This is representative of the unsatisfactory understanding of the effects of arsenic exposure on DNA methylation. Indeed, arsenic exposure provokes both global DNA hypomethylation, due to reduction in the levels of the universal methyl donor S-Adenosyl Methionine SAM, and hypermethylation of specific loci during carcinogenesis (Reichard & Puga, 2010; Ren et al., 2011). More fundamental studies are therefore needed to resolve the opposite effects of arsenic on DNA methylation (genome-wide vs locus-specific). Furthermore, SAM is also the methyl donor for histone methylation; thus, epigenetic crosstalks in arsenic TEI should be investigated.

The number of studies on transgenerational inheritance of metal exposure in rodents is limited, despite their wide distribution in our environment, their reported epigenetic inheritance in other animals (Carvan et al., 2017; Mu et al., 2021; Vandegehuchte, Lemièrre, Vanhaecke, Vanden Berghe, & Janssen, 2010) and epidemiological studies in humans (Maloney, Bayon, Zawia, & Lahiri, 2018; Sen et al., 2015). Cadmium and mercury co-administration in gestational mice was associated with reduced glucose tolerance up to the male F4 generation (Camsari et al., 2019). Lead gestational exposure in mice provoked

growth stunt in the F3 generation (Sobolewski et al., 2020). However, in both studies, potential epigenetic mechanisms for transgenerational inheritance were not studied. Overall, mercury, cadmium, lead, selenium, and aluminum are all prevalent environmental toxic metals and their potential TEI in Mammals needs to be urgently examined.

4.3 Diet

Early long-term epidemiological studies and some empirical observations were the first testimonies that parental nutrition affects the offspring health (Guo, Luo, & Lin, 2020; Soubry, 2015). In recent years, studies in animal models have shed light on how changes in parental diets can modulate germ cells metabolism and epigenomes and induce phenotypic changes in the offspring, particularly the development of metabolic diseases. Here, we discuss how high-fat diet, caloric restriction or diet composition can be transgenerationally-inherited in the offspring upon epigenetic changes in the germline.

Increasing prevalence of obesity has become a major global health concern. A critical question is whether obesity in parents can influence the development of metabolic diseases over several generations in the offspring. Modeling of obesity in rodent models, using high-fat diet (HFD), has revealed the existence of transgenerational epigenetic inheritance of parental diet, both maternally and paternally. Maternal HFD during pregnancy is known to increase the rates of obesity and metabolic syndrome in the first and second generations (Kaspar, Hastreiter, Irmeler, Hrabé de Angelis, & Beckers, 2020; Wu & Suzuki, 2006). However, maternal HFD can also be transmitted transgenerationally up to the third generation. Indeed, the F3 female offspring of mice fed with HFD before pregnancy showed increased body size and improved glucose tolerance, that were linked with dynamic patterns in imprinted genes expression (Dunn & Bale, 2011). More recently, the male F3 offspring of mothers perinatally fed with a HFD were shown to present an increased body weight and an impaired sensitivity to insulin (Sarker et al., 2018). These phenotypes were correlated with DNA methylation changes and signature DMRs in F2 spermatozoa (Sarker et al., 2018). Interestingly, observations regarding the phenotypes in the third generation vary between sexes (Sarker et al., 2018) and pre-conceptional versus gestational HFD also provoked different transgenerational phenotypes in the two above-mentioned studies. This indicates that multiple epigenetic mechanisms might be at play in maternal inheritance of HFD and more research is needed (Kaspar et al., 2020). Most studies on transgenerational inheritance of HFD have focused on the paternal route. The first study to address this question showed that female F1 descendent of male rat fed with HFD presented an impaired glucose-insulin homeostasis (Ng et al., 2010). This intergenerational phenotype for paternal inheritance of HFD in the rat was later shown to be transgenerationally transmitted by spermatozoa up to the second generation (de Castro et al., 2016). Indeed, HFD was associated with changes in F1 spermatozoa small RNAs consistent with their metabolic functions and the F2 phenotype (de Castro et al., 2016). Several studies have substantiated these findings in mice and shown that HFD provokes spermatozoa epigenome changes, in terms of DNA methylation and altered small RNA profiles (Chen, Yan, Cao, et al., 2016; Fullston et al., 2013; Wei et al., 2014). Since spermatozoa DNA methylation changes in response to HFD could not completely recapitulate the transcriptional outcomes in the offspring (Wei et al., 2014), attention is now shifting to the understanding of concerted epigenetic reprogramming in

spermatozoa following HFD. Another open question is whether dietary changes can restore spermatozoa epigenomes after weight loss.

