Review

MOLECULAR METABOLISM

Early life lessons: The lasting effects of germline epigenetic information on organismal development

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ABSTRACT

Background: An organism's metabolic phenotype is primarily affected by its genotype, its lifestyle, and the nutritional composition of its food supply. In addition, it is now clear from studies in many different species that ancestral environments can also modulate metabolism in at least one to two generations of offspring.

Scope of review: We limit ourselves here to paternal effects in mammals, primarily focusing on studies performed in inbred rodent models. Although hundreds of studies link paternal diets and offspring metabolism, the mechanistic basis by which epigenetic information in sperm programs nutrient handling in the next generation remains mysterious. Our goal in this review is to provide a brief overview of paternal effect paradigms and the germline epigenome. We then pivot to exploring one key mystery in this literature: how do epigenetic changes in sperm, most of which are likely to act transiently in the early embryo, ultimately direct a long-lasting physiological response in offspring?

Major conclusions: Several potential mechanisms exist by which transient epigenetic modifications, such as small RNAs or methylation states erased shortly after fertilization, could be transferred to more durable heritable information. A detailed mechanistic understanding of this process will provide deep insights into early development, and could be of great relevance for human health and disease.

Keywords Paternal effects; Epigenetics; Intergenerational; Sperm; Placenta

1. INTRODUCTION

Although biological inheritance is primarily driven by transmission of the genome from one generation to the next, additional "epigenetic" information can be passed on to future generations. Classic examples of epigenetic inheritance include relatively stable inheritance of gene silencing, as in the case of paramutation in maize [1], as well as short-term cases of programmed epigenetic inheritance which are erased each generation, as in the case of imprinted gene regulation in mammals and plants [2,3]. Beyond such largely environmentally-insensitive cases of epigenetic inheritance, it is increasingly clear that various perturbations can influence epigenetic modifications in germ cells, which can thereby transmit information about prevailing environmental conditions to future generations. This process is essentially a modern reappraisal of the once-discredited idea of the "inheritance of acquired characters", with subtle quantitative traits responding, over limited numbers of generations, to ancestral environments.

In mammals, it is now well-established that parental dietary challenges and other stressors can induce germline epigenetic alterations and thereby affect metabolic phenotypes in the next generation. However, the mechanistic basis by which these epigenetic modifications ultimately program altered metabolism in offspring remains obscure. In this review, we wish to focus on the "black box" between fertilization and the metabolically-reprogrammed adult animal. We will very briefly discuss parental dietary paradigms and their effects on the germline epigenome. We will then focus on potential mechanisms linking these germline epigenetic changes to the physiological changes observed in offspring.

2. EPIGENETIC INFORMATION CARRIERS IN THE GERMLINE

Not only is epigenetic information distributed to both daughter cells during mitotic division (as seen in cell state inheritance in multicellular organisms), but some epigenetic marks are inherited through the germline (reviewed in [4]); below, we will briefly cover the unusual aspects of the germ cell epigenome.

2.1. Chromatin

The nucleoprotein packaging of the sperm genome is remodeled extensively to ensure proper compaction. In many species, this compaction process involves replacing canonical histones with histone

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variants, transition proteins, and finally the small basic proteins known as protamines [5,6]. However, this global replacement process is not entirely exhaustive, with ~1–15% of histones being retained in the sperm of various mammals. The locations of retained histones in mammalian sperm remain somewhat unresolved, as many reports have mapped sperm histones to GC-rich promoters of early developmental genes [7–9], but with at least two studies instead finding histones retained primarily in large gene-poor regions [10,11]. This discrepancy appears to be at least partly explained by differences in stability between the bulk of sperm histones located in intergenic regions, and a subset of unusually-stable nucleosomes present at CpG islands. Importantly, evidence from both camps suggests that it is unlikely that a given nucleosome is ever retained uniformly at a particular locus. In other words, even in the case of histone retention at developmental promoters, ChIP-Seq detects signal for protamines as well. As it is highly unlikely that histones and protamines co-occupy the same locus simultaneously (and there is no reason to think that histone and protamine antibodies cross-react), this finding is best explained by a mix of sperm bearing histones at the promoter of interest, and sperm which have successfully replaced the histones at that location. As will also be discussed in the case of cytosine methylation changes, it is difficult to reconcile how epigenetic marks present in only a fraction of sperm could explain paternal effects which typically appear to be highly penetrant in the next generation. As a concrete example, if a paternal perturbation affects histone modifications at a locus where, say, 20% of sperm retain histones rather than protamines, the modifications in question can only be meaningful for 20% of offspring. Although counterarguments to this concern can be envisioned — perhaps the 80% of sperm lacking histones at that promoter are incapable of fertilization and thus irrelevant, or perhaps offspring phenotype integrates information from multiple independent loci — the issue of penetrance must be dealt with when considering the role of the sperm epigenome in paternal effects.

Nonetheless, there is some evidence supporting the idea that manipulating histone retention or covalent modification status can affect genomic functions in the zygote and beyond. In one prominent example, overexpressing the H3K4 demethylase KDM1A (LSD1) during spermatogenesis was shown to reduce global H3K4me2 levels in sperm, and led to impaired offspring development in three subsequent generations [12]. More recently, Lesch et al. showed that deleting the H3K27 demethylase KDM6A in the paternal germline resulted in increased tumor incidence in offspring, persisting through two generations [13]. While these and other related efforts [14] link chromatin-related mutations to offspring phenotypes, the relevant molecular carrier in sperm remains unclear — while cis-acting changes in histone levels or modifications at specific genomic loci in sperm could potentially influence local gene expression in the early embryo, it is also clear that deletion or overexpression of histone-modifying enzymes can affect other epigenetic modifications including sperm RNA levels [12] and cytosine methylation patterns [13]. It is therefore important to be aware that these or other molecular changes in mutant sperm could certainly drive offspring phenotypes even if sperm chromatin changes do not exert any direct effect on zygotic gene regulation.

