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Transgenerational epigenetic inheritance of diabetes risk as a consequence of early nutritional imbalances

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In today's world, there is an unprecedented rise in the prevalence of chronic metabolic diseases, including obesity, insulin resistance and type 2 diabetes (T2D). The pathogenesis of T2D includes both genetic and environmental factors, such as excessive energy intake and physical inactivity. It has recently been suggested that environmental factors experienced during early stages of development, including the intrauterine and neonatal periods, might play a major role in predisposing individuals to T2D. Furthermore, several studies have shown that such early environmental conditions might even contribute to disease risk in further generations. In this review, we summarise recent data describing how parental nutrition during development increases the risk of diabetes in the offspring. We also discuss the potential mechanisms underlying transgenerational inheritance of metabolic disease, with particular emphasis on epigenetic mechanisms.

Nutritional epigenomics: Transgenerational inheritance: Type 2 diabetes: Developmental programming

In today's world, we are seeing unparalleled increases in the prevalence of chronic metabolic diseases, including obesity, insulin resistance and type 2 diabetes (T2D). According to recent data from the World Health Organization[1] the global prevalence of diabetes in 2014 was estimated to be 9 % among people older than 18 years. This percentage equates to 350 million people worldwide[2,3], and the expectation is that this number might double between 2030 and 2040[4]. Although T2D is a treatable disease, it is associated with enormous morbidity and is expected to become the seventh leading cause of death worldwide by 2030[4]. In particular, diabetes increases the risk of other disorders with a high mortality rate, including CVD, hypertension, kidney failure and several types of cancers[5,6]. Thus, understanding the progression T2D and its associated metabolic disorders has become a major area of biomedical research, with the hope that research findings will lead to the development of novel treatments and, more importantly, preventative strategies.

It is recognised that both genetic and environmental factors contribute to the risk of T2D and its associated metabolic diseases. Genome-wide association studies have uncovered a fairly large number of loci that can contribute to the development of the disease[7]. However, their overall contribution to the risk of T2D is relatively small (5–10 %)[8]. For this reason, it is thought that the leading cause of the current T2D epidemic is the modern diabetogenic environment, characterised by an excessive energy intake and lack of physical activity[9]. Indeed, obesity is recognised as the primary risk factor for insulin resistance and T2D[10].

In addition to these lifestyle factors, it has recently been acknowledged that intrauterine and neonatal nutrition have long-lasting effects that influence the risk of obesity, insulin resistance and T2D[11]. A well-documented case exemplifying the long-term effects of intrauterine nutrition is the Dutch Hunger Winter[12]. The western Netherlands was affected by a period of famine at the end of the Second World War, during the

Abbreviations: HFD, high-fat diet; IUGR, intrauterine growth restriction; SAM, *S*-adenosyl-methionine; T2D, type 2 diabetes.

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winter of 1944–1945. Individuals exposed to famine *in utero* during the last two trimesters of gestation were born small for their gestational age. Strikingly, these low-birth-weight individuals were at increased risk for obesity, T2D and CVD as adults[13–16]. These observations have been largely replicated in many other populations worldwide[17,18]. Likewise, similar observations have been obtained from animal models, including rodents, sheep and non-human primates[19]. Together, these observations have been conceptualised in the developmental origins for health and disease hypothesis. This hypothesis proposes that environmental stressors (e.g. nutrition, toxicants, drugs, tobacco) during early life increase the risk for chronic non-communicable diseases in adulthood, such as obesity, insulin resistance, T2D and CVD[20]. In this review, we focus on the role of early nutritional imbalances on the later risk of T2D.

Remarkably, recent evidence suggests that long-term effects caused by nutritional imbalances during development can additionally lead to poor health in the following generation(s)[21]. For example, follow-up studies on the Dutch Hunger Winter showed that the offspring of women who had been exposed to famine *in utero* had increased neonatal adiposity. Furthermore, the offspring of men who were underfed *in utero* were more obese than those from a control population[14,22,23]. Nominally, the offspring of men and women exposed to intrauterine malnutrition constitute the second-generation offspring; the inheritance of environmentally induced phenotypes by the following generation is referred to as transgenerational effects. Data from another historical cohort in northern Sweden (the Överkalix cohort) have shown that overnutrition in boys can increase the risk of diabetes-related cardiovascular mortality in their grandchildren[24–27]. This is another well-documented example in which a nutritional imbalance during early development can trigger health problems in the following generation's offspring.

The key question is how transient nutritional alterations, occurring during specific periods of early development, can induce such long-lasting effects. It has been proposed that epigenetic mechanisms might mediate these transgenerational effects, given the fact that they: (i) respond to environmental challenges, including nutrition; and (ii) can remain very stable across life-spans. Also, it has been proposed that epigenetic marks might eventually be transmitted to following generations via the gametes[28]. This mechanism might provide the tool for the transmission of nutritionally induced diabetes risk across generations. This phenomenon is referred as epigenetic inheritance.

Epigenetic inheritance of diabetes risk from nutritional cues

Epigenetic inheritance in mammals has recently received an enormous amount of attention. This is because current dogma in the biological sciences states that genetic variants are the main carriers of information across generations[29]. Indeed, epigenetic mechanisms are not believed to play a role in mediating the inheritance of traits between generations because they are erased and reset during the process of gametogenesis and the first post-zygotic divisions (see section Transgenerational inheritance of diabetes by early nutrition). However, recent evidence from animal models suggests that epigenetic mechanisms might play a role after all[30,31]. If true, this could be of great relevance to fundamental biological thinking: epigenetic inheritance might provide a mechanism by which parents can transfer information about the environmental (nutritional) conditions they have encountered to their offspring[32]. The idea of the inheritance of acquired characteristics in mammalian systems remains a highly controversial topic. However, increasing evidence suggests that certain nutritionally acquired phenotypes can be passed on to the next generation (see following sections).

