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Intergenerational Programming of Metabolic Disease: Evidence from Human Populations and Experimental Animal Models

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SUMMARY

We are in the midst of unparalleled epidemics of obesity and type 2 diabetes - complex phenotypes originating at the intersection of genetic and environmental risk. As detailed in other chapters, evidence indicates that non-genetic, or environmental, risk may initiate during prenatal and early postnatal life¹. Striking examples in humans include the association of low birth weight (LBW) and/or accelerated early growth with increased risk of insulin resistance, obesity, type 2 diabetes (T2DM), and cardiovascular disease (CVD), and the close relationship between maternal obesity or diabetes with childhood obesity. In this chapter, we will focus on the intriguing emerging data from both humans and animal models which indicate that intrauterine and childhood exposures can also influence risk for diabetes and cardiovascular disease in subsequent generations. Understanding the mechanisms responsible for these effects is critical in order to develop effective metabolic and nutritional interventions to interrupt such vicious intergenerational cycles potentiating risk for metabolic disorders.

Intergenerational phenotypes can be defined as those which arise as a consequence of phenotypes in ancestors (e.g. parental, grandparental, or earlier generations) but are not mediated by inherited (genetic) primary DNA sequence alterations. In experimental models, phenotypes are usually considered to be intergenerational when effects of a direct environmental exposure persist for at least two subsequent generations.

Potential mechanisms contributing to intergenerational responses are complex (Figure 1). In human studies, intergenerational effects could be mediated by unrecognized effects of shared genetics, by selection biases, or by postnatal environments common to more than one generation (e.g. dietary patterns, socioeconomic, other family stressors). Beyond these environmental effects, potential mechanisms may differ depending on whether effects are transmitted through the maternal or paternal lineage. Maternal lineage effects could be mediated by genetic effects (DNA sequence of nuclear or mitochondrial DNA), maternal germ cell cytosolic or nuclear epigenetic effects, placental structure/function, or factors affecting the intrauterine or early postnatal environment (e.g. dysregulation of maternal metabolism, altered uterine structure/function, maternal behavior). For example, in mothers with diabetes, maternal glucose can cross the placental barrier and stimulate excessive insulin secretion by the developing fetus, potentially contributing to excessive growth and adiposity as well as dysregulation of hypothalamic development². Alternatively, both glucose and non-glucose fuels (e.g. maternal lipids³) and hormones may affect fetal metabolism via effects on placental nutrient transport and secretion and action of key hormones regulating whole-body metabolism, including leptin^{4,5}. By contrast, paternal lineage intergenerational effects observed in experimental models in which the father is removed immediately after breeding, must implicate genetic⁶, epigenetic, and/or placental developmental effects mediated by components of semen. These may include proteases, cytokines, and hormones (e.g. TGFβ) which could modulate female post-mating responses and immune responses and thus potentially also contribute to offspring phenotypes⁷⁻⁹.

Experimentally, it is challenging to clearly demonstrate germ cell-dependent epigenetic mechanisms mediating intergenerational effects. It is important to recognize that exposures which occur during critical periods of pregnancy affect not only the mother (F0), but also the developing germ cells in her offspring (first-generation, F1); these F1 germ cells may therefore have a *direct* impact on second-generation (F2) offspring. Thus, if exposure occurs during F0 pregnancy, demonstration of transgenerational germ cell epigenetic mechanisms requires phenotypes which can be observed in offspring of the F3 or subsequent generations.

