Understanding transgenerational epigenetic inheritance via the gametes in mammals

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Abstract | It is known that information that is not contained in the DNA sequence — epigenetic information — can be inherited from the parent to the offspring. However, many questions remain unanswered regarding the extent and mechanisms of such inheritance. In this Review, we consider the evidence for transgenerational epigenetic inheritance via the gametes, including cases of environmentally induced epigenetic changes. The molecular basis of this inheritance remains unclear, but recent evidence points towards diffusible factors, in particular RNA, rather than DNA methylation or chromatin. Interestingly, many cases of epigenetic inheritance seem to involve repeat sequences.

Heterochromatin
The portion of the genome that stays highly condensed throughout the cell cycle. It contains a high proportion of repetitive sequences, is gene-poor overall and is enriched for histone marks, such as histone H3 lysine 9 trimethylation (H3K9me3) and H4K20me3, as well as DNA methylation. Heterochromatin is generally associated with gene silencing.

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For the past 60 years, human genetic research has focused on DNA as the heritable molecule that carries information about phenotype from the parent to the offspring. Mutations in single genes or a small number of genes have been tightly linked to some phenotypes, but for most phenotypes the situation is more complex and, in many cases, environmental factors are involved. In these instances, genome-wide association studies (GWASs) have enabled the identification of SNPs that are weakly associated with increased disease risk, but the odds ratios are generally small, and it remains impossible to predict phenotype at an individual level.

In parallel, molecular biologists using animal models have realized that, in addition to DNA sequence, there are a number of other layers of information, termed epigenetic marks (BOX 1), that influence transcription. These epigenetic marks are fairly stable over the lifetime of an individual and have a role in determining phenotype. At some loci, the epigenetic marks are not tightly linked to the DNA sequence of the genome; both probabilistic and environmental events can influence the establishment of epigenetic states at these loci[1]. Considering the epigenome as well as the genome may allow us to develop better tools for predicting phenotype at an individual level.

Moreover, there is evidence that epigenetic marks can sometimes be transmitted from parent to offspring via the gametes, and studies have been published in the past couple of years that support this idea. In this Review, we describe the evidence for this form of inheritance, focusing on mammals but also looking at informative examples from other species. The molecular nature of the epigenetic marks that are inherited is unknown in most cases, but the recent emergence of high-throughput sequencing technologies makes this problem tractable. An emerging theme in cases of transgenerational epigenetic inheritance via the gametes (BOX 1) is the involvement of repeats and transposable elements, and recent progress in our understanding of the establishment of heterochromatin at repeats reveals the importance of RNA; this raises the possibility that RNA may have a role in transgenerational epigenetic inheritance via the gametes.

Evidence in mammals

Reprogramming of the epigenome. The epigenetic marks that are established in most tissues during an organism's lifetime are irrelevant with respect to the next generation. Only those of the mature gametes have the potential to contribute to the phenotype of the offspring. Moreover, there is considerable reprogramming between generations — and, in particular, of the gametic epigenome immediately after fertilization — to endow the cells of the early pre-implantation embryo with the capacity to differentiate into all cell types of a fully developed organism. Studies carried out in mice more than 30 years ago found that global DNA methylation levels, which were analysed using methylation-sensitive restriction enzymes, were much lower just after fertilization compared to those found in mature gametes and after implantation[2]. The idea that DNA methylation erasure and resetting is the basis of epigenetic reprogramming emerged from this finding[2]. However, our understanding of the function of DNA methylation

Histone modifications
Covalent alterations of histone tail residues that can alter chromatin structure. Modifications include phosphorylation, methylation, acetylation, sumoylation and ubiquitylation.

remains poor, and the common assumption that the sole role of DNA methylation is to control transcription no longer stands up to critical review (BOX 2). We now know of many other epigenetic marks, such as histone modifications, that could have an equally or more important role in transcriptional control, but the technical difficulty of analysing histone marks in small tissue samples, such as pre-implantation embryos, has limited progress in understanding their reprogramming. The crucial question in relation to transgenerational epigenetic inheritance via the gametes is whether or not there are parts of the epigenome, either DNA methylation or chromatin, that are instructive (that is, they drive transcription) and that at which the classic intergenerational reprogramming does not occur.

Parental imprinting. The first evidence for epigenetic marks that escape reprogramming in the early developing embryo came from the discovery of parental imprinting in mice[3–5]. A small group of genes was discovered with monoallelic expression that is dependent on the parent-of-origin of the allele. The maternal and paternal alleles of these genes are in different transcriptional states in the cells of the adult. This implies that different epigenetic states are established in the germline of the parents, inherited via the gametes and remembered across millions of cell divisions. Recent evidence suggests that the number of genes that are subject to some form of imprinting, previously thought to be around 100, may be much greater, at least in the brain[6,7].

At parentally imprinted genes, the alleles must undergo reprogramming each generation in the germline, depending on the sex of the individual. In other words, the memory only lasts one generation. For this reason, parental imprinting is not normally considered to be an example of transgenerational epigenetic inheritance via the gametes.

Non-Mendelian patterns of expression at transgenes. The original evidence that epigenetic information could be inherited through the gametes across more than one generation in mammals involved transgenes[8–13]. These studies reported that the transcriptional activity of some transgenes in mice was variable among inbred littermates and that the likelihood of activity was sometimes, and to some extent, inherited to the next generation (FIG. 1a). In other words, the offspring of mice in which the transgene was active were more likely to have an active transgene. Because these experiments were carried out in inbred backgrounds, an epigenetic mechanism was inferred. In some of these cases, transcriptional activity of the transgenes inversely correlated with DNA methylation levels at the transgene promoter[9,12,14]. Transgenes generally insert into the genome as multicopy arrays, and the larger the array, the greater the probability that the transgene will be transcriptionally silent, methylated and packaged into heterochromatin[15].

