

Paternal epigenetic inheritance

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Introduction

The experiences that an individual has over their life span (i.e., nutritional, hormonal, toxins, social, stress) can induce significant variation in phenotype. However, even when these experiences occur in a previous generation, there may be a significant influence on a broad range of outcome measures including growth/metabolism, immune function, neurodevelopment, and behavior. Thus, parental experience can induce variation in offspring phenotype, and in some cases, this variation may be observed in subsequent generations. When considering the mechanistic pathways linking parental

experience to offspring and grand-offspring outcomes, it is clear that mothers and fathers have unique routes through which these influences can be exerted, depending on the species-specific dynamics of reproduction and parental investment. In this chapter, we will highlight evidence for the influence of paternal experiences on phenotypes in subsequent generations and the potential mechanisms that may underlie this transmission. Paternal epigenetic inheritance has been suggested as a possible mechanism for these effects, and here we will explore the role of genetic and epigenetic factors in contributing to the influence of fathers. In addition, we will discuss the importance of paternal–maternal interplay in modulating the transmission of paternal effects.

Paternal intergenerational and transgenerational effects

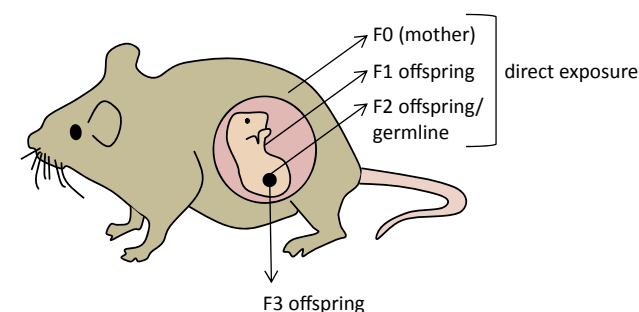
Effects of paternal experiences on offspring can be observed across species and taxa. In plants, paternal exposure to high versus low levels of light (100% vs. 73% full spectrum light) increases pollen production in the parental generation and seed mass in the offspring generation [1]. In insects, heightened gregarious behavior has been observed in the offspring of crowd-reared males [2]. In laboratory rats, male alcohol consumption before mating leads to reduced growth of offspring and altered neuro-behavioral development [3]. In humans, intrauterine growth retardation is associated with paternal smoking at the time of conception [4]. These examples illustrate the broad range of paternal environmental experiences, even occurring before conception, that shape offspring outcomes—indicating an intergenerational effect. This breadth can also be observed in studies that explore the persistence of these paternal effects into subsequent generations (among descendants where there has been no direct exposure to the environmental trigger), thus suggesting the transgenerational impact of paternal environments.

Nutritional effects

Epidemiological studies have demonstrated that the nutritional status of fathers and grandfathers can exert effects on metabolic functioning of sons (intergenerational) and grandsons (transgenerational). Archival data indicate that food availability during the prepubertal phase (8–12 years of age) of grandfathers is associated with mental health outcomes, increased risk of diabetes, cardiovascular disease, and mortality in grandsons [5–7]. In laboratory rodents, offspring and grand-offspring of rat dams that were fed a low-protein diet during gestation have hypertension [8]. Female offspring of male rats that were fed a high-fat diet have impaired insulin secretion and glucose tolerance [9]. Increased body length and reduced insulin sensitivity have also been observed in offspring (F1) and grand-offspring (F2) of dams that were fed a high-fat diet from preconception to the weaning period, and transmission of these effects to F3 generation female offspring has been observed through the patriline (Fig. 6.1) [10,11].

Drugs and toxins

The phenomenon of an experimentally induced paternal transgenerational effect was perhaps best originally demonstrated by studies of prenatal exposure to the pesticide vinclozolin. In rats, F3 offspring generated from a vinclozolin-exposed male (exposure occurring to F1 male in utero) have impairments in reproduction, altered anxiety-like behavior, stress sensitivity, and increased disease



Generation	Effect of F0 high fat diet exposure (pre-conception through to postnatal period)
F0	↑ body weight, glucose, & leptin
F1	↑ body length & weight; ↓ insulin sensitivity
F2	↑ body length; ↓ insulin sensitivity
F3*	↑ body length & weight (♀ only)

* effects only observed in paternal lineage (i.e. descendants of exposed F1 male)

FIGURE 6.1 Impact of maternal high-fat diet on subsequent generations of offspring.

In studies of the intergenerational and transgenerational epigenetic impact of environment on phenotype originating from in utero exposures, it is important to note that in addition to maternal exposure, the F1 fetus and germ line that will generate the F2 offspring have direct exposure to the environmental manipulation, whereas the continuity of the effects to the F3 generation is suggestive of the maintenance of changes in the F2 germ line and transmission to F3 offspring. In the case of maternal high-fat diet exposure in mice, altered phenotypes are observed to persist to the F3 generation when transmitted through the patriline (i.e., descendants of F1 exposed males).

Adapted from Dunn GA, Bale TL. Maternal high fat diet effects on third generation female body size via the paternal lineage. Endocrinology 2011;152(6):2228–36; Dunn GA, Bale TL. Maternal high fat diet promotes body length increases and insulin insensitivity in second generation mice. Endocrinology 2009;150(11):4999–5009.

risk (i.e., tumor formation, kidney disease, immune abnormalities) [12–15]. In mice, prenatal exposure to the endocrine-disrupting chemical bisphenol A (BPA) induces neuroendocrine changes and altered social behavior that have been observed to persist to the F4 generation of offspring [16]. In humans, high levels of consumption of nitrosamines (found in betel nuts) by fathers have been found to increase metabolic syndrome in offspring [17,18], and in mice, paternal preconception consumption of betel nuts increases the risk of developing hyperglycemia in offspring and their descendants [19].

Social environments

The social context of development, particularly contexts that impact stress responsivity, can have a significant impact on subsequent generations, and there is evidence for the patrilineal transmission of

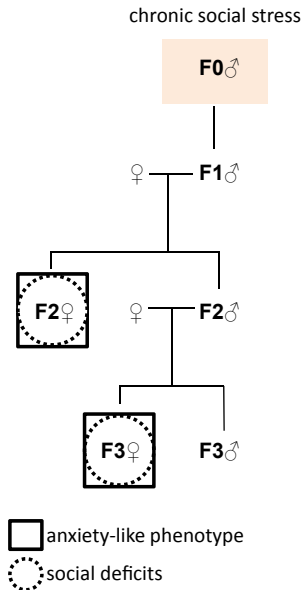


FIGURE 6.2 Impact of chronic social stress through the patriline.

Male mice exposed to chronic stress from adolescence through to adulthood sire F1 offspring that can transmit paternal effects to F2 and F3 offspring. In this case, deficits in social behavior and an anxiety-like phenotype are observed in F2 and F3 female offspring. Although F2 males do not manifest these phenotypes, they do transmit these behaviors to their own female offspring.