Human data supporting intergenerational transmission of metabolic phenotypes

Ever since Barker's landmark studies linking birth weight to later risk of cardiovascular disease and diabetes^{10,11}, the prenatal environment has been recognized as a risk factor for chronic diseases in offspring^{1,12}. This so-called developmental programming can initiate a vicious cycle of risk to subsequent generations, even in the absence of further environmental stressors¹²⁻¹⁴. In humans, this concept has been demonstrated most clearly in the case of maternal diabetes; infants of women with pre-existing or gestational diabetes have greater birth weight, adiposity, and subsequent risk for obesity and diabetes¹⁵⁻¹⁷. While shared high-risk genetics and family environment (nutrition, activity patterns) could of course accentuate these patterns, studies from the Pima population have demonstrated that offspring born to women with diabetes are consistently heavier than offspring born to the same mother before she developed diabetes^{18,19}, implicating strong non-genetic effects related to the diabetes metabolic environment. Unfortunately, these diabetes-exposed females are at higher risk for diabetes during adult life (and during pregnancy), thus perpetuating a vicious cycle of intergenerational risk²⁰. Similar patterns may also be operative in the setting of excessive gestational weight gain or obesity^{16,21,22}, as supported by data that children born before maternal bariatric surgery are at greater risk for obesity than offspring born after weight-loss surgery. Together, these data again indicate that prenatal exposure to an "obese" metabolic maternal environment confers risk not solely explained by shared genetics²³.

Similar, but more limited, human data demonstrate that early life nutritional exposures, including both *under*- and *over*nutrition, can affect not only the exposed individuals themselves, but also their offspring²⁴. For example, maternal undernutrition resulting from severe famine during the Dutch Hunger winter resulted in not only reduced birth weight of their babies (F1), but also increased adiposity during adult life in F1 females²⁵. Differential phenotypes persisted in the grandchildren of famine exposed mothers, as F2 offspring also had increased neonatal adiposity (ponderal index)²⁶. In other studies, women exposed *in utero* during the first and second trimesters to famine (F1) were more likely to give birth to LBW offspring (F2)^{27,28}, despite no further periods of nutritional stress. Similarly, in a South Indian cohort, strong correlations were described between birth weights of parents (F1) and of their offspring (F2)²⁹.

Like poor fetal growth, *excessive* fetal growth has also been linked to intergenerational effects to promote risk for chronic disease. In a Swedish birth registry study, women who were large for gestational age (LGA) at birth had a 2-fold higher risk of giving birth to LGA infants. This risk was further compounded by elevated maternal BMI³⁰. Thus, environmental stresses during pregnancy can increase disease risk, not only for one's children, but also grandchildren.

Beyond the prenatal period, early childhood may be another critical developmental window during which perturbations in nutrition can have intergenerational effects. For example, growth rates between 0-3 years in parents are significantly correlated with growth rates in their children³¹, and excessive weight gain during early childhood in parents is associated

with increased BMI of offspring, independent of parental adult BMI³². Moreover, historical studies of fluctuations in food availability in the Overkalix region of Northern Sweden have shown that food abundance and overfeeding during grandfathers' childhood (8-12 yrs) is linked to T2DM, CVD and reduced longevity in their grandsons³³⁻³⁶. Intriguingly, some authors have reported grandpaternal influences on obesity risk, with significant correlations between BMI of grandparents and grandchildren even when parents (in the intervening generation) were of normal weight³⁷.

Given the complexities of intergenerational phenotypes in humans, crisp experimental models are necessary to elucidate potential mechanisms. In the next section, we will review data from animal models highlighting paternal intergenerational effects of nutrition on birth weight, adiposity, glucose tolerance, and longevity³⁸⁻⁴⁶, and discuss implications for potential epigenetic mechanisms responsible for these phenotypes.

Animal models of intergenerational phenotypic transmission

Phenotypes which are "transmitted" across generations in the absence of DNA sequence differences implicate the presence of a cellular epigenetic mark which would influence transcription and developmental trajectories, but which would need to escape the reprogramming occurring during early embryonic development⁴⁶. Support for this concept was initially provided by the discovery of non-genetic but heritable differences in expression of engineered transgenes⁴⁷.

Two classic studies demonstrated non-genetic phenotypic transmission in mice at the agouti viable yellow (A^{vy})⁴⁸ and axin fused ($Axin^{Fu}$) loci. The agouti locus encodes for agouti, a protein promoting yellow coat color. Control of this locus is altered in mutant A^{vy} mice, in which an IAP element was inserted upstream of the agouti gene, promoting ectopic expression of agouti. However, mice carrying the A^{vy} allele can have a range of coat colors, despite genetic identity. This heterogeneity arises from differential expression of the agouti gene, which is associated with variable DNA methylation and increased H4K20me3 histone marks at the upstream IAP element^{49,50}. When the A^{vy} allele is transmitted by the mother, either by natural breeding or zygote transfer, a higher percentage of offspring mice than expected share her coat color. By contrast, this enrichment is not observed when the father transmits the A^{vy} locus. Thus, maternal intergenerational transmission can occur at the A^{vy} locus, potentially in part via chromatin remodeling^{49,50}.

