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REVIEW

Epigenetic Inheritance of Disease and Disease Risk

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Epigenetic marks in an organism can be altered by environmental factors throughout life. Although changes in the epigenetic code can be positive, some are associated with severe diseases, in particular, cancer and neuropsychiatric disorders. Recent evidence has indicated that certain epigenetic marks can be inherited, and reshape developmental and cellular features over generations. This review examines the challenging possibility that epigenetic changes induced by environmental factors can contribute to some of the inheritance of disease and disease risk. This concept has immense implications for the understanding of biological functions and disease etiology, and provides potential novel strategies for diagnosis and treatment. Examples of epigenetic inheritance relevant to human disease, such as the detrimental effects of traumatic stress or drug/toxic exposure on brain functions, are reviewed. Different possible routes of transmission of epigenetic information involving the germline or germline-independent transfer are discussed, and different mechanisms for the maintenance and transmission of epigenetic information like chromatin remodeling and small noncoding RNAs are considered. Future research directions and remaining major challenges in this field are also outlined. Finally, the adaptive value of epigenetic inheritance, and the cost and benefit of allowing acquired epigenetic marks to persist across generations is critically evaluated.
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INTRODUCTION: WHAT IS EPIGENETIC INHERITANCE?

Epigenetic processes intimately link environmental factors to our genetic code, by allowing outside events to leave biochemical footprints on our genome. Modern epigenetics can be defined as 'the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Bird, 2007) (see Box 1 for a detailed definition of epigenetics). Therefore, epigenetics offers mechanisms by which cells that are equipped with identical genetic information can acquire and maintain an individual molecular fingerprint reflecting not only the cell's history, but also programming its response to future events (Levenson and Sweatt, 2006). Two of the most prominent epigenetic mechanisms are DNA methylation (DNAme) and histone posttranslational modifications (HPTMs). DNAme is a modification of the DNA itself that occurs when a methyl group is transferred to the fifth position of the pyrimidine ring of cytosines in dinucleotide CpG sequences in mammals (Tost, 2009). HPTMs are modifications of protein histones rather than the DNA itself. Histones are associated with DNA to form nucleosomes in the chromatin and strongly influence chromatin structure. HPTMs are various and include acetylation, methylation, phosphorylation, sumoylation, ubiquitination, and others (Jenuwein and Allis, 2001). Together, DNAme and HPTMs can regulate gene expression by chromatin remodeling. Over the course of a lifetime, the epigenetic code—grossly defined as all chromatin modifications—changes dramatically. This is best exemplified in monozygotic twins whose epigenome is similar early in life but diverges extensively during development and adult life (Fraga *et al*, 2005).

Basic research has clearly demonstrated that transient or chronic environmental events can permanently alter the epigenetic code. Prime examples in the mammalian brain are the long-term epigenetic remodeling of the stress axis in response to stressful experiences early in life (Murgatroyd *et al*, 2009; Franklin *et al*, 2010), or the cascade of epigenetic events involved in the development of drug addiction (Kumar *et al*, 2005; Robison and Nestler, 2011) observed in experimental animals. Therefore, the marks accumulated and maintained within the epigenetic code throughout life carry important information about the interaction between the individual and its environment. In some instances, this information can be highly relevant for the offspring, and may theoretically provide an adaptive advantage (Harper,

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Box 1 Definition of Epigenetics

The term epigenetics refers to the study of mitotically and/or meiotically heritable changes in gene functions that cannot be explained by changes in DNA sequence (Russo *et al*, 1996; Allis *et al*, 2007). This definition emphasizes that epigenetic changes are heritable, which implies that any change in cells that do not divide, such as neurons in the adult brain, are excluded. More recent definitions of epigenetics are less stringent, and include 'all structural adaptations of chromosomal regions that can register, signal or perpetuate altered activity states' (Bird, 2007). Today, epigenetics can therefore be defined as 'the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA' (Goldberg *et al*, 2007). The most classical regulatory processes underlying epigenetic changes include DNA methylation, and posttranslational histone modifications.

2005; Jablonka and Raz, 2009). But at the same time, the epigenetic load accumulated over the course of a lifetime might also bring risk factors for disease, as many epigenetic changes are associated with cancer risk and predisposition to psychiatric disorders (Suter *et al*, 2004; Hitchins *et al*, 2007; Graff and Mansuy, 2009; Ptak and Petronis, 2010). It might therefore be beneficial to erase some of the epigenetic burden to give the new generation a *clean state*, while it might also be advantageous to keep and transmit other marks and give the offspring a *head start*.

This review describes prominent instances in which the epigenetic information acquired by the parent generation can be transmitted to the offspring. We highlight different possible routes of transmission and discuss which mechanisms might be in place to facilitate such transgenerational effects. We focus, in particular, on brain functions in mammals, and discuss the question of the transmission of epigenetic marks via the germline, a conceptually challenging mode of transfer of epigenetic information with major implications for disease risk. We also point out caveats inherent to studies of epigenetic inheritance, and highlight areas of interest for future research.

EPIGENETIC CONTRIBUTION TO HERITABILITY OF DISEASE RISK

Most of the complex diseases, including many psychiatric disorders, have a major heritable component (Kendler, 2001; Eichler *et al*, 2010). To date, despite the extensive use of high-throughput genetic screening techniques, the majority of disease heritability still remains unaccounted for (Altshuler *et al*, 2008; Manolio *et al*, 2009). The concept of 'missing heritability' has therefore gained great attention among geneticists (Manolio *et al*, 2009; Eichler *et al*, 2010). In humans, disease heritability can be estimated in several ways, but the most accurate estimates are provided by adoption studies in monozygotic twins. Because of their identical genetic make-up, such heritability estimates can distinguish biological from environmental factors (Visscher *et al*, 2008). However, if acquired epigenetic marks could also be transmitted from parents to offspring, heritability estimates would include both genetic and epigenetic contributions, and because of the complexity of the genome in humans, these factors are difficult to distinguish (Nadeau, 2009). In this review, inheritance will be considered as the transfer of phenotypic traits attributed to factors passed to the offspring through gametes, and include both genetic and epigenetic components. Thus far, the contribution of epigenetic mechanisms to the transmission of disease risk across generations has largely been ignored in studies of heritability (Jablonka and Raz, 2009). This is mainly because it does not concur with classic Mendelian inheritance and has thus received strong conceptual opposition. However, evidence for transgenerational effects that cannot be explained by Mendelian inheritance has accumulated (Jablonka and Raz, 2009; Franklin and Mansuy, 2010a). Early examples derived from mutant mouse lines in which the expression of a transgene was dependent on the parent of origin and associated with differences in DNAme (Hadchouel *et al*, 1987; Sapienza *et al*, 1987). A recent example with a link to brain function stems from research using a chromosome substitution mouse model, in which the Y-chromosome of a host strain (C57Bl6) is replaced with the Y-chromosome of a donor strain (129) characterized by high anxiety (Nelson *et al*, 2010). In these mice, C57Bl6 males carry either their natural Y-chromosome, or a 129 Y-chromosome. Following many generations of backcrossing to C57Bl6, when males are mated with C57Bl6 dams, females in the resulting litter are genetically identical and should thus be phenotypically similar, but differ in that their father carries either a C57Bl6 or a 129 Y-chromosome. This, however, greatly influences their behavior, and when tested on the open field and elevated plus maze, C57Bl6 females from fathers with a 129 Y-chromosome were more anxious than those from fathers with a C57Bl6 Y-chromosome. These striking results suggest that the Y-chromosome of a father can influence the female offspring, likely through epigenetic processes (Nelson *et al*, 2010).

Although evidence from transgenic mice or chromosome substitution models provides proof of principle that classical inheritance cannot explain all facets of heritability, these studies are not directly linked to human diseases. However, a few examples of epigenetic inheritance in humans have also been reported, in particular, examples of the impact of stress as a possible transgenerational risk factor for depression, or the effect of diet on the risk for cardiovascular diseases and diabetes across generations, and multigenerational effects of exposure to environmental toxins on cancer risk and anxiety disorders. We argue that transgenerational transmission of epigenetic information may account for some of the missing heritability of complex disorders, and that investigating the epigenetic contribution to psychiatric diseases may reveal new targets for treatment.