Adapted from Saavedra Rodríguez L, Feig LA. Chronic social instability induces anxiety and defective social interactions across generations. Biol Psychiatry 2013;73(1):44–53.

these environmental effects. Male mice exposed to maternal separation during postnatal development display social deficits and altered anxiety- and depressive-like behaviors that persist across generations [20]. Chronic social stress in male mice experienced during adolescence through to adulthood can induce social deficits and increased anxiety-like behavior in offspring and grand-offspring through the patriline. This transmission is intriguing because the males transmit the effects, but only female offspring manifest the behavioral deficits (Fig. 6.2) [21], illustrating a sex-specific impact of paternal experiences that is observed across experimental paradigms [15,22,23] and in human epidemiological data [5,24].

Paternal age

Paternal age is a significant predictor of offspring health and development [25]. Although chronological age is not paternal experience per se, older fathers will differ from younger fathers in cumulated life experiences. Moreover, paternal age at the time of reproduction, similar to other parental experiences, likely serves as an important life history variable. In humans, an increased incidence of birth defects and lower birth weight in offspring is predicted by advanced paternal age [26]. In addition,

increasing paternal age is associated with increases in externalizing behavior in childhood (i.e., hostile, unstable emotional responses, impulsiveness) [27], reduced IQ [28], and an increased risk of autism [29], schizophrenia [30], and bipolar disorder [31]. Consistent with findings in humans indicating an association between paternal age and offspring social development, studies in rats and mice indicate that social impairments in offspring are predicted by advanced father and grandfather age at conception [32,33]. In humans, paternal age effects also appear to be transmitted to subsequent generations. Increased grandparental age of the maternal grandfather (over 55 years of age) is associated with an increased risk of schizophrenia, and increased age of the maternal and paternal grandfather (>50 years of age) is associated with increased risk of autism spectrum disorder in grand-offspring [28].

Mechanisms of paternal intergenerational and transgenerational effects

The phenomenon of paternal effects in offspring and the transmission of these effects to subsequent generations have raised the critical question of mechanism. In species where fathers participate in the care of offspring (i.e., fish, birds, biparental mammals), there is an opportunity for males to directly influence the developmental experiences of offspring (i.e., nutritional, social, etc.), which may have long-term programming effects on phenotype [34,35]. However, in mammals, biparental or exclusive paternal care is relatively rare and thus cannot account for all of the paternal effects observed. Even in the case where paternal care is observed, this parent-offspring interaction may not account for the effects of fathers on offspring development. For example, children of alcoholic fathers exhibit hyperactivity and reduced cognitive performance, but only if the alcoholic father is also their biological father, indicating that these effects may be preconceptual in nature [36,37]. In CD1 mice (a species that provides some paternal care), the transmission of the effects of social stress across generations occurs even when males are removed shortly after mating to limit paternal contact and prevent postnatal interactions with offspring [21]. These findings provide support for the hypothesis that paternal environmental experiences are becoming embedded into the germ line through changes in the male gametes. The question of critical importance is the nature of those gametic “changes” and how they come to shape the development of subsequent generations.

Genetic modification

A traditional approach to understanding the inheritance of behavioral traits has focused on the transmission of DNA sequence variants across generations. In the field of developmental toxicology, there is evidence for an association between male exposure to various drugs/toxins and an increased occurrence of mutations, including numerical and structural chromosomal abnormalities, point mutations, copy number variant (CNV) changes, and duplications/deletions of microsatellites [38]. This mutagenic potency is a potential route of transgenerational influence of compounds such as vinclozolin [39], BPA [40], and betel nuts [41]. However, a commonly acknowledged average baseline mutational rate frequency in humans of 2.3×10^{-8} per nucleotide per generation appears to be too low to account for all of the observed phenotypic inheritance [42,43], though it should be noted that certain mutational events (e.g., CNVs) can occur at a much higher frequency than baseline and certain DNA regions (hotspots) may be particularly susceptible to de novo mutations [44,45]. Interestingly, even though the

increase in sporadic cases of genetic disorders is exponential with increasing age of the father, there is ambiguous evidence for an exponential increase in the mutational rate of sperm with increasing paternal age, suggesting the role of other mechanisms in these paternal age effects [42,46].

In addition to classic genetic mechanisms, altered telomere length has emerged as a potential route for environmentally induced variation in phenotype that may have implications for subsequent generations. Telomeres are DNA–protein complexes located at the ends of chromosomes, consisting of TTAGGG repeats of variable length that serve to maintain chromosomal stability [47]. Although telomere length decreases with each cell division, the length of telomeres can be maintained by the enzyme telomerase, which is typically highly expressed in rapidly dividing cells (i.e., stem cells, germ cells) [48]. Modulation of telomerase activity, and consequently telomere length, can have a significant impact on health and longevity [49], and there is increasing evidence for the impact of stressful life events on telomere length [50]. Interestingly, paternal age at conception is predictive of longer telomere length in offspring in humans, and this paternal age effect is additive with grandparental age effects [51] (Fig. 6.3). Unlike somatic tissues, in which telomere length decreases with age, in sperm there is increasing telomere length with increasing paternal age, which appears to be transmitted to offspring and grand-offspring (though birth cohort effects should be considered when interpreting this phenomenon) [51–53]. This phenomenon may be due to increase in telomerase activity within the testes and/or the selective survival of sperm with long versus short telomere lengths [54]. Variation in telomere length may be an important mechanism to consider in the context of paternal age effects, and as our understanding of the environmental regulation of telomerase activity becomes more advanced, this mechanism could serve as an important mediator of the transmission of paternal effects.

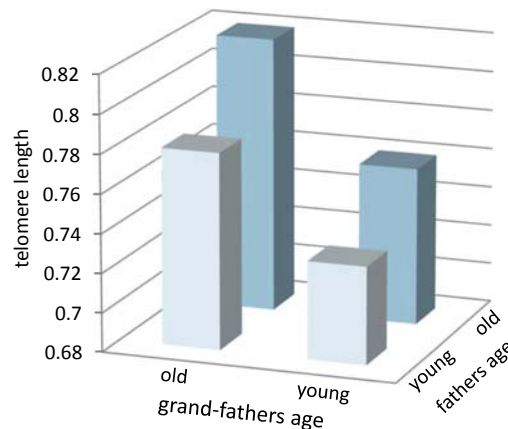


FIGURE 6.3 Telomere length in leukocytes of offspring as a function of paternal and grandparental age.

In humans, longer telomere length is observed in individuals who are born to older fathers (27–43 years of age) who are themselves the offspring of older fathers (30–54 years of age).

Adapted from Eisenberg DTA, Hayes MG, Kuzawa CW. Delayed paternal age of reproduction in humans is associated with longer telomeres across two generations of descendants. Proc Natl Acad Sci USA June 26, 2012;109(26):10251–6.