Subsequent studies demonstrated that expression at the A^{vy} locus could be influenced by the maternal diet during pregnancy^{51,52}. Specifically, supplementation with methyl donors, including folic acid, vitamin B12, choline, and betaine, increased methylation, silenced the A^{vy} locus, and darkened coat color⁵³. While coat color darkening persisted in F2 offspring⁵¹, it did not differ in the F3 generation⁵². Together these data indicate that diet can alter epigenetic marks at the A^{vy} locus in developing germ cells (yielding F2 mice) and that these marks can be at least partially retained during early embryonic reprogramming. However, further intergenerational transmission via the germ line does not continue to the F3 generation.

A second locus also exhibiting regulation by epigenetic marks is the Axin-fused ($Axin^{Fu}$) allele⁵⁴. When active, this locus confers a kinked tail phenotype. However, some $Axin^{Fu}$ mice do not exhibit this phenotype, indicating epigenetic silencing. Silencing is correlated with differential methylation of an IAP retrotransposon within this allele and altered expression of aberrant mRNA transcripts derived from this locus. Moreover, phenotypes can be inherited by offspring via either maternal or paternal lineages⁵⁴. Paternal lineage inheritance in this model suggests that sperm can potentially transmit epigenetic information, potentially via alterations in sperm methylation at this locus (which parallel

somatic methylation patterns), or via other marks which either are not completely cleared post-fertilization and/or influence subsequent zygote epigenetic marks. To add further complexity, intergenerational effects at this locus are influenced by background strain.

Recent studies have demonstrated that intergenerational inheritance in mice may extend beyond loci regulated by retrotransposons. For example, Nadeau and colleagues have demonstrated that Y chromosomal alleles can influence phenotypes in daughters (who of course do not maintain Y chromosome DNA) ⁵⁵, and that tumor susceptibility alleles in the parental lineage can influence phenotypes even in wild type offspring ⁵⁶. Similarly, wild-type offspring of mice with heterozygosity at the cKit locus demonstrate phenotypes similar to that of mutant parents ⁵⁷. Furthermore, paternal or grandparental alleles can reduce dietary obesity in obesity-sensitive C57BL/6J mice ⁵⁸. These parental genotype-based intergenerational effects have been proposed to explain “missing heritability,” or genetic susceptibility not explained by DNA sequence ⁵⁹. This may be a particularly important consideration in the case of complex polygenic diseases such as type 2 diabetes, in which GWAS thus far indicate that sequence variation may contribute only a relatively small portion of apparent disease risk.

Environmental exposures: nutrition

An increasing number of experimental models demonstrate that environmental exposures can also modulate metabolic phenotypes in offspring. Either maternal calorie or protein restriction, overnutrition, or insulin resistance during pregnancy can induce obesity and alter metabolism in offspring ^{43,60-67}. Early clues that these phenotypes might also be transmitted to subsequent generations, and potentially via epigenetic mechanisms, came from the seminal observations of Whitelaw and Waterland, as described above ^{53,54}.

One example of nutritionally-mediated transmission of metabolic phenotypes is provided from our laboratory's mouse model of prenatal undernutrition (UN), in which healthy dams (F0) are exposed to undernutrition during the last third of pregnancy (day 12.5-19.5, F0). Their offspring (F1) have a modest 15% reduction in birth weight and experience early postnatal “catch-up growth.” Despite normal chow diet after weaning and body weights equivalent to control mice, these UN-exposed mice go on to develop progressive glucose intolerance, insulin secretory dysfunction, reduced muscle mass, and obesity as adults. While the precise molecular mechanisms remain unclear, UN exposed mice have upregulation of lipogenic gene expression in both liver and adipose, increased adipocyte size, and reduced mitochondrial DNA content. We have recently demonstrated that UN-exposed mice also have reduced numbers of myogenic precursor cells ⁶⁸, suggesting the possibility that alterations in key stem cell populations may alter developmental trajectories and modify tissue structure/function and repair ⁶⁹. Many features of this phenotype, including glucose intolerance, obesity and lipogenic patterns, can be reversed by nutrient restriction of newborn mice, indicating plasticity of the early postnatal environment and providing a potential therapeutic window.