It is interesting that, in a number of instances, the transgenes that show transgenerational epigenetic inheritance via the gametes also show a degree of imprinting[8,9,11] — that is, expression status is influenced by the parent-of-origin of the allele as well as by the activity state of the locus in the parent. For example, the hepatitis B surface antigen transgene stays unmethylated and active when it has been paternally inherited, but it is methylated and silenced when it has been maternally inherited. Importantly, this silenced state cannot be reversed even when it has subsequently been passed through the male germline[8], and it is this that makes the behaviour of the hepatitis B surface antigen transgene different from classic parental imprinting. The fact that many of the transgenes that display transgenerational epigenetic inheritance also display some degree of gametic imprinting may help us to unravel the mechanisms that are involved in transgenerational epigenetic inheritance via the gametes.

Non-Mendelian patterns of expression at endogenous genes. Non-Mendelian patterns of expression have also been reported at endogenous genes, but only rarely. The best-characterized endogenous allele at which transgenerational epigenetic inheritance via the gametes has

Box 1 | Working definitions regarding epigenetics and epigenetic inheritance

Here we provide the definitions of key terms that we use in this Review. For some of these terms, alternative definitions may have been proposed elsewhere.

Epigenetics
This is the study of changes in gene expression that occur in the absence of changes in DNA sequence and that are fairly stable across the life of an individual. The term was first coined by Conrad Waddington to describe the fact that there must be something 'over and above' genetics that enables cells with the same genetic information to differentiate into various different cell types during development. At the time, there was no knowledge of the molecules involved. The problem with this definition is that it is based on what it is not: in this case, it is 'not the primary DNA sequence'. As the molecular nature of epigenetic processes becomes clearer, the word is likely to be replaced by more specific descriptors.

Epigenetic marks
These are molecular modifications to the DNA, such as the methylation of cytosine residues, or modifications of proteins that are associated with DNA, such as methylation of histones. Epigenetic marks are often, but not always, associated with changes in transcriptional activity. Originally, these modifications were called 'epigenetic marks' because they were found to underlie epigenetic phenomena (see above). We now know that some of these modifications are not as stable as was previously thought. For example, the pattern of cytosine methylation in the coding region of some genes changes during the cell cycle[115,116] (BOX 2); histone acetylation is extremely dynamic, and the acetylation state changes within the 2 hours of deposition of the acetyl group[117]. RNA is not usually considered to be an epigenetic mark, but it has been found to underlie some epigenetic processes.

Transgenerational (or intergenerational) epigenetic effects
These are regarded as effects on the phenotype (or on patterns of gene expression) that are detected across more than one generation and that cannot be explained by Mendelian genetics (or changes to the primary DNA sequence). This includes the effects of environmental exposures on adults that alter the phenotype of the developing embryo via the placenta or the newborn via the milk.

Transgenerational epigenetic inheritance via the gametes
This term refers to effects on phenotype (or on patterns of gene expression) that are passed from one generation to the next by molecules in the germ cells and that cannot be explained by Mendelian genetics (or by changes to the primary DNA sequence). We do not restrict the molecules to those that are usually regarded as 'epigenetic marks' but include RNA and proteins. The clause 'via the gametes' emphasizes the difference between this phenomenon and the effects of environmental exposure that alters phenotype via the placenta, and so on (see above).

Box 2 | **DNA methylation in eukaryotes is complex**

DNA methylation involves the methylation of cytosine residues mainly at CpG dinucleotides in mammals. In the 1980s, it was found that *in vitro* DNA methylation of promoters using purified DNA methylases resulted in transcriptional silencing[118]. The simplest conclusion from these studies is that DNA methylation is always associated with gene silencing, but this has turned out not to be true. Below we note some key findings that have revealed more complexities about the function of DNA methylation.

- Levels of C methylation vary considerably from species to species: in plants, it is up to 50%[119]; in humans, ~1% of Cs are methylated[120]; in fungi, it varies from 5% to almost undetectable[121], depending on the species; and it is ~0.1% in bees and *Drosophila melanogaster*[122,123].
- DNA methylation patterns vary across genomes. In mammals, most genes that have CpG-rich promoters lack methylation in this CpG-rich region even when the gene is silent (the globin genes in non-erythroid cells are an example)[124]. In cell lines and tumours, DNA methylation at CpG-rich promoters is often associated with transcriptional silencing; by contrast, a recent report identified thousands of heavily methylated CpG islands in oocytes and pre-implantation embryos, and these were preferentially located within active transcription units[125].
- In *Neurospora crassa* (filamentous fungi) mutation of the DNA methyltransferase *dim-2* eliminates DNA methylation but does not affect growth or sexual reproduction[126]. Furthermore, it was shown that DNA methylation depends on histone methylation[127].
- In female mammals, the inactive X chromosome is hypermethylated at only a subset of gene-rich regions and, unexpectedly, overall it is hypomethylated relative to its active counterpart[128].