Epigenetic modifications

Advances in our understanding of the plasticity, stability, and potential heritability of epigenetic modifications have turned the focus of paternal transmission of phenotype toward these gene regulatory pathways. However, it is important to note that these mechanisms are not independent of genetic variation, and there may be a dynamic interplay between environmentally induced epigenetic variation and genomic instability [55]. The key issues relevant to the study of paternal epigenetic inheritance is whether (1) male gametes have altered epigenetic characteristics following environmental exposures, (2) environmentally induced epigenetic variation in male gametes escapes pre- and postfertilization epigenetic reprogramming events, and (3) this inherited epigenetic variation accounts for the phenotypic variation observed in subsequent generations (Fig. 6.4). Evidence continues to emerge, which provides support for the hypothesis of an epigenetic basis for intergenerational and transgenerational paternal effects, though methodologically it has been challenging to demonstrate conclusively the occurrence of the full cascade of these events. A critical question within this framework is whether epigenetic information within the male gamete can be inherited, as there is evidence that epigenetic variation in both gametes is erased at the time of fertilization [56,57].

DNA methylation

DNA methylation is a process whereby cytosine residues are converted to 5-methylcytosine through the actions of DNA methyltransferases (DNMTs). Methylated DNA has reduced accessibility to transcription factors and recruits DNA binding proteins (methyl CpG-binding domain, MBDs) such as methyl-CpG-binding protein-2 (MeCP2) to help repress transcriptional activity [58,59]. DNA methylation is attractive as a possible mechanism of inherited biological transmission because of the mitotic heritability of DNA methylation patterns that are maintained through DNA replication and cell division. The heritability of DNA methylation patterns through meiosis and fertilization has been more controversial. After fertilization there is active demethylation followed by remethylation of the paternal genome within the zygote just before implantation [60]. Following sex determination during embryogenesis, DNA is remethylated in germ cells in a sex-specific manner. Although these events serve to erase parental DNA methylation marks acquired during the life span and ensure cell lineage-specific and sex-specific placement of methylation marks, it is evident that specific gene loci have the capacity to escape these waves of epigenetic reprogramming (Fig. 6.4). For example, variation in the DNA methylation status of an intracisternal-A particle (IAP) element, a long terminal repeat retrotransposon, can result in heritable phenotypic variability. When inserted into an exon of the agouti gene (*Avy*), variation in the DNA methylation status of this IAP results in a range of phenotypic characteristics, including coat color pigmentation and a predisposition for obesity, and this phenotypic variation is inherited through the gametes [61]. Similarly, when methylated, an IAP element inserted into the 5' region of the *AxinFu* allele results aberrant gene transcripts and a kinked-tail phenotype that can be inherited by offspring through both maternal and paternal lineages [62]. Interestingly, the epigenetic state of these repetitive elements can be modified by a broad range of environmental exposures, suggesting that epigenetic inheritance at these loci can be driven by environmental factors [61,63,64]. Evidence for altered DNA methylation of these elements in sperm in response to environmental exposures [65] is suggestive of the initiation of the cascade of events that lead to paternal epigenetic inheritance.

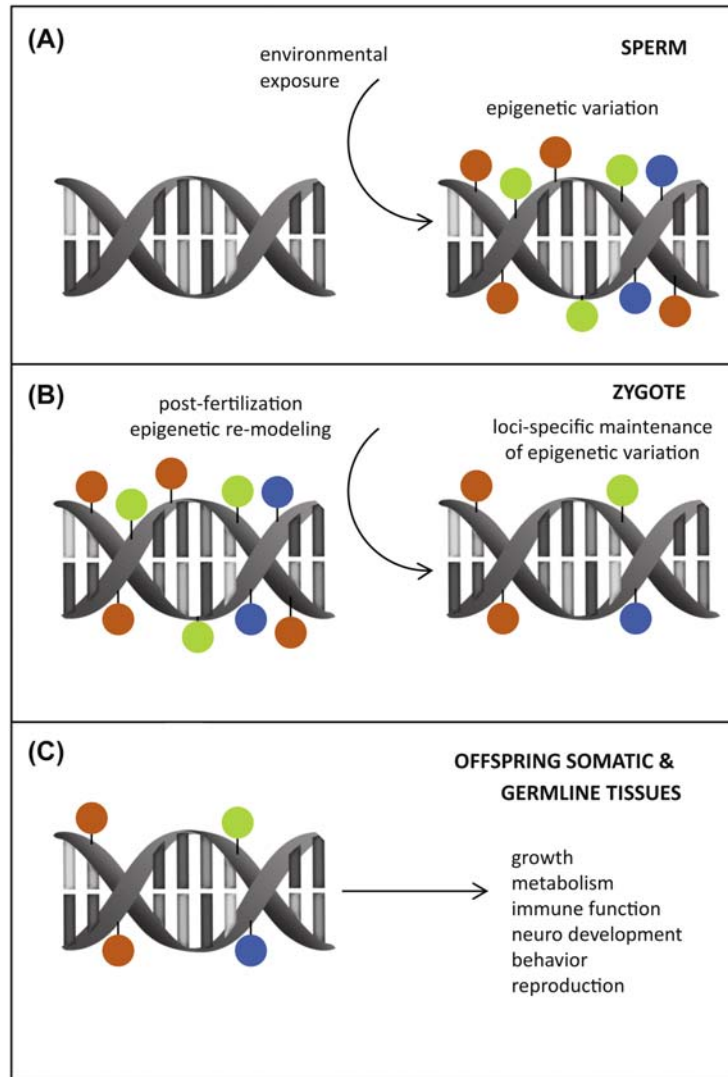


FIGURE 6.4 Hypothesized epigenetic pathway linking paternal experience to offspring phenotypes.

(A) Environmental experiences (e.g., nutrition, toxins, stress, social interactions) induce epigenetic variation in the sperm of exposed males. (B) This environmentally induced epigenetic variation is not completely erased during postfertilization epigenetic programming events that merge the maternal and paternal DNA within the zygote. (C) This inherited epigenetic variation induces phenotypic variation in offspring through effects on somatic tissue, and phenotypes may be transmitted to subsequent generations through inherited epigenetic variation in the germ line.

Similar to the case of IAPs, imprinted genes are loci that can retain epigenetic marks across generations, resulting in a parent-of-origin imprint that leads to epigenetic silencing of one of the parental alleles [66]. Like IAPs, gene expression and the DNA methylation status of imprinted genes in germ and somatic tissue can be modified by environmental factors. In humans, increased demethylation of two paternally imprinted genes, which are normally hypermethylated, *H19* and intergenic germ line derived differentially methylated region (*IG-DMR*), occurs in the sperm of fathers associated with chronic alcohol consumption [67]. In mice, superovulation (a procedure commonly used for assisted reproduction) reduces DNA methylation of the paternally imprinted *H19* gene but increases DNA methylation at the maternally imprinted small nuclear ribonucleoprotein polypeptide N (*Snrpn*) gene in the sperm of F2 offspring [68]. Although IAPs and imprinted genes represent an important set of targets for considering within the context of paternal epigenetic inheritance, approximately 100 genes have been identified that are neither repetitive elements nor imprinted genes and yet retain the gene promoter DNA methylation patterns present in either sperm or oocyte following postfertilization reprogramming [69]. Although reprogramming events occurring at a genome-wide scale may prevent the transmission of much of the parental variation in DNA methylation, it appears to be the case that numerous genes may permit epigenetic inheritance. A key question is regarding the degree of epigenetic plasticity of these target genes in response to paternal experiences and the role of altered DNA methylation in outcomes observed in offspring.