In this model, we tested whether effects of maternal nutrition could also be observed in her grandchildren by intercrossing F1 control (F1-C) and undernutrition-exposed (F1-UN) males and females, forming 4 groups of F2 offspring (Figure 2A). Second-generation F2 offspring of either F1-UN males or females also have increased adiposity, impaired glucose tolerance, and altered insulin secretion (Figure 2B/C), despite normal nutrition during the second-generation pregnancy ⁴³. Insulin resistance and glucose intolerance are even greater in F2 mice with nutritional exposure via both maternal and paternal lineages (Figure 2D). Together, these data demonstrate that metabolic phenotypes in F2 can be mediated by exposure of either their maternal or paternal grandmother to undernutrition. Of course,

although F1 females remained normoglycemic during pregnancy, we cannot completely exclude a possible contribution of subtle increases in F1 maternal adiposity to alter the F2 intrauterine metabolic environment. By contrast, paternal lineage transmission highlights the potential role of the germ cell as the most likely source of the intergenerational signal (see below).

Overnutrition has also been linked to offspring metabolic phenotypes. For example, high-fat feeding in mouse dams (F0) yields increased body length and insulin resistance in both children (F1) and grandchildren (F2) ⁷⁰. These phenotypes are transmitted to the F2 generation via both maternal and paternal lineages. By contrast, transmission of body length phenotypes to F3 offspring occurs via the paternal lineage only, with dominant effects in F3 females. Similarly, postnatal overnutrition, experimentally induced by litter size reduction at birth, can directly promote obesity, insulin resistance and glucose intolerance in pups ⁷¹. Such overnutrition-exposed males, when bred with control females, can also yield insulin resistance and glucose intolerance in their offspring and grand-offspring, further indicating that nutritional exposures during key developmental periods can affect the metabolic health of subsequent generations.

Other Exposures

Additional exposures may also produce epigenetic changes and yield phenotypes which can be transmitted to future generations. Transient exposure of pregnant female rats during mid-gestation (E8-14) to the antiandrogenic endocrine disrupter vinclozolin reduces fertility in not only male offspring (F1), but also in over 90% of males in subsequent generations (F2 to F4), despite no further exposures. These effects are transmitted via the paternal lineage and accompanied by alterations in paternal germ cell DNA methylation, particularly in repeat elements ^{72,73}. These data suggest that developing male germ cells are vulnerable to environmental exposures at discrete times of early development during which sex determination and global methylation reprogramming is occurring.

Maternal behavior during the early suckling period can also “program” epigenetic stress responses in offspring, potentially via sustained alterations in DNA methylation and histone acetylation at the glucocorticoid receptor (GR) promoter ⁷⁴. These changes, which persist into adult life, are likely mediators of reduced GR expression and altered HPA responses to stress in offspring and grand-offspring. Interestingly, these phenotypes can be reversed by trichostatin A, a histone deacetylase inhibitor, providing support for the potential reversibility of epigenetic marks.

“You are what your dad ate” ⁷⁵

Recent studies have solidified the concept that nongenetic, intergenerational transmission of metabolic phenotypes from fathers to their offspring can occur in mammals ⁴²⁻⁴⁵. In each of these models, male mice with either current or remote dietary exposures were bred to control females, and offspring phenotypes were compared to those of offspring of control males and females.

- Even a single bout of fasting in males prior to breeding with control females can reduce plasma glucose levels in both male and female offspring ⁴². Effects are even greater if breeder males are subjected to repeated fasting, with reductions in both glucose and body weight (in female offspring).
- Males with a remote history of undernutrition during their own intrauterine development, *but normal diet at breeding*, produce offspring with increased adiposity, impaired glucose tolerance, and altered insulin secretion, despite normal

nutrition during the second-generation pregnancy⁴³. As noted above, similar phenotypes are also observed in F2 mice derived from the maternal lineage, and offspring exposed to both maternal and paternal lineage undernutrition (i.e. in both their maternal and paternal grandmother) are even more glucose intolerant and severely insulin resistant.