Studying the potential role of epigenetic mechanisms in mediating the inheritance of complex traits in human subjects is extremely challenging. The main problem is that additional mechanisms, other than genetics and epigenetics, can contribute to the establishment of metabolic phenotypes in the offspring. These additional channels of inheritance include: (i) parental physiology; (ii) culture and behaviour; and (iii) transmission of environmental conditions[33]. In this regard, animal models can help to decipher whether epigenetic phenomena contribute to the inheritance of nutritionally induced metabolic phenotypes because it is possible to control, or eventually avoid, these additional confounders.

Here, we focus on the potential role of nutrition during development in mediating the inheritance of metabolic dysfunction via the epigenome. For this to occur the nutritional challenge should first modify the epigenome of the germline and/or mature gametes (see section Nutrition and epigenetic modifications in the gametes). Second, these epigenetic modifications must be passed to, and stably maintained in, the following generation (see section Epigenetic reprogramming). Before describing the available data, there are at least two important constraints of the experimental design that need to be considered in order to ascertain the epigenetic inheritance of complex traits in mammals: first, maternal and paternal inheritance should be distinguished. Second, knowing the structure of the pedigree is very important in delineating whether a phenotype is epigenetically transmitted.

Maternal v. paternal effects

Maternal effects. Maternal effects can be defined as the conditions under which the phenotype of the offspring is influenced by the maternal life history. A classic example of a maternal effect is gestational diabetes[34]. Many women experience transient gestational diabetes, which is characterised by hyperglycaemic episodes during gestation. Importantly, glucose is an essential nutrient for fetal growth that can cross the placenta through passive diffusion. The amount of glucose transferred from the diabetic mother to the fetus can be higher than in normal pregnancies. The net result is that

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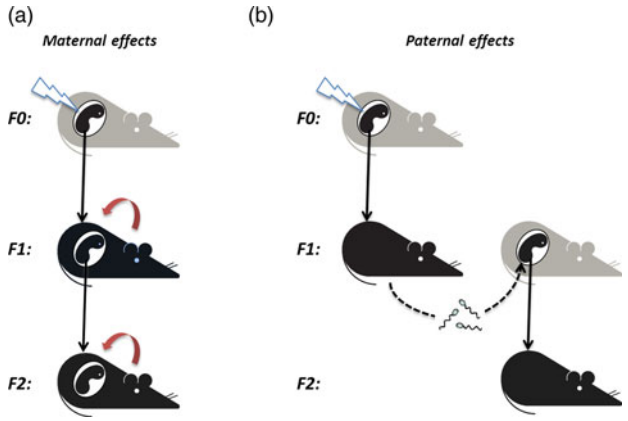

Fig. 1. (Colour online) (a) Maternal v. (b) paternal effects. (a) A gestating female (F0), carrying a female embryo/fetus, is exposed to a nutritional challenge. Consequently, the F1 female is at risk of developing diabetes. If she becomes pregnant, her offspring (nominally the F2) will be at risk of developing diabetes as well, because his/her metabolism will be compromised during gestation. (b) A gestating female (F0), carrying now a male embryo/fetus, is exposed to a nutritional challenge. The F1 male will be at risk of developing metabolic dysfunction in adulthood. In addition, his offspring will be at risk of metabolic dysfunction if the nutritionally derived signals are transferred to the next generation via the gametes.

newborns from women with gestational diabetes tend to be heavier and show greater adiposity at birth. Consequently, children from mothers with gestational diabetes display an increased risk of developing childhood obesity, insulin resistance and T2D later in life. Importantly, if the newborn is a girl, she in turn will have a high probability of developing gestational diabetes herself. Thus, her children (i.e. the grand-offspring of the first woman who developed gestational diabetes) will also be at risk of developing obesity and diabetes with ageing. This maternal cycle can be perpetuated over many generations (Fig. 1a). In addition to the paradigm of gestational diabetes, many other examples show the inheritance of diabetic phenotypes through the maternal lineage[35–39]. The transfer of phenotypic information through the maternal lineage is based on a complex interplay of several mechanisms, including genetics, epigenetics, mitochondrial DNA transfer, the *in utero* environment and, in human subjects, culture and behaviour[33].

Although epigenetic mechanisms can play a role in these maternal effects, it is very difficult to assess their contribution. Therefore, examples describing maternal effects are not considered in this review. However, it has to be emphasised that maternal effects have major implications for human health at large. From an epidemiological perspective, maternal health, including maternal nutrition, is clearly a major area in which action can be taken to promote the health of future generations.

Paternal effects. Paternal effects constitute a much simpler paradigm, since they avoid the confounding effects of the *in utero* environment and maternal physiology. As a definition, paternal effects refer to the situation in which the offspring's phenotype is influenced by the paternal life history. The inheritance of phenotypic variation via the paternal lineage occurs primarily through the information contained in the gametes: the genome and the epigenome (Fig. 1b). In addition, as we have previously described, in human subjects, fathers can influence their progeny through other mechanisms, such as behaviour, culture and/or maintenance of a particular environmental condition (e.g. food habits)[33]. Using experimental models such as rats and mice can minimise these confounding mechanisms. Accordingly, in these models, the sires can be removed from the cage upon pregnancy of the dam. Thus, paternal metabolism and behaviour do not contribute to the offspring's phenotype and the inheritance of nutritionally acquired phenotypes can be attributed, in part, to epigenetic mechanisms[40,41]. However, it has to be noted that additional carriers might contribute to such paternal effects, including paternal transfer of the microbiota or elements contained in the seminal fluid[42]. These mechanisms are not discussed further here.