2.2. DNA modification

Methylation of the C5 position of cytosine is the most common DNA modification in mammals, where it plays important roles in processes ranging from transposon silencing to imprinting, X-chromosome inactivation, and genomic stability (reviewed in [15,16]). Although cytosine methylation patterns can in principle be copied in perpetuity through the action of the maintenance methyltransferase Dnmt1, this is prevented by two waves of nearly-complete DNA methylation reprogramming that occur during primordial germ cell development, and again in the early embryo [2]. In the embryo, active demethylation of the paternal genome occurs soon after fertilization, contrasting with the apparently passive demethylation events on the maternal genome that occur over several cell divisions [17,18]. Nonetheless, a subset of genomic loci escape the reprogramming process, including imprinting control regions and certain evolutionarily young transposons (such as intracisternal A-particles, or IAP elements, in the mouse) [19]. The mechanistic basis by which these loci are protected from demethylation is an active area of investigation: in the case of imprinted genes, a pair of zinc finger DNA-binding proteins — ZFP57 and ZFP445 — appear to play essential roles in preventing demethylation of many imprinting control regions [20,21].

2.3. RNAs

Finally, germline RNAs are central to the best-understood trans-generational epigenetic inheritance paradigms in model organisms from plants to worms, and have emerged as primary candidates for mediators of paternal effect paradigms in mammals [22]. As with other epigenetic information carriers, small RNA populations are reprogrammed throughout development and maturation of germ cells. During testicular spermatogenesis, microRNAs, PIWI-interacting RNAs (piRNAs), and endogenous small interfering RNAs (endo-siRNAs) are dynamically expressed and play critical roles in normal development [23]. Most notably, the largely germline-specific piRNAs play well-known roles in protecting the germline from transposable element mobilization [24]. The sperm RNA payload is then extensively remodeled following the completion of testicular spermatogenesis but prior to mating: piRNAs, which are the major small RNA species in testicular sperm, are almost completely absent in ejaculated sperm, which instead carry an RNA payload comprised primarily of tRNA fragments (tRFs) derived from mature tRNAs [25–28]. Sperm also carry a variety of microRNAs, and several recent studies have revealed functional roles for sperm-delivered microRNAs in the early embryo [29–31].

In addition to these populations of small RNAs which have functional roles in the zygote, sperm carry a variety of other RNA species of unknown significance. For example, the majority of mRNAs and rRNAs present during the process of spermatogenesis exhibit varying degrees of degradation in mature sperm [32]; whether rRNA fragments and other partially-degraded RNAs have biological functions such as those attributed to tRNA fragments largely remains to be seen. In addition to the various digestion products of longer RNAs, circular RNAs (circRNAs) — circular species formed by back-splicing reactions [33] — are resistant to exonucleases and may therefore remain relatively intact throughout spermatogenesis and sperm maturation. Indeed, one of the first circRNAs discovered was a highly abundant testis-specific circRNA generated from the sex-determining region Y (*Sry*) gene [34]. CircRNAs have been linked to multiple functions, most notably acting in several contexts as microRNA "sponges" which effectively knock down microRNA function. For instance, *Sry* circRNA has been shown to reduce miR-138 expression [35], while circNAPEPLD, present in both mouse and human mature sperm, was shown to physically interact with multiple oocyte microRNAs [36].

2.4. Other paternal contributions to offspring

Beyond the three classic epigenetic inheritance pathways, a wide range of other factors could play potential roles in transmission of environmental information from father to child. For instance, although

the sperm genome is almost fully packaged with protamines, this packaging may be less homogeneous than commonly imagined, as protamines are subject to multiple covalent modifications [37], few of which have been characterized in any detail. In principle, environmental modulation of the protamine modification landscape could plausibly influence early embryo development or gene regulation. Additional factors carried by sperm that have the potential to modulate offspring development include transcription factors and other DNA-bound machinery, as well as signaling proteins which have the potential to exist in several alternate heritable prion folding states. Beyond the material carried by sperm, mating results in delivery of seminal fluid to the female reproductive tract. Seminal fluid, made up of secretions from accessory glands, is comprised of fructose, lipids, various ions such as zinc, copper and selenium, thousands of proteins, and both cell-free and vesicle-associated DNA, RNA, and microRNAs [38,39]. Seminal fluid is not just a nutrient-rich transport medium — it also initiates immune tolerance mechanisms in the female reproductive tract to allow for successful pregnancy [40—42]. Furthermore, seminal fluid composition can be modified in response to environment, and it was recently reported that seminal plasma obtained from males consuming control or low protein diet may impact offspring metabolism by altering the metabolic and immune environment of the maternal reproductive tract [43].

Although the majority of paternal effect studies have focused on the classic epigenetic information carriers in sperm (with few notable exceptions such as Watkins et al.), it is clear that the roles of other molecular carriers in sperm or seminal fluid must always be considered as potential mediators in paternal effect paradigms.

3. EFFECTS OF PATERNAL AND MATERNAL ENVIRONMENTAL CONDITIONS ON OFFSPRING PHENOTYPES

Armed with the knowledge that at least some epigenetic information in germ cells escapes erasure in the next generation, a large and increasing number of studies have explored the possibility that ancestral conditions might influence future generations. Here we will briefly survey paternal exposure paradigms (along with a few related maternal effect studies) that have been linked to changes in offspring phenotype, focusing on studies in inbred rodent model systems. In general, ancestral exposure studies typically focus on one of three broad environmental paradigms: altered diet/nutrition, toxin exposure, and stress.