- Males with diet-induced obesity produce female offspring with mild glucose intolerance, reduced insulin secretion, and altered pancreatic islet gene expression potentially linked to reduced methylation near IL13ra2⁴⁵.
- Males fed a low protein, high-carbohydrate diet produce offspring with increased hepatic expression of lipid and cholesterol synthesis genes. While overall DNA methylation in offspring was unchanged, there was modestly increased methylation in an intergenic CpG island located between PPAR α and Wnt7b⁴⁴. Interestingly, differential methylation was previously observed at the PPAR α promoter in offspring of female rats fed a low protein diet during pregnancy⁷⁶. Whether site-specific changes in methylation at this locus are functional for intergenerational phenotypes remains unclear. Alternatively, differences in developmental trajectories or systemic metabolism in low protein offspring could also contribute secondarily to expression patterns, and potentially, to methylation patterns as well. Additionally, the authors did not detect differences in sperm methylation, as assessed by MeDIP-seq, as a function of paternal diet.

Together, these data independently and strongly support that the dietary or metabolic history of males affects metabolism in offspring, even in cases of normal diet at breeding. These phenotypes are likely mediated by sperm, since the father is removed immediately after breeding, and in turn could be mediated by alterations in sperm function⁷⁷, semen proteins or hormones⁷⁻⁹, or potentially by epigenetic mechanisms in germ cells.

It is important to recognize that these models differ in design and potential conclusions (Figure 3). Experimental modulation of paternal nutrition prior to breeding is likely to have direct current impact on sperm (Figure 3B). By contrast, experimental modulation of maternal nutrition during pregnancy (Figure 3C), particularly during periods of germ cell development, actually has *direct* effects on 3 individuals: the mother (F0), her developing embryo (F1), and the developing germ cells in the F1 embryo which will ultimately contribute to F2 offspring. Strictly speaking, proof of “pure” intergenerational transmission by epigenetic mechanisms in germ cells requires demonstration of phenotypes in F3 generation animals not exposed to the initial stressor. While this is an important consideration in mechanistic studies, *direct* exposures of germ cells, either during early development or in response to current nutrition/metabolism, may have more robust impact and relevance for offspring and grand-offspring health.

Mechanistic insights into intergenerational transmission of metabolic phenotypes

Which mechanisms may contribute to paternally-mediated intergenerational transmission of metabolic phenotypes? As indicated above, in experimental models in which the father is removed after breeding, transmission must involve components of the sperm, including male germ cells or seminal fluid, as noted previously. In this section, we will primarily consider the potential role of epigenetic marks present in the male germ cell, including DNA methylation, histone modification, or small non-coding RNAs.

DNA Methylation

DNA methylation, long considered a “stable” epigenetic mark as it is maintained both during mitosis and meiosis, could potentially contribute to intergenerational inheritance. One example of epigenetic maintenance of stable transcriptional phenotypes is that of genomic imprinting, in which maternal and paternal alleles are differentially expressed based on parent-of-origin of the allele. Thus, epigenetic mechanisms responsible for imprinting, including DNA methylation at differentially methylated regions (DMR), have also been postulated to contribute to developmental programming. However, imprinting patterns are reestablished in each generation according to the gender of the individual, and thus could not be recapitulated in a gender-independent manner in subsequent generations. Moreover, recent data have demonstrated that global expression of imprinted genes is not altered in offspring mice in response to either prenatal or early postnatal nutrient stress of the father⁴⁴, mother^{78,79} or the grandmother⁷⁹. More global differences in methylation (not restricted to imprinted loci), hydroxymethylation⁸⁰, or other DNA modifications in male germ cells could also potentially contribute to intergenerational phenotypes. While Carone and colleagues were unable to detect differences in sperm methylation patterns as a function of current diet⁴⁴, we have observed site-specific differences in methylation at nonimprinted loci in sperm of males exposed to undernutrition during their gestation (Radford *et al*, manuscript under review). Maternal vinclozolin exposure has been shown to modestly alter methylation patterns at imprinted loci in offspring sperm e.g. reducing methylation at paternally imprinted loci and increasing methylation at maternally imprinted loci⁸¹. Together, these data indicate that mechanisms may differ in different exposure paradigms.