DIFFERENT MODES OF TRANSGENERATIONAL EPIGENETIC EFFECTS

Transgenerational epigenetic effects occur when an environmental trigger induces epigenetic changes that can be

observed in at least one subsequent generation. Three different routes of transgenerational epigenetic effects can be distinguished (Jirtle and Skinner, 2007; Youngson and Whitelaw, 2008). The first operates during prenatal life and involves fetal programming. With this route, environmental factors during pregnancy induce epigenetic changes in somatic cells of the developing fetus and affect its development. This may ultimately lead to specific phenotypic traits later in postnatal life. The second route operates through behavioral transfer. In this case, epigenetic changes are elicited and propagated from one generation to the next via behavioral or social interaction between parents and offspring. This route requires that the environmental factors inducing the epigenetic changes be present at each generation, and does not involve true inheritance. In contrast, the third route involves the germline, and does not require repetition of the trigger but the transfer of epigenetic changes in germ cells by sexual reproduction. Germline epigenetic inheritance is an intriguing mode of transmission that, however, remains controversial. This is because it contrasts with classical Mendelian genetics, and also because it remains mechanistically unresolved. The following section describes evidence for the three different routes of transmission.

Fetal Programming

Fetal programming is mediated by factors to which a developing embryo is exposed *in utero*. It involves epigenetic changes in somatic cells that modulate developmental processes and elicit specific features or alterations expressed by the organism after birth (Chen and Zhang, 2011). Typically, fetal programming implicates environmental stimuli that are evolutionary relevant. It is hypothesized to represent a form of adaptive response preparing an organism to future external factors that may be encountered after birth (Plagemann, 2005). The best-known examples of fetal programming are the influence of maternal diet on the physiology and health of the offspring, and the effects of prenatal stress on brain development and emotional reactivity. Because they occur *in utero*, such effects can also affect germinal cells in the embryo, and the following offspring. These cases then qualify as germline epigenetic effects and will be discussed later in this section.

Diet. Strong evidence in humans shows that *in utero* exposure to altered nutrition, whether under- or over-nutrition (eg, maternal obesity and high-fat diet), is a major risk factor for the development of obesity and diabetes in adulthood (Barker, 2004; Plagemann, 2005; Hanson and Gluckman, 2008; Seki *et al*, 2012). Similar effects have been observed in rodent models, and are associated with epigenetic changes in liver, pancreas, and fat tissue, as well as in brain regions involved in feeding and metabolism (Seki *et al*, 2012). The observed epigenetic alterations are changes in DNAme and HPTMs at multiple metabolism-related genes. A particularly insightful example comes from a rat model of intrauterine growth retardation (IUGR) that reproduces the metabolic syndrome and development of diabetes in human (Gatford *et al*, 2010). IUGR is induced by uterine artery ligation, and limits the supply of critical metabolic substrates to the fetus. It is associated with permanent reduction in the expression of *pancreatic and duodenal homeobox 1* transcription factor (*Pdx1*), a gene critical for pancreatic development. This is correlated with programming of an epigenetic cascade involving HPTMs and DNAme already *in utero* (Park *et al*, 2008). Following IUGR, histones 3 and 4 (H3 and H4) associated with the *Pdx1* promoter get deacetylated in the fetus. After birth, the *Pdx1* promoter recruits the histone deacetylase HDAC1, becomes further deacetylated, and acquires the repressive histone mark H3K9me2. This reduces binding of a key transcription factor (USF-1), followed by a strong increase in DNAme at the CpG island of the *Pdx1* promoter. This cascade ultimately leads to a marked decrease in *Pdx1* expression. This exemplifies the molecular complexity of the epigenetic cascade that can be initiated by an environmental trigger *in utero,* and lead to a stable gene expression profile in adulthood. Other instances of the epigenetic underpinning of fetal programming and the risk for metabolic disorders have recently been reviewed (Lillycrop, 2011; Seki *et al*, 2012).

Prenatal Stress. Another factor that prominently affects development is prenatal stress. Such stress can result from psychologically and/or physiologically threatening situations that a gestating mother may be exposed to (Chen and Zhang, 2011; Harris and Seckl, 2011). Prenatal stress has been associated with several behavioral alterations such as temperamental problems, impaired cognition and attention, and symptoms of autism, depression, and schizophrenia during childhood and adulthood in humans. Similarly, impaired cognition, increased stress responsiveness and depression, and anxiety behaviors have been observed in animal models of prenatal stress (O'Donnell *et al*, 2009; Charil *et al*, 2010; Harris and Seckl, 2011). In rats, the heightened stress response observed later in life depends on maternal corticosteroid release during gestation, since adrenalectomy of the dam prevents the effects of prenatal stress (Barbazanges *et al*, 1996). Conversely, glucocorticoid injections during pregnancy mimic the effects of prenatal stress. Changes in gene expression and DNAme of the glucocorticoid stress-hormone receptor were observed, together with alterations in serotonergic signaling, depressive behavior, and increased stress responsiveness (Mueller and Bale, 2008). Other prenatal stressors such as infections, starvation, or hypoxia have similarly been linked to depression and neurodevelopmental disorders like schizophrenia, ADHD, and autism (Bale *et al*, 2010; Ronald *et al*, 2010). The transgenerational germline effects of prenatal stress exposure (Morgan and Bale, 2011) and the potential epigenetic mechanisms involved are further described below.

Social/Behavioral Transfer

In most mammals, the first and most formative environmental experiences of the newborn occur through the interactions with the mother. The quality of maternal care has long been recognized as a major factor determining the psychological development and well-being of the offspring (Belsky *et al*, 1991). The quality of maternal care is itself susceptible to environmental factors such as food supply, social hierarchy, and stress. Some epigenetic mechanisms account for the long-lasting effects of mother–infant interactions, and provide a means for the acquired information to be passed from mother to offspring across generations (Weaver, 2007; Champagne, 2008).

In rats, maternal care is reflected by the time a mother spends licking, grooming, and nursing her pups. The amount and quality of care strongly affect cognitive and emotional development of the pups (Champagne *et al*, 2003). Rat dams—as well as human mothers—have different mothering skills and provide variable level and quality of maternal care to their offspring. Dams can be divided based on their maternal care into high, mid, or low licking/grooming (LG) mothers. Compared with pups raised by high-LG mothers, pups raised by low-LG mothers have increased stress responsiveness, associated with elevated activity of the hypothalamus–pituitary–adrenal (HPA) stress axis. When adult, the neglected rats respond to stressful situations with prolonged secretion of the stress hormone corticosterone. They have less glucocorticoid receptor (GR) mRNA and protein in the hippocampus, but more corticotrophin-releasing hormone (CRH) mRNA in the hypothalamus (Meaney, 2001a; Champagne, 2008). Behaviorally, the offspring of low-LG mothers have higher anxiety and impaired learning when adult. These symptoms and the persistent alteration of the stress axis have been associated with epigenetic mechanisms (Weaver, 2007). After the first week of life, pups of high-LG mothers have increased NGFI-A expression and binding to the GR promoter, a process involved in the regulation of GR expression, compared with low-LG offspring (Weaver *et al*, 2007). However, the increase in NGFI-A expression is only transient and is not observed in adulthood. It therefore does not account for the persistent effect on GR expression. Instead, DNAme of the GR gene, in particular at a single CpG site within the NGFI-A response element, is strongly increased 1 week after birth through to adulthood in low-LG offspring (Weaver *et al*, 2004). This results from a succession of epigenetic events that are differentially regulated by maternal care. In high-LG offspring, the sharp increase in NGFI-A expression during the first postnatal week triggers a cascade of downstream events initiated by the recruitment of CREB-binding protein (CBP), a protein with histone acetyltransferase activity, after NGFI-A binding to GR promoter. Enrichment of CBP in the GR promoter region and increased H3K9 acetylation, a permissive HPTM, are detected in the brain of high-LG offspring. This suggests that high expression and binding of NGFI-A might prevent DNAme in this region during a critical developmental window. In contrast, in low-LG offspring, the reduced binding of NGFI-A to the promoter might expose the chromatin to the DNAme machinery and induce hypermethylation at this locus. *In vitro* assays indeed show that increased promoter methylation prevents NGFI-A binding. Therefore, once a DNAme pattern is established, it can prevent NGFI-A binding later in life, and reduce GR expression (Weaver *et al*, 2007).