There are now several strands of evidence indicating that levels of DNA methylation in sperm are altered in response to a number of qualitatively different exposures (e.g., drugs, toxin, social experience) applied at range of developmental time points, and these epigenetic effects are consequently observed in offspring. Nutritional and toxicological manipulations in males have been demonstrated to induce variation in DNA methylation in offspring. In rats, female offspring born to males that were fed a high-fat diet in adulthood have impaired pancreatic function (e.g., reduced insulin secretion and glucose tolerance and β -cell dysfunction), and this phenotype is associated with changes in the expression of genes associated with insulin regulation and glucose metabolism as well as DNA methylation changes proximal to the transcriptional start site of the interleukin 13 receptor alpha 2 (*Il13ra2*) gene in the pancreas [9]. In mice, offspring of males that were fed a low-protein diet during the postweaning period have altered DNA methylation within the putative enhancer for peroxisome proliferator-activated receptor alpha (*Ppara*) gene in hepatic tissue [70]. Hippocampal genome wide DNA methylation and cytosine promoter region methylation of the calcium-activated potassium channel subunit beta-2 (*Kcnmb2*) is increased in the offspring of male mice placed on a high-methyl donor diet (folic acid, L-methionine, choline, zinc, betaine, and vitamin B12) before mating [71]. It is presumed that these epigenetic changes in offspring derive from environmentally induced epigenetic alterations in sperm, and there is evidence for the impact of diet on DNA methylation within the sperm [72,73]. Variation in sperm DNA methylation, both globally and at target loci, has been observed in rodents following exposure to pollutants [74–76]. Male rats that are exposed to alcohol during in utero development show a significant deficit in proopiomelanocortin (POMC) function, a hypothalamic peptide that regulates energy homeostasis, stress responsivity, immune function, and brain reward systems, and these deficits are associated with increased DNA methylation of a proximal region of the *Pomc* promoter in the brain. Significantly, these changes in DNA methylation are also observed in the sperm of alcohol-exposed males (F1) and their sons (F2) and grandsons (F3) but not in controls or their descendants [77]. In rodents, paternal exposure to alcohol or cocaine is

associated with significant decreases in mRNA levels of DNMTs in the testes and sperm [67,78,79]. This effect on DNMTs may account for the reduced DNA methylation of the imprinted *H19* and *IG-DMR* genes observed in humans that engage in heavy drinking [67]. In utero vinclozolin exposure induces DNA methylation changes in sperm cells that persist to F3 generation males. Global screens of DNA methylation of sperm reveal multiple differentially methylated sites in promoters at imprinted and imprinted-like genes in the male germ line that likely have the capacity to induce global changes in gene expression and propagate the effects of vinclozolin across generations [12,80].

The effects of social experiences of males during development have also been shown to induce changes in DNA methylation in both brain and germ tissues. Analyses of brain tissue indicate that male mice that experience postnatal maternal separation have elevated DNA methylation in some loci, such as *MeCP2* and cannabinoid receptor type 1 (*Cbr1*) genes, but decreased DNA methylation in others, such as the corticotropin-releasing factor receptor 2 (*Crr2*) gene. Moreover, these changes are also observed in the sperm of maternally separated males and the cortex and sperm of the offspring of these males [20] (Fig. 6.5). These paternal effects on *MeCP2* may have consequences for the epigenetic programming of multiple gene targets through the role of this methyl-binding protein in gene silencing. Environmental targeting of MBDs and DNMTs in male gametes may be a critical step in the process of paternal epigenetic inheritance.

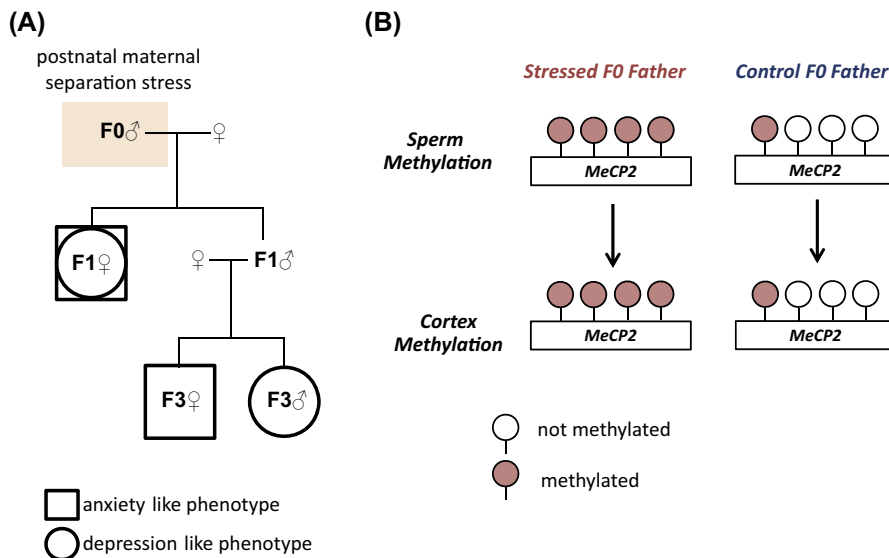


FIGURE 6.5 Transmission of the effects of postnatal maternal separation.

(A) Increased indices of anxiety- and depressive-like behavior are observed in the female offspring (F2) of males (F1) that experienced maternal separation. These behavioral effects are also observed in F3 offspring (patrilateral transmission). (B) Within the *MeCP2* gene, increased DNA methylation is observed in the sperm of maternally separated males (F1) and in the brain (cortex) of offspring of these males (F2).

Adapted from Franklin TB, Russig H, Weiss IC, Gräff J, Linder N, Michalon A, et al. Epigenetic transmission of the impact of early stress across generations. *Biol Psychiatry* 2010;68(5):408–15.

The epigenetic effects of paternal age in offspring can be observed within target genes and at a genome-wide level. In mice, offspring of older fathers have global DNA hypermethylation and gene-specific DNA methylation changes in the brain at sites that regulate the expression of *Gnas-Nesp*, *GnasXL*, zinc-finger protein regulator of apoptosis and cell cycle arrest gene (*Zac1*), and paternally expressed gene 3 (*Peg3*) [81]. In humans, DNA methylation changes have been shown to accumulate with increasing paternal age in multiple tissues including the gametes [82]. Paternal age related changes in the DNA methylation of *FOXP1* in sperm are also observed in fetal cord blood of offspring [83]. Parental age has been associated with genome-wide changes in DNA methylation in cord blood samples of newborns though the impact of parental age on this outcome is stronger for analyses of maternal age than paternal age [84]. In mice, there are broad changes in DNA methylation in the sperm of older fathers [85], and some of these epigenetic changes are also observed in the hippocampus of offspring [86].