been reported is agouti viable yellow (A^{vy})[16]. The A^{vy} allele arose 50 years ago as a result of an intracisternal A particle (IAP) retrotransposon insertion upstream of the *agouti* locus. Although the A^{vy} locus is unique in the mouse genome, the IAP element is present in thousands of copies. The agouti protein indirectly results in yellowness of the coat and, if it is overproduced, it has some other phenotypic consequences, including obesity. Inbred mice that carry this allele show variable coat colours, ranging from yellow to mottled (yellow and brown patches) to pseudoagouti (brown)[17,18]. At the A^{vy} locus, transcriptional control of the *agouti* coding sequence is driven by promoter elements in the retrotransposon[18]. The DNA methylation state of the IAP promoter at A^{vy} inversely correlates with transcriptional activity[16]. More recently, histone marks that are associated with transcriptional activity have been reported at the locus in yellow mice, and those that are associated with silencing have been reported at the locus in pseudoagouti mice[19]. The range of coat colours in offspring is unaffected by the coat colour of the sire following transmission of A^{vy} through the male germline, suggesting that the epigenetic marks are cleared following passage through the male germline[16] (FIG. 1b). Following transmission of A^{vy} through the female, yellow dams produce a higher percentage of yellow offspring than pseudoagouti dams do[16] (FIG. 1b). This suggests that there is a failure to clear the epigenetic marks that are established at the A^{vy} locus in the germline of the dam: that is, there is transgenerational epigenetic inheritance via the female gamete.

Because transgenerational epigenetic inheritance was only seen following maternal transmission of the A^{vy} allele, there was a need to rule out maternal effects that could occur after fertilization, so fertilized eggs were removed from yellow dams and transferred to pseudopregnant pseudoagouti dams[16]. The higher percentage of yellow offspring was still seen. We can conclude that some factor in the egg of yellow mothers is different to that of pseudoagouti mothers and is responsible. In the past, we have assumed that this is the chromatin or the DNA methylation state at the A^{vy} locus, but it could be some diffusible factor in the cytoplasm.

Another endogenous allele that has been reported to show transgenerational epigenetic inheritance via the gametes in the mouse is axin fused ($Axin^{Fu}$)[20]. Alleles such as A^{vy} and $Axin^{Fu}$ are called metastable epialleles: 'metastable' to emphasize the probabilistic nature of their expression and 'epiallele' to emphasize that the allelic forms differ with respect to epigenetic state, rather than DNA sequence.

A number of different strategies have been used to try to identify other metastable epialleles in the mouse, and a handful have been found[21,22]. All metastable epialleles, at least in mice, are associated with the recent insertion of a repetitive element[23]. Until recently, there has not been evidence of metastable epialleles in humans. This may be, in part, because of the difficulties that are associated with studying epigenetics in an outbred population. A recent study identified a handful of human genomic regions that exhibit inter-individual epigenetic variation that occurs systemically (that is, similarly in all tissues within an individual). The researchers showed a link between the establishment of epigenetic state at these loci and the season of conception and have called these regions 'putative metastable epialleles'[24].

There are two reports that abnormal epigenetic states induced by nuclear transfer can be passed on to the next generation in the mouse[25,26]. The researchers found that following the transfer of the maternal pronucleus from one strain to the enucleated egg of another strain, the expression of a handful of genes — including those encoding the major urinary proteins (MUPs) and the olfactory marker protein (OMP) — that are normally active in both strains was reduced. When male mice resulting from nuclear transfer were backcrossed to one of the original inbred strains, the offspring had reduced expression of MUPs and OMP: that is, the repression was paternally heritable. In the case of the MUP genes the repression correlated with increased DNA methylation, suggesting that transcriptional silencing was involved. Interestingly, MUPs are members of a recently evolved gene family and, like transgenes, are present as tandem copies of nearly identical sequence.

Environmental effects

Enviromentally induced epigenetic changes. One of the most exciting findings about the behaviour of metastable epialleles is that the probability of expression can be influenced by the environment[27]. In the case of A^{vy}, the decision about whether the locus will be 'on' or 'off' is made in the early post-implantation embryo[28]. The percentage of yellow pups decreases as a result of exposure of the dam to a diet that is rich in methyl groups (for example, from folic acid, betaine or vitamin B12), genistein or ethanol[27,29–32]. Environmental epigenomics is becoming an area of great interest for those working

Intracisternal A particle
(IAP). A long terminal repeat (LTR)-containing retrotransposon of mice that resembles a retrovirus but has a defective *env* gene.

Genistein
An isoflavone found in plants that acts as an antioxidant and binds to the oestrogen receptor, hence its classification as a phytoestrogen.