The pedigree structure

The second important point to address is whether the paternal exposure to nutritional challenges occurred either *in utero* or postnatally[43]. This distinction is extremely significant in interpreting the available pedigrees and suggesting potential mechanisms of transgenerational inheritance.

In utero exposure. In the first scenario, a female is exposed to an adverse environment during gestation (Fig. 2a). Therefore, two generations, the F0 gestating mother and her F1 embryo/fetus, are exposed to a given nutritional challenge at the same time. Furthermore, the cells that will constitute the germline of the F1 generation are also exposed to the same nutritional challenge (or, in a broad sense, to any environmental trigger). The germline from the F1 generation will actually be involved in producing the F2 generation. Therefore, according to some authors, this paradigm is defined as a multigenerational effect, rather than a transgenerational effect, because multiple generations are exposed to a given condition during a specific time period[43,44]. In this scenario, phenotypic consequences in the F2 generation might be explained by factors other than epigenetics, such as sperm viability, sperm selection, DNA alterations in germ cells and so on. Thus, to fully implicate epigenetic mechanisms with no other confounders, an analysis of the F3 generation is required. The F3 generation is the first on without direct exposure to the external triggers, since the F2 germ cells will not have been previously exposed.

Postnatal exposure. In the second scenario, the exposure to nutritional challenges occurs postnatally, either during the neonatal period or later in life (Fig. 2b). Under this paradigm, a male (F0) is originally exposed to an environmental factor. Hence, the F0 germline, which will give rise to the F1 generation, is also nutritionally compromised. Consequently, the transmission of metabolic phenotypes to the F1 generation can be explained, in part, by the direct effects of environmental cues on the development, maturation and/or stability of the germline

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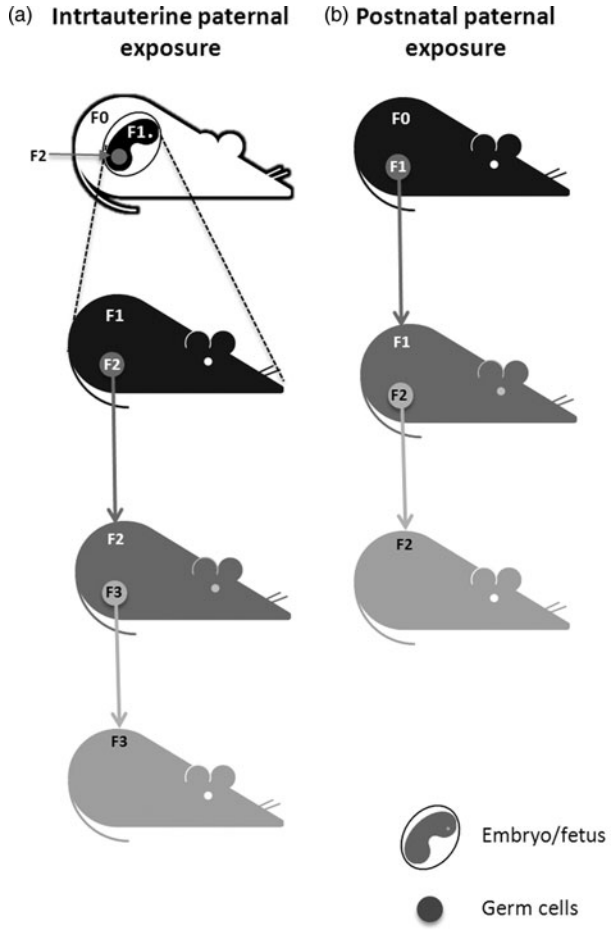

Fig. 2. Pedigree structure. (a) *In utero* paternal exposure. A female is exposed to a nutritional challenge during gestation. Under this paradigm, up to three generations are exposed to the environmental cue: the F0 gestating female, her F1 offspring and the germ cells of the offspring that will eventually give rise to the next-generation offspring (F2). Thus, any metabolic effect up to the F2 generation will be considered a multigenerational effect. Transgenerational effects appear when the metabolic consequences are transmitted to the F3 generation because the germline from the F2 generation has not been previously subjected to the nutritional inputs. (b) Postnatal paternal exposure. A founder male (F0) is exposed to a nutritional challenge postnatally. Therefore, his germline, which will generate the first-generation offspring (F1), is also exposed to this cue. Under this paradigm, metabolic effects up to the F1 generation are considered multigenerational effects. If the metabolic consequences manifest in the following-generation offspring (F2), then the metabolic challenges to F0 founder males will result in transgenerational effects.

of exposed individuals. Again, epigenetic factors might play a role in determining the phenotypes of the F1 generation. However, to be able to demonstrate transgenerational epigenetic inheritance and completely rule out a direct effect of the environment on germ-cell quality, an analysis of phenotypes in the next generation, F2, would be required.

In summary, the setting of the appropriate pedigrees is extremely important in order to be able to implicate the role of epigenetics on transgenerational effects. While this is difficult to control in human subjects, specific breeding can be performed in animal models to fulfil both criteria: (i) paternal inheritance through (ii) an appropriate pedigree where phenotypes can be searched up to the F2 or the F3 generation.