Inadequate protein intake is one example of malnutrition and can be modeled by feeding rodents with low-protein diet. One study found that the F1 generation of male mice fed with a low-protein diet had reduced DNA methylation levels in the liver (Carone et al., 2010). These epigenetic signatures correlated with increased expression of genes involved in lipid and cholesterol biosynthesis (Carone et al., 2010). However, a later study with the same mice exposure paradigm showed that this intergenerational phenotype was not associated with changes in spermatozoa methylome (Shea et al., 2015), pointing towards the existence of other epigenetic mechanisms such as histone PTMs or small RNAs (Yoshida et al., 2020). In addition, a rat maternal low-protein diet also resulted in altered DNA methylation in the liver of male pups, due to decreased expression of the DNA methylation machinery (Gong, Pan, & Chen, 2010). These studies indicate that low-protein diet results at least in intergenerational phenotypes in rodents. Epidemiological studies in humans show how maternal low protein diet is associated with the lean variant of type 2 diabetes (Vipin, Blesson, & Yallampalli, 2022). Considering the worldwide increase in vegetarian and vegan diets, which are typically associated with low protein consumption (Vipin et al., 2022), more data will be needed to determine whether low protein diet associate with inter- and transgenerational epigenetic inheritance of metabolic disorders.

Undernutrition in early life may affect adult health. The famine period in the Netherlands during World War II, known as the Dutch Hunger Winter of 1944–1945, has allowed to study the intergenerational effect of parental nutrition on the metabolic status of grandchildren in humans (Lumey, Stein, & Susser, 2011). In this historical cohort, famine exposure during pregnancy (daily average of 900 kca/day or less) was associated with an adverse metabolic profile (suboptimal glucose handling, higher body mass index, etc.) and a higher risk for developing schizophrenia in the F1 offspring (Lumey et al., 2011). Adult offspring (F2) of gestationally exposed F1 fathers, but not mothers, were also found to present higher body mass index, suggesting a paternal intergenerational inheritance over two generations (Veenendaal et al., 2013). Subsequently, an methylome-wide approach by reduced representation bisulfite sequencing (RRBS) in the blood of F1 individuals demonstrated the presence of DMRs associated with the phenotypic outcome of early-life exposure to famine (Tobi et al., 2014). Studies in rodents support that gestational undernutrition in mice provokes intergenerational developmental reprogramming, with the apparition of DMRs in the F1 spermatozoa methylome (Radford et al., 2014) (Radford et al., 2014). Male mice undernutrition also resulted in spermatozoa global demethylation and an offspring with reduced postnatal weight (McPherson et al., 2016). However, the only evidence of TEI of undernutrition was found in the rat (Nowacka-Wozzuk, Szczerbal, Malinowska, & Chmurzynska, 2018). Indeed, the F3 fetuses of pregnant females fed with a restricted diet presented lower hepatic levels of histone deacetylases (HDAC1 and SIN3A) but the increased H3 acetylation was not statistically significant (Nowacka-Wozzuk et al., 2018). Altogether, more studies will be needed to pinpoint the epigenetic mechanisms of inter- and transgenerational inheritance of under- and malnutrition.

The impact of diet onto developmental processes needs to be placed in the larger context of the role of metabolism in early development and of the interplay between metabolism and epigenetics (Dahan, Lu, Nguyen, Kennedy, & Teitell, 2019; Verdikt & Allard, 2021). In this regard, another aspect of nutrition that has been explored is the change in diet composition, in particular, how micro-nutrient deficiency can impact gametes epigenomes and alter the offspring development. Folate is a metabolite that serves as a methyl-donor and its levels influence the availability of SAM (Clare, Brassington, Kwong, & Sinclair, 2019). Female mice carrying mutations in genes involved in the folate metabolism gave rise to an offspring with congenital malformations and tissue-specific loss of DNA methylation (Padmanabhan et al., 2013), demonstrating the importance of folate metabolism in maternal epigenetic inheritance. Folic acid supplementation is often recommended in early pregnancy and studies in humans indicate that changes in maternal plasma folate levels are associated with epigenome-wide DNA methylation variations in newborn cord blood (Caffrey et al., 2018; Joubert et al., 2016). In a recent study in the mouse, paternal inheritance of folate deficiency was shown to occur through dysregulation of H3K4me3 in spermatozoa (Lismer et al., 2021). Importantly, regions with altered H3K4me3 profiles in spermatozoa corresponded to developmental loci, were retained in the zygote and overlapped with differential zygotic gene expression (Lismer et al., 2021). Further research will need to determine whether folate deficiency-induced histone retention interplays with DNA methylation in transgenerational epigenetic inheritance. Another open question is whether the epigenetic modes of inheritance of folate deficiency vary in a sex-specific manner, considering the different role of chromatin methylation in gametogenesis.