Posttranslational histone modifications

Posttranslational modification of histone proteins is another mechanism through which epigenetic control of gene expression can be achieved. Depending on the amino acid site and the type of modification (acetylation, phosphorylation, mono-, bi-, or tri-methylation), these posttranslational marks can either repress or enhance gene expression by changing the density of DNA packaging and the accessibility of transcriptional machinery to the DNA [87]. Because of the dynamic nature of histone modifications and the dramatic reorganization of the chromatic structure at the time of fertilization, the role of these epigenetic factors in paternal epigenetic inheritance has not been explored to the same degree as DNA methylation. Within sperm, DNA is primarily packaged within protamines rather than histones, and at the time of fertilization, the protamines are replaced with maternally derived histones [88]. However, in humans, 10%–15% of paternal histones are retained, and these “persisting histones” may be positioned within genomic regions that have a significant influence on the early stages of development [89,90]. In rodents, paternal cocaine exposure before mating can reduce body weight, impair cognitive performance, and increase indices of hyperactivity and depressive-like behavior of offspring [78]. Reduced cocaine self-administration has been observed in the offspring of cocaine-exposed male rats, and within the prefrontal cortex, there are elevated levels of brain-derived neurotrophic factor (BDNF, mRNA, and protein) amongst cocaine-sired males. Interestingly, preconceptual cocaine exposure was associated with elevated histone acetylation within the *Bdnf* promoter in the testes and sperm of exposed males and in the prefrontal cortex of their offspring [91], suggesting the possibility of inherited histone modifications. Offspring of male transgenic mice with reduced H3K4 dimethylation in sperm exhibit developmental disruption and this effect persists across generations [92]. Transgenerational effects of vinclozolin may also involve altered histone retention in sperm and work in concert with other epigenetic changes to alter phenotype [93].

Small RNAs

Epigenetic regulation of gene expression and the translation of RNA transcripts can also be achieved through small RNAs. For example, microRNAs (miRNAs) can cause posttranscriptional gene silencing by base pairing with target messenger RNAs (mRNAs) to regulate gene expression [94]. At fertilization, both sperm and oocyte transmit various cytoplasmic RNAs (e.g., mRNAs, endogenous

small-interfering RNAs, miRNAs, and PIWI-interacting RNAs [piRNAs]) that play key roles in initializing development. Although sperm RNA content is much lower, many of these RNAs induce oocyte activation and signaling in the early zygote [95], and there is increasing evidence that these RNAs could be involved in paternal epigenetic inheritance [96].

MicroRNAs

The critical importance of sperm miRNAs to spermatogenesis and early oocyte development has been demonstrated through direct manipulation of specific miRNAs and through inhibition of DICER, which prevents the maturation of miRNAs [95,97]. A classic example illustrating the role of RNA-mediated inheritance comes from work on paramutation—a phenomenon in which the interaction between two homologous alleles of a single locus results in heritable variation. This phenomenon is well described in plants [98], with increasing examples observed in vertebrate and nonvertebrate animals [99,100]. One example of paramutation involves the *Kit* gene that encodes a tyrosine kinase receptor and is involved in the synthesis of melanin. Mice that are heterozygous for a mutation of the *Kit* gene have reduced *Kit* mRNA expression and distinctive white pigmentation in the feet and tail. This phenotype is also observed in the wild-type descendants of heterozygous crosses: a resulting paramutated phenotype [101]. These wild-type offspring have altered levels of *Kit* mRNAs, as well as other abnormal RNA transcripts in the testes and sperm, and injection of *Kit* mRNA from heterozygotes or miRNAs against *Kit* mRNAs into fertilized eggs can reproduce the paramutation phenotype [101] (Fig. 6.6). Similarly, injection of the cardiac-specific miRNA, miR-1, into fertilized eggs induces anatomical and physiological signs of cardiac hypertrophy in the resulting offspring [102], whereas injection of miR-124, a miRNA critical for brain development, results in offspring with increased growth rates [103]. Both manipulations modify the expression of genes known to be targeted by these respective miRNAs during development and in adulthood.

Paternal transmission of environmentally induced phenotypes via altered miRNA content has emerged as a plausible molecular pathway linking paternal experiences and offspring development. In mice, paternal exposure to irradiation leads to upregulation of miRNAs from the miR-29 family in the exposed male and upregulation of miR-468 in thymus in the offspring of exposed males [104]. Paternal exposure to stress during in utero development has been found to reduce sexual differentiation between males and females, and in mice, male offspring of stressed males have significant reductions in miR-322, miR-574, and miR-873, an effect that shifts the levels of these miRNAs to be more similar to those of control females [105,106]. Direct manipulation in the zygote of miRNAs identified as being upregulated in the sperm of stressed males can alter stress responsivity of offspring and thus may account for the transmission of paternal stress across generations [107]. Similar transmission effects have been observed when injecting RNAs from the sperm of male mice that experienced social stress during the postnatal period into the fertilized oocytes of nonstressed parents suggesting the mediating role of miRNAs in paternal epigenetic inheritance.

Evidence for the impact of paternal environmental exposures on sperm miRNA is accumulating across species. Paternal stress applied during adolescence or adulthood in mice has been shown to elevate levels of specific miRNAs in the sperm (i.e., miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, miR-698) [106]. Exposure to chronically elevated glucocorticoid levels in mice alters sperm miRNA profiles and offspring behavior across multiple generations [108]. Exposure of males to the polycyclic aromatic hydrocarbon benzo[a]pyrene has been found to alter the expression of several miRNAs in offspring, leading to both up- and downregulation

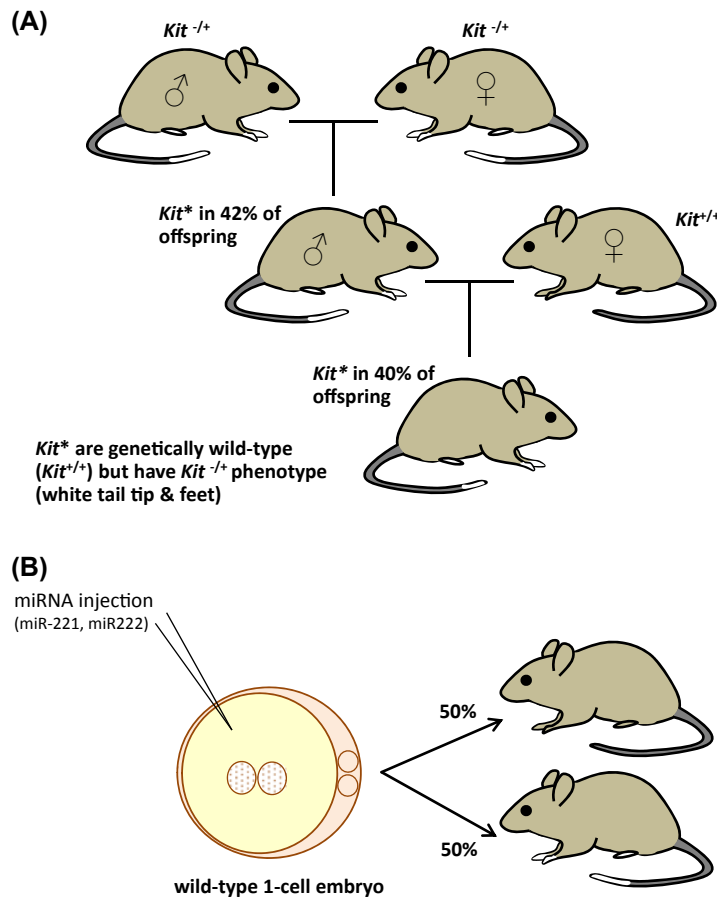


FIGURE 6.6 Transmission of *Kit*^{+/+} phenotype and the role of RNAs in offspring phenotype.