Nutrition and epigenetic modifications in the gametes

Many studies have shown that nutritional challenges, including protein deprivation, global energy restriction and chronic high-fat feeding, can induce epigenetic modifications[45–47]. For example, *S*-adenosyl-methionine (SAM), which is the universal methyl donor, is required for DNA and protein methylation[48]. SAM is synthesised in the methionine cycle from several precursors that are provided by the diet: methionine, folate, choline, betaine and vitamins B_2, B_6 and B_{12}[45,49–51]. Therefore, it has been proposed that reduced availability of methyl donors will result in low SAM synthesis and global DNA hypomethylation. Accordingly, diets deficient in some of the afore-mentioned methyl donors (i.e. no folate, no choline and very low methionine) lead to global DNA hypomethylation in rodents[50,51]. Likewise, low-protein diets can reduce the availability of the methionine precursor homocysteine and lead to low SAM content and global DNA hypomethylation[52]. Whether a high methyl-donor intake results in global DNA hypermethylation is as-yet undetermined. Finally, vitamins B_6 and B_{12} might determine global DNA methylation not only through their role as precursors to SAM bioavailability, but also as cofactors involved in regulating the activity of the enzymes of the methionine cycle.

Nutritional factors can also influence histone covalent modifications, such as histone methylation and histone acetylation[47,53,54]. For example, similarly to DNA methylation, histone methylation depends on the availability of methyl donors from the diet. Again, the production of SAM from its precursors is a critical step in appropriate histone methylation. Histone acetylation, on the other hand, depends on the opposing activities of histone deacetylases and histone acetyl-transferases. There are three classes of histone deacetylases (I, II and III). Classes I and II histone deacetylases are inhibited by short-chain carboxylic acids and polyphenols, whereas class III histone deacetylases, also known as sirtuins, require NAD+ as a cofactor. Therefore, dietary exercises that influence the intracellular concentration of short-chain carboxylic acids and/or NAD+ will potentially result in histone modifications that might ultimately change patterns of normal gene expression. At this point, it is not known whether dietary factors can influence other histone marks. However, it is plausible that this might be the case, given the fact that nutrients have a wide range of implications in the cell.

There are many examples of dietary challenges during early development leading to changes in DNA methylation and/or histone modifications[47]. Most of these studies have focused on analysing epigenetic modifications in target metabolic tissues, such as β-cells, the liver, adipose tissue, skeletal muscle or the hypothalamus. The question, then, is whether this also happen in germ cells or mature sperm, because these cells are the actual carriers

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of information across generations. The issue of whether early nutritional challenges can influence the epigenome of these cell types has only recently been addressed.

As we review in more detail in the last section of this article, there is now evidence that *in utero* global energy restriction influences the pattern of DNA methylation in the sperm of adult male mice[55,56]. It is not known whether these changes are established *in utero*, during germ-cell development, or later in life, when the mice develop progressive metabolic dysfunction. Likewise, it has not yet been determined whether these modifications are a consequence of SAM bioavailability and/or changes in the activity of enzymes involved in the methionine cycle. The role of nutrition in modifying the sperm methylome deserves future investigation. In another example, protein malnutrition in adult male mice has also been seen to cause widespread small changes in DNA methylation in mature sperm[57]. Again, the potential mechanisms are not clear. However, this experimental design indicates that methylation of the male gametes might be modulated during adulthood. Therefore, the window within which dietary factors can influence the epigenome is not restricted to the early developmental stages, but might extend throughout life. In agreement with this, paternal pre-diabetes has been reported to alter overall methylation patterns in adult male mice[58].

In summary, recent evidence suggests that the epigenome of the male germ cells/mature gametes is largely responsive to nutritional cues. These effects might occur in response to both *in utero* nutritional events and adult nutrition. At the moment whether these epigenetic modifications occur in response to the same pathways as in somatic cells remains unknown. The key question now is whether these modifications, occurring in the spermatozoa, are: (i) stably maintained; (ii) successfully transmitted to offspring; and (iii) influence the phenotype of offspring.

Epigenetic reprogramming

As stated previously, it has been proposed over the last decade that, in addition to the genome, the epigenome can also contribute to the inheritance of phenotypes[33,59]. Nevertheless, although very attractive, this concept remains extremely controversial[60,61]. The main problem is that there are natural barriers aimed precisely at preventing the transfer of epigenetic modifications across generations. First the germ cells, and later the early pre-implantation embryo, undergo massive epigenetic reprogramming[62]. Specifically, the entire genome is almost completely demethylated during gametogenesis and re-established in the mature gametes. In addition, 90 % of histones in male gametes are replaced by protamines. After fecundation, the male and female pro-nuclei are again almost completely demethylated and new epigenetic marks are reinstated during the first post-zygotic divisions[63]. These two reprogramming events are necessary to maintain the pluripotency of the zygote and ensure proper embryonic development. But the important point is that, given these processes, any epigenetic modifications induced in the gametes by the environment are very unlikely to survive this global epigenomic resetting and thus be passed on to the following generation.