Chromatin Remodeling

Beyond DNA methylation, chromatin remodeling mechanisms have been implicated in intergenerational transmission given the importance of chromatin organization in sperm⁸². Although histones are exchanged for protamine during spermatogenesis, a small percentage of nucleosomes are retained in human sperm. These retained nucleosomes and associated histone modifications are enriched at loci regulating genes controlling post-fertilization development, including imprinted genes, miRNA clusters, and HOX genes. Thus, sperm chromatin patterns are likely to be important for developmental trajectories and could potentially mediate intergenerational effects of environmental stimuli.

One example implicating chromatin modification in intergenerational transmission comes from the *C. elegans* model, which lack DNA methylation. Worms with mutations in an H3K4me3 regulatory complex have increased lifespan⁸³. Interestingly, wild type offspring of heterozygous worms (F2) also demonstrate increased longevity, and this phenotype persists in wild type progeny for at least two more generations before normalizing in F5 offspring⁸⁴. Increased longevity in wild type descendants is also paralleled by altered gene expression, with enrichment for H3K4me3-marked genes. Together, these data demonstrate transgenerational inheritance of not only longevity, but also histone marks and gene expression. Similarly, H3K4me3 patterns mediated by the Set1/MLL complex can be established in the maternal germ line and inherited for at least several cell divisions⁸⁵. While this phenotype requires the presence of functional female germ cells, it remains unclear whether this is a cell-autonomous effect, or an indirect effect of germ cells on regulation of systemic metabolism, and whether effects are specific to maternal or paternal lineages. Moreover, it remains unclear whether these marks are primary, or pathogenic. Similar data from *Drosophila* also support a role for chromatin marks in intergenerational phenotypes. Heterochromatin disruption can be induced by heat or osmotic stress in a p38- and dATF2-dependent manner. These patterns are also observed in the next generation, and further amplified and extended by repeated stress exposures in subsequent generations⁸⁶. In

mammals, there is significant overlap between H3K4 methylation patterns in sperm and 4-8 cell stage embryos, suggesting that these histone marks could be retained in the early embryo and potentially contribute to expression patterns and early developmental trajectories⁸². Limited data are available from rodent models of intergenerational transmission; Carone et al assessed H3 abundance and H3K27me3 marks, finding few changes related to current paternal diet⁴⁴.

Noncoding RNA

Recent data have also highlighted the potential role of alterations in small non-coding RNA species, such as microRNA (miRNA) or piwi-interacting RNA (piRNA), as mediators of intergenerational effects. In *C.elegans*, siRNA-induced phenotypes can be inherited by some of their progeny for more than 5 generations^{87,88}. Buckley and colleagues recently demonstrated that such intergenerational inheritance patterns require germ cell Hrde-1, a member of the argonaute family which interacts with siRNA to direct gene silencing⁸⁹ and can also act downstream of piRNA. Several recent publications have further demonstrated that piRNA can mediate multigenerational inheritance^{90,91} via silencing of transposons and a subset of genes in germ cells⁹². Through their interactions with Argonaute family proteins, piRNA can also promote nuclear gene silencing, perhaps via deposition of the repressive H3K9me3 mark in the germline^{89,93}. Depending on the study methods, silencing can be observed for up to 5 generations⁹⁰, even in the absence of the original stimulus, before reverting to the original phenotype⁹³. Such mechanisms may be beneficial, at least in worms; this pathway appears to be required for normal germ cell viability⁸⁹ and resistance to viruses⁹⁴. In *Drosophila*, piRNA effects may depend on parent of origin⁹⁵.