4.4 Substances use and abuse

The evidence for epigenetic modifications following exposure to drugs of abuse, and their consequences on rewiring neuronal circuitry to maintain addiction, is well documented (Nestler, 2014). In recent years, several studies have started to address whether drugs of abuse can alter gametes epigenomes and the consequences for transgenerational inheritance (Wanner, Colwell, & Faulk, 2019; Yohn, Bartolomei, & Blendy, 2015; Zeid & Gould, 2020). Inheritance of drug abuse is particularly confounded by genetic and non-genetic (social, cultural, and environmental) factors (Yohn et al., 2015). Hence, modeling parental inheritance to drug abuse in rodent models provides mechanistic bases, i.e., the identification of epigenetic biomarkers, to study the relevance of TEI of drug abuse in human populations.

Ethyl alcohol (ethanol) effects are mediated through interference with several neurotransmitter systems (Chastain, 2006). Alcohol exposure provokes epigenetic and transcriptional changes in the brain, particularly in regions critical to addictive behaviors (Berkel & Pandey, 2017). Mechanistically, alcohol was shown to reduce the production of SAM provoking global hypomethylation in rat liver (Lu & Mato, 2005) as well as cause histone hyperacetylation by directly providing the acetyl groups through ethanol metabolism (Mews et al., 2019), although a tissue-specific understanding of epigenetic remodeling following alcohol exposure is currently lacking. In addition, alcohol exposure provokes genome-wide alteration in DNA methylation patterns in germ cells and several occurrences of intergenerational inheritance of these changes have been found both in rodent models and in human cohorts (Yohn et al., 2015). The risks of maternal alcohol abuse during pregnancy

are well-established; however, gestational exposure to alcohol (prenatal ethanol exposure, PrEE) also provokes phenotypic and epigenetic changes across generations. For instance, in a rat model for fetal alcohol exposure, male from the F3 generation presented significant reduced expression of the proopiomelanocortin gene in the hypothalamus, concomitant with increased DNA methylation on its CGIs (Govorko et al., 2012). The fact that F3 male but not female progeny presented these changes support a paternal inheritance, although epigenome-wide studies in the brain are needed to confirm the observation. A similar paternal inheritance of gestational exposure to ethanol was shown in the mouse, where the F3 offspring presented an altered development of the neocortex, associated with a decreased expression of all three DNA methyltransferases and a global neocortical hypomethylation (Abbott et al., 2018). A study in the same transgenerational mouse model for fetal alcohol exposure confirmed that the developmental changes were associated with altered behavioral phenotypes (Bottom et al., 2022). Overall, these studies highlight the importance of paternal inheritance in the transmission of alcohol exposure. Considering recent works showing that chronic ethanol exposure alters spermatozoa small ncRNAs, and their nucleoside modifications (Rompala et al., 2018), further works will need to assess the contribution of pre-conceptional ethanol exposure to TEI of alcohol abuse.

The abuse of opioids, including the illegal drug heroin and pain relievers such as morphine, fentanyl, codeine, and oxycodone, has emerged as a major health threat over the last decade (Gilardi, Augsburger, & Thomas, 2018). Prevalence of opioid medication signifies that exposure in pregnant mothers has increased (Gilardi et al., 2018). While accumulating data evidence that gestational exposure provokes intergenerational phenotypic and epigenetic changes (Gilardi et al., 2018; Zeid & Gould, 2020), there are currently no studies examining epigenetic transgenerational effects of opioids beyond the F1 generation. This is true for another illicit drug: cocaine. Indeed, epigenetic reprogramming in male germ cells has been demonstrated in response to cocaine exposure (González et al., 2020; Swinford-Jackson et al., 2022) and intergenerational inheritance has been reported (He, Lidow, & Lidow, 2006; Swinford-Jackson et al., 2022; Vassoler, White, Schmidt, Sadri-Vakili, & Christopher, 2013). However, future studies will need to address whether pre-conceptional or gestational cocaine exposure can be transmitted through epigenetic remodeling of germ cells across generations.