However, this view has changed over the last few years. First, there is now evidence to support the hypothesis that some epigenetic marks can actually resist either the germline or the post-zygotic reprogramming events. One well-known example is parental imprinting[64,65]. Imprinted genes are a small group of genes, the expression of which depends on the parent of origin of the allele[66]. The process of imprinting is mediated by specific epigenetic marks, primarily DNA methylation and non-coding RNA. The imprinting control regions can survive the epigenetic resetting of the early zygote[67], thus constituting a proof-of-principle that at least a few parental epigenetic marks can be inherited and maintained in the next-generation offspring. The question is whether other loci, different from those controlling imprinting, behave similarly. In this regard, a series of studies has systematically mapped DNA methylation dynamics in germ cells during the process of gametogenesis. Collectively, they have shown that 90 % of the genome is almost completely demethylated. However, a significant fraction of the genome remains substantially methylated during all stages of germ-cell development until mature gamete formation[68–74]. These regions included predominantly intracisternal A particles of transposons. Intracisternal A particles are a family of retrovirus-like genetic elements coding for virus-like particles found regularly in early rodent embryos. They are typically heavily methylated. Hypermethylation maintains them in an inactive state and, therefore, avoids their transposition during gametogenesis and early embryonic development, which would cause novel mutations. In addition to these transposable elements, about CG rich regions (200 CpG islands) also show variable degrees of stable methylation (>40 % methylation). These regions could resist DNA methylation reprogramming in primordial germ cells, and it has been proposed that they could be potential carriers of epigenetic inheritance[74].

In addition to DNA methylation, histones and non-coding RNA might also play a role. For example, the vast majority of histones are replaced by protamines in the mature sperm. However, a small fraction (2–4 %) of the mouse genome retains some histones[75,76]. Their function is not yet known but, since histones are sensitive to environmental cues, they could act as carriers of environmentally acquired epigenetic information across generations. Finally, a plethora of non-coding RNA have been recently included as part of the epigenetic machinery. Indeed, despite the fact that sperm is transcriptionally inactive, it contains a whole set of RNA, including messenger (mRNA), long non-coding RNA, microRNA (miRNA), PIWI-interacting RNA (piRNA) and endogenous interfering RNA[77,78]. The biological function of these RNA is still unclear, but it has been proposed that they might play an important role during early embryogenesis and could therefore constitute an additional layer of epigenetic information[78]. Some functional insights with relevant implications for the offspring have been described for miRNA and piRNA. For example, miRNA have been reported to mediate transgenerational

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Table 1. Representative examples of multigenerational/transgenerational inheritance of diabetes risk

Nutritional paradigm	Pedigree	Epigenome in the offspring	Sperm epigenome	References
Prenatal paternal nutrition				
In utero undernutrition	Multigenerational (effects until F2)	Altered methylation of the Lxra gene in liver of the offspring (F2)	Modest changes in DNA methylation of the Lxra locus	(56)
In utero undernutrition	Multigenerational (until *F2*)	No changes in methylation in liver and brain from F2 embryos	Over 100 loci showed differential patterns of DNA methylation	(55)
Maternal high fat feeding	Transgenerational (effects until *F3*)	One CpG site in the promoter of the Ghsr gene in liver was differentially methylated	Not reported	(90,91)
Maternal diabetes (*in utero* hyperglycaemia)	Multigenerational (until F2)	An intragenic DMR that controls the imprinting of the Igf2/H19 locus in islet cells was hypermethylated (in F1 and F2). Igf2/H19 expression was reduced	Not reported. Intriguingly, expression of Igf2/H19 was reduced in sperm samples from F1 males	(92)
Postnatal paternal nutrition				
High fat feeding	Multigenerational (until F1)	Altered methylation in the promoter region of the *Il13ra2* gene in islet cells of the offspring (F1)	Not reported.	(85)
High fat feeding	Transgenerational (until F2)	Not reported	Not reported	(84)
Low protein diet	Multigenerational (until F1)	Widespread changes in DNA methylation (10–20 %) in liver from the offspring. Hypermethylation of an intergenic region associated to PPARα	Global DNA methylation was similar between low-protein and control samples. The retention of specific histone marks (H3K27me3) was reduced in association with specific genes in sperm samples of low-protein mice	
Paternal pre-diabetes	Transgenerational (until F2)	More than 8000 regions (including 5′UTR, 3′UTR, coding sequences and intronic regions) appeared to be differentially methylated in the islets from the offspring. At the single locus *Pik3ca* and *Pik3r1* were hypermethylated and *Ptpn1* hypomethylated in islet cells and blastocysts of the offspring	*Pik3ca* and *Pik3r1* were hypermethylated in sperm samples of the pre-diabetic sires	(58)

DMR, DNA methylation region; *Ghsr*, growth hormone secretagogue receptor gene; *Igf2/H19*, insulin growth factor-2 and H19; *Pik3ca*, phosphatidylinositol 3-kinase catalytic subunit α; *Pik3r1,* phosphatidylinositol 3-kinase regulatory subunit 1; *Ptpn1,* protein tyrosine phosphatase non-receptor type 1; UTR, untranslated region.

epigenetic inheritance at a specific locus (the *Kit* locus) in mice[79,80]. On the other hand, piRNA are primarily expressed in the reproductive organs and are highly abundant in sperm[81]. piRNA contribute to the establishment of parental imprints and epigenetic silencing of retrotransposons[82,83]. Thus, it has been proposed that piRNA are involved in the establishment of epigenetic marks during the process of reprogramming in germ cells and, hence, piRNA-mediated DNA methylation could be a potential mechanism by which epigenetic information is carried to the next generation[67]. While these are extremely attractive propositions, further experimental support for these processes in mammalian systems is required.

In summary, there is evidence to support the idea that nutritional cues can alter the epigenome of gametes. In addition, some of these epigenetic marks might survive the reprogramming events that occur during gametogenesis and the first post-zygotic divisions. Therefore, there is a theoretical framework to suggest the possibility of epigenetic inheritance of phenotypes in response to nutritional cues. In the following section, we summarise the studies that strongly support the idea of transgenerational epigenetic inheritance of diabetes risk. These studies fulfil the conditions that we have so far described in this review: (i) paternal inheritance of diabetes through (ii) an appropriate pedigree structure in which (iii) the epigenome has been analysed in the sperm (see Table 1). We review models in which the nutritional challenge occurs during early developmental stages, in the context of the developmental origins for health and disease hypothesis. Additional examples in which the nutritional exposure occurs during adulthood are also briefly summarised[57,84,85].