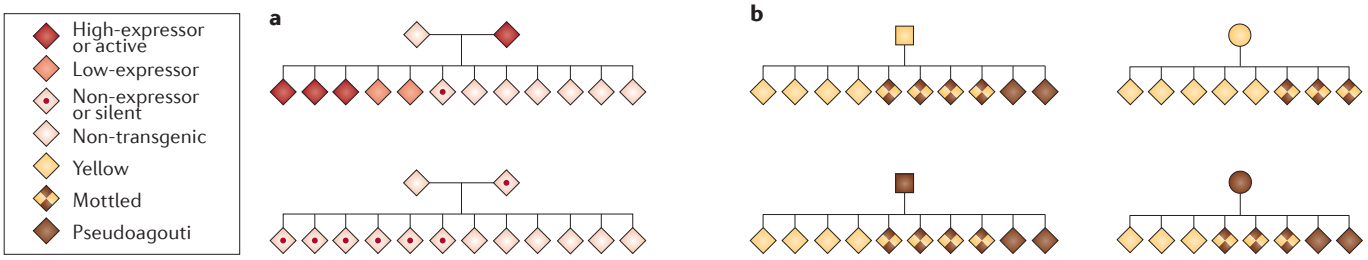

Figure 1 | **Transgenerational epigenetic inheritance at transgenes and metastable epialleles.** **a** | The phenotype of the parent affects the phenotype of the offspring at a transgene. A high-expressor will produce mostly high-expressing offspring, although some offspring may show increased or total silencing (upper panel). A non-expressor will produce only non-expressing offspring (lower panel). **b** | The phenotype of the agouti viable yellow (A^{vy})/agouti (*a*) sire does not affect the phenotype of the offspring following a cross to congenic *a*/*a* females; yellow and pseudoagouti sires produce the same proportion of coat colours in their offspring (left panel). The phenotype of the A^{vy}/*a* dam affects the phenotype of the offspring; yellow dams produce a higher proportion of yellow offspring than pseudoagouti dams (right panel). There is some memory of the epigenetic state of the maternal A^{vy} locus in the offspring. All offspring shown are A^{vy}/*a*. Part **a** is adapted, with permission, from REF. 12 © (2000) Springer Verlag. Part **b** is adapted, with permission, from REF. 16 © (1999) Macmillan Publishers Ltd. All rights reserved.

in many fields of medicine[33], although current evidence that the environment can permanently influence the epigenome in humans is not extensive. For example, only studies involving monozygotic twins have reported a small number of DNA methylation differences in peripheral blood within twin pairs[34–38]. The finding that the establishment of the epigenome can be influenced by environment, in combination with the finding that a few epigenetic marks escape reprogramming between generations, raises the possibility that environmentally induced epigenetic marks could be inherited to the next generation. If this were true, it would profoundly change our understanding of inheritance.

Assessing inheritance of environmentally induced epigenetic effects through the germline in mammals. When considering whether environmentally induced epigenetic marks could be inherited to the next generation, care must be taken to ensure that any environmentally induced heritable phenotype is truly dependent on passage through the germline. For example, it has been known for decades that rats that are nurtured by stressed mothers are more likely to be stressed[39]. This phenotype is perpetuated across generations and involves the setting of a 'stressed' state by the hypothalamic–pituitary–adrenal axis (HPA axis) in the pup[40]. Molecular studies have identified an epigenetic change (namely, DNA methylation) at the glucocorticoid receptor in the hippocampus of the pups[40]. It is an example of a transgenerational epigenetic effect that is not transgenerational epigenetic inheritance via the gametes (BOX 1). This experiment encouraged many researchers to look for epigenetic marks in adults that may be indicators of environmental events *in utero* or in early development but in which passage of a molecule through the gametes is not involved. To discriminate between gametic and non-gametic inheritance when transgenerational effects are observed down the maternal line, cross-fostering or embryo transfer to non-exposed dams is required[41]. However, although it is difficult to rule out confounding non-gametic inheritance when studying transgenerational epigenetic inheritance via the female gamete, it may still occur.

Transgenerational epigenetic effects reported through the paternal line are less likely to be confounded by non-gametic inheritance because the males contribute less to the environment of the fetus and newborn. The first study to support the idea that environmentally induced epigenetic changes could be inherited via the gametes in mammals reported that epigenetic changes, which were induced by a fungicide, were inherited for at least four generations through the male germline in rats. Female rats that were exposed to vinclozolin during pregnancy produced male offspring with abnormal spermiogenesis[42]. This phenotype was faithfully passed down the male germline. Epigenetic changes in the sperm were reported[43]. The authors found that one of the sites that had initially been identified as displaying a heritable epigenetic change is associated with a copy number variation. No genetic changes were found at the other sites, but the authors suggested that genome-wide DNA analysis is required. They concluded that vinclozolin is likely to cause both genetic and epigenetic changes.

The finding that the establishment of the epigenetic state at the A^{vy} locus in offspring is influenced by maternal diet (see above) and that epigenetic states established at this locus are not completely cleared on passage through the female[16] encouraged two independent groups to investigate whether these diet-induced epigenetic changes were passed on to the next generation. The two groups carried out the experiments in slightly different ways and came to different conclusions[44,45]. Some public debate about this has occurred[46], and we await further studies with this model.

Low birth weight in humans is associated with an increased risk of obesity, diabetes and cardiovascular disease during adult life[47,48], and it has been proposed that this programmed disease risk may be passed on to subsequent generations[49]. There are a number of reports of the effects of nutritional changes in the

Hypothalamic–pituitary–adrenal axis
(HPA axis). A set of interactions between the hypothalamus, the pituitary gland and the adrenal glands that control reactions to stress.

Glucocorticoid receptor
The receptor to which cortisol and other glucocorticoids bind. It is expressed throughout the body and controls transcription of many genes involved in development, metabolism and the immune response.

Hippocampus
A neurogenic region of the forebrain that has important functions in learning and memory.

Vinclozolin
A fungicide used on vines, fruits and vegetables. It is associated with the development of testicular tumours. There is some evidence that it is carcinogenic and can act as an endrocrine disruptor.