3.1. Paternal dietary exposures

A large number of studies have investigated the effect of paternal diets on F1 and F2 offspring in mice and rats. Perhaps the best studied dietary perturbation is consumption of a high-fat diet, which programs a coherent pattern of phenotypes in the next generation, including abnormalities in glucose tolerance, body weight, fat distribution, and reproductive health [44—52]. Interestingly, many of these phenotypes, including effects on glucose tolerance, glucose uptake, and weight gain, can be ameliorated when fathers on high fat diets are also forced to exercise [46,53]. The next-most common dietary paradigms used in paternal effect studies are related to undernutrition: paternal consumption of a low protein diet has been shown to program changes in cholesterol and lipid metabolism, glucose control, and other cardiovascular parameters in F1 offspring [43,54—56]. Other undernutrition paradigms with documented effects on progeny phenotypes include caloric restriction [57] and intermittent fasting [58].

In addition to dietary perturbations employed from weaning onwards, a large number of studies have explored the effects of in utero nutritional challenges on offspring metabolism. Here, pregnant females are subject to nutritional challenges during gestation — common perturbations include starvation or low protein diets [59—61], as well as overnutrition with high fat diets [62—64] — and male offspring born after these interventions are then used as the P0 paternal generation. In these studies, the use of in utero nutritional challenges is motivated by the fact that key aspects of the germline epigenome (such as cytosine methylation patterns) are established in primordial germ cells during fetal development [2]. As with the dietary challenges experienced during adolescence and adulthood, these in utero nutritional challenges affect a similar array of phenotypes from glucose control to cholesterol metabolism to various behavioral phenotypes. An interesting area for future studies will be to determine whether prenatal and postnatal nutritional perturbations have similar, or contrasting, effects on offspring metabolism.

3.2. Maternal dietary exposures

Maternal environmental exposures both during and prior to gestation alter the course of embryonic development and can result in lasting phenotypic changes that persist into adulthood. It has long been clear that poor nutrition during gestation — often induced either by starving a pregnant female or ligation of the uterine artery — results in long-term metabolic deficits, a phenomenon that motivates the "Developmental Origins of Health and Disease", or DOHaD, hypothesis [65—69]. Similarly, numerous studies of maternal *over*nutrition (e.g. high fat diet consumption) during gestation reveal a clear pattern of metabolic dysregulation in offspring, ultimately resulting in childhood obesity and increased insulin resistance [51,70—72]. Of course, altering nutrition during gestation directly impacts the growing fetus, and even pre-gestation diets can indirectly impact fetal nutrition by causing long-term changes to the maternal metabolism. Although understanding how fetal nutrition exerts long-term effects on a child's later metabolic proclivities is an active and important area of study, our focus here is instead on the more opaque question of whether the oocyte epigenome plays any role in programming offspring metabolism.

A small subset of maternal effect studies have used oocyte or embryo transfer techniques to identify pre-gestational dietary effects that act on the next generation via the germline. Sasson et al. generated embryos from mating control or high fat-fed females with control males, then transferred embryos from high fat diet-fed mothers to control females for gestation and vice versa [73]. These experiments determined that pre-gestational high fat diet resulted in impaired fetal growth and placental development, with offspring exhibiting decreased birth weight but few other metabolic abnormalities. Interestingly, pre-gestational exposure to high fat diet suppressed detrimental metabolic consequences of transfer to a high fat-consuming uterine environment, suggesting that mismatch between an embryo's metabolic history and its gestational environment exerts greater long-term effects than the gestational environment *per se*. A slightly different approach was taken by Huypens et al., who generated embryos via in vitro-fertilization (IVF) using sperm, oocytes, or both from high fat diet animals. The resulting embryos were transferred to foster mothers, demonstrating that both paternal and maternal diet influence offspring metabolic function [51]. Notably, both maternal and paternal high fat diet cause the same phenotypes (with the maternal influence being consistently stronger than the paternal influence) with both effects occurring in the same direction. This fascinating discovery raises a key mechanistic question: how do signals encoded in the oocyte and sperm epigenomes, which are massively distinct from one another, somehow convergently program similar phenotypic outcomes?

3.3. Ancestral exposure to traumatic experiences

Exposure of fathers to traumatic situations, generally experienced early in life, has been repeatedly documented to drive various behavioral and even metabolic alterations in unexposed offspring. Typical paternal stress paradigms include social defeat stress [74], unpredictable maternal separation and maternal stress (MSUS — [75,76]), social instability [77,78], and chronic variable stress in which males are subjected to unpredictable daily stressors ranging from moist bedding material, to social defeat, to placement of novel items in the cage [79—82]. Males subjected to MSUS in childhood sired offspring that produced lower levels of the stress-responsive hormone corticosterone and engaged in fewer anxiety-like behaviors as measured by elevated plus maze, light dark box, and forced swim test [80]. Beyond these anxiety-related behaviors, MSUS offspring may also inherit deficits in memory formation processes, as Bohacek et al. observed that MSUS offspring could not differentiate between a familiar and novel object 24 h after initial exposure, and spent less time frozen in response to a conditioned fear stimulus [83]. Furthermore, long-term potentiation was impaired in MSUS offspring, whereas long-term depression was improved over controls, although this phenotype lasted for only one generation. Of note, not only does paternal stress influence stress-related behaviors in offspring, but there is some evidence that F1 offspring also exhibit metabolic phenotypes, such as increased insulin sensitivity [76] or altered dietary preferences [74].