Animal models of transgenerational inheritance of metabolic risk

Transgenerational inheritance of diabetes by early nutrition

Intrauterine undernutrition. *In utero* energy restriction in mice (50 %) has been reported to result in intrauterine

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growth restriction (IUGR) and low birth weight[86,87]. As can happen in human subjects, IUGR male mice developed obesity and glucose intolerance with ageing. Strikingly, the offspring (IUGR-F1), but not the grand-offspring (IUGR-F2), of male mice exposed to intrauterine undernutrition also developed glucose intolerance as adults[88]. Therefore, by definition, this is a model of multigenerational transmission of disease risk (Fig. 2). However, paternal transmission of disease risk strongly suggests epigenetic inheritance via the gametes.

Two independent studies have directly addressed whether (i) *in utero* undernutrition modifies epigenetic marks (DNA methylation) in the mature spermatozoa of IUGR-F1 males that are (ii) later transmitted into the offspring (IUGR-F2) and (iii) might contribute to the development of metabolic phenotypes[55,56]. First, in a transcriptomic survey, Martinez *et al.* reported that *in utero* energy restriction in F1 male mice influenced the expression of 256 genes in the livers of second-generation offspring[56]. Many of these genes were involved in regulating lipid metabolism. Among them, the transcription factor Lxra, involved in regulating fat-cholesterol metabolism, was reduced in adult liver samples from IUGR-F2 mice. This alteration may be explained, in part, by significant hypomethylation of a canonical CpG island that encompasses part of the first exon and the first intron of the gene. The key question was whether this epigenetic mark was inherited from the father or, instead, appeared secondarily as IUGR-F2 mice developed metabolic abnormalities. Strikingly, the authors found that the differential methylation of Lxra was already established in the mature sperm of the progenitors (i.e. IUGR-F1 males) and prominently in the fetal liver of IUGR-F2 mice. Thus, this work is among the first to show a line of continuity of a given epigenetic mark in two consecutive generations that can also contribute to explaining the metabolic phenotype. Although epigenetic inheritance is strongly suggested, it has to be noted that a few caveats exist. First, it is possible that the mark in the sperm was completely erased in the early embryo and then reappeared secondarily in the fetal liver as development progressed[89]. Second, the percentage of methylation change in sperm samples was about 5 %. However, the penetrance of the phenotype ranged between 40 and 60 %. Thus, the small change in DNA methylation cannot fully account for the phenotypic effects, implying that other molecules might mediate non-genomic inheritance of diabetes risk in this model, including histones and/or non-coding RNA[42].

In agreement with this view, an independent study addressed the methylation profile in sperm samples from IUGR-F1 male mice[55]. In line with the previous work, prenatal undernutrition influenced the pattern of sperm methylation, with more than 100 regions showing differential methylation as compared with controls. Nevertheless, the methylation marks that were found in F1 sperm did not persisted in the fetal liver and brain of the following generation (F2). Whether these marks reappear later in life or at any other developmental stage has not yet been studied. Interestingly, the expression of some genes, which lay in the vicinity of the methylation marks found in the sperm of the F1 generation, were differentially expressed in somatic tissues of the F2 generation. The authors proposed that the methylation marks in the gametes might serve as a platform that 'contribute[s] to the intergenerational transmission of environmentally induced disease'[55]. Together, these two studies[76,77] suggest that another molecular driver(s) might play a role in transmitting information across generations, with DNA methylation acting as a secondary mark that stabilises the information postnatally.

Maternal obesity and/or exposure to high-fat feeding. In mice, a maternal high-fat diet (HFD) during pregnancy has been reported to increase the risk of obesity and metabolic dysfunction in the offspring and following generations (Table 1). In a prominent example, founder female mice were maintained on an HFD from 4 weeks prior to pregnancy until the end of lactation[90]. The male offspring of the HFD-fed dams (nominally, the F1 generation) displayed an increased body length, obesity and mild insulin resistance as adults. Furthermore, the second-generation offspring also developed an increased body length and insulin resistance[90]. Nutritionally induced transmission of metabolic phenotypes up to the F2 generation through the paternal lineage strongly suggests a contribution of epigenetic mechanisms. To further assess whether transgenerational effects are mediated through germline-derived epigenetic factors, Bale *et al.* analysed the phenotypes in the following generation (F3)[91]. Strikingly, F3 female offspring of F2 males still showed an increased body length and weight. This is a relevant model in which real transgenerational effects have been detected.

Next, the authors explored whether epigenetic mechanism are involved in these transgenerational effects. The expression of the growth hormone secretagogue receptor gene, the protein product of which is involved in somatic growth, was moderately deregulated in liver samples from F2 mice[90]. At least one CpG site within the promoter region of the gene was significantly demethylated, suggesting that altered expression of growth hormone secretagogue receptor is due in part to this epigenetic modification. It is unclear, however, whether this epigenetic signature was actually inherited or whether it appeared secondarily as the mice developed metabolic alterations.

In summary, paternal inheritance of such complex traits in this model strongly implicates epigenetic mechanisms passing from the F2 to the F3 generations through stable marks in the germline. Nevertheless, at this point, the molecular carrier of phenotypic inheritance remains uncharacterised. Epigenetic analysis in germ cells and mature spermatozoa is warranted to fully implicate transgenerational epigenetic inheritance in this model.