With the growing use of medical and recreational cannabis, consumption of cannabis products has dramatically increased in recent years, including by expecting mothers (Carrier, Huestis, Zaami, Pichini, & Busardò, 2020; Volkow, Han, Compton, & McCance-Katz, 2019). More than 100 phytocannabinoids have been isolated from *Cannabis sativa*, with (–)-trans-9-tetrahydrocannabinol (9-THC) being the predominant phytocannabinoid responsible for the psychoactivity of cannabis (Andre, Hausman, & Guerriero, 2016). As a result, the level of 9-THC in cannabis has reached up to 20% of total compounds (Chandra et al., 2019). In recent years, several studies have shown that effects of gestational 9-THC exposure are intergenerationally inherited in rodents. Indeed, the descendants of rats exposed to 9-THC present functional alterations in brain regions controlling compulsive and goal-directed behaviors (Prini et al., 2018; Szutorisz et al., 2014; Vassoler, Johnson, & Byrnes, 2013; Watson et al., 2015). Furthermore, 9-THC also has a clearly described impact on the epigenome, especially on DNA methylation and histone PTMs both in the brain (Prini et al., 2018; Prini, Penna, Sciuccati, Alberio, & Rubino, 2017; Szutorisz & Hurd, 2018; Watson et

al., 2015) and in spermatozoa (Murphy et al., 2018; Osborne et al., 2020; Schrott & Murphy, 2020). In particular, one study focusing on cross-comparison of spermatozoa methylome in 9-THC-exposed rats and human cannabis users showed that similar pathways were altered (Murphy et al., 2018). These DMRs could further serve as biomarkers when investigating the existence of TEI for cannabis in Mammals.

Finally, accumulating data indicate intergenerational epigenetic inheritance following exposure to tobacco smoke (Hammer et al., 2018). In particular, prior evidence has demonstrated that maternal smoking contributes to asthma and poor lung function in the offspring (Magnus et al., 2015; Rehan, Liu, Sakurai, & Torday, 2013). Whether transgenerational inheritance of asthma occurs in humans is more conflicting. Extensive population-based studies have found a significant correlation between a grandmother's smoking exposure and asthma in grandchildren, independent of the mother's smoking exposure (Li, Langholz, Salam, & Gilliland, 2005; Magnus et al., 2015). Mechanistically, the transgenerational effects of smoking on asthma susceptibility could arise from differential DNA methylation in fetal oocytes (Li et al., 2005). In this regard, newborn cord blood samples from the Norwegian Mother and Child Cohort Study have confirmed the existence of altered methylation patterns in newborns born to women who smoked (Joubert et al., 2012). Additionally, a Swedish Birth Cohort study observed higher DNA methylation of insulin-like growth factor 2 (IGF2) in newborns whose mothers smoked; this was correlated to the paternally expressed IGF2 with male offspring preferentially affected (Krauss-Etschmann, Meyer, Dehmel, & Hylkema, 2015). Thus, epigenetic mechanisms may play a role in explaining the increasing incidence of asthma in the offspring of smoking mothers. However, whether epigenetic changes in the germline arise from nicotine, the active component of tobacco, or other chemical constituents of tobacco is difficult to assess retroactively (Zeid & Gould, 2020). Studies in rodents have shown that nicotine can remodel the chromatin through DNA methylation and changes in histone PTMs (Levine et al., 2011; Toledo-Rodriguez et al., 2010). Intergenerational epigenetic inheritance of nicotine exposure has been reported in mice (Dai et al., 2017). However, to date, few studies have addressed the mechanisms of epigenetic inheritance to nicotine exposure in rodents further than the second generation. The F2 offspring of male mice exposed to nicotine displayed behavioral changes that might be due to the observed changes in P0 spermatozoa methylome (McCarthy et al., 2018). This warrants further research to understand the epigenetic mechanisms of paternal inheritance to nicotine exposure. Given the rise in the use of smokeless tobacco products, electronic cigarettes in particular (McCarthy et al., 2020), another open question is whether nicotine and other components, such as flavoring agents, synergize in the epigenetic remodeling of germ cells. Data in a mouse intergenerational model for electronic cigarette showed that co-exposure to nicotine and saccharin in the P0 provoked memory deficits in the F1 whereas individual exposure to either substance alone did not (McCarthy et al., 2020). Overall, as for other drugs of abuse, research on TEI of nicotine is still in its early stage. An additional difficulty for nicotine, as well as cannabis and opioids, is to adapt the dose and delivery mode in rodent models as to mimic the best the reported uses in human populations.

5. Harnessing in vitro models for gene-environment interaction studies

Despite multiple lines of evidence for transgenerational inheritance in rodents, our understanding of the epigenetic mechanisms involved in phenotypic inheritance across multiple generations remains limited. Furthermore, the existence of epigenetic inheritance of phenotypes further than the second generation in humans remains controversial. Here we discuss the current limitations in studies on the impact of the environment on germs cells. We further highlight the potential of in vitro germ cell models and in vitro gametogenesis for TEI studies.