3.4. Ancestral toxin exposure

Finally, we will briefly note the wide range of studies reporting intergenerational effects of a variety of bioactive small molecules, ranging from endocrine-disrupting fungicides to drugs of abuse such as alcohol and nicotine. Most notably, toxin exposure as an epigenetic stressor has been extensively studied in the context of endocrine-disrupting chemicals used in pesticides, fungicides, and herbicides. In one of the earliest documented paternal effect studies, exposure of pregnant rats to high levels of vinclozolin or methoxychlor during fetal gonadal development was reported to cause decreased sperm number and motility in F1, F2, F3, and even F4 generations, with eight percent of males, especially older males, developing infertility [84]. Other toxins, such as dioxins, bisphenol A, carbon tetrachloride, and pollutants contained in jet fuel have also been linked to offspring phenotypes ranging from reproductive health to metabolism [85—87]. Beyond the various environmental contaminants studied, a number of efforts have focused on the effects of drugs of abuse on future generations. For example, paternal ethanol consumption affects a range of phenotypes, including HPA axis responsiveness and alcohol preferences, in F1 offspring [88]. Paternal exposure to nicotine, cocaine, and THC have all also been linked to offspring phenotypes including altered behaviors [89—91], resistance to xenobiotics [92], and altered glucose control [92].

4. ENVIRONMENTAL EFFECTS ON THE GERMLINE EPIGENOME

Despite the now overwhelming evidence supporting the idea that ancestral environments can influence phenotypes in future generations, the mechanistic basis for ancestral effects remains mysterious. One key issue that has not been entirely resolved in all paradigms is the question of whether paternal effects are mediated by the sperm epigenome or by non-germline information carriers such as seminal fluid composition or microbiome transfer. Several studies have recapitulated at least a subset of paternal effect phenotypes through assisted reproduction (artificial insemination, IVF, or intracytoplasmic sperm injection) using purified gametes [25,27,43,51,93]. Nonetheless, seminal fluid composition may also play some role in modulating offspring phenotypes, as Watkins et al. reported increased weights not only in offspring born following artificial insemination using low protein sperm (with control seminal plasma), but *also* in offspring generated using control sperm and low protein seminal plasma [43]. In addition, not all paternal effect paradigms have been recapitulated using purified gametes: effects of social defeat on offspring, for example, were lost following IVF in rats [74]. It will therefore be important in other systems to verify or reject the hypothesis that the sperm epigenome is responsible for paternal programming.

Based on the positive results supporting germline transmission in several paternal effect systems, along with the fact that the germline epigenome remains the *a priori* most likely information carrier of paternal exposure history, a large number of studies have examined environmental effects on sperm, with a bewildering array of reported modifications to the sperm epigenome. Although, as noted above, genetic studies have linked global changes in histone modifications in sperm to altered phenotypes in future generations, relatively few studies have examined changes in histone retention/modification in response to environments (see, eg, [94,95]). Although histone retention and modification cannot be ruled out as mediators of paternal effect paradigms, given the paucity of existing studies on the topic we simply reiterate here the need to account for the low penetrance of histone retention in sperm in future efforts in this domain.

4.1. Cytosine methylation

Sperm were long believed to carry no functional RNAs, and the very few histones escaping replacement were also generally viewed as unlikely to be functional in the early embryo. The vast majority of paternal effect studies have therefore focused on the sperm cytosine methylome, given the well-established role for cytosine methylation as epigenetic information carrier in mammals. We briefly highlight several examples to illustrate commonalities in the current literature on environmental regulation of the sperm methylome. First, in the prominent paternal effect paradigm based on in utero undernutrition, low-resolution genome-wide analyses identified ~100 loci, primarily in intergenic regions, hypomethylated in sperm of undernourished males [96]. As is typical for reported environmental effects on sperm methylation, quantitative followup revealed ~10—20% changes in methylation (e.g. a decrease from 40% to 20% methylation) at between one and nine neighboring CpGs. Importantly, only a small subset of these methylation changes persisted into adulthood, or were correlated with changes in expression of nearby genes, further underlining the question of how these modest methylation changes could influence offspring phenotype. Effects of similar magnitude are quite abundant in the literature, with 10—20% methylation changes identified in sperm obtained from males subject to dietary [50], toxin-induced [97], or even odorant-paired stress [93] paradigms. The common theme of modest methylation changes in sperm means that environmental conditions are driving relatively subtle shifts at any one locus in each sperm. This presents a mechanistic challenge based on the "digital" nature of sperm DNA methylation. Haploid sperm carry a single copy of each cytosine in the genome, and only one sperm contributes its genome to any given offspring. As a single cytosine can only occur in one of only two states — methylated or unmethylated — a given CpG cannot be 65% methylated in a single sperm. This means that a 10% methylation change in a sperm sample indicates a change from, say, 2 of 10 sperm bearing a methyl group to 3 of 10 sperm bearing a methyl group at that cytosine. Thus, modest methylation changes should only affect the *penetrance* of a phenotype within a litter, rather than affecting the majority of offspring across litters as observed in many paternal effect paradigms.

Given this "digital sperm" problem, studies reporting cytosine methylation changes at various repeat elements seem more promising for future investigation. For instance, sperm from mice subjected to a low protein diet from gestation through pre-weaning age exhibited subtle changes in DNA methylation at ribosomal DNA (rDNA) [61], with rDNA copies bearing a particular polymorphism (CpG-133^{A}) exhibiting more significant methylation changes than other rDNA copies. Related findings include reports of high fat diet driving increased DNA methylation at satellite repeats (centromeres/telomeres) in rats [98], a global decrease in repeat element cytosine methylation in low protein sperm [43], as well as human epidemiological studies linking phthalate exposure to DNA methylation at LINE-1 elements in sperm [99]. Countering these studies, we note that extensive whole genome bisulfite sequencing in our own laboratory revealed no significant impact of either low protein or high fat diet on repeat element methylation status [100]. Rather, we identified a significant confounding influence of repeat copy number differences between even closely-related animals, which initially lead to the artifactual identification of dietary effects on rDNA and other repeat methylation.

