Review

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Nongenetic inheritance and transgenerational epigenetics

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The idea that inherited genotypes define phenotypes has been paramount in modern biology. The question remains, however, whether stable phenotypes could be also inherited from parents independently of the genetic sequence *per se*. Recent data suggest that parental experiences can be transmitted behaviorally, through *in utero* exposure of the developing fetus to the maternal environment, or through either the male or female germline. The challenge is to delineate a plausible mechanism. In the past decade it has been proposed that epigenetic mechanisms are involved in multigenerational transmission of phenotypes and transgenerational inheritance. The prospect that ancestral experiences are written in our epigenome has immense implications for our understanding of human behavior, health, and disease.

Evidence for nongenetic multigenerational transmission of parental experience

New adaptive phenotypes can emerge as a result of natural selection of genetic variants. Natural selection is highly inefficient and slow in responding to immediate environmental challenges. It is well known that physiological systems can respond and adapt to new changes in real time, but the question remains whether there are nongenetic processes that could establish stable phenotypes and whether these can be inherited through germline transmission across generations. Biological examples have been documented of phenotypic plasticity emerging in relatively fast time-scales, and of frequencies that are orders of magnitude higher than can be explained by natural selection (see e.g., [1]).

Epidemiological evidence from two important multigenerational studies has brought to the fore the prospect of epigenetic memory of ancestral dietary distress in humans. First, Pembrey and colleagues [2–4] examined records of the multigenerational Överkalix cohorts in Northern Sweden using harvest and birth and death records. Variation in the food supply during the early life of paternal grandparents was associated with variation in mortality rate (and diabetic deaths) in their grandchildren. There were striking sex-specific transmissions, such that the food supply of the paternal grandfather was associated with the mortality rate of grandsons only, while the early-life food supply of the paternal grandmother was only associated with the mortality rate of granddaughters [2–4]. Interestingly, the effects were seen only when exposures occurred before puberty, supporting the hypothesis that reprogramming of gametes was involved. Similarly, in the UK Avon Longitudinal Study of Parents and Children (ALSPAC48) cohort, growth effects associated with paternal smoking were only observed when paternal smoking took place before puberty [3].

A second landmark study showed that children of mothers exposed to the Dutch famine of 1944 during the last trimester of pregnancy and the first months of life were less obese than controls, whereas exposure in the first half of pregnancy resulted in higher obesity rates than in controls [5]. Famine exposure early in pregnancy was associated with hypermethylation of the imprinted insulin-like growth factor 2 (IGF2) receptor gene *IGF2R* 60 years later, pointing to the possibility that DNA methylation might be involved [6]. Examination of the F2 generation revealed higher weights and body mass index (BMI) in adult offspring of prenatally exposed F1 fathers than in offspring of unexposed F1, but this effect was sex-specific and was not found in offspring of prenatally exposed mothers [7].

Nongenetic transmission of memory of parental experience could happen at several time scales (Figure 1). First, it could be transmitted through *in utero* programming of the developing F1 embryo, as well as through postnatal parental behavior. Second, it could be transmitted from F1 gametes, which were exposed *in utero* to maternal experiences, to F2 offspring. Third, a true transgenerational transmission of ancestral memory via the unexposed F2 gametes to the F3 offspring.

The first two modes of nongenetic transmission could be explained by several known mechanisms which are triggered in response to exposures and that target either the embryo or gametes during embryogenesis. It is more difficult to understand how marks of exposure in gametes are replicated, escape programming during primordial germ cell differentiation [8] and early embryogenesis [9,10], and are transmitted across many generations that were not subjected to the same experiences. Real transgenerational nongenetic inheritance could potentially result in a stable new trait. Therefore, a provocative question is whether

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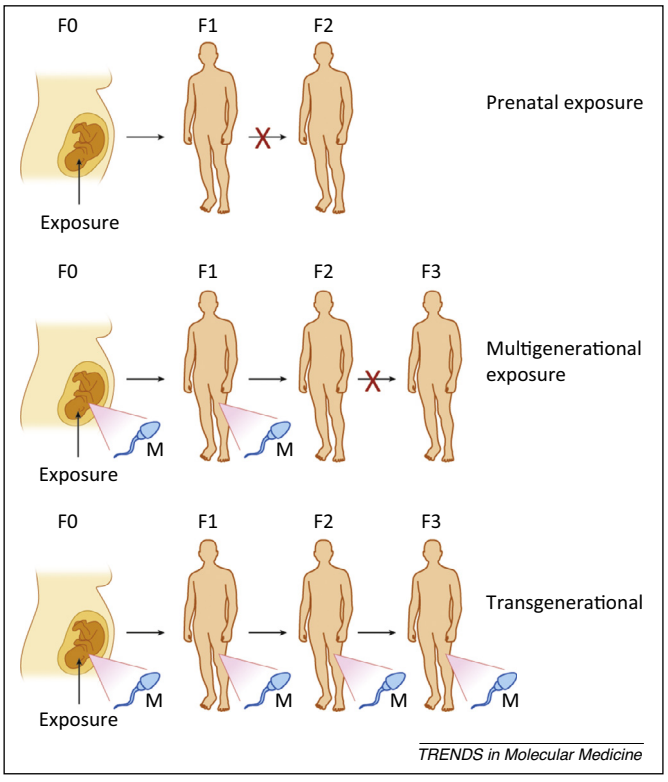

Figure 1. Modes of inheritance across generations. Three modes of multiple generational transmission of parental experience. Top row: prenatal exposure. The F0 mother is exposed during pregnancy with F1. F1 is programmed in response to exposure in different tissues but not the germline. Programming will alter the F1 phenotype but this will not be transmitted to next generation. Middle row: multigenerational exposures. The F1 generation is exposed during gestation and the developing germ cells are modified. The modified sperm of F1 will affect F2 development and phenotype. However, reprogramming of this modification during primordial germ cell differentiation of F2 will prevent transmission of the phenotype to F3. Bottom row: transgenerational inheritance. The F1 gametes are modified during gestation and exposure of the F0 mother. The modified F1 sperm affect F2 development. If gamete modification is not erased during primordial germ cell differentiation the sperm of F2 are modified as well and will transmit the phenotype to F3.

such mechanisms might play a role in 'rapid evolution' of traits in response to new experiences and environments at rates that are orders of magnitude faster than natural selection of stochastically arising genetic alterations.