5.1 Current limitations to TEI studies in mammals

Beyond epigenetic inheritance, studies on the impact on the environment on subsequent generations must account for other mechanisms that may be interrelated, including genetic changes and non-genetic changes on the individual, ecological, cultural, or social levels (Danchin et al., 2011). Traditional Mendelian inheritance postulates that phenotypes are inherited based on the transmission of DNA across generations and that abnormal DNA sequences lead to abnormal phenotypes (Senaldi & Smith-Raska, 2020). Epigenetic inheritance builds on this genetic model of inheritance and adds that the chromatin structure of gametes, not just their DNA sequence, also contributes to the offspring phenotype. However, an under-appreciated dimension in inheritance studies is how genetic and epigenetic information are interrelated. For instance, DNA sequence variations affect DNA methylation, not just in CpG loci, but genome-wide (Hannon et al., 2018; McRae et al., 2014). In fact, genetic heritability was shown to be the major cause of similarity in DNA methylation levels in a human cohort (McRae et al., 2014), although the mechanisms at play or the contribution of non-genetic inheritance were not assessed. Another dimension is the evolution of epigenomes over time. Indeed, aging is associated with changes in the chromatin structure both globally and locally (Kane & Sinclair, 2019). Hence, aging should be taken into account when studying interactions between the environment and epigenomes (Barrere-Cain & Allard, 2020; Bertozzi et al., 2021). Finally, non-genetic factors also contribute to epigenetic inheritance of phenotypes. In social animals, the inherited environment is shaped by social status, cultural norms and acquired behaviors (Danchin et al., 2011). For instance, in humans, socioeconomic status, political orientation or religious practices may influence a child's environment, with consequences on their epigenome and phenotype (Danchin et al., 2011). Overall, the epigenome is shaped by its interaction with the genome and the inherited environment. These confounding factors should be considered in the context of an extended theory of inheritance. In addition, studies aiming at proving TEI in animal models must account for genetic biases (by controlling the genetic background) and acknowledge the extent of non-genetic inheritance.

Another major issue in TEI studies is a technical one. Indeed, while epigenetic studies in male germ cells can be easily performed, considering the relatively large amount of material and accessibility, the small number of oocytes in vivo prevent detailed molecular analyses. In fact, the only epigenetic evidence of inheritance of phenotypes across generations following environmental exposure in rodents are paternal, mostly associated with detectable changes in spermatozoa methylome and ncRNAs profiles (Table 1). The development of

new low-input chromatin analyses has allowed to further our understanding of the epigenetic mechanisms regulating the homeostatic development of oocytes (Skene & Henikoff, 2017; Xu & Xie, 2018). It is therefore likely that the same techniques will reveal molecular mechanisms of maternal inheritance of environmentally-induced phenotypes.

5.2 In vitro germ cell models

In parallel to the development of low-input chromatin techniques, epigenetic inheritance studies can benefit from in vitro models for germ cells development. Here, we briefly summarize the existing models and highlight how in vitro gametogenesis can be harnessed in studying the impact of the environment on germ cells epigenome.

The generation of functional mature and fertile gametes in vitro requires the reconstitution of the multi-stepped development of germ cells in vivo. Mouse embryonic stem cells (mESCs) are pluripotent stem cells derived from the inner cell mass of blastocysts and thus constitute an immortalized representation of the preimplantation embryo (Evans & Kaufman, 1981; Nichols & Smith, 2009). In early forays into in vitro gametogenesis, direct derivations of mPGCs from mESCs were found to be poorly efficient, with the induced cells not contributing to healthy offspring (Hayashi, Kawaguchi, Durcova-Hills, & Imai, 2018). The first successful reconstitution of the fetal mouse germ cell specification pathway in vitro was made over 10 years ago. In this system, the so-called mouse PGC-like cells (mPGCLCs) are obtained in a two-step differentiation from mESCs to a mouse epiblast-like intermediate (mEpiLCs), therefore mimicking the stepwise differentiation of mPGCs (Hayashi et al., 2018; Hayashi, Ohta, Kurimoto, Aramaki, & Saitou, 2011). Mouse PGCLCs have been found to accurately recapitulate early developmental processes and epigenetic remodeling of mouse PGCs (Hayashi et al., 2011; Ohta et al., 2017; von Meyenn et al., 2016). In particular, using transgenic ESCs harboring fluorescent reporters for germline markers, properly-specified mPGCLCs can be isolated by flow cytometry (Ohinata, Sano, Shigeta, Yamanaka, & Saitou, 2008). The scalability of this PGCLCs differentiation protocol allows, in turn, the rapid generation of a large number of cells, making of it a powerful system for the interrogation of underlying transcriptional and epigenetic mechanisms. In this regard, we have recently explored the developmental trajectory of mPGCLCs following exposure to BPA and showed a dose-dependent effect on mPGCLCs proliferation in XX but not XY cells (Ooi, Jiang, Kang, & Allard, 2021). Furthermore, mPGCLCs transcriptome revealed a dysregulation of X-linked genes and retrotransposons (Ooi et al., 2021). These results represent the first characterization of the effect of a toxicant exposure on fetal germ cells, highlighting their potential for TEI studies.