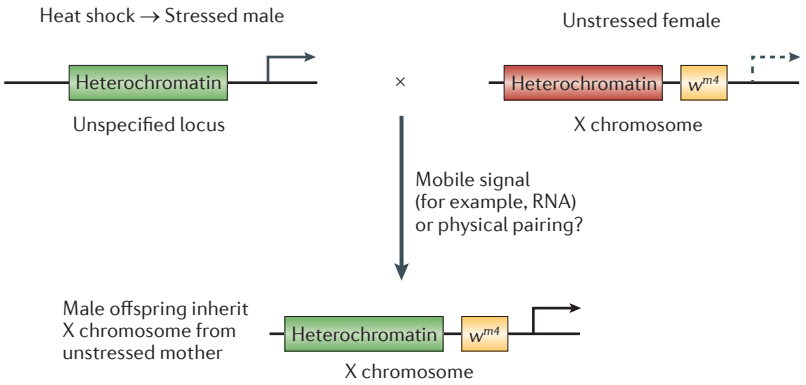

Figure 2 | **Heat-shock-induced disruption of heterochromatin in *Drosophila melanogaster*.** Wild-type *D. melanogaster* have red eyes, and this is the result of expression at the *white* locus. When the *white* gene is placed adjacent to pericentric heterochromatin, called w^{m4}, occasional spreading of the heterochromatin across the gene results in silencing and white flecks in the eye. This is called position effect variegation (PEV)[129,130]. Seong and colleagues[58] showed that heat shock of fly embryos decreases the amount of white in the eyes of the adults, and this is inherited for at least one (and often two or three) generations following both paternal and maternal transmission. The X chromosome harbours w^{m4}. In females that have not been exposed to heat shock, heterochromatin is intact and the gene is 'off'. In crosses between an 'unstressed' female and a 'stressed' male, male offspring inherit the X chromosome harbouring w^{m4} from the female but, in these offspring, the heterochromatin is disrupted and the gene is 'on'. The disruption of the heterochromatin could be the result of *trans*-acting molecules, such as RNA, or physical pairing between the heterochromatic regions of the X chromosomes and disrupted heterochromatic regions on other chromosomes. Disrupted heterochromatic regions (which have less heterochromatin) are depicted in green. The figure is based on data from REF. 58.

parents or grandparents on the phenotype of the next generation[50–52]. For example, offspring of women who were pregnant during the Dutch famine in the Second World War were found to be at a higher risk of impaired glucose tolerance in adulthood[51]. Although DNA methylation differences were found in adult females who had been exposed to famine *in utero*[53], it is not known whether these differences are present in their germline or indeed whether these differences drive the abnormal phenotype rather than simply being associated with that phenotype. Using a mouse model of maternal undernutrition during pregnancy, reduced birth weight, impaired glucose tolerance and obesity have been reported in both the first- and second-generation offspring[54]. However, in the absence of cross-fostering, it remains unclear whether this involves passage of information through the gametes.

A recent study in rats has shown that when sires are fed a high-fat diet, female offspring show a subtle pancreatic phenotype as well as concomitant changes in the expression of some genes involved in insulin regulation and in glucose metabolism compared with those that are sired by males fed a normal diet[55]. The sires only spent a couple of days in the cage with the dams, so the opportunity for effects to be passed on to the offspring in any way except via the gametes is minimal. A methylation change at a single cytosine close to the transcriptional start site of interleukin 13 receptor alpha 2 (*Il13ra2*) was detected in the affected tissue (that of the pancreas), and this gene had the highest expression change (1.7-fold). Whether the methylation changes are seen in the sperm of the rats that were fed a high-fat diet is unknown; the transgenerational effect could be mediated by chromatin or RNA changes in the sperm or even some factor (or factors) in the semen.

Another study looking at the effects of paternal exposure to a low-protein diet in mice found that both male and female offspring showed detectable changes in expression of some lipid and cholesterol genes in the liver[56]. Again, male mice only spent 1–2 days in the cage with the females. When the researchers looked at genome-wide DNA methylation in the offspring, subtle changes were found, including one at an intergenic CpG island that is adjacent to peroxisome proliferator-activated receptor alpha (*Ppara*). Interestingly, DNA methylation changes at the *Ppara* promoter had been reported some years earlier in the offspring of rats that had been fed a low-protein diet during pregnancy[57]. Neither of these studies reported an effect on obesity or insulin resistance, but such an effect could be subtle. There is currently no knowledge of the underlying molecular differences in the sperm of the exposed sires. The studies described above reignite the idea that transgenerational epigenetic inheritance via the gametes could be occurring at endogenous alleles in mammals, and we await follow-up studies with interest.

When considering these phenomena in mammals, it is also interesting to note that transgenerational inheritance of an environmentally induced epigenetic mark has recently been reported in *Drosophila melanogaster*. The study found that changes in eye colour induced by heat stress could be inherited by the offspring[58] (FIG. 2). This case involved silencing at an epigenetically sensitive allele of the *white* gene. The effect occurs in *trans* (FIG. 2), and the authors favour the idea that RNA molecules are involved[58].

Epigenetic marks and inheritance

We have described studies that suggest that transgenerational epigenetic inheritance via the gametes can occur, but we have not described studies that have rigorously established the molecule that is involved in the transfer of information from one generation to the next. In this section, we consider the possible involvement of epigenetic marks (BOX 1): that is, DNA methylation and histone modifications; in the next section, we consider RNA and *trans* effects.

DNA methylation. In order for epigenetic marks to be involved in transgenerational epigenetic inheritance via the gametes, the epigenetic marks must avoid being cleared during both early development and in the germline. DNA methylation has always been considered to be a likely candidate. As mentioned previously, global DNA methylation levels are much lower at or around fertilization compared with those that are found in mature gametes and at implantation[2]. This finding has been confirmed more recently using immunohistochemistry with methylcytosine-specific antibodies and genome-wide bisulphite sequencing[59]. But some classes of retrotransposons — in particular, intracisternal A-type

Peroxisome proliferator-activated receptor alpha (PPARα). A nuclear receptor and transcription factor involved in lipid metabolism.