It should be noted, however, that nongenetic multigenerational inheritance is not necessarily mediated by gametes. Multigenerational transmission could occur when the phenotypic response to the ancestral parental experience creates and sustains across generations the same social and physical environments that triggered the phenotype in the first place. For example, offspring of maternal adverse experience (such as violence, stress, poverty) which triggers a pattern of behavior in the offspring (e.g., aggression) would create the same adverse experience (aggression) for their offspring, leading to perpetuation of the phenotype across generations (Figure 2). The perpetuation of such traits could be interrupted by changing the parental environment or through cross-fostering [11,12].

Although epidemiological and animal experiments have provided data that support each of the different forms of nongenetic inheritance, in the absence of a plausible mechanism the interpretation of these data remains a matter of intense controversy. Moreover, questions are raised regarding the robustness of the data and its reproducibility.

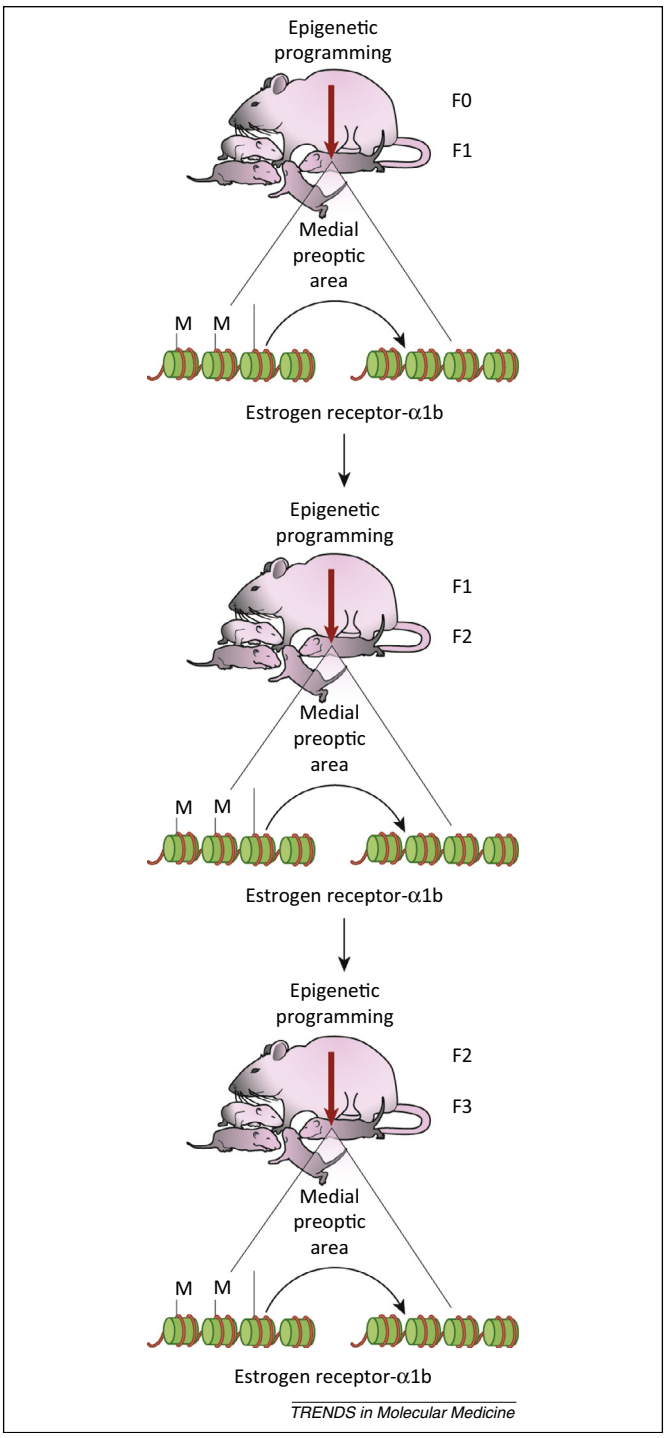

Figure 2. Gamete-independent multigenerational transmission. Maternal care in rodents programs stress-responsivity of the offspring as well as their maternal phenotype through the epigenetic modulation of genes such as that for estrogen receptor α1b in the medial preoptic area in the brain. Maternal care behavior of F0 will program genes in the brain of F1, which develops a maternal care phenotype as a result of this epigenetic programming that parallels F0 maternal care behavior. When F1 matures it epigenetically programs F2, which develops a maternal care behavior as a result that parallels F1. F2 similarly programs F3 and alters its brain gene program.

The cardinal issue is defining a fundamental common mechanism, or several different mechanisms, that can explain the different modes of nongenetic inheritance. Progress in epigenetics in the past decade has started to provide plausible mechanisms for the different forms and timescales of nongenetic inheritance.