Because it is elicited by maternal behavior, the altered promoter methylation can be maintained across generations (Weaver *et al*, 2004). Cross-fostering prevents such transfer and when the offspring of low-LG mothers are raised by high-LG surrogate mothers, the methylation level of the GR promoter is restored. In this case, DNAme is directly associated with the behavior of the rearing mother, but is independent from the biological mother. It is a clear example of transgenerational transmission of an epigenetic mark through a maternal-dependent mode of behavioral transfer.

Germline Transmission

Germline transmission of epigenetic information requires that the epigenetic marks be first established in the germ cells of the parent generation, maintained through meiosis, then passed to the offspring. Experimentally, such epigenetic germline inheritance is not easily distinguished from non-germline epigenetic inheritance, mostly because maternal interventions are difficult to rule out as a source of transmission (Champagne, 2008). To eliminate this possibility, breeding studies based on males (when a treated male is paired with a naive female to get a patriline) are necessary. Indeed, most studies on germline transmission have focused on patrilines, and relatively little is known about matriline transmission (Weiss *et al*, 2011). However, although rat and mouse males do not contribute to the raising of pups, patrilineal designs must be interpreted with caution, because maternal investment can vary depending on the physical characteristics of the mate. For example, a female may vary her level of care to her pups depending on the fitness or attractiveness of her mate (Gowaty *et al*, 2007; Curley and Mashoodh, 2010). Since little is known about how females can sense and evaluate health and fitness of mates, patriline studies cannot fully rule out maternal effects. Cross-fostering, although laborious, can potentially clarify such uncertainty. Furthermore, a control of intrauterine environment may also be needed in some cases, and *in vitro* fertilization (IVF) and/or embryo transfer methods can be envisaged to exclude this possibility. However, IVF and preimplantation embryo transfer can both elicit alterations of the epigenome (Grace and Sinclair, 2009), which could potentially interfere with germline transmission of the epigenetic marks under investigation. These limitations need to be considered when interpreting data on germline epigenetic inheritance. Despite these limitations, and the apparent antidogmatic nature of the concept, there is now substantial evidence that transmission of acquired

traits can occur through germline-dependent epigenetic marks and not just through classical Mendelian heredity.

CURRENT EVIDENCE FOR GERMLINE EPIGENETIC INHERITANCE IN MAMMALS

Transposable elements

Studies in *agouti viable yellow* (A^{vy}) mice and *axin-fused* ($Axin^{Fu}$) mice have provided initial evidence for epigenetic inheritance through the germline in mammals (Morgan *et al*, 1999; Rakyan *et al*, 2003). These mice are unique in that they carry transposable elements in one allele of the agouti or axin locus that can be silenced by methylation. These epigenetically regulated alleles, called metastable epialleles, are linked to specific phenotypic traits. When hypomethylated, they are responsible for a phenotype characterized by a yellow coat (A^{vy}, as compared with the agouti brown-colored coat of wild-type mice) or a kinked tail ($Axin^{Fu}$, as compared with a straight tail in wild-type mice) (Rosenfeld, 2010). Intermediate levels of methylation lead to phenotypes with varying yellow patches of fur or different degree of kinked tail (Wolff *et al*, 1998; Rakyan *et al*, 2003; Rosenfeld, 2010). The alterations in DNAme also coincide with changes in HPTMs at the differentially methylated regions, hinting at an interplay between DNAme and HPTMs in gene regulation (Dolinoy *et al*, 2010). Although genetically identical, the different methylation of the metastable epialleles can be passed to the offspring through the matriline in the case of A^{vy} (Morgan *et al*, 1999), and through both matriline and patriline for $Axin^{Fu}$ (Rakyan *et al*, 2003; Fernandez-Gonzalez *et al*, 2010). The most exciting finding, however, was that feeding of a diet rich in methyl donor to pregnant dams could shift the phenotype of the offspring toward silenced epialleles. This not only demonstrated that DNAme does play a role in phenotype transmission, but also that external conditions can shift the phenotype of the offspring (Wolff *et al*, 1998). Because of conflicting results, however, the idea that such a diet-induced shift can be inherited across several generations is still controversial. When pseudoagouti females were chosen for breeding and were fed a diet supplemented with methyl donor, a transgenerational phenotypic shift toward the 'silent', methylated phenotype could indeed be demonstrated across two generations (Cropley *et al*, 2006; Cropley *et al*, 2012). If, however, mice carrying the weakly methylated allele (yellow or motteled phenotype) were chosen for breeding, no transgenerational shift in phenotype was observed (Cropley *et al*, 2007; Waterland *et al*, 2007a). The possibility that the observed transgenerational effect is due to the *in utero* effects of the methyl donor directly on germ cells in the developing embryos needs to still be addressed (Waterland *et al*, 2007b). Support for the notion that diet can induce changes in germline epigenetic programming and inheritance comes from studies using $Axin^{Fu}$ mice. In a given mouse, DNAme at the $Axin^{Fu}$ allele in male sperm correlates with the methylation level of somatic cells (Rakyan *et al*, 2003). DNAme in the sperm determines the penetrance of the kinked-tail phenotype (Fernandez-Gonzalez *et al*, 2010), such that high DNAme is associated with penetrance of the silent phenotype, whereas hypomethylation is associated with penetrance of the kinked-tail phenotype. DNAme might, however, not be the mark directly responsible for transmission. A recent study showed that $Axin^{Fu}$ methylation is lost after fertilization, but that the penetrant phenotype in the preimplantation embryo correlates with the histone marks dimethylated lysine 4 on histone 3 (H3K4me2) and acetylated lysine 9 on histone 3 (H3K9ac) (Fernandez-Gonzalez *et al*, 2010). These marks are indicative of active chromatin, and might later prevent the establishment of DNAme, thus perpetuating the epigenetic information across generations (Delaval *et al*, 2007). Despite these findings, mouse models with inserted transposable elements are specific to this species and may provide an artificial system. Nevertheless, although no comparable retroviral inserts exist at the human orthologs of the *Agouti-related protein* and *Axin1* genes (Rosenfeld, 2010), transposable elements are common in the human genome and might play a role in epigenetic inheritance (Lane *et al*, 2003), a possibility that remains to be demonstrated.

Stress

Stressful conditions are negative environmental factors with potentially a strong impact on behavior across generations. In the context of psychology and neuroscience, stress can be described as the state of arousal of an individual in response to an aversive situation (Kim and Diamond, 2002). Severe stressors can affect brain functions and mental health throughout life (Lupien *et al*, 2009). In particular, when experienced early in life, stressful and traumatic events are major risk factors for the development of behavioral and emotional disorders later in life (Heim *et al*, 2008). Many of these behavioral disorders also have strong heritable components, which often cannot be explained by genetic factors alone. A few studies have now begun to examine the possibility that stressful events experienced by one generation can also affect the offspring, even if the offspring itself has not been exposed to any stressor.

Prenatal stress. As early as during development *in utero*, exposure to stress can have long-term effects on brain functions. When pregnant dams are exposed to stressful conditions during gestation, their male offspring is dysmasculinized (shortened anogenital distance, reduced male-typical copulatory behavior) and is highly sensitive to stress (Ward, 1972; Morgan and Bale, 2011). The offspring of prenatally stressed males, born and raised by nonstressed females, also tend to have increased stress sensitivity and dysmasculinization (Morgan and Bale, 2011). In the brain of the offspring one day after birth, a shift toward a female-like expression pattern is observed for several genes important for neurodevelopment, and several microRNAs are also significantly reduced. These modifications are transmitted through the patriline, raising the possibility that miRNAs

present in the sperm may play a role in the transgenerational transmission of the behavioral changes induced by stress. More studies are however needed to address this important question.