Whether similar mechanisms are responsible for mammalian intergenerational paradigms remains untested. However, piRNA have also been implicated in de novo methylation at both transposons⁹⁶ and differentially methylated regions in mammals^{97,98}. Wild-type offspring of mice with heterozygosity at the cKit locus⁵⁷, which display phenotypes similar to heterozygotes, have altered sperm RNA expression patterns; such phenotypes can be recapitulated by injection of miRNA targeting Kit into one-cell embryos of wild type mice. These and subsequent studies at the Cdk9⁹⁹ and Sox9 loci¹⁰⁰ also suggested that experimental modulation of RNA could act as signals to propagate phenotypes across generations. These data are also reminiscent of early studies in mouse hybrid zygotes which demonstrated effects on growth and gene expression of offspring when the maternal cytoplasmic genotype differed from the maternal nuclear genotype¹⁰¹. These data suggest that components of the maternal cytoplasm (potentially including mitochondria, proteins, and cytosolic RNAs) could influence nuclear phenotypes in the zygote. Whether similar effects of small noncoding RNA in sperm could also regulate mammalian epigenetic patterns and early development and potentially contribute to intergenerational patterns is unknown at present and an important question for future research.

Epigenetic phenotypes in humans

Few studies thus far have examined epigenetic phenotypes in humans in relation to early-life exposures. Not surprisingly, DNA methylation patterns are quite concordant in monozygotic twins, given their genetic identity¹⁰². Concordance is even more striking in monozygotic monochorionic twins who separate later in development than monozygotic dichorionic twins¹⁰². Moreover, the identification of loci with interindividual epigenetic variation across multiple tissues also suggests that differential patterns may arise during very early development. With aging, such differences in DNA methylation and histone acetylation between monozygotic twins become more prominent¹⁰³, likely reflecting the combined impact of both prenatal and postnatal environmental stimuli in modulating epigenetic marks.

Several lines of evidence indicate that nutritional exposures may play a prominent role in modulating epigenetic marks in humans. In the Dutch Hunger Winter model, individuals who were exposed to famine during early prenatal life had less DNA methylation at the maternally imprinted IGF2 locus at age 60, as compared with their unexposed siblings ¹⁰⁴. Subsequent studies demonstrated that methylation differences at additional loci could be gender-specific and dependent upon the gestational timing of famine exposure ¹⁰⁵. In rural Gambia, seasonally-determined nutritional availability at the time of conception is linked to DNA methylation patterns ¹⁰⁶, again highlighting the importance of developmental timing for nutritional epigenetic marks. Recently, targeted analysis of CpG methylation in human umbilical cord tissue samples demonstrated that methylation 5' to the start sites for RXRA and eNOS was associated with childhood fat mass, and linked to maternal carbohydrate intake ¹⁰⁷. These two CpG methylation marks, together with gender, contributed substantially to variance in fat mass at age 9 (over 25%). While cause-and-effect relationships cannot be ascertained from these data, these data do indicate that early-life exposures are associated with altered DNA methylation in humans. Whether methylation is the inciting or responsible epigenetic mechanism, or just a detectable mark of prior stress, remains unclear. Finally, despite the parallels between such detectable epigenetic marks following nutritional exposures in humans, rodents, and lower organisms, we cannot determine whether these same mechanisms contribute to intergenerational inheritance in humans.

Caveats and unanswered questions

If we are to implicate epigenetic perturbations in the male germline as effectors of an environmental stimulus and its intergenerational transmission to offspring, these “memory marks” must either survive the dramatic changes in DNA methylation and histone modification occurring shortly after fertilization, or influence this process. This “erasure” of epigenetic marks may be beneficial to ensure pluripotency during development but is clearly incomplete; for example, imprinted loci resist genome-wide epigenetic reprogramming and thus maintain parent-of-origin marks. Differential imprinting does not appear likely to mediate intergenerational phenotypes ^{44,78,79}, but there may be other genomic features similarly capable of maintaining differences during early post-fertilization development. These could potentially include (a) sites of retention of histone marks ⁸⁵ or DNA methylation (e.g. repetitive IAP elements ¹⁰⁸), (b) targets of non-coding RNA, or (c) loci stably associated with repressive polycomb or trithorax proteins ¹⁰⁹. Further complexity is suggested by the emerging literature of additional DNA modifications, including hydroxymethylation ¹¹⁰, which could potentially be regulated by the cellular metabolic environment. Detailed critical analysis of these exciting possibilities in mammalian systems will be required to elucidate molecular mechanisms responsible for intergenerational phenotypes.