Epigenetic mechanisms as possible mediators of responses to the environment

Epigenetics refers to mechanisms of long-term or stable regulation of gene expression programs that do not involve a change in gene sequences. Differences in epigenetic programming between different tissues in the same individual, or in the same tissue between different individuals, can result in alteration in gene expression programming that could cause phenotypic differences in the absence of a genetic difference [13–16]. The idea that epigenetic variance could create phenotypic differences between individuals in the absence of genetic changes positions epigenetics as a possible mechanism for nongenetic transmission. The term 'epigenetics' was coined by Waddington more than half a century ago to account for both plasticity and canalization as possible mechanisms for cellular differentiation and organogenesis [17]. To function in embryogenesis as the definers and guardians of cellular identities, epigenetic mechanisms should be mitotically heritable. If indeed epigenetic mechanisms are required for maintaining the unique identity of cell types, they would need to be highly resistant to change as well as being conserved across individuals, and should accurately reflect predefined and evolutionarily programmed developmental strategies. Nevertheless, research in the past decades suggests that, despite of the robustness of these developmental mechanisms, they are highly responsive to external signals, particularly during early life [18,19]. Thus, epigenetic processes might be involved not only in creating differences between tissues but also among individuals. Epigenetic mechanisms include DNA methylation [20], chromatin structure and modifications [21], and noncoding RNAs such as siRNA, miRNA, piRNA, and long noncoding RNA [22–24].

DNA methylation and heritability

Cytosine bases in vertebrate DNA are enzymatically modified by the DNA methyltransferase enzymes which catalyze the transfer of a methyl moiety from the methyl donor *S*-adenosylmethionine (SAM) to the 5′ position on the cytosine ring [25–27]. The methyl cytosine moiety could be further modified by hydroxylation [28] catalyzed by the ten-eleven translocation (TET) oxidases [29]. DNA methylation and hydroxymethylation are part of the covalent structure of DNA and are therefore the most proximal and certain epigenetic mechanisms. Thus, the DNA molecule itself contains both genetic and epigenetic information. DNA methylation patterns vary from tissue to tissue, and even within tissues, thus conferring upon DNA a particular cellular identity [30]. Recent genome-wide mapping of DNA methylation states at single-nucleotide resolution confirmed that DNA methylation states are rearranged during stem cell differentiation and development [31–33].

DNA methylation can alter the state of gene expression through several mechanisms. The mechanisms involved in silencing of gene expression by DNA methylation include direct interference with the binding of transcription factors to recognition elements that contain a CG dinucleotide [34], or through recruitment of methylated DNA binding factors [such as the methylated DNA-binding domain (MBD)-containing proteins] [35] that in turn attract chromatin-inactivating complexes including histone deacetylases and histone methyltransferases [36,37]. However, the role of methylated DNA-binding proteins in silencing gene expression is unclear, and genome-wide analyses showed that these proteins can bind to both unmethylated active and methylated inactive genes [38]. Methylation in the body of the gene is believed to play a positive role in gene activity by an unknown mechanism [39–41].

One of the interesting properties of DNA methylation is its heritability. Methylation in vertebrates occurs mainly in the palindrome dinucleotide sequence 5′CG3′. Therefore, when DNA is replicated the nascent CG site will be found across a methylated CG in the parental strand [42,43]. DNA methyltransferase 1 (DNMT1) is a maintenance methyltransferase which is highly specific for hemimethylated CGs generated during DNA replication, and faithfully copies CG methylation from the parental to the daughter strand [44], perpetuating the DNA methylation state. Maintenance methylation of several repetitive sites may also require the *de novo* methyltransferases DNMT3A and DNMT3B [45,46], which biochemically do not differentiate between hemimethylated and fully unmethylated sites [47]. DNA methylation of CG sequences is an established biochemical mechanism for replicating an epigenetic mark, and is therefore well positioned to serve as an epigenetic signal that maintains cellular identities for the entire life-course. Could the same mechanism of heritability be extended to meiosis, and thereby enable nongenetic inheritance across generations?

The fact that DNA methylation can be replicated places it as an excellent candidate for serving as a transgenerational epigenetic mark. However, it was long believed that although DNA methylation is faithfully conserved in differentiated cells it is completely erased in primordial germ cells [48] as well as shortly after fertilization and conception [10,49]. This erasure was believed to establish a clean slate for developmental patterning of DNA methylation during embryogenesis. If the erasure of DNA methylation is complete, then transgenerational transmission through DNA methylation marks would be impossible. The idea that DNA methylation is erased in the gametes during primordial germ cell differentiation has been an important reason for the initial rejection of the idea of transgenerational transmission of DNA methylation marks in sperm [10,49]. However, there is evidence from animal models for persistent DNA methylation changes in sperm that are transgenerational. It is now clear that, although the erasure of DNA methylation during primordial germ cell differentiation and post fertilization is fairly extensive, it is not complete. Particular repetitive sequences, such as intracisternal A particle (IAP) elements, escape DNA methylation erasure, while imprinted genes (genes that show allele specific methylation dependent on the parental origin) escape erasure following fertilization [8,50]. It is possible that other genomic sequences escape erasure as well, and that these sites might have evolved to play a role in bearing transgenerational epigenetic marks.

Histone modification and heritability

Chromatin states are determined by chemical modifications of the N-terminal tails of histones by methylation [51], phosphorylation, acetylation [52], sumoylation [53], and ubiquitination [54]. Histone acetylation is catalyzed by histone acetyltransferase (HAT) and removed by histone deacetylase enzymes (HDAC) [55]. Acetylation of histones is believed to associate with the active state of genes through increasing accessibility to DNA [56,57]. Histone methylation at lysine residues is catalyzed by lysine histone methyltransferases (HKMT) [58], and histone demethylases [59]. Trimethylation of lysine 4 on histone 3 (H3me3K4) is associated with promoter regions of active genes, and monomethylation at histone 3 at lysine 4 (H3me1K4) is associated with enhancers. Histone methylation could also act as a suppressive signal of gene expression; for example, H3me2K9 and H3meK27 [60]. It is still unclear whether there is a biochemical mechanism that faithfully replicates discrete chromatin states and how this is accomplished [61].