Postnatal early-life stress. The negative effect of postnatal stress and the involvement of epigenetic processes have also been demonstrated in experimental animals. In a series of studies, our lab has shown that severely stressful experiences early in postnatal life affect behavior across several generations. Using a mouse model combining unpredictable maternal separation and maternal stress to mimic early traumatic life events, we showed that the stressed pups (F1 generation) develop depressive phenotypes in adulthood, and display an altered response to novelty, deficits in risk assessment behaviors, and impaired social behaviors (Franklin *et al*, 2010; Weiss *et al*, 2011). When these F1 animals were bred to naive controls, and the F2 offspring was further bred similarly, both F2 and F3 mice exhibited behavioral symptoms comparable to F1 animals (Franklin *et al*, 2010; Franklin *et al*, 2011; Weiss *et al*, 2011). Furthermore, in these animals, several molecular pathways were perturbed across generations. For instance, serotonergic signaling was altered and serotonin receptor 5HT1A binding was reduced in several brain regions (Franklin *et al*, 2011). The reduction in 5HT1A autoreceptors in the dorsal raphe coincided with increased serotonin release in the frontal cortex, an area strongly innervated by serotonergic neurons from the dorsal raphe. The transmission of stress-induced changes across generations was observed through both males (Franklin *et al*, 2010) and females (Weiss *et al*, 2011). In this model, transmission through several generations is particularly remarkable, because F3 mice and the germ cells they originate from were never exposed to stress, and must therefore have inherited behavioral alterations acquired by their fathers through epigenetic processes. Indeed, initial evidence suggested that epigenetic mechanisms in germ cells are at work. Thus, in F1 sperm, there were differences in DNA methylation at the promoter regions of several candidate genes involved in stress, emotionality, or epigenetic regulation, including corticotrophin-releasing factor receptor 2 (*CRFR2*), cannabinoid receptor 1 (*CB1*), and methyl-CpG binding protein 2 (*MeCP2*). Similar alterations also occurred in the brain of F2 progeny, coinciding with the inherited behavioral phenotype. In the brain, the changes in DNA methylation paralleled changes in gene expression for all candidate genes. In the matriline, the phenotype could not be reversed by cross-fostering, ruling out maternal care as the primary mediator of transmission. Together, these data provide evidence for a relationship between the alteration of DNAme and transmission of behavioral traits induced by early-life stress across generations. Specifically, they suggest that early-life stress experienced by the parent or grandparent generation may contribute to inherited risk for the appearance of depression in the offspring.

A similar observation has been reported in a rat model of maternal abuse, in which pups were exposed to a stressed dam displaying poor and abusive maternal behaviors (Roth *et al*, 2009). Rats exposed to abusive mothers express lower level of brain-derived neurotrophic factor (*BDNF*) mRNA in the prefrontal cortex, coinciding with increased DNAme of the *BDNF* gene. Although weakened, this effect could be passed on to the offspring through the mother, but could not be completely reversed by cross-fostering, indicating that additional factors are at play, possibly transmission of the epigenetic information through the germline.

Stress during adulthood. Recent work has suggested that the transgenerational effects of severe stress may not be restricted to stressful events experienced prenatally or early in life. It was reported that chronic social defeat, a severe form of stress to which mice were exposed during adulthood, induces a phenotype marked by stress vulnerability, depressive and anxiety behaviors in the offspring (Dietz *et al*, 2011). Further to breeding through the patriline, IVF technique was also used. Thus, two-cell embryos generated from oocytes of superovulated donor females and sperm of stressed or control males were implanted into naive pseudopregnant females. Interestingly, many of the stress-induced behavioral alterations were not observed in IVF-generated offspring, although the depressive phenotype still persisted (Dietz *et al*, 2011). In a way, the persistence of the depressive phenotype makes a strong case for epigenetic germline transmission independent of maternal care. Furthermore, as IVF is known to affect epigenetic reprogramming differently from natural sexual reproduction, it is not surprising that it may result in the loss of some of the behavioral effects (Grace and Sinclair, 2009). In summary, stressful life experiences can profoundly affect the organism throughout development, and several studies now indicate that detrimental effects can also be observed in the nonstressed offspring of stressed mice. These effects are, at least to some degree, likely transmitted through the germline and may involve mechanisms such as DNAme and small noncoding RNAs.

Evidence from the human literature provides support for an impact of stress across generations, but also demonstrates the difficulty of untangling such effects in human populations. Recent studies have demonstrated a higher prevalence of depression and anxiety disorders in descendants of holocaust survivors than in a control population (Yehuda *et al*, 2008). Furthermore, the lifetime risk for posttraumatic stress disorder (PTSD) in the offspring depends on the presence of maternal and, to a lesser extent, paternal PTSD. Similarly, parental PTSD is associated with a lower level of plasma cortisone in the offspring (Yehuda *et al*, 2007). However, these studies cannot determine whether the differential risk for psychiatric illness in the children results from transmission through the parental germline or through differences in parenting. PTSD in the offspring could also result from a genetic predisposition. Animal models are therefore necessary to better delineate the contribution of epigenetics to the inheritance of disease risk. Hopefully, these models will identify molecular targets that can then be assessed in human populations.

Environmental toxicants

Strong evidence for transgenerational inheritance of environmentally-induced phenotypes comes from the endocrine disruptor vinclozolin, an antiandrogenic compound used as a fungicide for agricultural fruit crops. Exposing mice repeatedly to high doses of vinclozolin during mid-gestation increases the risk of infertility, tumor formation, kidney disease, immune abnormalities, and anxiety behavior across four generations (Anway *et al*, 2005; Jirtle and Skinner, 2007). These effects correlate with abnormal DNA methylation in the sperm across generations (Anway *et al*, 2005). A recent epigenome-wide approach identified 16 genes that show alterations in DNA methylation in their promoter regions in the sperm of third-generation offspring (F3) of animals exposed to vinclozolin (Guerrero-Bosagna *et al*, 2010). This shows the potentially widespread epigenomic effects of vinclozolin across generations. In addition, this study also identified a consensus DNA sequence that is more prevalent in the promoter regions of the genes that are significantly altered and in imprinted genes, compared with the prevalence at random promoters throughout the genome (Guerrero-Bosagna *et al*, 2010). Another study focused specifically on the effects of vinclozolin on imprinted genes in sperm. It showed reductions in DNAme at several paternally imprinted genes, and increased DNAme at some maternally imprinted genes (Stouder and Paoloni-Giacobino, 2010). It is thus possible that specific sequence patterns, including sequences featured in imprinted genes, are responsible for rendering a given region of the genome more prone to respond to environmental factors and/or more resilient to the erasure of epigenetic marks during developmental reprogramming. Because anxiety was also affected across generations, a genome-wide study looked at gene expression in brain samples of F3 offspring (ie, two generations removed from vinclozolin exposure). Widespread differences in gene expression were observed in the hippocampus and amygdala, brain regions important for cognitive functions and mood regulation (Skinner *et al*, 2008). Concomitantly, anxiety was decreased in F3 males but increased in F3 females. These results suggest that vinclozolin exposure causes epigenetic changes that persist across generations and that can alter the gene expression profile as well as behavioral outcomes of the offspring. However, the transgenerational effects of vinclozolin repeatedly observed by two labs (Jirtle and Skinner, 2007; Stouder and Paoloni-Giacobino, 2010) have been criticized, because the high dose of vinclozolin used is not physiologically relevant to humans. Furthermore, two other groups have failed to replicate the transgenerational effects at a lower dose or even at the same dose (Schneider *et al*, 2008; Inawaka *et al*, 2009). These discrepancies are still a matter of debate, and highlight the need for further investigation.