On a practical level, a key challenge is to better define the roles of nutrition, obesity and metabolic fitness in determining optimal function of parental germ cells. While diet and exercise can improve sperm motility, DNA damage, ROS, and mitochondrial potential in obese mice ¹¹¹, it will be important to translate these studies to humans in order to identify the optimal intake of specific macro- or micronutrients (and total calories) and physical activity which maximizes germ cell function and preconception health.

Summary

Together, data from both human and animal model studies demonstrate that the early life environment, via both metabolic and epigenetic mechanisms, can initiate a vicious cycle of metabolic risk for offspring, grand-offspring, and subsequent generations (Figure 4). Two

considerations arise from this perspective. Firstly, paradigms for complex diseases with prominent environmental risk factors must include the possibility that inheritance of disease risk in families may be related not only to genetics and shared environments, but also mediated by epigenetic information accumulated over generations. Secondly, increased awareness of the important role of both maternal *and* paternal germ cells in contributing to offspring phenotypes should promote the design and evaluation of strategies to improve metabolic health - not only in women of reproductive age, but also in all potential fathers. Such efforts are critical if we are to improve health and reduce disease risk for our children and grandchildren.

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Maternally-Mediated Intergenerational Effects	Paternally-Mediated Intergenerational Effects
Shared genetics (nuclear DNA sequence)	Shared genetics (nuclear DNA sequence)
Shared postnatal environment (diet, stress, socioeconomic factors)	Shared postnatal environment (diet, stress, socioeconomic factors)
Mitochondrial DNA	
Germ cell epigenetic effects <ul style="list-style-type: none"> • nuclear • cytosolic 	Germ cell epigenetic effects <ul style="list-style-type: none"> • nuclear • ?cytosolic
Placental structure/function	Placental structure/function
Intrauterine and early postnatal environment <ul style="list-style-type: none"> • Dysregulation of maternal nutrition or metabolism • Altered uterine structure/function • Maternal behavior • Alterations in maternal nursing behavior or milk composition 	Semen proteins, cytokines, and hormones

Figure 1.
Potential mediators of maternal and paternal intergenerational phenotypic transmission.

Figure 2.

Maternal nutritional exposures during pregnancy can alter growth patterns and induce increased adiposity and glucose intolerance in both her offspring and grandoffspring. Such effects can be transmitted via both paternal and maternal lineages. (A) Breeding strategy assess phenotypes in F2 mice via the paternal-only (blue), maternal-only (green), or both paternal and maternal (red) lineages. (B) Adiposity, as assessed by DEXA analysis of fat mass, is increased in F2 mice with either paternal, maternal, or both lineage histories. (C) Glucose tolerance is impaired in F2 mice with paternal (left), maternal (middle), or both (right) lineages. (D) Insulin resistance is present in F2 mice only when grandmothers from both maternal and paternal lineages have a history of experimental nutritional exposures.

Figure 3.

Diverse nutritional exposures, either direct or indirect, can modulate offspring metabolic phenotypes and contribute to paternal lineage intergenerational transmission of phenotypes. (A) demonstrates normal development and postnatal nutrition in control males, yielding healthy offspring. (B) Direct effects on sperm can be mediated by alterations in **current** paternal diet, including high fat or low protein diets, while (C) indirect effects may be mediated by prior **history of intrauterine exposure** to maternal caloric restriction, even with normal postnatal nutrition. Indirect effects may be mediated via exposure of germ cells of the developing embryo. Both can result in increased metabolic risk in offspring. Such paternal-lineage risk must be conferred via sperm, potentially via alterations in DNA methylation, chromatin modeling, or non-coding RNA. Mechanisms may be direct (on In turn, alterations in gene expression and metabolic risk in offspring indicates persistence of epigenetic marks & altered chromatin structure or effects on early postimplantation embryos altering developmental trajectories. Reproduced from ⁷⁵.

Figure 4.

Genetics, the intrauterine environment, and postnatal growth synergize to increase lifelong risk of obesity & diabetes. Vicious cycles of metabolic risk can be initiated by paternal lineage epigenetic effects on germ cells (red dotted lines) or maternal effects on both the intrauterine environment & germ cells (blue dotted lines).