In the original mPGCLCs protocol, XY cells were shown to properly undergo spermatogenesis following transplantation in the seminiferous tubules of neonatal mice, whereas XX cells aggregated with female gonadal somatic cells in reconstituted ovaries also underwent maturation to oocytes (Hayashi et al., 2011). Since then, multiple studies have attempted to recreate the entire process of gametogenesis in culture. Today, in vitro full reconstitution of mouse spermatogenesis and oogenesis is a reality (Hikabe et al., 2016; Komeya, Sato, & Ogawa, 2018; Luo & Yu, 2022), with the micro-environment of the gonads and communication with somatic cells being replicated in organoids (Yoshino et al.,

2021). However, transposition to human gametogenesis has been hindered by ethical and technical limitations (Luo & Yu, 2022). For instance, human PGCLCs (hPGCLCs) can be obtained from human induced-pluripotent stem cells (hiPSCs), however, proper induction cannot be confirmed by the production of fertile offspring as done in the mouse, for obvious ethical reasons (Luo & Yu, 2022). Furthermore, development of germ cells require cocultures of hPGCLCs with human embryonic somatic gonadal cells, which again, cannot be easily obtained for ethical and technical reasons (Luo & Yu, 2022). With the advent of organoid research (Yoshino et al., 2021), reconstituted gonads will provide a platform to study the molecular mechanisms of mammalian germ cells differentiation. These in vitro gametogenesis systems can also be harnessed for TEI studies, by allowing the expansion of initial material for downstream epigenetic interrogation techniques.

6. Conclusion and future challenges

Epigenetic mechanisms represent a formidable tool for organisms to evolve with their environment. Whether transgenerational inheritance of environmental cues across multiple generations occurs in Mammals is still a very much debated topic. Despite the epidemiological evidence in humans and phenotypical evidence in rodent models that we have described, research on the molecular mechanisms of TEI is still in its infancy and multiple open questions remain.

Future studies will need to define what are the epigenetic mechanisms of transgenerational inheritance. We have shown that oftentimes, changes in male germ cells methylome are pointed as a mode of inheritance. However, we have also discussed how DNA methylation and other epigenetic mechanisms, such as histone PTMs, for instance, are coordinated in homeostatic development of germ cells. Hence, understanding how environmental cues alter epigenetic crosstalks in the germline will be critical for TEI studies. Similarly, deciphering the role of sex specificity in epigenetic remodeling in response to the environment will shed light on conflicting results regarding maternal versus paternal modes of inheritance.

Another dimension is an ethical and practical one: if we are to prove epigenetic remodeling in human germ cells and in human embryos, we will need to rely on models. Most studies have been performed in rodent models, which, ultimately do not recapitulate fully the extent of epigenetic remodeling occurring in humans. In addition to these animal models, we have highlighted the relevance of using in vitro gametogenesis to unravel the molecular mechanisms of epigenetic inheritance in response to environmental changes.

Overall, while the study of transgenerational inheritance in response to changes in our environment is still in its early stages, the development of new tools, techniques and models opens the way for exciting future research that may reshape once more our definition of “epigenetics” in an extended theory of inheritance.