*Maternal diabetes (*in utero *hyperglycaemia).* As we have previously described, gestational diabetes is strongly associated with a higher risk of obesity and diabetes in the offspring (see section Epigenetic

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inheritance of diabetes risk from nutritional cues). In mice, gestational diabetes has been reported to impair insulin secretion in the offspring (F1), leading to glucose intolerance[92]. Impaired β-cell function was attributed, in part, to reduced expression of the imprinted genes encoding insulin growth factor-2 and H19. Indeed, the expression of both genes showed a negative correlation with the level of methylation of a specific intragenic differential DNA methylation region of this locus. In addition, the authors reported that the diabetic phenotypes were transmitted to the following generation through the paternal lineage. The offspring of males previously exposed to *in utero* hyperglycaemia also showed impaired glucose-stimulated insulin secretion and glucose intolerance. Again, impaired β-cell function could be attributed to hypermethylation of the DNA methylation region and a concomitant reduction in the expression of insulin growth factor-2 and H19. These data strongly suggest epigenetic inheritance of DNA methylation marks from one generation to the next via the spermatozoa. Intriguingly, the expression of these two imprinted genes was significantly reduced in sperm samples from F1–gestational diabetes males. However, the methylation status of the DNA methylation region in sperm was not reported. Therefore, although the data are suggestive of epigenetic inheritance, at this moment we cannot ascertain whether DNA methylation (or other factors) truly plays a role in this model.

Transgenerational inheritance of diabetes after exposure to paternal nutritional challenges during adulthood

In addition to the paradigms in which paternal nutrition is compromised during development, there are a few examples in which adult paternal nutrition might have transgenerational consequences and where the sperm epigenome has been analysed (Table 1). These are summarised later.

Paternal obesity/high-fat feeding. Two studies have reported that HFD-induced paternal obesity provoked glucose intolerance in the offspring[84,85]. In one of these studies, HFD-fed founder males (F0) developed obesity, impaired glucose tolerance and insulin resistance[85]. Furthermore, a paternal HFD impaired insulin secretion and glucose tolerance in the female offspring (F1). Next, the authors determined that the expression of seventy-seven genes was altered in the pancreatic islets of F2 females. Among them, the gene encoding the IL 13 receptor α (*Il13ra2*), which can influence β-cell function, showed the greatest fold difference compared with control islets. The methylation of one cytosine (–960) in the promoter region of this gene was increased as compared with controls. This corresponded to a putative binding site for transcription factors that can actually regulate the expression of the gene. However, a functional relationship of this methylation in influencing gene expression was not provided. The authors of this study proposed that altered methylation in the islet cells of F2 rats might be inherited from the fathers via the germline. However, two important considerations have to be taken into account before considering epigenetic inheritance in this model. First, the sperm methylome was not analysed in this study. Second, F2 females had developed impaired glucose tolerance and impaired insulin secretion by the age of 12 weeks, and the islets included for analysis of the methylome were collected from 13-week-old rats. Thus, progressive metabolic dysfunction might secondarily influence patterns of methylation in the islets of F2 rats. Further investigation is required to elucidate these issues.

In the second model, adult male mice were fed an HFD containing a relatively moderate amount of energy from fat (21 %)[84]. The diet induced obesity in founder F0 mice in the absence of any other additional components of the metabolic syndrome, including insulin resistance, dyslipidaemia or impaired β-cell function. Strikingly, paternal high-fat feeding induced glucose intolerance and insulin resistance in the offspring (F1). Furthermore, paternal HFD in F0 founder mice also induced metabolic abnormalities in their granddaughters (F2 females) through the male line (F1 male offspring). These real transgenerational effects (Fig. 2), which were passed through the male line, strongly suggest the transmission of epigenetic signals from F0 grandfathers through F1 fathers to F2 females. Nevertheless, to our knowledge, no molecular analyses have been reported in this model to underpin the epigenetic mediators of such effects. While awaiting elucidation of the molecular mechanisms, it is important to note that paternal impaired glucose homeostasis or diabetes was not a prerequisite for passing ancestral phenotypes to the offspring.

Paternal low-protein feeding. Similar to the previous paradigms, Carone *et al.* studied the impact of a paternal low-protein diet on the offspring[57]. Paternal low-protein feeding in adult founder mice resulted in upregulation of the genes involved in fat and cholesterol biosynthesis in the livers of the offspring (F1). This is another example of the paternal diet influencing the offsprings' metabolism by altering the expression of specific metabolic genes. Furthermore, global DNA methylation showed widespread modest changes (10–20 %) in liver samples from F1 offspring. At the single locus level, it was found that DNA methylation of an intergenic region upstream of the gene encoding PPARα was increased by 30 %. PPARα is a key transcription factor that regulates lipid oxidation and that could explain, in part, the deregulation of fat and cholesterol biosynthesis.

Next, the investigators studied whether these epigenetic marks were already present in sperm samples from the low-protein-fed founder males. First, DNA methylation of the PPARα locus was unaltered in sperm samples from the F0 low-protein-fed mice. Second, global sperm DNA methylation, analysed via methylated DNA immunoprecipitation and high-throughput sequencing (MeDIP-Seq), was largely similar between groups. Thus, in agreement with previous models, the authors concluded that the sperm methylome is not the likely carrier of epigenetic information between

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generations[42,55,89]. Therefore, other epigenetic mechanisms might mediate such paternal effects, with RNA and/or chromatin modifications being likely carriers[67]. In agreement with this, a genome-wide histone retention assay found that the specific mark H3K27me3 was reduced at the promoter regions of some genes (*Maoa* and *Eftud1*) in sperm samples from mice fed a low-protein diet[57]. However, to fully implicate these histone marks as carriers of information, it is necessary to demonstrate that they are passed to the following generation and that they remain stable in tissues from F1 males and females.