Transgenerational effects in response to exposure to environmental toxicants during pregnancy have also been observed for other substances such as the synthetic estrogen diethylstilbestrol (DES), the environmental estrogen bis-phenol A (BPA) (Salian *et al*, 2009), the contaminant dioxin (TCDD) (Bruner-Tran and Osteen, 2011), or the pesticide methoxychlor (MXC) (Stouder and Paoloni-Giacobino, 2011). Several studies have demonstrated that DES can increase the incidence of tumors in the offspring and grand-offspring (Walker, 1984; Walker and Haven, 1997; Newbold *et al*, 2006). Data suggest that transmission involves the germline, although factors coming from the maternal environment also influence the phenotype (Walker and Kurth, 1995). Despite the observation that epigenetic alterations such as changes in DNAme occur in animals exposed to DES *in utero* (Li *et al*, 1997; Li *et al*, 2003; Bromer *et al*, 2009; Sato *et al*, 2009), little is known about the epigenetics of transgenerational transmission (LeBaron *et al*, 2010), a topic that will hopefully be examined in the future.

Drugs

Alcohol consumption is known to affect epigenetic mechanisms in several organs. A recent transcriptome study in human *post-mortem* brain samples compared alcoholics with age-matched controls and detected profound (epi)-genome-wide effects of alcohol abuse (Ponomarev *et al*, 2012). A notable difference was that endogenous retroviral sequences, which are normally silenced by DNAme, were less methylated, coinciding with dramatically increased transcription of their host genes. As retroviral sequences can maintain DNAme throughout gametogenesis and fertilization, it will be highly interesting to determine whether the offspring of alcoholic parents still carry elevated DNAme at retroviral sequences. Based on the example of the Agouti viable yellow (A^{vy}) mouse model in which a retrotransposon can be silenced by DNAme and inherited through the germline (Morgan *et al*, 1999), it may also be the case in humans.

Pregnancy is a particularly sensitive period, and *in utero* exposure to alcohol has teratogenic effects on the fetus, partially linked to epigenetic mechanisms (Shukla *et al*, 2008; Guerri *et al*, 2009). In mice, exposing A^{vy} dams to ethanol increases methylation at the A^{vy} allele, silences agouti expression, and thereby shifts the offspring phenotype toward agouti colored coat (Kaminen-Ahola *et al*, 2010). Alcohol exposure *in utero* also reduces DNAme at the differentially methylated domain of the paternally imprinted growth-related gene H19 in the sperm of exposed mice (F1) (Stouder *et al*, 2011). Most notably, a similar decrease at the same CpG sites is observed in the brain of the offspring (F2). It is conceivable that changes in DNAme at imprinted genes can hijack the ability of imprinted genes to escape the global epigenetic reprogramming event after fertilization, thus allowing them to be carried over to the next generation. These findings are in agreement with human data showing that compared with nondrinkers, moderate and heavy drinkers show subtle reductions in DNAme at the *H19* imprinted gene in sperm (Ouko *et al*, 2009). Furthermore, mice sired by males acutely exposed to alcohol show a range of developmental disturbances ranging from reduced litter

size and birth weight, to behavioral alterations such as lower fearfulness and higher aggression (Jamerson *et al*, 2004; Meek *et al*, 2007). Similarly, studies in humans suggest that paternal alcohol consumption has detrimental effects on cognitive performance in their children (Tarter *et al*, 1989), an effect that could be because of epigenetic alterations.

Cocaine is another drug that can affect the epigenome, and its use in animal models has been a valuable paradigm to study the epigenetic underpinning of drug addiction in animals (Robison and Nestler, 2011). Elegant studies in rats have revealed that cocaine has a profound effect on chromatin remodeling in several brain areas involving reward circuits such as the nucleus accumbens (Kumar *et al*, 2005; Renthal *et al*, 2009; Maze *et al*, 2010). Although no studies have yet been published on transgenerational effects, some evidence already points to measurable behavioral alterations in the offspring of male rats exposed to prolonged cocaine self-administration paradigms (McCarthy *et al*, 2011; Vassoler *et al*, 2011). It will thus be exciting to observe future developments in this field.

In summary, the potent and persistent impact of drugs of abuse on behavior, such as compulsive drug seeking and relapse, clearly imply epigenetic mechanisms. These mechanisms may also be at play in the germline, and interfere with normal embryonic epigenetic reprogramming, such as genomic imprinting or the silencing of retroviral sequences. Future studies will be important to determine if this is the case.

Enriched conditions

Brain functions generally benefit from stimulating environmental conditions, a concept that was initially demonstrated experimentally by Donald Hebb in 1947 (Hebb, 1947). He reported that rats that were raised free-roaming in his house outperformed rats raised in cages on problem-solving tasks (Hebb, 1947). Since then, numerous animal studies have confirmed that rodents raised in environments with ample sensory, motor, social, and cognitive stimulation have improved cognitive performance compared to animals raised in standard environments (Nithianantharajah and Hannan, 2006). In rats and mice, larger cages equipped with running wheels and frequently changing toys typically provide enrichment. Epigenetic mechanisms play a role in the long-term cognitive effects induced by such stimulating early environments. For example, total level of H3 and H4 acetylation and methylation is increased in the hippocampus and cortex of enriched mice, and the cognitive enhancement observed in these mice can be mimicked by injections of histone deacetylase (HDAC) inhibitors (Fischer *et al*, 2007). The potential transmission of such effects has also been reported in mice (Arai *et al*, 2009). In one study, enhanced memory performance and synaptic plasticity resulting from exposure to enriched conditions for 2 weeks during postnatal life (aged 2–4 weeks) was transmitted to the offspring. However, transmission occurred only through the mother, not the father. Importantly, it was independent of maternal care, as the effect of enrichment persisted even after cross-fostering (Arai *et al*, 2009). The epigenetic mechanisms underlying the observed effects have not yet been investigated, but may involve persistent modifications such as DNAme. Because such mechanisms may be at play to allow transgenerational transmission of epigenetic marks acquired in response to adverse, stressful life events (Franklin *et al*, 2010), similar mechanisms might also allow for positive, stimulating effects to be carried across generations. This would open the door for behavioral interventions to possibly counteract negative life experiences and allow for compensation through stimulating environments.

Diet

Currently, the best evidence for the occurrence of transgenerational effects induced by environmental factors in humans comes from studies investigating nutrition and food availability. A well-known example is the Dutch famine of 1944–/1945. During the Second World War, Nazi-led Germany imposed a food embargo on the Netherlands, leading to a devastating 5-month famine. Mothers who were pregnant during this time gave birth to children who had reduced birth weight and developed a host of clinical disorders during adulthood ranging from obesity to glucose intolerance and coronary heart disease (Susser and Stein, 1994; Kyle and Pichard, 2006). Surprisingly, the grandchildren of mothers exposed to the famine during gestation showed similar effects. This raises the possibility that starvation during pregnancy might have induced epigenetic changes in the germline of the exposed embryos that were then passed on to their own children (Lumey and Stein, 1997; Stein and Lumey, 2000; Painter *et al*, 2008). Other groups have reported similarly intriguing effects. For example, food supply during the slow-growth period (the time preceding the prepubertal peak in growth velocity) is associated with mortality risk ratios of the offspring in a sex-specific manner (Bygren *et al*, 2001; Pembrey *et al*, 2006; Kaati *et al*, 2007). Food supply of the paternal grandmother was associated with the mortality risk of the granddaughters (because of cardiovascular disease and diabetes) (Kaati *et al*, 2007), whereas food supply of the paternal grandfather was associated with the mortality risk of the grandsons (Pembrey *et al*, 2006; Kaati *et al*, 2007). Of course, studies in humans are merely observational and, in the mentioned cases, retrospective in nature. Therefore, they cannot provide information on the mechanisms underlying the observed effects.

Work in rodents similarly suggests that during pregnancy, alterations in diet affect the offspring across several generations. This possibility has already been realized more than 30 years ago. When rats were fed low-protein diet across many generations, their offspring presented complex behavior alterations such as deficits in home-orienting behavior and visual discrimination compared with rats malnourished only for one generation (Stewart *et al*, 1975; Galler, 1980, 1981). Furthermore, although returning the multi-generation food-deprived rats to normal diet for several generations

was able to reverse some of the deficits, certain behavior alterations persisted even after two generations of normal feeding (Galler, 1981; Galler and Seelig, 1981). The partial reversibility suggests that no genetic change due to genetic drift underlie these phenotypes. However, genetic drift cannot be ruled out for effects that persisted despite being placed on normal diet. Revisiting some of these early studies with modern genetic tools might prove extremely interesting, although such longitudinal breeding designs are very time consuming.