(A) *Kit*^{-/+} mice have a pigmentation phenotype consisting of a white tail tip and feet. Crosses between *Kit*^{-/+} mice generate a proportion of offspring (42%) who have a *Kit*^{+/+} genotype yet exhibit the *Kit*^{-/+} phenotype and are thus considered paramutated (*Kit*^{*}). Crossing *Kit*^{*} with *Kit*^{+/+} mice generates a proportion of offspring (40%) with a partial *Kit*^{-/+} phenotype. (B) Disruption to RNA in one-cell embryos of wild-type genotype using microRNA (miRNA) leads to 50% of offspring exhibiting the *Kit*^{-/+} phenotype, suggesting the role of miRNAs in the transgenerational inheritance of the *Kit*^{-/+} phenotype.

Adapted from Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, Cuzin F. RNA mediated non mendelian inheritance of an epigenetic change in the mouse. *Nature* May 25, 2006;441(7092):469–74.

of miRNAs in the developing embryo [109]. In humans, paternal smoking induces changes in the miRNA content of sperm, and the specific miRNAs targeted by this environmental exposure likely play a role in embryonic development [110]. miRNA levels in male germ cells appear to be environmentally sensitive and developmentally relevant and, thus, can serve as a mechanism through which the experiences of fathers can alter offspring phenotypes.

PIWI-interacting RNAs

Another class of small noncoding RNAs that might prove to be important in paternal transgenerational inheritance is piRNAs. Unlike miRNAs, which are highly expressed in somatic tissues, piRNAs are thought to be expressed primarily in germ cells and germ line tissues. Their primary role appears to be in the silencing of transposons in the male germ line, which is accomplished through piRNA interactions with P-element induced wimpy testis gene (PIWI) proteins (e.g., MILI, MIWI and MIWI2) that maintain DNA methylation at long interspersed element-1 and IAP elements [111]. Further, piRNA PIWI protein complexes recruit DNMTs to methylate the differentially methylated region of the RAS protein specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) gene during epigenetic reprogramming in primordial germ cells [112]. Therefore, an additional role for piRNAs may be to establish parental imprints at some imprinted loci. Studies in *Caenorhabditis elegans* indicate that piRNAs may be required to maintain a memory of environmentally induced changes in gene expression across generations [113]. In mice, altered expression of piRNAs in sperm has been associated with early life stress [114] and diet [115]. Older paternal age (compared with younger age) in mice is associated with a downregulation of piRNAs in sperm [86]. Therefore, piRNAs may represent a molecular signal to confer epigenetic information (particularly at repeat elements and imprinted genes) across generations.

Transfer RNAs

Transfer RNAs (tRNAs) are typically 76–90 nucleotides in length and play a critical role in translation through their interaction with mRNA and amino acid sequences during protein synthesis. Fragments of tRNAs (28–34 nucleotides) generated from the 5' ends of these molecules have been found to be altered in expression in sperm following exposure of male mice to both a high-fat diet [116] and a low-protein diet [117]. These tRNA fragments increase in abundance during sperm maturation and may be more significantly impacted by dietary manipulations in comparison with other small RNAs in sperm [116]. Moreover, isolating tRNA fragments from sperm that have been upregulated by a high-fat diet in mice and injecting those tRNA fragments into a normal zygote leads to emergence of a glucose intolerance phenotype in F1 offspring [116]. Evidence for the propagation of paternal sperm mediated effects via tRNA fragments provides yet another critical pathway to explore when considering the mechanisms of intergenerational and transgenerational paternal effects.

Paternal effects on maternal investment

Direct epigenetic transmission via the germ line is a tantalizing mechanistic hypothesis for understanding the link between environmentally induced paternal phenotypes and offspring characteristics. The plausibility of this hypothesis is supported by the impact on offspring of direct manipulation of the epigenetic state of sperm or zygote [103,107,114] and by the conserved epigenetic variation observed in sperm and offspring tissues as a consequence of paternal environmental exposures [20,118]. However, it is important to consider alternate or complementary pathways through which these effects can be achieved, particularly in the context of mammalian paternal effects. In mammals, development occurs within an in utero and postnatal environment consisting of intense mother–infant interactions, and the earliest stages of embryonic development are dependent on oocyte factors. Dissociating the

effects of paternal epigenetic factors from maternal influences is methodologically and conceptually challenging. In vitro fertilization (IVF) is one approach that can provide insight into the direct inheritance of epigenetic variation through sperm, though potential disruption to epigenetic programming may be a concern when using this procedure [119]. In the case of the altered expression of miRNAs in embryos generated from the sperm of males exposed to benzo[a]pyrene, the effects observed were based exclusively on IVF, and so there is no comparison to effects achieved through natural mating [109]. Interestingly, though males that experience chronic social defeat stress have offspring that exhibit heightened anxiety- and depression-like behaviors, these stress-related phenotypes are not completely transmitted to offspring generated through IVF [120] (Fig. 6.7). Similarly, the *Kit* paramutation phenotype can be prevented from generational transmission in mice by manipulating the breeding design [100]. These findings suggest that in addition to the direct inheritance of effects through the male germ line, there may be maternal factors that are important to consider within the context of intergenerational and transgenerational paternal effects.

These maternal influences are perhaps not surprising within the fields of ecology, animal behavior, and evolutionary biology, where it has been well acknowledged that females can dynamically adjust their reproductive investment based on mate phenotype and/or quality. Maternal investment adjustments in response to mate quality can come in the form of “differential allocation” or “reproductive compensation.” The differential allocation hypothesis states that females paired with high-quality (typically attractive) males should increase their investment in offspring if the cost of reproducing

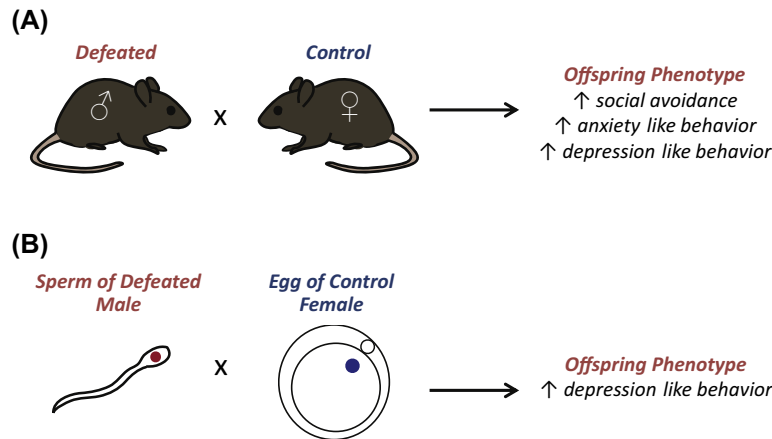


FIGURE 6.7 Role of maternal factors in the transmission of environmentally induced paternal effects.