Paternal pre-diabetes. Wei *et al.* have recently reported an extremely interesting mouse model of pre-diabetes[58]. They generated a model of pre-diabetes by treating mice with an HFD and low doses of streptozotocin. As expected, founder males exposed to this treatment developed increased body weight and adiposity, insulin resistance and glucose intolerance. Strikingly, the offspring of pre-diabetic males also developed glucose intolerance and insulin resistance. Next, the authors showed that 402 genes were differentially expressed in islet cells of the offspring of pre-diabetic males. In parallel, genome-wide DNA methylation analysis was also undertaken in the same samples. More than 8000 regions (including 5′UTR, 3′ UTR (UTR; untranslated region), coding sequences and intronic regions) appeared to be differentially methylated in the islets from the offspring. These changes did not globally correlate with patterns of expression. Nevertheless, at a single locus, the authors reported a substantial increase in methylation in association with reduced expression of genes encoding phosphatidylinositol 3-kinase subunits (phosphatidylinositol 3-kinase catalytic subunit α and phosphatidylinositol 3-kinase regulatory subunit 1). Likewise, expression of the protein tyrosine phosphatase non-receptor type 1 gene was significantly increased, while cytosine methylation was reduced. These three genes regulate insulin signalling and their deregulation might contribute to impaired β-cell function. At this point, it can be suggested that paternal pre-diabetes influences the epigenome (DNA methylation), which can in turn drive the expression of key genes in the pancreatic islets of offspring.

The key question, again, is whether these epigenetic signatures were inherited from the pre-diabetic founder mice. The authors reported that paternal pre-diabetes substantially altered DNA methylation patterns in sperm samples. This is a new example showing that the sperm epigenome is largely responsive to environmental cues. Strikingly, a substantial fraction of the hypermethylated (39 %) and hypomethylated (36 %) intragenic regions overlapped between the sperm and the islet cells. Interestingly, the methylation of phosphatidylinositol 3-kinase catalytic subunit α and and phosphatidylinositol 3-kinase regulatory subunit 1, but not of protein tyrosine phosphatase non-receptor type 1 gene, was also altered in the same direction as in the islets. This line of continuity for many epigenetic marks across two generations strongly suggests epigenetic inheritance. To further confirm this issue, the researchers also analysed the methylation of these two targets in embryonic day 3·5 blastocysts. Again, both phosphatidylinositol 3-kinase catalytic subunit α and and phosphatidylinositol 3-kinase regulatory subunit 1 showed increased methylation in the pre-diabetic line.

In summary, this study provides the strongest data to date to support the epigenetic inheritance of diabetes risk (or phenotypic variation in general) in mammals. Further experimental paradigms will be necessary to determine whether this is an isolated example or whether it constitutes a more general phenomenon.

Conclusion

To conclude, both human and experimental data provide compelling evidence supporting the hypothesis that paternal nutrition and/or metabolic modifications might influence the epigenome in the offspring and, in some examples, even the grand-offspring. Whether the epigenetic modifications detected in the offspring are truly inherited from their progenitors, or instead develop secondary to metabolic dysfunctions that are progressively acquired with ageing, is currently unknown. This has become a very active and intensive area of research, and we expect that the coming years will deliver substantial data to truly prove (or disprove) that epigenetic mechanisms play a relevant role in the inheritance of complex diseases in human subjects.

Only a few animal models, developed in very well-controlled experimental settings, have suggested that epigenetic marks can be inherited in offspring via the gametes and influence the physiology of the offspring (Table 1). This effect appears to particularly arise when nutritional challenges occur during early development, at the time of germ-cell maturation[47]. To note, the reported dietary challenges include energy restriction, high-fat feeding, low-protein feeding. A major site of future research will be to determine the specific components of the diet that make the major contributions in modifying the epigenome. For example, we have described that the bioavailability of precursors of SAM (betaine, choline, vitamin B_2, B_{12}) might influence global and locus-specific patterns of DNA methylation. Likewise, there is on-going research studying the impact of micronutrients present in many products, such as polyphenols, curcumin, etc., in modulating the epigenome. We anticipate that this understanding will be of potential clinical relevance because it might be possible to design nutritional interventions, especially during early development, aimed to set the epigenome in a 'healthy' state. And, according to the developmental origins of health and disease hypothesis, establishing the appropriate epigenetic marks might have long-lasting effects in promoting a healthy life span.

The actual molecular carrier(s) of epigenetic information are poorly characterised and remain a matter of intense debate[61,67]. For example, most animal studies have primarily focused on analysing the methylome. This is probably because DNA methylation is the easiest and best-characterised epigenetic mechanism. It is likely

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that the analysis of genome-wide histone marks and the whole transcriptome, including non-coding RNA, will soon complement the current DNA methylation data. In fact, some authors have proposed that RNA might be the main epigenetic carrier of information between generations[67].

To conclude, to fully confirm (or refute) that epigenetic mechanisms play a role in the inheritance of complex traits in mammals, a careful and detailed analysis of epigenetic marks should be conducted not only in gametes, but also in the early blastocyst, the embryo and, ideally, somatic tissues from adult individuals.

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Conflicts of Interest

None.

Authorship

M. R. K., R. D. and S. R. wrote sections of the manuscript. J. C. J. supervised the writing and assembled the final version.

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