As already discussed earlier, food restriction or overfeeding of the dam (F0) during gestation affect birth weight of the offspring (F1), and later in life both paradigms lead to similar high-risk phenotypes for the development of metabolic imbalances including obesity and impaired glucose tolerance. Even when F1 animals then receive normal diet throughout their life, their F2 offspring still exhibit, depending on experimental protocol, altered birth weight, impaired glucose tolerance, and reduced insulin secretion (Benyshek *et al*, 2006; Dunn and Bale, 2009; Jimenez-Chillaron *et al*, 2009; Ng *et al*, 2010; Pentinat *et al*, 2010; Dunn and Bale, 2011). In some reports, these changes have also been observed in the F3 generation (Benyshek *et al*, 2006; Dunn and Bale, 2011). Molecularly, these transgenerational effects are associated with altered gene expression in the liver across three generations (Carone *et al*, 2010; Hoile *et al*, 2011). Some of the genes with altered expression pattern in F1 and F2 also had changed DNAme, and changes in HPTMs were observed as well (Burdge *et al*, 2007; Lillycrop *et al*, 2007; Lillycrop *et al*, 2008). A recent genome-wide study has analyzed epigenetic alterations in the sperm of male mice fed a low-protein/high-sugar diet (Carone *et al*, 2010). No change in DNAme was detected in the sperm, but differences in gene expression were observed in the liver of the offspring. This may indicate that DNAme is not directly responsible for the altered expression, or that potential changes may not have been detected because of insufficient sensitivity of the used immunoprecipitation technique. Furthermore, other epigenetic processes were found to be altered, in particular the histone mark H3K27me3 that was decreased at the promoter of several genes in the sperm of malnourished males. These results demonstrate that the epigenetic landscape of sperm cells is sensitive to dietary factors, and that changes in this landscape can exert transgenerational effects (Carone *et al*, 2010). Several studies have identified target genes associated with transgenerational metabolic alterations (eg, *GR*, *PPARα*, and *11βHSD1*) (Burdge and Lillycrop, 2010); thus, future work should use sensitive high-resolution techniques to investigate whether DNAme and/or HPTMs are modified at these genes in the germline. Another promising approach is to systematically analyze the epigenetic composition of sperm derived from males fed high- or low-fat diet because these males were shown to yield offspring with impaired metabolic functions (Carone *et al*, 2010; Ng *et al*, 2010). Such studies could further be conducted in a translational approach and compare the effects of diet in rodents and human subjects.

POTENTIAL MECHANISMS OF GERMLINE TRANSMISSION

The concept that acquired epigenetic marks can be inherited transgenerationally through the germline has been, until recently, fraught with major conceptual challenges. DNAme or HPTMs were not considered stable enough in gametes to allow for transgenerational transmission. Since germline transmission can best be investigated by studying the patriline, much of the research has focused on changes occurring in sperm cells. In the paternal genome, DNAme is dynamically regulated and goes through several waves of establishment and erasure. It first undergoes a major reset after fertilization in the preimplantation embryo, which is likely a prerequisite for totipotency (Feng *et al*, 2010). Further to demethylation, most histones linked to DNA are replaced by protamines during spermatogenesis, which implies that epigenetic modifications of histone tails are largely lost. However, it is now becoming increasingly clear that epigenetic marks in germ cells are more stable and persistent than initially assumed. A recent genome-wide DNA methylation study revealed that a substantial number of genes retain parental DNAme in promoter regions after fertilization, which is an important factor for the transgenerational transmission of DNAme (Borgel *et al*, 2010). Similarly, not all histones in sperm are replaced by protamines and those that are maintained likely keep their PTMs and can be transferred to the oocyte (Hammoud *et al*, 2009; Brykczynska *et al*, 2010). Further to DNAme and HPTMs, new data also point to a role for noncoding RNAs in the transgenerational information transfer through the germline (Johnson *et al*, 2011a). Together, these findings provide potential mechanistic pathways possibly underlying germline transmission of epigenetic marks.

DNA methylation

DNAme is a strong candidate mechanism for the transgenerational transmission of acquired epigenetic information. A key argument is its involvement in genomic imprinting (Paoloni-Giacobino and Chaillet, 2006; Sha, 2008). Although most genes are expressed from both parental alleles, imprinting is a mechanism to silence one parental allele, resulting in expression of only the allele from the other parent. Therefore, by definition, imprinting is a form of epigenetic information transfer from parents to offspring. Most imprinted genes (~100 currently known) contain imprint control regions, which show differential DNAme between the maternal and paternal alleles (Sha, 2008; DeVeale *et al*, 2012). Sex-specific imprints are established during oogenesis and gametogenesis, and importantly they retain their methylation marks during the wave of demethylation that occurs shortly after fertilization (Sha, 2008; Weaver *et al*, 2009).

Furthermore, it has recently been suggested that DNAme at nonimprinted regions may also be retained across generations at a much larger scale than previously anticipated. First, DNAme is maintained throughout embryonic development

at intracisternal A particles (IAPs), the abundant retrovirus-like long-terminal repeat transposable elements in the mouse genome (Lane *et al*, 2003). Genes that carry such repeat-rich sequences might be targets for transgenerational epigenetic regulation (Ruden *et al*, 2008). Beyond imprinting and repeat-elements, an epigenome-wide immunoprecipitation approach for methylated cytosines determined that a group of >200 genes retain promoter methylation in the preimplantation and postimplantation embryo (Borgel *et al*, 2010). The few genes that have been individually confirmed so far suggest that DNAme from the maternal oocyte is maintained, but maintenance of male germline methylation is also possible. Importantly, such studies are limited by the affinity of antibodies, and thus regions with smaller CpG content or lower levels of DNAme cannot be sampled (Weber *et al*, 2005). Therefore, if specific methylation marks at individual CpG sites (eg, transcription factor biding sites in target genes) were to be transmitted, they would evade detection, thus still leading to an underestimation of the total number of inherited DNAme marks. The fact that certain DNAme marks can be maintained from one generation to the next, combined with the knowledge that methylation marks can be modulated by environmental factors such as stress (Murgatroyd *et al*, 2009; Franklin *et al*, 2010), exercise (Guo *et al*, 2011), or memory formation (Miller and Sweatt, 2007), make DNAme an interesting mechanism to explain transgenerational epigenetic transmission of acquired information.

Histone posttranslational modifications

In somatic cells, HPTMs influence chromatin structure and play a major role in regulating gene expression. In sperm cells, however, most histones are replaced by protamines. This dramatically compacts the genome to fit into the small sperm head, and results in the loss of epigenetic modifications that were on the removed histones. However, it has been known for several decades that a sizable proportion of histones are retained in sperm cells (Gusse *et al*, 1986; Gatewood *et al*, 1990). Although this proportion is modest in mice (1–2%), in humans an estimated 4–15% of histones are retained (Hammoud *et al*, 2009; Johnson *et al*, 2011a). Only recently has it been demonstrated, however, that HPTMs can also be maintained in the sperm and transferred to the oocyte where they likely play a critical role in embryonic development after fertilization (Hammoud *et al*, 2009; Brykczynska *et al*, 2010). Specifically, in humans and mice, dimethylated lysine 4 on histone 3 (H3K4me2) and trimethylated lysine 27 on histone H3 (H3K27me3) can be retained in sperm. H3K4me2 is associated with genes relevant for spermatogenesis, while H3K27me3 is associated with developmental regulatory genes. Furthermore, there is also a clear correlation between H3K27me3 present at transcription start sites of genes in sperm and gene expression in early embryos, raising the possibility that this histone mark may contribute to paternal transmission of epigenetic information across generations, a possibility that still needs to be proven (Brykczynska *et al*, 2010). It is becoming increasingly clear that the retention of histones is tightly regulated in sperm. The density of GC-sequences determines the retention of histones in sperm across the genome (Vavouri and Lehner, 2011). Therefore, histones are preferentially retained in CpG islands around transcription start sites, suggesting that epigenetic information at a large number of genes could potentially be retained throughout fertilization. Furthermore, because of the interaction between repressive histone modifications and DNAme, the retention of histones at CpG-rich regions likely accounts for the low level of DNAme of CpG islands established during embryogenesis and maintained throughout life (Vavouri and Lehner, 2011). In addition to histones, it is of course also conceivable that protamines may have important regulatory functions in sperm cells beyond their key role in genome compaction (Balhorn, 2007).