(A) Under natural mating conditions, offspring of males that were chronically socially defeated over a period of 10 days during adulthood show increased social avoidance following a single aggressive encounter, increased anxiety (reduced exploration of open arms in an elevated plus maze), and increased depression-like behavior (shorter latency to immobility and reduced sucrose consumption in males). (B) Offspring generated by in vitro fertilization show minimal transmission of these phenotypes, with offspring only exhibiting increased depression-like behavior (reduced latency to immobility).

Adapted from Dietz DM, Laplant Q, Watts EL, Hodes GE, Russo SJ, Feng J, et al. Paternal transmission of stress induced pathologies. *Biol Psychiatry* 2011;70(5):408–14.

is high[121]. An alternative strategy is provided by the compensation hypothesis, in which females paired with unattractive or nonpreferred males increase their investment toward offspring to compensate for any disadvantages they may inherit from their father [122]. These hypotheses have been tested in a number of species across a wide variety of taxa with support emerging for both hypotheses. Overall, it is evident that female investment likely arises as an interaction between her own life history and the quality of her mate. Significantly, in many of these cases, the female's level of reproductive investment appears to be based on observed phenotype or perceived quality that is not attributable to genetic differences between potential mates. For example, in inbred (genetically identical) laboratory mice, females that are mated with males that have experienced social enrichment across their life span show elevated levels of maternal nursing and pup licking/grooming toward their offspring compared with those mated with socially isolated males [123]. In contrast, females mated with males exposed to chronic stress through reduced premating food intake engage in elevated food intake during pregnancy and display more frequent postnatal nurturing of offspring, suggestive of reproductive compensation [124].

In the context of paternal effects, an important question arises regarding the mechanisms by which the acquired phenotypes of males could influence the maternal investment of their offspring. One possibility is that females may detect/perceive differences in the phenotypic quality of males, inducing a preference for particular males, which then determines the level of perinatal care and resources that his offspring receive. The role of female preference within paternal effects is suggested by studies in house mice where females were given a free choice to show a preference for or against individual males and then mated with either a preferred or nonpreferred male. Females mated with a preferred male gave birth to larger litters, and these offspring were more socially dominant, better nest builders, exhibited more freezing behavior in a predator-avoidance test, and had reduced mortality rates compared with the offspring of females who mated with nonpreferred males [125]. Using embryo transfer in mice, it has been demonstrated that though direct effects of paternal chronic food restriction on offspring behavior can be observed, a female's mating experience with a food-restricted male and the compensatory changes in pre- and postnatal maternal investment that this experience triggers is a critical modulator of paternal effects [124]. (Fig. 6.8)

Thus, mate preference may be a significant predictor of maternally driven paternal effects and subsequent offspring development, as in utero food restriction [126], premating food restriction [124], predator odor exposure [127] and vinclozolin exposure [128] have been shown to shift female preferences toward males from nonexposed lineages. Male phenotype could also have direct effects on the female (i.e., shifting reproductive behavior, hormones, or stress physiology) during the mating period which can go on to have effects on the level of maternal investment. Importantly, these effects could occur independently of mate preference-based decisions. Although there is less evidence for this phenomenon, it is known that some experiential factors (e.g., stress) can shift mating strategies and the sexual behavior of males (e.g., rates of intromission), which can have consequences for successful pregnancy and parturition [129].

Although direct paternal genetic/epigenetic inheritance and maternal influences are typically explored separately as potential mechanisms of paternal effects, the interplay between these mechanisms is likely a significant predictor of offspring phenotype. Offspring may trigger differential levels of prenatal and postnatal maternal investment via the effects of paternally inherited genetic/epigenetic variation. Paternally expressed genes are likely to play an important role in this proposed pathway as they are highly expressed in the placenta, critical to normal growth and development of the fetus, and

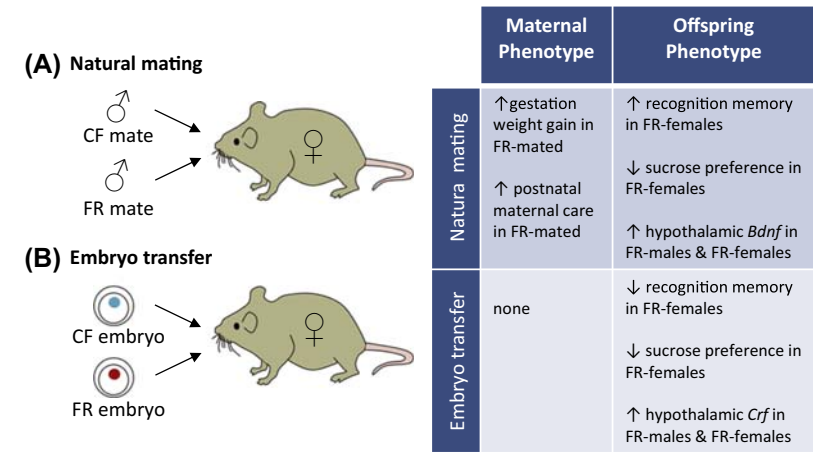


FIGURE 6.8 Paternal transmission of food restriction effects is impacted by mothers.

Using embryo transfer and natural mating conditions in mice, male pre-mating food restricted (FR) and control fed (CF) effects were examined in mothers and offspring. Females mated with FR males exhibited increased gestational weight gain and increased postnatal maternal care (compared to CF-mated females). This effect was not observed when embryo transfer was used (i.e., when females did not have a mating experience with FR males). Behavioral effects of paternal FR were observed only in female offspring, with natural mating associated with impaired object recognition and embryo transfer associated with enhanced object recognition in FR-sired offspring. Decreased sucrose preference was observed in female offspring of FR males regardless of rearing condition. Gene expression changes in offspring were observed in both male and female offspring of FR males and varied dependent on rearing condition.