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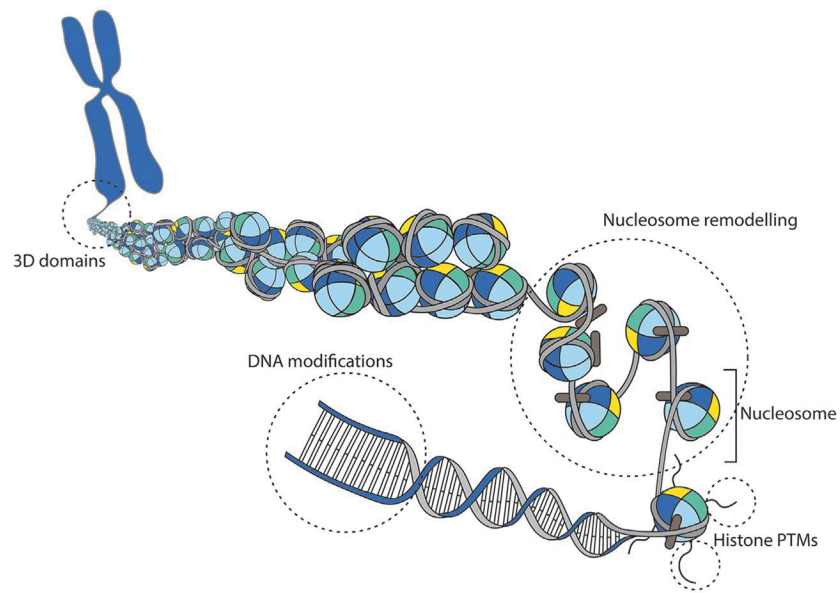


Fig. 1. Epigenetic Regulation of Chromatin Structure. The nucleosome is the basic structural unit of chromatin and consists in 146 base pairs of DNA tightly packaged around an octameric protein core of histones. Epigenetic mechanisms include the positioning and remodeling of nucleosomes, post-translational modifications of histones, chemical modifications of DNA itself and higher organization of three-dimensional chromatin domains.

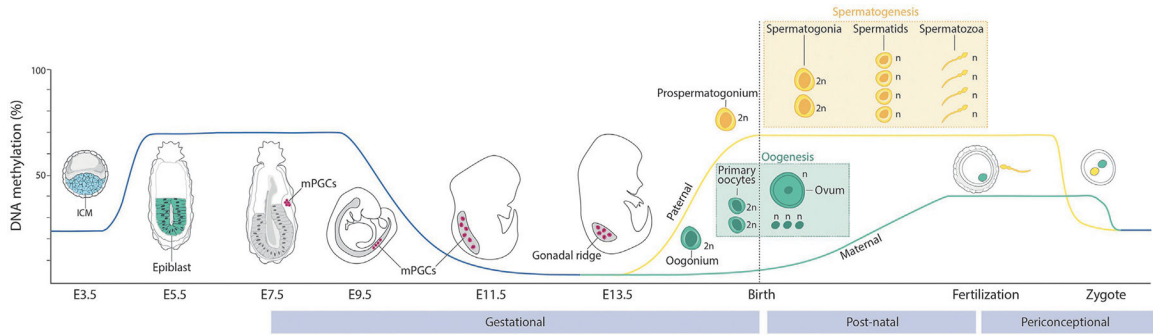


Fig. 2. Epigenetic Remodeling during germline development offer unique exposure windows for inheritance. Variations in DNA methylation levels (%) are shown during germline development in the mouse. Spermatogenesis and oogenesis are summarized and depicted in respective frames. Windows of susceptibility for epigenetic memory to environmental exposures are shown for the gestational, post-natal and periconceptual periods, respectively. $2n$ = diploid, n = haploid.