Nonetheless, environmental effects on repeat element methylation status bear further investigation both because repeat elements represent some of the rare genomic loci that escape methylation erasure upon fertilization, and because repeat element methylation provides a plausible mechanism for small methylation changes to affect offspring with high penetrance (see below).

4.2. RNAs

The largely fruitless search for cytosine methylation changes capable of eliciting offspring phenotypes, along with the central role for small RNAs in well-established transgenerational epigenetic inheritance paradigms in other organisms, has motivated an increasing focus on small RNA levels in mammalian sperm. A key feature driving the recent surge of interest in sperm RNAs in paternal effects is the relative ease of functional testing of small RNAs as causal agents of paternal effect paradigms: microinjection of purified or synthetic small RNAs into control zygotes is far more tractable than modulating histone retention or cytosine methylation in a locus-specific manner (although CRISPR-based targeting does make the latter approach more feasible than in the past).

Focusing first on dietary paradigms, numerous studies have documented dietary effects on the sperm RNA payload, although as in the case of methylation profiling, these studies often disagree in detail. For example, in the case of high fat paradigms, dietary intervention has been reported to result in: 1) increased expression of several microRNAs and 5' tRFs (most notably let-7c, tRF-Glu-CTC and tRF-Glu-TTC) in rats [52]; 2) increased levels in tRF-Gly-GCC and miR-10a/b, accompanied by a decrease in tRF-Glu-CTC [101]; 3) increased levels of tRF-Gly-GCC, tRF-Gly-CCC, and tRF-His-GTG [46]; 4) altered levels of miR-503, miR-456b, miR-542, and miR-652 [102]; 5) increased levels of miR-19b and miR-29a [103]; and 6) widespread changes in tRF and microRNA levels, including increased miR-10a/b, miR-122, and decreased let-7c/f/a/b, as well as changes in the covalent nucleotide modifications associated with tRFs [25]. These discrepancies presumably reflect some combination of known and currently-enigmatic technical and biological differences, ranging from differences in RNA extraction and cloning protocols (size range of gel-purified RNAs, for example) to differences in the endemic microbiota across animal facilities.

Early life stressors have also been linked to diverse changes in the sperm RNA payload in many studies: 1) MSUS was reported to drive changes in both microRNA levels (miR-375, miR-200b, miR-672, and miR-466) as well as levels of several mRNAs and lincRNAs [75,76]; 2) paternal chronic stress was shown to affect expression of nine microRNAs (miR-193, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, miR-532, and miR-698) [80]; 3) cortisol injections led to altered levels of nearly 200 small RNAs, including ~100 microRNAs and ~60 tRFs [104]; 4) social instability in mice led to decreased levels of miR-34c and miR-449a in sperm, and, intriguingly, the same microRNAs were also less abundant in human sperm samples from adult survivors of adversity early in life [78]. As with the dietary paradigms above, the small RNA changes reported in the sperm of stressed males differ dramatically from report to report, although here the paradigms differ much more substantially in detail than for the various high fat diet protocols. The diversity of small RNA changes may therefore simply reflect differences between conditions — e.g. social instability as opposed to cortisol injections — in terms of their effects on the recipient.

These and many other findings using various paternal effect models, from low protein diet to pesticide exposure to ethanol consumption, suggest that the sperm RNA payload is surprisingly plastic. Importantly, in the case of sperm RNAs, it has been possible to directly test whether the RNAs in question are sufficient, when microinjected into control zygotes, to program offspring phenotypes. A number of studies have reported successful induction of specific phenotypes — often a limited subset of the phenotypes induced by the relevant paternal environments — following injection of various RNA populations. For example, Gapp et al. reported that injection of total RNA purified from MSUS sperm into control zygotes resulted in glucose intolerance following restraint stress, and depression-like behaviors including increased floating in a forced swim test, in adult offspring [75]. Some of these effects were later reported following injection of gel-purified long (>200 nt) RNAs from MSUS sperm, suggesting that mRNAs or lincRNAs could play some role in programming offspring phenotypes [76]. Other studies have used more defined populations of small RNAs — gel-purified tRFs isolated from high fat sperm were able to direct altered glucose metabolism in one study [25]. Similarly, tRFs gel-purified from the sperm of males born to high fat mothers were sufficient (when injected into control embryos) to induce increased locomotion after amphetamine exposure, as well as increased preference for sucrose and high fat diet [64]. More specifically still, small numbers of synthetic RNAs have been used in some microinjection studies, with nine microRNAs (miR-193, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, miR-532, and miR-698) causing altered blood brain barrier permeability in injected animals [81], while microinjection of miR-19b resulted in decreased glucose tolerance in offspring [103].

Taken together, these studies provide strong support for the potential of sperm RNAs to act as the key molecular mediators by which paternal environmental conditions influence metabolic and other phenotypes in F1 offspring. However, none of these studies address a central mechanistic question — how do small RNAs or other epigenetic changes in sperm alter early development so as to modulate phenotypes present in adults? This question is further emphasized by the discrepancies between the various studies detailed above. Below, we address what we view as the least-studied link in models connecting paternal experiences to offspring physiology.

5. HOW DOES EPIGENETIC INFORMATION PRESENT AT FERTILIZATION MODULATE LATER ORGANISMAL PHENOTYPES?

Whatever changes to the sperm epigenome prove to be causal for paternal effects on offspring phenotypes, two key mechanistic

questions remain almost entirely unaddressed. The first, which we will not discuss here (see [105] for a recent review), is the question of how signaling pathways in the male germline link paternal experiences to changes in the sperm epigenome. We instead focus on the second question: how do changes in epigenetic information present at fertilization ultimately modulate coherent physiological phenotypes that manifest much later in development?