If chromatin modification states serve as cross-generational epigenetic signals there should be a mechanism that links parental exposures to discrete changes at particular addresses in chromatin in specific tissues. Very recent data generated using *Caenorhabditis elegans* provide evidence for transmission of male gamete-mediated chromatin states through several rounds of replication, although it remains to be seen if such a mechanism is conserved in mammals [61].

Small noncoding RNA (sncRNA)

There are several classes of sncRNAs, including miRNAs (hairpin RNAs with imperfect complementarity to multiple targets), siRNAs (with perfect complementarity to targets) [62] and PIWI-interacting RNAs (piRNAs) [63,64] (which specialize in targeting transposon transcription in germ cells and are of particular interest for gamete-mediated transgenerational epigenetic inheritance [65]). There is strong evidence that noncoding RNAs play a role in transgenerational silencing [66]. In addition to the conserved post-transcriptional roles of miRNA and siRNA in RNA interference (RNAi) resulting in decreased gene expression [62], siRNA and piRNA play a nuclear epigenetic role by targeting specific loci for either histone [67–69] or DNA methylation. The unique sequence identity of sncRNAs, and the complementarity with both mRNA and DNA, positions these molecules as excellent candidates for delivering epigenetic grooming enzymes to particular loci. The fact that sncRNA can be released from one cell and be systematically distributed [70–72] creates an opportunity for delivering signals from a tissue that senses the experience (such as the brain) to germ cells.

The first demonstration of a nuclear epigenetic role for siRNA was the co-transcriptional silencing (in *cis*) of repeats in heterochromatin that was discovered in the yeast *Schizosaccharomyces pombe* [67–69]. Sequence specificity of silencing is achieved by targeting the siRNA to nascent complementary RNA molecules as they are being transcribed on their genomic loci, thus delivering the siRNA effector molecule the Argonaut 1 (Ago1) protein in close proximity to the silenced locus. Ago1 interacts with the H3K9 methyltransferase Clr4, recruiting it to the locus resulting in silencing through histone H3K9 methylation [67–69].

SiRNA and miRNA may also direct DNA methylation, a mechanism which was first proposed in *Arabidopsis thaliana* [73,74]. miRNA-directed DNA methylation points to a potential mechanism for site-specific gene silencing that would be stable both mitotically and meiotically. piRNAs are transcribed from piRNA clusters and play an important role in silencing transposable elements, as first described in *Drosophila* [75]. The piRNA effector protein PIWI interacts with heterochromatin protein IA, and this interaction is required for silencing of transposons. There are two PIWI homologs in the mouse (MILI [63] and MIWI2 [76]) which are involved in silencing of transposons in germ cells. MIWI2, which is expressed in the nucleus, is believed to be the effector molecule that targets *de novo* methylation to transposons; however, no direct interaction has been demonstrated with a *de novo* DNMT. piRNA pathways are also utilized in the mouse germline to silence parentally imprinted genes (Box 2) [77,78].

Box 1. Diet and DNA methylation in the multigenerational agouti mouse model

The first demonstration that epigenetic mechanisms, and particularly DNA methylation, connect parental experience and behavior and lifelong offspring phenotypes involved the impact of maternal diet on offspring phenotype. The viable yellow agouti [*A*(vy)] mouse harbors a transposable element in the agouti gene which controls its expression and is regulated by DNA methylation, which silences the agouti gene leading to a brown coat phenotype. Methyl supplementation of maternal diets during pregnancy resulted in differences in methylation of the transposable element and expression of the agouti gene in the offspring, resulting in a brown coat phenotype [116].

Genistein, at levels comparable encountered in humans consuming high-soy diets, also increased methylation of the transposable element and shifted the coat color of heterozygous viable yellow agouti [*A*(vy/a)] offspring toward pseudo-agouti (brown) [117]. Maternal exposure to bisphenol A, a chemical found in plastics, led to loss of methylation and shifted the coat color of the offspring to yellow; this loss of methylation could be blocked by maternal dietary supplementation with methyl donors or genistein which prevent the hypomethylation induced by bisphenol A in the offspring [118]. Interestingly, although these maternal dietary modulations have a large impact on the immediate F1 generation offspring, Waterland *et al.* observed that supplementation across successive generations did not result in cumulative effects, and therefore suggested that these were examples of multiple generational exposure rather than transgenerational [119]. However, Cropley and colleagues showed that combining methyl donor supplementation with selection for a silent *A*(vy) allele progressively increases the prevalence of the associated phenotype in the population over five generations, suggesting true transgenerational inheritance [120]. Because this model utilizes a gene controlled by a methylation-sensitive regulatory region, the dietary supplements that have an effect on this phenotype are either inhibitors or enhancers of enzymatic DNA methylation reactions during embryogenesis, a period of time when broad reprogramming of DNA methylation takes place naturally. Does this mechanism extend to a wider scope of experiences that are not direct chemical inhibitors or donors of DNA methylation/reaction? In the context of nutrition, the question is whether exposures to different kinds of diets, irrespective of whether these diets are methyl rich or inhibitory of DNA methylation enzymes *per se*, can reprogram DNA methylation in the offspring in a physiologically relevant manner.