Altogether, the highly regulated maintenance of histones in sperm and the transmission of these histones and their PTMs to the zygote may play an important role in gene regulation in the developing embryo. This makes HTPMs a potential candidate for transgenerational transmission of epigenetic information that needs to be further studied in the future (Puri *et al*, 2010).

Small noncoding RNAs in sperm

RNAs have long been thought to be absent in mature, transcriptionally quiescent sperm (Krawetz, 2005). However, owing to recent methodological advances, it is now firmly established that small amounts of RNA are present in the sperm (Krawetz, 2005; Johnson *et al*, 2011a). A big proportion of sperm RNA consists of fragmented remnants of ribosomal RNA that has been cleaved to prevent spurious translation (Johnson *et al*, 2011b). However, mRNAs and small noncoding RNAs such as microRNA (miRNA), piwi-interacting RNA (piRNA), or small nucleolar RNA (snoRNA) have also been identified (Miller and Ostermeier, 2006; Krawetz *et al*, 2011). One possibility is that these RNAs contribute to chromatin remodeling in the sperm, providing a molecular toolbox for transgenerational epigenetic inheritance. This idea is supported by the fact that a proportion of sperm RNAs associate with the sperm nuclear matrix (Lalancette *et al*, 2008), and that miRNAs detected in mature sperm mainly align to promoter regions, raising the speculation that they may bind paternal DNA and either recruit gene silencing machinery (Kim *et al*, 2008) or prevent histone to protamine transition during sperm nuclear remodeling (Johnson *et al*, 2011a).

Additionally, a fertilizing human sperm can carry an estimated 10–20 fg of RNA to the oocyte (Johnson *et al*, 2011a), thus constituting a direct signal that carries information from one generation to the next. Although miRNAs typically act to repress translation by targeting the 3′UTRs of their target mRNAs (Ghildiyal and Zamore, 2009) the role of sperm-delivered miRNAs in the oocyte remains somewhat controversial, because endogenous miRNAs have been shown to only poorly repress target mRNAs in the oocyte

(Ma *et al*, 2010). However, recent studies have found that individual sperm-borne miRNAs, miR-134A and miR-34c, regulate key genes in the zygote and are crucial for normal embryo development (Pang *et al*, 2011; Liu *et al*, 2012). In addition, piRNAs are known to play a role in germ cell development, especially in the silencing of transposons by guiding DNA methylation (Aravin *et al*, 2008; Law and Jacobsen, 2010). Just recently, the presence of piRNAs in mature sperm was recognized, when >1000 piRNA sequences common to three mature human spermatozoal small noncoding RNA libraries were detected (Krawetz *et al*, 2011). Thus, if piRNAs were delivered from sperm to oozyte, they could play a role in establishing DNA methylation marks.

Paramutation. All studies in support of transgenerational epigenetic effects discussed so far have mainly considered DNAme or HPTMs as likely candidate mechanisms for transmission across generations. However, recent findings have raised the possibility that sperm RNA may also contribute. Historically, the first clear demonstration that epigenetic transgenerational inheritance can occur came from plant models, specifically maize (Brink, 1973). It has since been shown that multiple mechanisms involving a plant-specific RNA-dependent RNA polymerase, DNAme at noncoding tandem repeats, and siRNAs likely mediate the observed transgenerational epigenetic effects (Chandler, 2007; for review, see Stam, 2009; Chandler, 2010).

In mammals, the first study on RNA-mediated transgenerational inheritance has focused on the mouse *Kit* gene, which encodes a tyrosine kinase receptor (Rassoulzadegan *et al*, 2006). Although a loss-of-function mutation leads to death shortly after birth in homozygotes, mice heterozygous for a *Kit*-null insertion mutant are viable but have an altered pigmentation pattern. Instead of pink feet and pink tails that are characteristic of wild-type mice, heterozygous *Kit* mutant mice have white feet and white tails. Surprisingly, most wild-type offspring of these heterozygous mice also showed an altered pigmentation pattern (white tail). Despite carrying two wild-type alleles, *Kit* mRNA levels were reduced to a level similar to heterozygous mice. In the sperm of heterozygous mice, much higher levels of *Kit* RNA were detected than in wild-type controls. Transmission of the white-tail phenotype was thus suggested to occur via the presence of abnormal RNA in the sperm. This hypothesis was corroborated by the fact that the phenotype could be transmitted to the next generation when total RNA from *Kit* mutants or miRNAs targeting the *Kit* mRNA (miR-221 or miR-222) were injected into wild-type fertilized eggs (Rassoulzadegan *et al*, 2006).

A similarly striking effect was also reported for miR-1 and its mRNA target *Cdh9*, a key regulator of cardiac growth, overexpression of which leads to cardiac hypertrophy in mice. miR-1 was shown to increase *Cdh9* mRNA expression, and microinjection of this miRNA into fertilized eggs was associated with transmission of cardiac hypertrophy across three generations, via the matriline as well as the patriline (Wagner *et al*, 2008). This demonstration of transgenerational epigenetic inheritance suggests that RNA, RNA fragments, or miRNAs present in the fertilized egg likely mediate the transmission of information across generations. An important parameter in mammals that makes the possibility that RNAs are involved in transmission plausible is that RNAs contained in sperm are delivered to the oozyte upon fertilization. Thus, this putative mode of transgenerational transmission of information has great potential, but given the important implications and issues that it raises, it will need to be carefully analyzed and confirmed in the future.

RNAi in Caenorhabditis elegans. Because transgenerational studies in rodents are hampered by the relatively slow reproductive cycle (eg, breeding mice across three generations can take more than 1 year), studies in more rapidly reproducing organisms have long been the preferred choice of many geneticists. Within the past few months, several highly interesting papers have been published that demonstrate transgenerational epigenetic inheritance through RNA-dependent pathways in the nematode *C. elegans*.

When *C. elegans* is infected by a virus, it mounts an immune response by generating virus-derived small-interfering RNAs (viRNAs) to silence virus expression. Surprisingly, this acquired defense has been shown to be transmitted to the offspring, so that when future generations get infected with the same virus, they can suppress its effect even if they lack the machinery necessary to mount the original virus response (Rechavi *et al*, 2011). Using an RNA sequencing approach, it was determined that small viRNAs complementary to the virus genome, which were generated upon first exposure to the virus, were indeed transmitted through sexual reproduction across at least three generations, indicating transmission of an acquired phenotype across generations. A previous study had already demonstrated a curious phenomenon of transgenerational memory transfer in *C. elegans*. It showed that after exposing several generations of worms to favorable odor cues, the offspring showed a memory in the form of increased migration when the odor was presented. This effect was extremely persistent, and was detectable for at least 40 generations after exposure to the odor had ceased (Remy, 2010).

Just this year, another study in *C. elegans* proposed the idea of an interplay between small RNAs and HPTMs in the context of epigenetic inheritance. Specifically, it was observed that upon triggering a siRNA response in the parent generation, the silencing histone mark H3K9me3 was induced, and was present in the offspring (Gu *et al*, 2012). Because both the small RNA itself and the histone mark could still be detected in the next generation, it is not yet clear which of the two signals is required for the transgenerational transfer. Inheritance of chromatin modifications in a germline-dependent manner across generations was also reported in *C. elegans* and proposed to determine longevity of the offspring (Greer *et al*, 2011). Here too, it is conceivable that small RNAs are involved in this phenomenon. The mechanisms that would allow siRNAs to modify histones are currently unknown, but it can be anticipated

that exciting work from this field will soon provide new insights into these mechanisms. Of course, it is entirely possible that mechanisms uncovered in the nematode system do not translate to mammals; however, as many of the observed mechanisms have already been described in plants (Bayne *et al*, 2010; Suter and Martin, 2010), they may have been evolutionary conserved also in mammals.