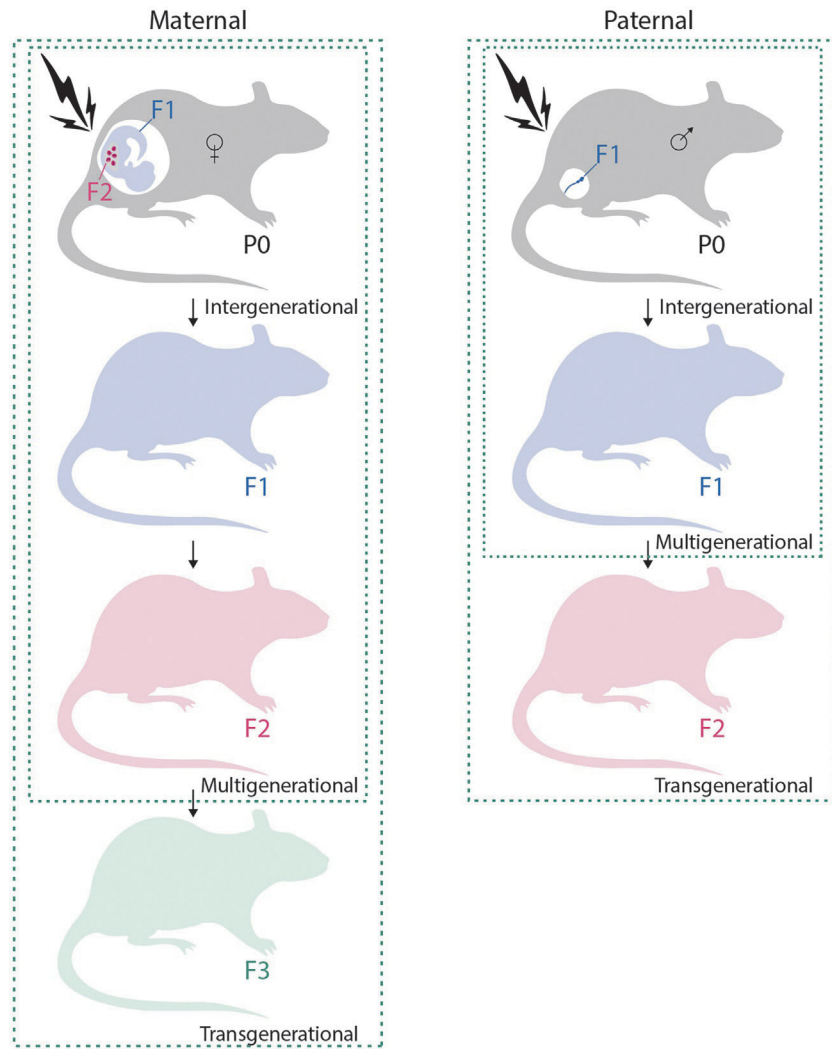


Fig. 3. Modes of Inheritance following Environmental Exposure. Exposure in gestational females (P0) also exposes the embryo (F1) and its germ cells (F2). Therefore, only the third generation is the one where the observed phenotype is not due to direct exposure and can truly be transgenerational. For post-natal exposure, as presented under the paternal scheme, exposure affects the F1, hence, the second generation is the one where transgenerational phenotypes can be observed.

Table 1

Evidence of transgenerational epigenetic inheritance to environmental cues in rodents.

Animal Model	Environmental cue	Offspring phenotype	Epigenetic mark	Mode of exposure	References
Rat, Mouse	Manufactured chemical (Vinclozolin)	Impaired male fertility, increased adult onset disease	DNA methylation	Maternal (gestational)	Anway, Cupp, Uzumcu, and Skinner (2005); Anway, Memon, Uzumcu, and Skinner (2006); Anway, Leathers, and Skinner (2006); Guerrero-Bosagna, Settles, Lucker, and Skinner (2010); Guerrero-Bosagna et al. (2012)
Rat, Mouse	Manufactured chemical (Dioxin)	Impaired female fertility, increased adult onset disease	DNA methylation	Maternal (gestational)	Bruner-Fran and Osteen (2011); Manikkam, Tracey, Guerrero-Bosagna, and Skinner (2012)
Rat, Mouse	Manufactured chemical (BPA)	Impaired female fertility, increased adult onset disease	DNA methylation	Maternal (gestational) Paternal (pre-conceptual)	Manikkam, Tracey, Guerrero-Bosagna, and Skinner (2013); Ziv-Gal, Wang, Zhou, and Flaws (2015); Saidur Rahman, Pang, Ryu, Park, and Pang (2020)
Mouse	Manufactured chemical (Atrazine)	Impaired male fertility	H3K4me	Maternal (gestational)	Hao et al. (2016)
Mouse	Metal (Arsenic)	Impaired male fertility	DNA methylation	Maternal (gestational)	Yin et al. (2021)
Rat	Metal (Arsenic)	Impaired fertility	DNA methylation	Maternal (pre-conceptual) and Paternal (pre-conceptual)	Nava-Rivera et al. (2021)
Mouse	Diet (HFD)	Increased male body weight, impaired insulin sensitivity	DNA methylation	Maternal (gestational)	Sarker et al. (2018)
Rat	Diet (HFD)	Reduced birth-weight, female increased resistance to HFD-induced weight gain	Small RNAs	Paternal (pre-conceptual)	de Castro et al. (2016)
Mouse	Diet (HFD)	Increased obesity, insulin resistance	DNA methylation and small RNAs	Paternal (pre-conceptual)	Fullston et al. (2013); Wei et al. (2014); Chen et al. (2016)
Rat	Drugs (Alcohol)	Altered hypothalamic functions	DNA methylation	Maternal (gestational)	Govorko, Bekdash, Zhang, and Sarkar (2012)
Mouse	Drugs (Alcohol)	Altered neocortical development and behaviors	DNA methylation	Maternal (gestational)	Abbott, Rohac, Bottom, Paladia, and Huffman (2018); Bottom, Kozanian, Rohac, Erickson, and Huffman (2022)
Mouse	Drugs (Nicotine)	Reversal learning deficit	DNA methylation	Paternal (pre-conceptual)	McCarthy et al. (2018)