Box 2. Germline silencing by RNA-mediated DNA methylation

Because small noncoding RNAs (sncRNAs) can be distributed across cell barriers [70–72], sncRNA responses triggered in a tissue that reacts to experience could potentially be redistributed to gametes and transferred to the offspring. However, the involvement of sncRNA in nongenetic germline-mediated inheritance across more than two generations requires a mechanism for memorizing the initial effect of the RNA in the gametes that would resist epigenetic reprogramming in primordial germ cells. Parental imprinting and transposon silencing that are mediated by sncRNAs do not resist germ cell reprogramming. In fact they are developmentally reprogrammed by *de novo* induction of sncRNAs [78]. How could new parental experiences trigger a '*de novo*' induced sncRNA event that would persist in the progeny for many generations?

One possible mechanism is replication of new noncoding RNAs by RNA polymerase. In *Caenorhabditis elegans* long-term silencing of viral DNA through the RNAi machinery is transmitted in a non-Mendelian manner and requires the RNA-dependent RNA polymerase rrf-1 [121]. Another possible mechanism is that sncRNAs might induce specific changes in DNA methylation in vertebrates, and histone methylation in other animals, which might maintain a silencing event initially triggered by RNA through several generations, if indeed these DNA methylation and histone methylation marks resist epigenetic reprogramming in primordial germ cells and after fertilization. Such a chromatin/DNA-based mechanism could potentially perpetuate the initial RNA signal even after the original RNAs are depleted. For example, in *C. elegans* a piRNA-dependent foreign RNA can trigger highly-stable long-term silencing lasting at least 20 generations. The inheritance of the phenotype becomes independent of the original piRNA, but requires the maintenance of chromatin silencing [65].

Non-gamete mediated multigenerational transmission of parental experience and its epigenetic underpinning

If multigenerational transmission of ancestral experiential memory evolved to increase survival and fitness, such a mechanism should be able to modulate phenotypes crucial for survival, such as reproduction, mate selection, diet and feeding habits, and flight from threat. It is plausible then that nongenetic inheritance would function at different timescales depending on the nature of the ancestral experience. Maintaining plasticity in response to dynamic environments requires generation-limited and reversible reprogramming (Box 1). By contrast, a permanent change in habitat requires a stable multi-generational phenotypic transformation (Box 2).

Parental experiences and epigenetic-mediated multigenerational transmission

It has been long known both in human and rodents that the quality of perinatal care is associated with trajectories of physical and mental health later in life. For example, natural variation in maternal care between high licking-and-grooming (high-LG) mothers and low licking-and-grooming (low-LG) rat mothers is associated with phenotypic differences in the response to stress and anxiety in their adult offspring [79,80] (Figure 2). Cross-fostering experiments demonstrated that that these differences were not genetic (and are not gamete-mediated) because they were driven by the fostering mother [80]. The glucocorticoid receptor (GR), encoded by *Nr3c1*, is expressed at lower levels in the adult offspring of low-LG mothers, and these animals exhibit a heightened stress response [79,80]. Weaver *et al.* demonstrated differences in DNA methylation and histone acetylation in the *Nr3c1* promoter in response to differences in maternal LG behavior that emerged early in life and remained stable into adulthood, regulating expression of *(Nr3c1)* GR [11]. Later studies showed that the differences in DNA methylation are not limited to *Nr3c1*, but that hundreds of genes show differential expression between offspring of high- and low-LG mothers [81] and that differences in DNA methylation span broad genomic regions and gene networks, including the protocadherin (*Pcdh*) gene family [82]. The maternal effect on offspring phenotype could be reversed by epigenetic manipulations. Methionine is a precursor of the DNA and histone methylation cofactor SAM, and injection of methionine reverted high-LG offspring to exhibit stress and anxiety behaviors resembling low-LG offspring [83], whereas TSA (HDAC inhibitor) injection reverted low-LG adult offspring to behaviors that resembled high-LG animals, suggesting a causal relationship between DNA methylation and transmission of the phenotypes [11]. Interestingly, these phenotypes could be transmitted across several generations in the absence of gamete transmission because the offspring of high-LG mothers exhibit high-LG maternal care behavior, and this in turn epigenetically programs their offspring. In support of this hypothesis, female offspring of high LG mothers exhibit differences in the state of methylation of the estrogen receptor (ER) α1b promoter and estrogen receptor-α expression in the medial preoptic area (MPOA); differences in ERα receptor expression in the MPOA are associated with differences in their maternal behavior [12].

What is the mechanism that transmits signals from maternal behavior to the offspring epigenome in a given tissue? Firing of 5-HT receptors in the neonatal hippocampi in response to maternal LG triggers a signaling pathway resulting in cAMP-mediated induction of the transcription factor nerve growth factor (NGF)-induced clone A (NGFIA), which then delivers the transcription factor CREB binding protein (CBP) [84] and methyl-CpG binding domain protein 2 (MBD2) to the *Nr3c1* promoter [85]. This provides a plausible paradigm for a molecular chain of events between parental behavior and programming of the offspring genome in target tissues at specific genomic loci. The epigenetic response to parental experience engages known physiological systems that evolved to respond to experience, and this response targets particular functional gene pathways relevant to parental behavioral signals. Such a mode of multigenerational reprogramming is stable on the one hand, but plastic and reversible on the other, and is attuned to changes in the social environment.

It is extremely difficult to prove causation in human studies of DNA methylation.

It is possible to compare evolutionarily conserved responses in humans with animals that could be tested in randomized controlled experiments. Elevated DNA methylation in the *Nr3c1* promoter has been reported in adult humans who were abused as children [86]. Similarly to observations from rat studies, the differences in DNA methylation covered broad genomic regions [82] and the span of the *Pcdh* gene family locus was differentially methylated in humans who were subjected to child abuse [19], consistent with the hypothesis that response

of neonates to parental deprivation, or abuse, is evolutionarily conserved [19].