EVOLUTIONARY BENEFIT, DISEASE RISK, AND DRUG DEVELOPMENT

Evidence in favor of epigenetic inheritance from basic and clinical research is mounting. Rapid progress has been made in the understanding of the epigenetic mechanisms involved in establishing and maintaining epigenetic marks. At this point, sound evidence exists for the lifelong epigenetic programming established already *in utero* as well as epigenetic programming as a result of mother–infant interactions. In addition, the concept of germline epigenetic inheritance has become mechanistically plausible, and its occurrence in mammals probable. Given these developments, it is of course important to consider why mechanisms in support of transgenerational transmission of acquired epigenetic marks do exist. Obviously, an advantage of epigenetic inheritance over classical inheritance is that a rapid, targeted adaptation to new environmental challenges can be passed from one generation to the next (Harper, 2005; Jablonka and Raz, 2009). This may better prepare the offspring for challenging environmental conditions they might encounter during their lifetime. For instance, increased insulin sensitivity in the offspring of mothers who encountered scarce food supply might promote adipogenesis, thus providing nutritional reserves to protect the brain after weaning (Godfrey *et al*, 2010). Thereby the offspring is better equipped to deal with an environment in which food resources are similarly scarce. If, however, environmental conditions are markedly different than 'anticipated', the individual would be 'mismatched' and have a phenotype not appropriate for that environment. Thus, increased insulin sensitivity in an environment with abundant food resources could lead to obesity and diabetes. The same concept could also explain why alterations in response to highly stressful life circumstances become maladaptive, if the environment encountered by the offspring is not the adverse environment encountered by the parents. If mechanisms for epigenetic inheritance are indeed in place, it is also possible that certain unrelated factors that share some of the same molecular pathways might exert undesirable transgenerational effects. This could explain why certain compounds such as alcohol or environmental toxicants, which profoundly alter the epigenetic make-up, could lead to disease across generations (Jirtle and Skinner, 2007; Franklin and Mansuy, 2010a).

Importantly, the notion of epigenetics blurs the old boundaries between nature (genetic factors) and nurture (environmental factors) (Meaney, 2001b). This will play an important role in our understanding of disease and disease risk, as it is likely that complex interactions arise between our genetic make-up, the potential inheritance of certain epigenetic marks through the germline, and the epigenetic marks acquired in early development and later in life (Petronis, 2010). Despite the fact that these novel concepts further convolute an already complex system, epigenetic mechanisms also hold a great promise for diagnostics and drug development. On the one hand, epigenetic marks may be able to inform us about disease susceptibility of a given individual. For example, higher methylation levels at specific sites of the glucocorticoid stress hormone receptor have been detected in the brains of suicide victims, with different methylation profiles associated with the victims' history of childhood abuse (McGowan *et al*, 2009). In addition to diagnosis, epigenetic marks are interesting candidates for drug treatment, as epigenetic changes are—in stark contrast to gene mutations or single-nucleotide polymorphisms—reversible. Epigenetic drugs that broadly target DNAme (eg, DNA methyltransferase inhibitors) as well as HPTMs (eg, HDAC inhibitors) have already been approved for treatment of several forms of cancer (Stresemann and Lyko, 2008; Rodriguez-Paredes and Esteller, 2011). However, because of the broad and nonspecific nature of these drugs, and unwanted side effects, several more targeted epigenetic modifiers are currently under intense research investigation and in clinical trials (Hamm and Costa, 2011). For etiologically complex disorders such as most psychiatric diseases, research is still in the early stages. Several drugs that are already well accepted for treatment of depression seem to engage epigenetic mechanisms, specifically HPTMs (Tsankova *et al*, 2006; Covington *et al*, 2009). To date, most psychiatric diseases or their corresponding animal models have been linked to epigenetic dysregulation (Graff and Mansuy, 2009; Ptak and Petronis, 2010; Day and Sweatt, 2012), and thus we expect that the next years will bring major advances in the contribution of epigenetic mechanisms to disease etiology. One key point will be to understand how epigenetic marks can be induced or manipulated at specific target sites with tissue or even cell-type specificity. Although still poorly understood, the body effectively employs such mechanisms to establish tissue-specific epigenetic marks dependent on environmental experiences (Graff and Mansuy, 2009; Franklin and Mansuy, 2010b; Guo *et al*, 2011). For epigenetic drugs to act with high specificity and without broad side effects, these lessons still need to be learned from basic research.

FUTURE RESEARCH DIRECTIONS

Clearly, the study of transgenerational epigenetic effects is still at the beginning, and much work will be needed to provide firm evidence for the proposed mechanisms. It is a challenging field of research given the difficulties in study design, duration of experiments, technical and methodological challenges, and the requirement for

highly interdisciplinary expertise. However, new concepts are expected to quickly emerge in this rapidly expanding field, and it will remain a major challenge to integrate new findings into existing conceptual frameworks. A current example is the discovery of the presence of high level of 5-hydroxymethylcytosine (5hmC) in the brain (Kriaucionis and Heintz, 2009). This is particularly exciting, because DNA hydroxymethylation (DNAhme) is another direct epigenetic modification of the DNA. This has led to two not mutually exclusive theories that DNAhme may be an intermediate state in DNA demethylation, or may be an epigenetic modification with important regulatory functions on its own (Branco *et al*, 2012). The possibility that it is involved in DNA demethylation is appealing because the mechanisms of demethylation are still unknown (Ooi and Bestor, 2008). But the possibility that it has an independent role is also very interesting, and initial evidence has shown that DNAhme is involved in the epigenetic reprogramming following fertilization (Iqbal *et al*, 2011; Wossidlo *et al*, 2011). However, nothing is known about its possible contribution to transgenerational epigenetic inheritance, and there is yet no evidence that environmental factors can affect DNAhme. Studies addressing this possibility are expected in the near future.

Another milestone in epigenetics in general, and transgenerational epigenetics in particular, is the development of methods for manipulating certain epigenetic modifications with high specificity. It is currently not possible to provide direct evidence that a specific DNAme mark in sperm cells *causes* a specific phenotype in the offspring. For such demonstration, it would be necessary to reproduce the methylation profile (erase or add methyl residues) at specific loci or even specific CpG site(s), and then show that similar marks are present (or absent) in the offspring. Such a technical breakthrough would provide a great conceptual advance and also propel drug development forward for the design of specific epigenetic drugs. The design of drugs that can specifically interfere with DNA methylation without inducing point mutations would for instance be highly desirable.

Beyond the new challenges that arise from novel discoveries in epigenetics research and technical limitations to be overcome, the advent of more powerful and sensitive (epi)genome-wide screening techniques is expected to revolutionize the ability to study environmental regulation of epigenetic marks and transgenerational transmission. For example, it will be critical to determine with high resolution—across the genome—how DNAme and HPTMs in mature sperm can respond to environmental challenges, and which alterations can persist throughout fertilization and embryo development. Similarly, the identification and characterization of minute levels of RNA in sperm by next-generation sequencing will shed new light on the precise role of RNAs in transmitting information across generations, and whether and how the composition of RNAs in sperm can respond to environmental events. Of course, these technological advances place unprecedented demands on bioinformatics and also on our ability to integrate the vast amount of data in meaningful ways (Hawkins *et al*, 2010).

Ultimately, to make significant headway in understanding transgenerational epigenetic effects in complex psychiatric disorders, substantial collaboration between experts from several fields will be necessary. Reproductive biologists will have to team up with developmental biologists and neuroscientists, supported by the necessary facilities to provide expertise in and access to next-generation epigenomic methods and data processing.

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DISCLOSURE

The authors declare no conflict of interest.

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