Bisulphite sequencing Treatment of DNA with sulphite ions increases the relative resistance of the conversion of methylcytosine to uracil compared with cytosine. PCR amplification and sequencing of the DNA following conversion shows a thymine where a cytosine was located, whereas persistence of a cytosine reflects its methylation in the starting DNA sample.

particles (IAPs) — have been found to remain methylated in mature gametes and early pre-implantation embryos in mice[60]. Similar findings were reported in primordial germ cells[61,62] and, more recently, using genome-wide analyses, these findings have been confirmed[63]. Another study[64] used methylated DNA immunoprecipitation (meDIP) followed by hybridization to promoter arrays to look at DNA collected from a range of developmental stages — from mature gametes, the morula, the blastocyst and so forth. This study identified ~100 non-imprinted, non-repetitive genes at which promoter DNA methylation does not change, which is consistent with an escape from post-fertilization DNA methylation reprogramming[64]. These studies demonstrate the principle that some loci escape DNA methylation reprogramming and so suggest the potential for DNA methylation to be involved in the transmission of epigenetic information through the gametes.

Changes in DNA methylation have been associated with many cancers, and transgenerational epigenetic inheritance via the gametes has been suggested as the explanation for the inheritance of colorectal cancer in some cases of this disease that involve epimutations in *MLH1* and *MSH2*. However, in the case of *MSH2*, the epigenetic changes turned out to be associated with mutations in *cis*[65,66] and, in the case of *MLH1*, it has also been difficult to rule out genetic changes and to determine whether methylation is transmitted in the gametes or is triggered post-fertilization[67–72].

A study carried out to determine whether DNA methylation at the A^{vy} locus could explain the transgenerational memory of coat colour reported that the methylation at the promoter was completely absent in blastocysts from pseudoagouti dams[28], making it an unlikely candidate. However, this analysis only investigated CpG methylation, and now we know that Cs can be methylated at non-CpG sites. For example, in human embryonic stem cells, 25% of the methylated C residues are found at CHG and CHH (where H is A, T or C)[73]. Most studies using bisulphite sequencing only report the methylation at Cs that are part of CpG dinucleotides. Presumably, researchers will now go back and reanalyse data to look at these other sites. It has also recently been discovered that Cs can be hydroxymethylated, formylated and carboxylated[74–77]. Despite the fact that the levels of these modifications are low, they could turn out to play some part in transgenerational inheritance.

Chromatin proteins. For many decades, it was thought that all histones were cleared from the DNA in mature sperm and replaced by protamines. However, we now know that ~1–2% of the haploid genome in mice and ~4% of that in humans remains packaged into nucleosomes in sperm[78,79]. Recent genome-wide analysis of the histone marks in human and mouse spermatozoa has revealed that some genes retain extensive histone H3 lysine 27 trimethylation (H3K27me3) at their promoters — a mark that is associated with silencing. These genes are enriched for ones that are not expressed during gametogenesis and/or early development[78,79]. Although the authors raise the possibility that this mark is retained and could carry epigenetic information from one generation to the next[79], an alternative explanation is also possible: the mark simply correlates with transcriptional silencing that is re-established so rapidly that the period in which the mark is 'missing' has not been detected. Studies in early mouse pre-implantation embryos have shown that Polycomb repressive complex 1 (PRC1) components derived from the maternal genome are transferred to the paternal genome after fertilization, providing an opportunity for the direct effects of maternal heterochromatin on silencing in the zygote[80].

There is an increasing awareness of the dynamic nature of chromatin. For example, heterochromatin protein 1 (HP1) binds *in vivo* with a residence time of only a few minutes[81], and nucleosome turnover has been shown to be extremely rapid[82], making the notion that histone marks could be a vehicle for transgenerational epigenetic inheritance via the gametes less attractive. Furthermore, not all histone modifications direct the transcriptional activity of the underlying gene[83]. For example, in organisms as diverse as yeast and humans, gene activity has been found to be associated with gradients of some histone modifications with a 5′ to 3′ polarity along the gene, suggesting a transcription-coupled generation of the histone marks, rather than vice versa.

RNA and other *trans* effects

Molecular biologists studying transgenerational epigenetic inheritance via the gametes have generally focused on the nucleus. However, recent studies suggest that *trans*-acting and/or non-nuclear factors could be involved. For example, RNA, which occurs inside and outside the nucleus.

Evidence of* trans *effects. An important example that demonstrates the occurrence of *trans* effects is paramutation — a phenomenon in which homologous DNA sequences communicate in *trans* to establish meiotically heritable expression states. It has been looked at in most detail in some rare instances of non-Mendelian inheritance of phenotypes in a number of plant species[84]. The epigenetic state at an allele that is subject to paramutation is influenced by the epigenetic state of the homologous allele. After it has been established, this new epigenetic state is heritable and stable for many generations in some cases[85]. Because the *trans* effect is between homologous chromosomes, it has been suggested that its basis could be the transitory physical interaction of non-homologous chromosomes[86], sometimes known as 'chromosome kissing'[87]. However, genetic screens have been carried out and reveal the involvement of the RNA interference (RNAi) machinery, implying a role for small RNAs[88,89] (discussed further below). In the light of several examples discussed in this Review involving repeat sequences, it is interesting to note that the sequences involved in many cases of paramutation contain direct or inverted repeats[90].

The focus on the nucleus has been particularly true among those scientists researching transgenerational epigenetic inheritance in mammals. However, reports of 'paternal effect genes' provided early evidence for

Epimutations
Mitotically heritable changes in epigenetic state but not gene sequence. Epimutation usually takes place by an abnormal increase or decrease in the methylation status of a gene. The heritability of epimutations across generations is currently under debate.

MLH1
MutL homologue 1, colon cancer nonpolyposis type 2 (*Escherichia coli*) is a human gene coding for a protein that has an important role in DNA repair.

MSH2
MutS homologue 2, colon cancer nonpolyposis type 2 (*Escherichia coli*) is another human gene coding for a protein involved in DNA repair.