Natural disasters also offer a unique opportunity for a randomized study in humans. To test whether maternal exposure to objective stress during pregnancy would result in alterations in DNA methylation in offspring, DNA methylation in T cells derived from children whose mother was pregnant with them during the Quebec ice storm of 1998 was analyzed. A correlation was observed between the level of DNA methylation in several genes and the level of objective stress that the mother suffered during pregnancy [87]. These data strongly support the hypothesis that, in humans as well as in rodents, maternal experiences can be translated into epigenetic changes in offspring genomes.

Placenta-mediated transmission

The placenta is able to register maternal stress and is involved in the transmission of phenotypes to the fetuses that remain into adulthood [88,89]. Maternal dietary exposures have long been known to impact upon programming in the offspring. Particularly important is the link between maternal gestation diabetes in humans and increased risk for development of type 2 diabetes in the offspring. Several studies have recently demonstrated genome-wide alterations in DNA methylation in placentae, particularly in metabolism-associated genes, of babies born to mothers with gestational diabetes [90–93].

Gamete-mediated multigenerational transmission of parental experience

Exposure of either male or female gametes could change their epigenetic state and lead to phenotypic changes in the offspring that develop from these gametes (Figure 1). Although the main focus to date has been on the effect of gestational exposure of gametes, spermatogenesis continues throughout adult life, and it is therefore possible that preconception adult paternal experience might have an impact on the offspring. The question is whether these exposures are limited to chemical exposures or whether behavioral experiences might also be transmitted through the gametes to the next generation (Box 3).

Hormones, and particularly stress hormones, may link behavioral experience and tissues outside the brain such as sperm, which has been shown to contain glucocorticoid receptors [94–96]. Interestingly, Belyaev and colleagues demonstrated in the early 1980s that hydrocortisone injections of male mice that carried a mutation in the (fused) *Fu* gene (*Axin1*) could result in a decrease in the number of phenotypically *Fu* offspring. Their experiment ruled out differential death of gametes or embryos that expressed the *Fu* gene, and they concluded that this might be caused by a decrease in penetrance, although the mechanism was unknown at the time [97]. Interestingly, the gene can pass from an active to inactive state at a high rate, which is consistent with epigenetic regulation [98]. Exposure of adult male mice to synthetic glucocorticoids was examined to determine any effect on the phenotype of offspring conceived after exposure [99]. Differences in mineralocorticoid receptor (*Nr3c2*; MR), estrogen α receptor (*Nr3a1*; ERS1), and glucocorticoid receptor (*Nr3c1*; GR) expression in the hippocampus and kidney were observed in these offspring. Because the only vehicle of transmission between the fathers and their offspring was sperm, this study demonstrates that glucocorticoids can modulate sperm even in adult animals and that this could be transmitted to the next generation. While the study did not document gene-specific changes in sperm DNA methylation, a global change in non-CG methylation was noted (methylation of cytosines outside the consensus CG dinucleotides) [99].

SncRNAs may also link the brain and sperm. Male offspring of mice subjected to prenatal chronic variable stress during pregnancy develop a dysmasculinized and stress-sensitive phenotype, and transmit mainly the phenotype of dysmaculinized gene expression to the next generation (F2), supporting germline-mediated transmission of this phenotype [100]. Examination of miRNA expression in the brain identified two miRNAs that showed significant paternal stress effects [100]. These miRNA both regulate β-glycan mRNA, which was previously implicated in release of gonadal hormones. In this study the effects on F3 were not examined, and it is therefore unclear whether the effects are transgenerational.

Box 3. Gamete-mediated multigenerational transmission of parental preconception dietary experience and exposure to cocaine

Adaptive feeding behaviors and metabolic phenotypes are crucial for survival, therefore it is plausible that multigenerational inheritance of metabolic responses exist. In support of this hypothesis, feeding male mice with chronic high-fat diets results in reprogramming of pancreatic β cell functions in their female offspring: early onset of impaired insulin secretion and glucose tolerance with normal adiposity. A few hundred genes showed altered expression in β islets and a change in methylation of the *Il13ra2* gene in β-islet cells was demonstrated [122]. Wide changes in the transcriptome also occur in retroperitoneal white adipose tissue, and several gene networks were commonly affected in both tissues [123]. The data are consistent with alterations in early developmental networks. Because this phenotype is paternally transmitted it must involve sperm-mediated transmission. However, while the data demonstrate a sex-specific transgenerational epigenetic response to parental dietary challenge, it remains unclear whether the DNA methylation changes occurred in sperm, and whether these particular changes are functionally relevant. How were the changes in sperm, if any, transmitted to the pancreas? And could these epigenetic and phenotypic differences be transgenerationally transmitted and escape erasure during primordial germ cell reprogramming?

Male mice trained to self-administer cocaine sired male offspring who developed a response to cocaine phenotype that was characterized by delayed acquisition and reduced maintenance of cocaine self-administration [124]. Brain-derived neurotrophic factor (BDNF) was expressed at higher levels in the offspring prefrontal cortex (PFC), and the promoter of the gene displayed increased histone acetylation in the PFC. There was no noted difference in DNA methylation. Remarkably, there was also increased histone acetylation in the promoters of BDNF in the sperm of the father. The key question, however, is how was histone acetylation in BDNF in sperm triggered by cocaine self-administration, which is believed to be mostly appraised and processed in the brain? In addition, there is no known mechanism for propagation of histone acetylation. How was this acetylation signal transmitted from the sperm to the brain in the offspring through all the stages of differentiation, organogenesis, and neurodevelopment? Again it is unclear whether the effect is transgenerational and is only mediated by exposed paternal sperm, or whether it could be passed down to progeny via the offspring sperm [124]. In any case, these data raise the provocative prospect that male drug abuse pre-conception will have long-term phenotypic consequences in children who were never exposed to the drug [125].