Adapted from Mashoodh R, Habrylo IB, Gudsnuik KM, Pelle G, Champagne FA. Maternal modulation of paternal effects on offspring development. Proc Biol Sci March 14, 2018;285(1874).

influence the level of postnatal mother–infant interactions. For example, insulin growth factor 2 (*Igf2*), an imprinted gene that is paternally expressed, enhances offspring growth by increasing nutrient supply through fetal signals to the placenta [130]. Paternally expressed genes such as *Peg3* and paternally expressed gene 1 (*Peg1*)/*MEST* also regulate postnatal mother–infant interactions, promote the suckling behavior of pups, and have been found to be altered in expression in the maternal hypothalamus in mice following mating with chronically stressed males [124,131,132]. These findings are particularly interesting in light of the epigenetic plasticity of these genes in response to paternal environmental exposures.

Issues and future directions

The paternal transmission of environmentally induced phenotypes across generations (in the absence of paternal interactions with offspring) had been previously viewed as an interesting but challenging phenomenon because of our limited understanding of the potential mechanisms that could mediate this transmission. However, advances in our understanding of the dynamics of genetic and epigenetic plasticity combined with a more complex appreciation of the behavioral and molecular events that

influence reproduction has generated a broad range of hypotheses regarding the mechanisms driving paternal effects. Although still interesting and challenging, now there are many experimental strategies that can be implemented to further our understanding of the transmission of environmentally induced traits, and comprehensive studies using these strategies will be particularly valuable in elucidating the mechanisms that underlie the inheritance of epigenetic information. Based on the current state of evidence supporting the inheritance of epigenetic variation, there are several important issues to consider:

1. *Epigenetic variation versus genetic variation*: The reliable transmission of genetic variation is a central tenet of Mendelian genetics and a primary basis for our prediction of gene frequencies in ancestors and descendants. When considering the transmission of epigenetic variation, it is difficult to diverge from the rules governing inheritance that have developed within the framework of genetics. However, epigenetic variation can be highly dynamic, and it is this plasticity that allows for the fine tuning of phenotype through developmental experiences. Reconciling this plasticity with intergenerational stability is challenging, and it is perhaps the case that we must create a new set of rules and ways of thinking about the transmission of epigenetic variation. This is particularly important when considering the consistency over generations of epigenetic modifications. Although molecular imprints, such as DNA methylation, may have the capacity for transmission through the germ line, there are many opportunities for passive loss of these epigenetic marks during cell division, even if they have escaped postfertilization erasure. There will also be increased likelihood of reversal of the epigenetic imprinting of the genome in cases where the phenotypic consequences are maladaptive or disruptive to reproduction. When genetic variants reduce fitness, there is likely to be removal of that genetic variant from the gene pool due to selective breeding. This can also occur in the case of epigenetic variants that impair fertility or alter the attractiveness/fitness of a potential mate; however, removal of the epigenetic variant could also be achieved by the enzymatic processes that dynamically regulate DNA methylation, posttranslational histone variation, and RNA production and maturation. Ultimately, this is an issue of reconciling the characteristics of plasticity and stability that have been observed amongst epigenetic mechanisms.
2. *Inheritance of phenotype is the product of multiple mechanistic pathways*: Our unique characteristics are the product of multiple biological pathways that are influenced by the quality of the environment at both an individual (i.e., family, social, nutritional) and global (i.e., cultural, climate) level. Epigenetic inheritance is one of many pathways that work in concert with other mechanisms to shape phenotype. In addition, it may be the case that a paternal genetic effect in one generation has phenotypic consequences for subsequent generations through epigenetic mechanisms [133,134]. Although it may be difficult to dissociate these multiple influences, it is important conceptually to think inclusively rather than exclusively regarding the mechanisms of inheritance [135]. This inclusive approach may help to interpret the complex phenotypic outcomes and the often inconsistent expression of these phenotypes in subsequent generations that have been observed in studies of parental environmental exposures. Integration of multiple interacting inheritance pathways may be particularly important when interpreting observed multigenerational effects in humans. Much of the experimental support for paternal epigenetic inheritance emerges from studies in rodents, invertebrates, and plants, which differ from humans in their epigenetic reprogramming events during reproduction. Although multigenerational

phenomena have been clearly observed in humans associated with paternal experiences, the assumption that epigenetic inheritance accounts for this observation needs thoughtful critique.

3. *Intergenerational and transgenerational adaptive responses and the Lamarckian perspective:* In parallel with our expanding understanding of the molecular events that permit the transmission of paternal effects across generations, it is important to continue to develop broader evolutionary explanations for why these effects occur. Although nothing in biology is more provocative than suggesting that Jean-Baptiste Lamarck may have been right, it is important to understand the rationale for the Lamarckian notion of the inheritance of acquired characteristics. Lamarck theorized that under specific environmental conditions, there would be phenotypic adaptations that would suit those environments best—improving the fitness and reproductive capacity of the individual. If these conditions were to persist across many generations and both males and females of a mating pair were exposed to these conditions, Lamarck predicted that these adaptations would become heritable, thus lessening the developmental burden of offspring who would need to shift phenotype to survive and reproduce. The adaptiveness of intergenerational and transgenerational effects has been speculated, and in the case of paternal effects in species that do not engage in paternal care of offspring, the transmission of phenotypically relevant epigenetic information through the germ cells may provide a mechanism for improving the fitness of offspring based on the experience of fathers. An elegant example of this transmission is evident in studies of the enhanced wound healing observed in the descendants of males exposed to liver damage [136]. Viewing offspring phenotypes through this lens may enhance our ability to predict and detect the outcomes associated with paternal environmental exposures.

List of abbreviations

AVY	Agouti gene
AXINFU	Axin fused gene
BDNF	Brain derived neurotrophic factor
BPA	Bisphenol A
CB1	Cannabinoid receptor type 1
CF	Control fed
CNV	Copy number variant
CRFR2	Corticotropin releasing factor receptor 2
DAH	Differential allocation hypothesis
DNMT	DNA methyltransferase
Endo-siRNAs	Endogenous small interferingRNA
FOXK1	Forkhead box protein K1
FR	Food restricted
GNAS	Guanine nucleotide binding protein alpha stimulating gene
IAP	Intracisternal A particle
IG-DMR	Intergenic germ line—derived differentially methylated region
IGF2	Insulin growth factor 2
IL13Rα2	Interleukin 13 receptor alpha 2
IVF	In vitro fertilization
KCNMB2	Calcium activated potassium channel subunit beta 2
KIT	Tyrosine kinase receptor gene

LINE-1	Long interspersed element 1
MBD	Methyl CpG binding domain
MECP2	Methyl CpG binding protein 2
MEST	Mesoderm specific transcript gene
miRNA	MicroRNA
MPFC	Medial prefrontal cortex
PAC	Paternal age at conception
PEG1	Paternally expressed gene 1
PEG3	Paternally expressed gene 3
PIWI	P element induced wimpy testis gene
piRNA	PIWI interacting RNA
POMC	Proopiomelanocortin
PPARα	Peroxisome proliferator activated receptor alpha
RASGRF1	RAS protein—specific guanine nucleotide releasing factor 1
SNRPN	Small nuclear ribonucleoprotein polypeptide N
tRNA	Transfer RNA
ZAC1	Zinc finger protein regulator of apoptosis and cell cycle arrest gene

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