5.1. Long-term copying of cis-acting epigenetic information

The simplest models linking the sperm epigenome to offspring phenotype involve efficient copying of cis-acting epigenetic modifications on the paternal genome from fertilization through adulthood. For example, one could imagine diet or stress signaling leading to increased cytosine methylation in sperm at the enhancers of genes encoding metabolic signaling proteins (ranging from circulating metabolic signals such as insulin or leptin, to their receptors, to metabolic transcription factors such as SREBP or various NHRs). Faithful copying of methylation patterns by Dnmt1-mediated methylation in every cell generation would maintain the new methylation status in key metabolic tissues, ultimately affecting expression of the relevant protein and thereby modulating organismal physiology. Analogous mechanisms can be envisioned for the long-term copying of chromatin states by histone modifying enzymes that are recruited to newly-synthesized histones every S phase by modifications on old histone proteins.

However, all extant paternal effect systems in mammals suffer from two major roadblocks to such models. The first is the near-global reprogramming of the epigenome that occurs shortly after fertilization. The counter-argument to this is, of course, to focus on the rare subset of loci that escape these reprogramming events. The second problem with cis-acting epigenetic changes as mediators of paternal effect paradigms is the modest quantitative changes thus far reported for environmental effects on the sperm epigenome. A multitude of studies have documented significant cytosine methylation changes in sperm in response to dietary, stress, and toxin exposure paradigms, but to our knowledge all such changes are confined to no more than ~10–20% changes in cytosine methylation levels. As discussed above, both histone retention and all reported changes in cytosine methylation are therefore unable to explain penetrant effects of paternal environments on the majority of offspring.

These considerations motivate a greater emphasis on environmental effects on repeat elements in the sperm genome, where distributed changes of small average magnitude can nonetheless affect every sperm in an ejaculate. In other words, a repeat-associated CpG that is 80% methylated in a sperm sample, when present in a repeat element occurring 1000 times in the genome, could either reflect 1) a sperm sample composed of 80% of sperm carrying 1000 methylated repeats and 20% of sperm bearing 1000 unmethylated repeats, or, more likely, 2) a sperm population in which every sperm carries 800 methylated repeat copies and 200 unmethylated repeat copies. In the latter case, a shift from 80% to 90% methylation would enable penetrant control of offspring phenotype, as every sperm could carry quantitative information encoded in the number of methylated repeat copies, enabling "digital" sperm to exert "analog" control over repeat expression in the embryo. Repeat element methylation also bypasses the other major mechanistic concern with the germline methylome, as (some) repeat elements are among the privileged loci which escape demethylation in the early embryo.

Several issues dampen enthusiasm for methylation at repeat elements as carriers of paternal environmental information in extant paradigms, not least including extensive whole genome analyses which did not find any significant changes in repeat element methylation in response to low protein or high fat diets [100]. In addition, the mechanism by which offspring would integrate methylation across repeat populations — how would a shift from 800 methylated IAP copies to 900 methylated IAP copies per sperm cause changes to glucose control in children? — presents challenges of its own.

5.2. Transforming short-lived signals into long-term epigenetic information

Turning next to germline RNAs, it is important to recognize that, unlike model organisms such as plants and nematodes, mammals do not encode any recognizable RNA-dependent RNA polymerase. In the absence of a clear mechanism for long-term maintenance of altered RNA levels, small RNAs in germ cells must act rapidly in the early embryo before they are lost to degradation and dilution.

The conceptually simplest mechanism for small RNAs in the zygote to exert long-term molecular effects in the offspring would be for the small RNAs to direct local changes to cytosine methylation or chromatin states, which could then be copied over longer time scales after dilution of the inciting RNA species. Abundant precedent for this type of mechanism exists in non-mammalian species, as small RNAs are known to direct cytosine methylation [106] and heterochromatin [107] to specific genomic loci based on homology-dependent targeting to nascent transcripts [108]. In mammals, RNA-mediated targeting of silencing machinery to the genome is primarily thought to occur in the context of piRNAs targeting transposon methylation in the testis, but it is plausible that similar mechanisms operate in the early embryo as well. In one study supporting this idea, Grandjean et al. reported that microinjection of miR-124, which targets the Sox9 transcription factor, into fertilized oocytes led to a 30% increase in pup size across three generations of offspring [109]. This giant phenotype in offspring was associated with increased repressive histone marks (H3K9me2 and H3K9me3) at the *Sox9* locus in embryos following miR-124 microinjection, suggesting an RNA-mediated chromatin remodeling event that is then passed through subsequent divisions during early development.

Alternative mechanisms for fixation of small RNA-mediated information transfer which do not require local targeting to the genome can be envisioned as well. Indeed, the most likely hypothesis for paternal microRNA function during early development is that these microRNAs play a role in degradation or translation of maternally-deposited mRNAs soon after fertilization. Thus, microRNA-dependent repression of key epigenetic regulators (e.g. Dnmt3a) could potentially alter overall cytosine methylation "tone" during global re-methylation in the preimplantation embryo. Global differences in cytosine methylation could then be propagated throughout later development long after degradation or dilution of the sperm-delivered microRNA. Some literature support for this idea exists. For example, in their study on sperm microRNAs as mediators of paternal stress effects, Bale and colleagues found that microinjection of nine synthetic microRNAs led to decreased expression of several genes, including the histone deacetylase *Sirt1*, in the early embryo [81]. Similarly, we have recently found that the 5' tRF-Gly-GCC regulates levels of histone mRNAs [110] and thus global chromatin packaging. These and other effects of small RNAs on expression of chromatin regulators suggest the potential for paternal information to direct widespread changes in the ability of DNA-modifying enzymes to access the genome, which could then be "fixed" for longer-term copying via effects on the re-methylation process. Of course, the follow-up mechanistic questions — how would a small global change in methylation lead to physiological changes in offspring later in life? — have yet to be explored.