In a more recent study, males were exposed to chronic stress either before or after puberty. The offspring of both groups of such stressed mice exhibited a blunted hypothalamic–pituitary–adrenal axis response, but no other notable behavioral measures. Because these effects were transmitted by paternal sperm it is hypothesized that the sperm of the fathers could be epigenetically programmed even during adulthood, as has been suggested from the effects of glucocorticoid treatment of adult mice [99]. Nine miRNAs were differentially expressed in the sperm of stressed mice, some of which target important developmental genes, including *Dnmt31* [101].

Gamete-mediated transgenerational inheritance of ancestral behavioral, toxic, and addictive experiences

There is increasing evidence for nongenetic gamete-mediated transgenerational inheritance of responses to several types of paternal experiences, including diet, stressful and adverse social experiences, and exposure to toxins and drugs of addiction, which is mediated via inheritance of epigenetic states. There is also new evidence of transgenerational transmission of ancestral experience of organ injury (Box 4).

Environmental toxin exposure

There is a significant body of evidence for multigenerational epigenetic inheritance in plants, worms, and fungi [102,103]. The first evidence in mammals for a *bona fide* (F3 transmission; Figure 1) transgenerational epigenetic transmission came from experiments exposing pregnant rats to the endocrine disruptor vinclozolin during the gestational period of gonadal sex determination [104]. Exposed offspring exhibited reduced fertility and sperm counts. Importantly, the effect lasted through to the F4 generation. DNA methylation is the only epigenetic mechanism known to be copied through cell divisions and, if DNA methylation plays a role in transmission through generations, changes in DNA methylation should be detected in sperm. In this study, DNA methylation differences in sperm were detected by a combination of methylation-sensitive restriction enzyme digestion and PCR amplification in F1 mice, and one region was shown to be different up to the F3 generation, suggesting that this DNA methylation difference escaped primordial germ cell (as well as early developmental) reprogramming [104]. Although the doses used were higher than usually found in the environment, the study provided first proof of principle that mechanisms for transgenerational transmission of environmental exposures are associated with changes in DNA methylation in sperm. Sex-specific changes in the transcriptome were observed in the brain, as well as differences in anxiety-related behaviors, suggesting that there is a mechanism that transmits the epigenetic signal from sperm to brain during development [105].

Further work has investigated the phenotypic scope of genome-wide changes in DNA methylation in sperm through generations and expanded this line of analysis to other environmental toxicants [106]. For example, the plastic-derived endocrine disruptor compounds bisphenol-A (BPA), bis(2-ethylhexyl)phthalate (DEHP), and dibutyl phthalate (DBP) triggered pubertal abnormalities, testis disease, obesity, and ovarian disease (primary ovarian insufficiency and polycystic ovaries) in animals of the F3 generation, while kidney and prostate disease were observed only in the F1 generation [106]. 197 differential DNA methylation regions (DMR) in gene promoters were revealed in the F3 generation sperm epigenome, and some of these promoters were previously shown to be associated with the pathologies triggered by exposure [106]. Similar conclusions were derived using jet fuel [107] and dichlorodiphenyltrichloroethane (DDT) [108].

The differential DNA methylation regions identified for different environmental exposures do not overlap. However, they all have a common structural feature – the differential methylation sites occur in regions with a very low density of CGs [109].

Interestingly, exposure to endocrine disruptors defines not only phenotype but also mate preference in a sex-dependent way: females three generations removed from the exposure discriminate and prefer males who do not have a history of exposure, whereas similarly epigenetically imprinted males do not exhibit such a preference [110]. Such a mechanism could have an impact on how a history of environmental exposures might affect the evolution of species [110].

Early-life stress and social adversity

Depressive phenotypes and altered responses to adverse environments are phenotypes that are triggered in male mice by maternal chronic and unpredictable separation during early life [111]. These phenotypes are then transmitted to their offspring over up to two generations and, because these phenotypes are transmitted from the fathers despite the fact that the mothers are normal, they must result from sperm-mediated transmission [111]. There is sexual dimorphism and 'skipping a generation' in the transmission of the phenotypes to the F2 and F3 generation, an observation that has been repeated in several studies of epigenetic transgenerational transmission in

Box 4. Transgenerational transmission of ancestral experience of organ injury

An unusual example of transgenerational transmission of ancestral experience is the observation that an experience of liver injury in rodent male ancestors reduces liver fibrogenesis in F2 male offspring. Molecularly, it is characterized by elevation in hepatic expression of the antifibrogenic factor peroxisome proliferator-activated receptor γ (PPAR-γ) and reduction in the levels of the profibrogenic factor transforming growth factor β1 (TGF-β1). DNA methylation differences are detected in both genes in the liver of F2 offspring of injured ancestors, showing persistence of the DNA methylation mark in the liver. This study addresses one of the mysteries of sperm-mediated epigenetic transmission; how does a target tissue which experiences such exposure communicate this exposure to the sperm? Transfer of serum from the injured rat to the uninjured rats induced a modest increase in PPAR-γ-associated H3K27me3, and a 15-fold enrichment of H2A.Z in the uninjured rat sperm, suggesting that the injured livers secrete a soluble factor that alters chromatin in sperm; however, the nature of this factor remains unknown. Moreover, it is still unknown how the sperm modification state is replicated, escapes reprogramming, and is translated through embryogenesis and liver development to a particular DNA methylation alteration in the offspring liver [126].