Polycomb repressive complex 1
(PRC1). Silencing of the homeotic genes in development requires the Polycomb group proteins (PcGs). PcGs form two distinct multiprotein complexes, PRC1 and PRC2.

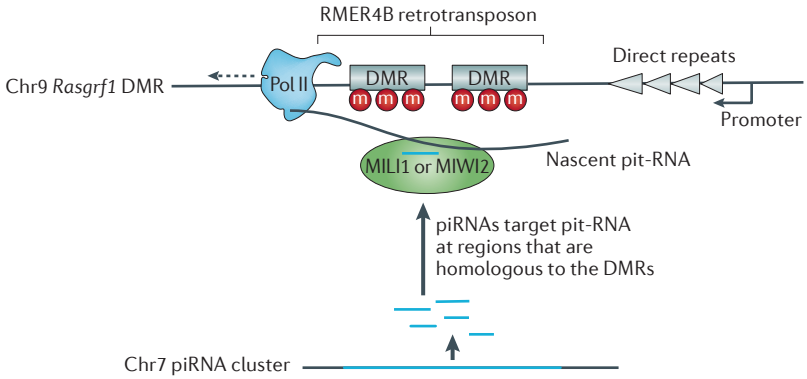

Figure 3 | **Imprinting of the *Rasgrf1* differentially methylated region via piRNA-directed DNA methylation.** In mice, PIWI-interacting RNAs (piRNAs) are generated from the chromosome 7 (Chr7) piRNA cluster and bound by the PIWI family proteins MILI1 (also known as PIWIL2) and/or MIWI2 (also known as PIWIL4). The piRNA–MILI1 and/or piRNA–MIWI2 complexes then target the nascent antisense RNA that is transcribed through the RAS-protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) differentially methylated region (DMR). This RNA is designated here as piRNA-targeted non-coding RNA (pit-RNA). The DNA methyltransferases DNMT3A, DNMT3B and DNMT3L form a complex that is recruited in a process that involves the piRNA complex and is responsible for methylation of the *Rasgrf1* DMR that spans the RMER4B retrotransposon. m, DNA methylation; Pol II, RNA polymerase II. The figure is based on data from REF. 104.

trans effects in mice. In this particular context, paternal effects are phenotypic effects that are observed in wild-type offspring of sires that are heterozygous for a mutation: that is, the effects are seen despite the fact that the offspring did not themselves inherit the mutant allele[91,92]. For example, using coat colour as a readout for expression from the A^{vy} locus, haploinsufficiency for modifiers of epigenetic reprogramming in sires was found to alter the coat colour of wild-type offspring that had inherited the A^{vy} allele from the dam[91]. These findings suggest that some '*trans*-acting factor' introduced into the zygote along with the paternal gamete influences the establishment of epigenetic state at a gene present on the maternal set of chromosomes (in this case, the A^{vy} allele). There are two possible explanations for *trans* effects of this type: first, the involvement of diffusible factors such as RNA or protein, and second, chromosome kissing[87].

RNA in gametes and roles in silencing transposons. The highly condensed sperm nucleus is transcriptionally and translationally inert and contains little cytoplasm, but RNA populations have been detected in mature sperm[93], and sperm-borne RNA has been detected in the zygote[94]. In addition to mRNAs, long non-coding RNAs (lncRNAs) and small RNAs of various classes have been found. These include microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs), and these classes of small RNAs can be involved in gene silencing. Oocytes also contain large amounts of RNA of all classes[95–97]. Indeed, the egg has evolved special strategies to maintain a store of RNA that enables the zygote to function in the absence of transcription until the two-cell stage.

MicroRNAs
(miRNAs). Evolutionarily conserved small non-coding RNAs (~22-nucleotides long) that silence gene expression by degrading or inhibiting translation of mRNA transcripts in a sequence-specific manner.

Endogenous small interfering RNAs
(endo-siRNAs). Small RNAs that originate, in a Dicer-dependent manner, from long double-stranded (sense–antisense or hairpin) precursors. Initially mainly thought of as a mechanism of host defence against exogenous double-stranded RNA, endo-siRNAs are now known to also regulate endogenous mRNAs in mouse oocytes and *Caenorhabditis elegans*.

PIWI-interacting RNAs
(piRNAs). Small (24–31 bp) RNAs that are associated with PIWI-clade proteins of the Argonaute family. They ensure genome stability in the germline of flies, mice and zebrafish by silencing transposable and repetitive elements.

It is also interesting that it has been shown in plants and worms that small RNA molecules can travel between cells[98]. Transposable elements are the targets of siRNA-mediated silencing in animals, fungi and plants, and there is evidence that some small RNAs can be involved in silencing transposable elements in adjacent germline cells. For example, a recent study in *Arabidopsis thaliana* has found a population of siRNAs from *Athila* retrotransposons generated in the vegetative nucleus of pollen that may have a role in silencing the *Athila* retrotransposons in the adjacent sperm cells[99].

Following from this, it is easy to envisage how small RNAs could act in *trans* at fertilization as mobile signals that influence the silencing of genetic elements containing retrotransposons, such as A^{vy}, introduced on the complementary haploid genome. In mouse oocytes, endo-siRNAs are also known to be required for retrotransposon silencing[96,97,100]. The large amounts of these endo-siRNAs in the oocyte could theoretically direct repeat silencing in the early embryo as well.