Based on these and other studies, there is some precedent for small RNAs, which in mammals are not copied by RNA-dependent RNA

polymerases, to establish longer-lived epigenetic modifications, either at specific targeted loci or via more diffuse control of epigenetic "tone".

5.3. Long-term effects of altered preimplantation growth or cell fate allocation

Finally, we consider indirect mechanisms by which small RNAs or other epigenetic changes in the germline could drive metabolic changes in offspring. A curious feature of paternal effect studies is that that the majority of phenotypes typically documented in the F1 generation — altered glucose control, cholesterol metabolism, and anxiety-related behaviors — are well-known sequelae of altered placental provisioning during fetal development [111]. The potential exists for paternally-delivered epigenetic information to somehow influence placental development, with long-term metabolic effects stemming from subsequently altered fetal nutrition. Indeed, altered placental growth or function have been documented in several parental effect studies, as embryos derived from high fat-fed mothers, but then transferred to control recipients, were associated with diminished placental weights [73], while paternal low protein diet was shown to affect the uterine inflammatory environment [43].

As with the other models above, the mechanistic basis by which small RNAs or other epigenetic marks in sperm might affect placental development is an open one. However, one recurring observation in the paternal effect field is that paternal dietary challenges often affect the pace of preimplantation development, as both high fat and low protein diets have been linked to lower cell numbers in offspring blastocysts [27,49]. One possibility is that, following transit through the oviduct, blastocysts of different sizes implanting into the uterus lead to differences in placental development. Alternately, early changes to key regulatory factors such as CARM1 [112] in the four-cell embryo could drive changes in the ratio of trophectoderm to inner cell mass cells of the later blastocyst.

Whatever the mechanism, there are several appealing features to models in which paternal effects are exerted via altered placental development. The first, as noted above, is the curious concordance (at least when viewed coarsely) between F1 phenotypes affected in paternal and maternal effect studies, which is readily explained by such models. The second is that cell fate allocation to inner cell mass and trophectoderm, respectively giving rise to the embryo proper and to extraembryonic tissues, is the first cell fate decision in mammals and occurs very early — it is clearly defined in the blastocyst, but can be foreshadowed already in four-cell stage blastomeres. Thus, for epigenetic marks that do not persist, whether through RNA dilution or DNA demethylation, early functions can impact cell fate decisions with much longer-term sequelae.

6. OUTSTANDING MYSTERIES

As should be clear, there is presently no clearly-established mechanistic pathway linking a specific sperm or oocyte epigenetic modification with a coherent metabolic output in children. Below, we discuss several key features of paternal effect studies which must be addressed in order to fully understand the mechanistic link between the zygote epigenome and altered offspring physiology.

6.1. Specificity of the F1 phenotype for the ancestral perturbation

Given that it is now nearly indisputable that paternal experiences can program altered phenotypes in children, in our view the central question in the field of intergenerational epigenetic inheritance concerns what we will call the "bandwidth" of sperm. Loosely considered, the question is how much environmental information sperm is capable of delivering to the next generation. In a formal sense, this would mean how much mutual information [113] exists between the "exposome" of parents and the "phenome" of offspring. However, although high-dimensionality characterization of animal physiology (the phenome) is possible with great effort, it is challenging to envision (much less carry out) parametrizing the possible world of experiences to which a parent has been subjected. Instead, a more tractable question to ask is how specific a programmed phenotype is for the ancestral environmental to which it is a response. In other words, do all paternal effect paradigms represent a pleiotropic intergenerational stress response, or do diverse molecular perturbations — cadmium, fatty acids, endocrine disruptors, etc. — all direct specific and distinctive responses in children?

Two case studies in the paternal effects literature, both leveraging environmental stimuli chosen based on defined ligand-receptor interactions, illustrate extreme views of the question of sperm bandwidth. In the first study, we examined the effects of paternal nicotine exposure on offspring phenotypes [92]. We found that offspring of nicotine-exposed fathers were significantly protected from toxic levels of nicotine, but that this did not result from specific downregulation of nicotinic acetylcholine receptors, as offspring were also resistant to toxic levels of cocaine. Resistance to these distinct toxins was accompanied by enhanced xenobiotic clearance and upregulation of the hepatic xenobiotic response program. Even more surprisingly, paternal programming of enhanced xenobiotic resistance was observed in animals consuming both nicotine and the nicotine receptor antagonist mecamylamine, demonstrating that nicotine sensing by fathers does not require nicotine signaling, and instead presumably reflects a more nonspecific xenobiotic sensing program. Adding to this view of a relatively nonspecific information transfer system, offspring of nicotine-exposed males also exhibited metabolic phenotypes, with increased hepatic expression of lipid metabolism genes as well as diminished glucose tolerance. Taken together, these findings are inconsistent with models in which nicotine exposure initiates a signaling pathway that leads to nicotine-specific information (e.g. cytosine methylation at promoters associated with nicotinic acetylcholine receptors, or siRNAs derived from these receptors, etc.) in the germline. This idea — that paternal effects tend to influence one or perhaps a handful of pleiotropic phenotypic programs in offspring — is supported by many other paternal effect studies, where many potentially distinct stimuli (endocrine disruptors, high fat diet, maternal separation, etc.) all affect the same spectrum of phenotypes (glucose tolerance, HPA axis reactivity).