animals and in humans. For example, in the forced swim test the males (but not females) were different from controls in the F2 generation, but females (and not males) were different in the F3 generation. The authors tested the hypothesis that changes in DNA methylation in F1 sperm might mediate the nongenetic transmission by showing that a few genes (such as methyl CpG binding protein 2, MeCP2) were differentially methylated in the F1 sperm and in the F2 brain [111]. This remarkable observation suggests that the maternal separation altered DNA methylation in sperm, and that this pattern survived reprogramming during early development and was replicated during brain development in the F2 generation. The DNA methylation pattern was partly maintained in the sperm of the F2 generation, which is a prerequisite for transgenerational transmission via DNA methylation [111]. These data show that a behavioral exposure could alter DNA methylation in the sperm, which points to the possibility that behavioral experiences of ancestral generations can be registered in the epigenome and be maintained across both reprogramming during early development and during primordial germ cell differentiation, as well as during organogenesis. It is nevertheless difficult to conceive a mechanistic framework for these events, and it is particularly difficult to understand how experiences registered in the brain might be transmitted to the sperm. Moreover, it is unknown how methylation patterns in sperm could guide specific methylation states in the brain.

One possible mediator of response to behavioral signals is noncoding RNA. Recent work using a model of early-life trauma in mice that involves unpredictable maternal separation combined with unpredictable maternal stress shows transgenerational transmission of altered behavioral and metabolic phenotypes. This work identified noncoding RNA expressed in the sperm as a mediator of nongenetic transmission [112]. A definitive proof that sperm RNA mediates transgenerational transmission was provided by showing that injecting sperm RNAs from traumatized males into fertilized wild type oocytes reproduced the alterations in behavior in the offspring. Subsequently, differences in miRNA expression were seen in hippocampus and serum, but not sperm, in the F2 generation, suggesting that the miRNA is not transmitted transgenerationally in the sperm. At the F3 generation differences in phenotypes were noted, but no miRNA differences were detected. Other epigenetic mechanism(s) may have embedded the original miRNA response in the sperm epigenome, and these mechanisms must be accurately replicated through meiosis and mitosis. It is tempting to suggest that the initial miRNA signal results in a change in DNA methylation in the F2 sperm, that is then maintained and replicated through meiosis and mitosis ([113] for a recent thorough discussion of epigenetic regulation of heritable RNA).

Transgenerational transmission of odor fear conditioning

The olfactory system is crucial for species survival, associating odors with distinct threats and developing a characteristic adaptive and protective behavioral response. It stands to reason that transmission of ancestral memory of association between smell and danger would increase the survival of a species. The question is whether acquisition of a fear response to a predator smell is solely defined by natural selection, or whether epigenetic mechanisms have evolved to address this challenge. Dias and Ressler trained mice in one generation to associate a specific odor (acetophenone), with mild foot-shocks as a fear threat. Remarkably, their study shows that when mice are trained with acetophenone the F1 and F2 generations respond with a heightened startle response to acetophenone (but not propranolol), and when the ancestors are trained with propranolol their descendants respond to propanolol and not to acetophenone. The authors show that the response is transmitted through either the male or female germline for up to two generations, suggesting that sperm and egg DNA register the exposure as an epigenetic mark. *In vitro* fertilization using sperm from a specific-odor conditioned mouse results in increased size of odor-specific glomeruli in the olfactory bulb [114].

Sperm DNA methylation is a plausible heritable epigenetic mark on DNA and is an excellent signal to mediate this mode of transmission. The gene encoding the olfactory receptor that the mice were trained to associate with a fearful experience is differentially methylated in the sperm, and this differential methylation remains in F1 and F2 generations, indicating that it escapes both the post-fertilization and primordial germ cell erasures of DNA methylation as a true transgenerational epigenetic mark [114]. Surprisingly, the same receptor is not differentially methylated in the target tissue, raising the question of how the DNA methylation signal in the sperm is transmitted during neurodevelopment and is translated into differential expression relevant to the brain phenotype? Moreover, how does an epigenetic mark in sperm dictate the formation of the complex network that associates fear and smell in particular neurons in a defined anatomical distribution in the brain during neuronal development? This question has been common in almost all transgenerational data. The scope and the relative impact of this mechanism in introducing new traits in vertebrate and human evolution remains unknown, and these data have far-reaching implications. It should be of no surprise that the paper describing transgenerational transmission of odor fear conditioning has been recently challenged [115]; however, there was no experimental evidence for this challenge. Replication with other fear-conditioned odor paradigms and other strains of mice or perhaps even other species will be crucial for the general acceptance of these provocative results.

Concluding remarks and future perspectives

Many questions remain regarding the strength and significance of transgenerational phenotypes and further replication is required. Particularly important is estimating how widespread transgenerational nongenetic inheritance is in humans. There are lingering doubts about whether stable transgenerational effects are truly epigenetic, or whether they are genetic differences that are misconstrued as nongenetic inheritance. With accumulating evidence, confidence in this process is increasing. However, despite

Box 5. Outstanding questions

- How does experience sensed by the brain (or other tissues) of the parent lead to an epigenetic change in the gamete?
- What defines the specificity of the response in the gamete genome, and how does it register a complex experience that requires fine-tuning of multiple gene expression circuits in multiple tissues (e.g., reprogramming associated with fear conditioning or metabolic control)?
- How does the epigenetic change escape reprogramming and how is it replicated across generations?
- How is the epigenetic change in sperm translated to a network of epigenetic instructions during embryonic development, leading to the establishment of particular gene expression programs in particular tissues?

advances in understanding the epigenetic biochemistry and developmental mechanisms involved, there are fundamental mysteries that are difficult to unravel with current understanding (Box 5).

Although epidemiological and animal experiments have provided support for the different forms of nongenetic inheritance, in the absence of a plausible mechanism the interpretation of these data remains a matter of intense controversy. The cardinal issue is defining a fundamental common mechanism (or several different mechanisms) that can explain the different modes of nongenetic inheritance.

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