Roles of piRNAs in gametes. piRNAs have a role in the silencing of mobile genetic elements in many eukaryotic organisms[101] and, in *D. melanogaster*, PIWI has been shown to influence the epigenetic phenomenon position effect variegation (PEV)[102]. Although some aspects of the mechanisms of piRNA biogenesis remain unclear, they are produced from piRNA clusters that, at least in *D. melanogaster*, lie at heterochromatin–euchromatin boundaries in the most repeat-rich regions of the genome. They are specifically expressed in reproductive organs and are found in fairly high levels in spermatocytes[101,103]. Interestingly, in mammals, piRNAs have recently been shown to play a part in the establishment of parental imprints[104]. A study in mice found that piRNAs are required for the establishment of the DNA methylation at the RAS-protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) locus in the paternal germline[104]. A retrotransposon sequence within a non-coding RNA that spans the differentially methylated region of this imprinted gene is targeted by piRNAs generated from a different locus (FIG. 3). These experiments reveal a clear role for piRNAs in the establishment of DNA methylation imprints during reprogramming in primordial germ cells (PGCs). One scenario in this case is that RNAs are involved in establishing the epigenetic mark, and then DNA methylation carries the information in the gametes; another scenario is that piRNAs are also carried in the gametes and influence DNA methylation in the offspring.

piRNAs have also been shown to be involved in the phenomenon of hybrid dysgenesis. Hybrid dysgenesis refers to the fact that when wild *D. melanogaster* are crossed to laboratory strains, an incompatibility is sometimes observed: progeny from laboratory males mated to wild females develop normally, but those from the reciprocal cross display abnormalities. The underlying cause has been traced to the mobilization of transposons that are present in the genome of the wild strain but that are absent in the laboratory strain[105]. The differential behaviour of the reciprocal crosses implies the existence of a

Position effect variegation (PEV). This term describes a type of phenotypic variegation among cells of the same type that is the result of mosaic silencing of a particular gene. The variegation in these cases is due to the position of the gene adjacent to a heterochromatic region of the chromosome.

maternal factor that influences the ability of the progeny to silence inherited elements[106]. We now know that these factors are piRNAs[107].

MicroRNAs. In general, miRNAs direct post-transcriptional repression by pairing to the mRNAs of protein-coding genes, but they have also been found in the mammalian nucleus, where their function remains unclear[108,109]. miRNAs that are present in sperm or oocytes could, by reducing the levels of their target mRNAs in the zygote, indirectly alter the epigenetic state of the developing embryo.

miRNAs have been implicated in transgenerational epigenetic inheritance at the *Kit* locus in mice[110]. When breeding mice to be heterozygous for a mutant allele designated *Kit*^{tm1Alf}, an under-representation of offspring with a wild-type phenotype was found. Significantly more offspring than expected showed white tail tips and white feet, a phenotype that is usually associated with heterozygosity for the mutant allele. In fact, genetically wild-type mice were born at the expected frequency, but a proportion of them had retained the phenotype of their heterozygous parents, suggesting that transgenerational epigenetic inheritance via the gametes was involved. In the wild-type offspring with the mutant phenotype, *Kit* mRNA levels were found to be reduced (to a level similar to that expected in heterozygotes), and *Kit* RNA molecules of abnormal size were detected in the testes of mutant sires. Additionally, *Kit* mRNA was found in the mature gametes of the heterozygous males but not the wild-type males[110]. Two miRNAs (miR-221 and miR-222) that had previously been identified as having the potential to target *Kit* mRNA[111] were injected into wild-type zygotes, and this resulted in mice with the white-tail phenotype[110]. Similar paramutation-like phenomena have been reported by the same group at some other loci in the mouse[112,113]. To our knowledge, these are the only experiments that directly link a molecule of any kind to transgenerational epigenetic inheritance via the gametes. It is worth noting that the mutant allele *Kit*^{tm1Alf}, at which the observation of transgenerational epigenetic inheritance was made, carries a transgene insertion and that transgenerational epigenetic inheritance via the gametes is not observed at other null *Kit* alleles.

Conclusions and perspectives

The number of alleles at which it has been shown that the epigenetic state, independent of the underlying genotype, is inherited across generations remains small and, when it does occur, the process shows incomplete penetrance (only a proportion of offspring are affected). Indeed, transgenerational epigenetic inheritance via the gametes is likely to be rare. It seems counterintuitive to evolve a widespread system to inherit marks that are normally considered to have evolved to enable complex cell types to emerge from one genome. In the gametes, there is only one copy of a particular gene. If that is 'marked' in such a way that that is not cleared (that is, the mark is inherited), then this mark would affect the gene's activity in all cell types of the developing embryo, affecting the organism's ability to develop all of the correct cell types. As such, the probability of a positive outcome (such as being better adapted to a changed environment) is outweighed by the probability of a negative effect (such as failure to develop). For example, a recent study in *Caenorhabditis elegans* highlights the importance of reprogramming between generations. Mutants that are null for the H3K4me2 demethylase *spr-5* (the mammalian orthologue is *KDM1A* (also known as *LSD1*)) exhibit progressive sterility over many generations[114]. This sterility correlates with the misregulation of genes in spermatogenesis and the transgenerational accumulation of H3K4me2, suggesting that H3K4me2 needs to be cleared between generations.

Nevertheless, transgenerational epigenetic inheritance via the gametes has been shown to occur in some parts of the genome across eukaryotes. It seems to occur mainly at retrotransposons and other repeated elements, and it is true that these are the areas of the genome about which we know the least. Whereas DNA methylation is still the most popular candidate for the molecular basis of transgenerational epigenetic inheritance via the gametes, data collected in various situations provides mixed support for this view. Perhaps future studies should focus less on looking for an epigenetic mark that is retained across generations and more on looking for processes (or factors) that disrupt or enhance the re-establishment of silent heterochromatin between generations. RNA may be the best candidate.

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