One remarkable study provides a counterargument to the "low bandwidth" view of the sperm epigenome. Here, Dias and Ressler leveraged the specificity of olfactory receptors for specific odorants, exposing male mice to foot shocks paired with one of two odorants — acetophenone and propanol [93]. Astonishingly, offspring of these males exhibited greater sensitivity in odor-potentiated startle assays for the specific odorant used in the paternal generation — offspring of acetophenone-exposed males were themselves more sensitive to acetophenone, but exhibited the same sensitivity to propanol as control animals (and vice versa). Consistent with this, neurons expressing a transgene based on *Olfr151*, the acetophenone receptor, were more abundant in acetophenone-exposed offspring. Mechanistically, the authors reported alterations in cytosine methylation at the *Olfr151* locus in sperm of acetophenone-exposed males, but these were very low magnitude changes (~5% or less for all but one CpG) and thus present the challenges described above — the "digital sperm" problem — regarding the ability of low magnitude methylation changes to affect offspring phenotype with high penetrance. Whatever the actual germline

mechanism linking odor exposure in males to offspring odor sensitivity, the finding reported by Dias and Ressler, if generalized, would very strongly support the idea that sperm transmit high-resolution environmental information to children. It will be of great interest to see whether the reported findings can be recapitulated, and whether they generalize to other odorants and to other bioactive ligands.

The question of how much information is transmitted from fathers to children is perhaps the most important question in the paternal effects field. Not only is the answer to this question interesting teleologically and for epidemiology, but it has clear implications for the mechanistic basis for paternal effects. Faithful ligand-specific responses in progeny [93] cannot be explained by mechanisms in which small RNAs simply influence placental development, and almost certainly require some form of sequence-specific epigenetic modulation: cytosine methylation or histone packaging changes at the locus encoding the relevant receptor, or siRNAs targeting the RNA in question. Alternatively, although the fact that many distinct paternal exposures modulate common phenotypes such as glucose control could potentially be explained by locus-specific information transfer — high fat diet could target methylation at the gene encoding insulin, nicotine could target mTOR, etc. — these observations are more simply explained by paternal exposures convergently affecting placentation or epigenetic "tone".

6.2. Diverse RNAs implicated in paternal effect studies

Related to the question of sperm bandwidth is the increasingly disparate set of epigenetic changes which have been *functionally* linked to offspring phenotypes. Here, we ignore changes in cytosine methylation and histone retention, which have, to our knowledge, yet to be tested functionally using targeted epigenome editing technologies. In contrast, thanks to the relative ease of manipulating RNA pools, a number of studies have shown that microinjection of sperm RNAs — either purified from sperm, or individual synthetic RNAs — into zygotes can cause phenotypic changes in offspring. The RNAs identified in these studies are diverse, including gel-purified long RNAs [76], gel-purified tRNA fragments [25,64], and synthetic microRNAs including miR-19b [103] and a mixture of nine (distinct) microRNAs [81]. Although not all of these studies focused on the same phenotypes, it is illuminating to note that offspring glucose control has been reported to change in response to both gel-purified tRFs [25,64] and to synthetic miR-19b [103], while insulin tolerance was affected by both of these RNA populations as well as gel-purified sperm long RNAs [76]. Taken at face value, reconciling these findings requires that multiple distinct inputs converge, at some stage after fertilization, to affect a pathway ultimately leading to altered glucose tolerance. Convergent signaling could occur immediately, with, say, miR-19b and sperm tRFs both targeting the same specific maternal transcript for degradation, or it could occur further downstream, with targeting of distinct molecular pathways converging to affect trophectoderm cell number and later placental development. In addition to motivating the search for the point where distinct RNA signals converge in the early embryo, these findings also further emphasize the need to more systematically explore phenotypes induced by these different RNAs — do miR-19b and sperm tRF injections both cause an identical suite of offspring phenotypes? Or do resulting offspring share a glucose/insulin tolerance phenotype, but differ in their blood pressure or xenobiotic resistance, etc.?

6.3. Sex-specific F1 phenotypes

Finally, we briefly discuss the intriguing mystery of why offspring phenotypes in paternal effects studies are often (albeit not universally) only observed in either sons or daughters. Prominent examples include effects of paternal high fat diet being confined to daughters in several studies [45,52], or nicotine exposure driving xenobiotic resistance only in sons [92]. As we have previously discussed this in greater detail [92], we only briefly reiterate a handful of potential explanations here as some explanations have important implications for the relevant germline epigenetic information carrier. Most obviously, sex-specific phenotypes could be programmed in fathers via cis-acting epigenetic marks on the sex chromosomes. Alternatively, females and males could differ in their capacity to copy epigenetic information during development, as the process of X inactivation in females utilizes a great deal of epigenetic machinery (cytosine methylation, Polycomb-mediated chromatin regulation, SMC proteins, etc.), so epigenetic changes in the early embryo could be copied more faithfully in one sex or the other. Finally, and perhaps most likely, faithfully-copied epigenetic landscapes could exhibit sexually-dimorphic effects on phenotypes. As with the other unsolved mysteries above, the ultimate answer to the question of why paternal effects are manifest only in one gender could illuminate the mechanistic basis for ancestral epigenetic control of metabolic phenotypes.

7. CONCLUSIONS AND OUTLOOK

Over the past decade, well over one hundred studies have shown that paternal and maternal environmental conditions can program changes in a variety of offspring phenotypes, most commonly including metabolic phenotypes such as glucose tolerance, cholesterol metabolism, and many others. Although it is increasingly clear that the sperm epigenome carries at least some of the information from father to child, and several studies have implicated sperm RNAs as the key carriers of paternal information, the mechanistic basis for these parental effect systems otherwise remains obscure. Identifying the pathways from paternal environments to offspring phenotypes promises not only to deepen our understanding of early development and of epigenetic inheritance mechanisms, but also to provide plausible targets for interventions to modulate disease susceptibility in human populations. To this end, we believe that a key goal for future research must be to demystify the "black box" between the zygotic epigenome and the later development of a coherent physiological response in adult offspring.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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