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Review
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Review

A history of why fathers' RNA matters†

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Abstract

Having been debated for many years, the presence and role of spermatozoal RNAs is resolving, and their contribution to development is now appreciated. Data from different species continue show that sperm contain a complex suite of coding and noncoding RNAs that play a role in an individual's life course. Mature sperm RNAs provide a retrospective of spermatogenesis, with their presence and abundance reflecting sperm maturation, fertility potential, and the paternal contribution to the developmental path the offspring may follow.

Sperm RNAs delivered upon fertilization provide some of the initial contacts with the oocyte, directly confront the maternal with the paternal contribution as a prelude to genome consolidation. Following syngamy, early embryo development may in part be modulated by paternal RNAs that can include epidydimal passengers. This provides a direct path to relay an experience and then initiate a paternal response to the environment to the oocyte and beyond. Their epigenetic impact is likely felt prior to embryonic genome activation when the population of sperm delivered transcripts markedly changes. Here, we review the insights gained from sperm RNAs over the years, the subtypes, and the caveats of the RNAs described. We discuss the role of sperm RNAs in fertilization and embryo development, and their possible mechanism(s) influencing offspring phenotype. Approaches to meet the future challenges as the study of sperm RNAs continues, include, elucidating the potential mechanisms underlying how paternal allostatic load, the constant adaptation of health to external conditions, may be relayed by sperm RNAs to affect future generations.

Summary Sentence

A historical perspective of the discovery and delivery of sperm RNAs that initiate development is presented. How these RNAs provide a record of, and components essential to fertility, embryo development, and offspring's phenotype are discussed.

Key words: sperm RNA, recurrent miscarriage, transgenerational epigenetics, allostatic load.

Abbreviations

ACTB :	Actin beta
AGO :	*Argonaute*
BMI :	*Body mass index*
CARM1 :	*Histone-arginine methyltransferase CARM1*
CD45 :	*Leukocyte common antigen*
circRNA :	*circular RNA*
DGCR8 :	*DiGeorge Syndrome Critical Region Gene 8*
Dicer :	*Endoribonuclease Dicer*
DNMT1 :	*DNA methyltransferase 1*
DMNT3 :	*DNA methyltransferase 3*
E2R :	*Ubiquitin Conjugating Enzyme E2 R2*
EGA :	*Embryonic genome activation*
EGFP :	*Enhanced green fluorescent protein*
FPKM :	*Fragments Per Kilobase of transcript per Million*
GAS5 :	*Growth arrest specific 5*
H3K27 :	*Histone 3 lysine 27*
H3K4 :	*Histone 3 lysine 4*
Hdac11 :	*Histone deacetylase 11*
HPA :	*Hypothalamic-pituitary-adrenal*
IAP :	*Intracisternal A Protein*
ISH :	*In situ hybridization*
ITGB :	*Integrin subunit beta 1*
Kit :	*KIT proto-oncogene receptor tyrosine kinase*
LGC1 :	*Low glutelin content 1*
LINE :	*Long interspersed nuclear element*
LINE1 :	*Long interspersed element 1*
Lnc2 :	*lncRNA transcript 2*
Lnc3 :	*lncRNA transcript 3*
lncRNA :	*long noncoding RNA*
LTR :	*Long terminal repeat*
MII egg :	*Meiosis II egg*
m5C :	*5-methylcytidine*
m2G :	*N2-methylguanosine*
MERVL :	*Mouse Endogenous retroeLement*
MILI :	*Piwi like RNA-mediated gene silencing 2*
MIWI :	*Piwi like RNA-mediated gene silencing 1*
MIWI2 :	*Piwi like RNA-mediated gene silencing 4*
miRNA :	*micro RNA*
mRNA :	*messenger RNA*
MYC :	*MYC Proto-Oncogene, BHLH Transcription Factor*
NGS :	*Next Generation Sequencing*
PARN :	*poly(A)-specific ribonuclease*
piRNA :	*Piwi-interacting RNAs*
Piwi :	*P element-induced wimpy testes*
PLCZ :	*Phospholipase C zeta*
PNLDC1 :	*PARN like, ribonuclease domain containing 1*
POU5F1 :	*POU class 5 homeobox 1*
PRM1 :	*Protamine 1*
PRM2 :	*Protamine 2*
PTPRC :	*Protein tyrosine phosphatase, receptor type, C*
RASGRP1 :	*RAS guanyl releasing protein 1*
RISC :	*RNA-induced silencing complex*
RM :	*Recurrent miscarriage*
RPKM :	*Reads Per Kilobase of transcript per Million*
rRNA :	*ribosomal RNA*
SINE :	*Short interspersed nuclear element*
siRNA :	*small interfering RNA*
sncRNA :	*small noncoding RNA*
sno75 :	*piwi RNA 75*
SOX2 :	*Sex-determining region Y box 2*
RE :	*RNA element*
TCEP :	*Tris (2-carboxyethyl) phosphine hydrochloride*
TE :	*Transposable element*
TNP2 :	*Nuclear transition protein 2*
tRNA :	*transfer RNA*
TRAIL :	*Tumor necrosis factor (TNF) superfamily member 10*
3' UTR :	*3' untranslated region*

Introduction

Although spermatozoa were thought for many years to merely contribute their half of the genome to the offspring, it is now appreciated that sperm delivers its entire structure, from which selective components are used to build a healthy child [1]. These include phospholipase C zeta (PLCZ) and other factors that yield a pulsatile Ca^{2+} response, and in humans, the sperm centriole organizing center [reviewed in 2]. In contrast, certain structures including the mitochondria are ubiquitinated and targeted for degradation [reviewed in 2]. The suite of RNAs that a sperm carries is also part of the package. The RNAs can reflect the fidelity of spermatogenesis that impacts embryo development [3, 4], that, in turn, may affect offspring phenotype [5–8]. This has prompted the field to begin to dissect their role in the fetal origins of adult disease, a concept laid out in the Barker hypothesis [9] that continues to be tested as our environment changes. Health maintenance reflects a biological endpoint termed allostatic load [10], that is both directed by, and responds to, past and present events [11, 12]. A system overload, even by one too many inputs, leaves the potential for adverse health effects to arise. The relationship between the allostatic load and offspring health has recently been implicated in epidemiological studies and recapitulated in mouse models [13–15], with sperm RNAs as potential messengers between generations.

In this review, we will provide a historical perspective of sperm RNAs, and discuss the characteristics of this unique population and the caveats of studying sperm RNAs. Their contribution to the oocyte and their role in embryo development is considered. We conclude with a discussion of how sperm RNAs may respond to their environment epigenetically relaying the father's experience to the offspring.

History of sperm RNAs

Sperm RNAs had been considered an artifact and, if present, as having no role in fertilization and obviously not embryo development. The hypothesis was founded upon the extrusion of RNAs from the cell as part of the residual body during spermatogenesis and their lack of intact ribosomal RNA (rRNAs) [16]. This led to the conclusion that if any RNA remained, it was residual, possessing no function in the absence of a complete transcript. This has not stood the test of time. With marked technological advances that the field has experienced over the years, the presence and some of the roles of sperm RNAs (Figure 1) are now established with possibly others on the horizon.

Early studies suggested that spermatozoal RNAs were present in the epididymis and ductus deferens from mouse sperm, but were dismissed as reflective of mitochondrial contamination [17]. It was not until the late 1980s that the presence of sperm RNAs was beginning to be considered by various independent techniques e.g., using immunogold staining in ferns [18], and finally in humans and rats

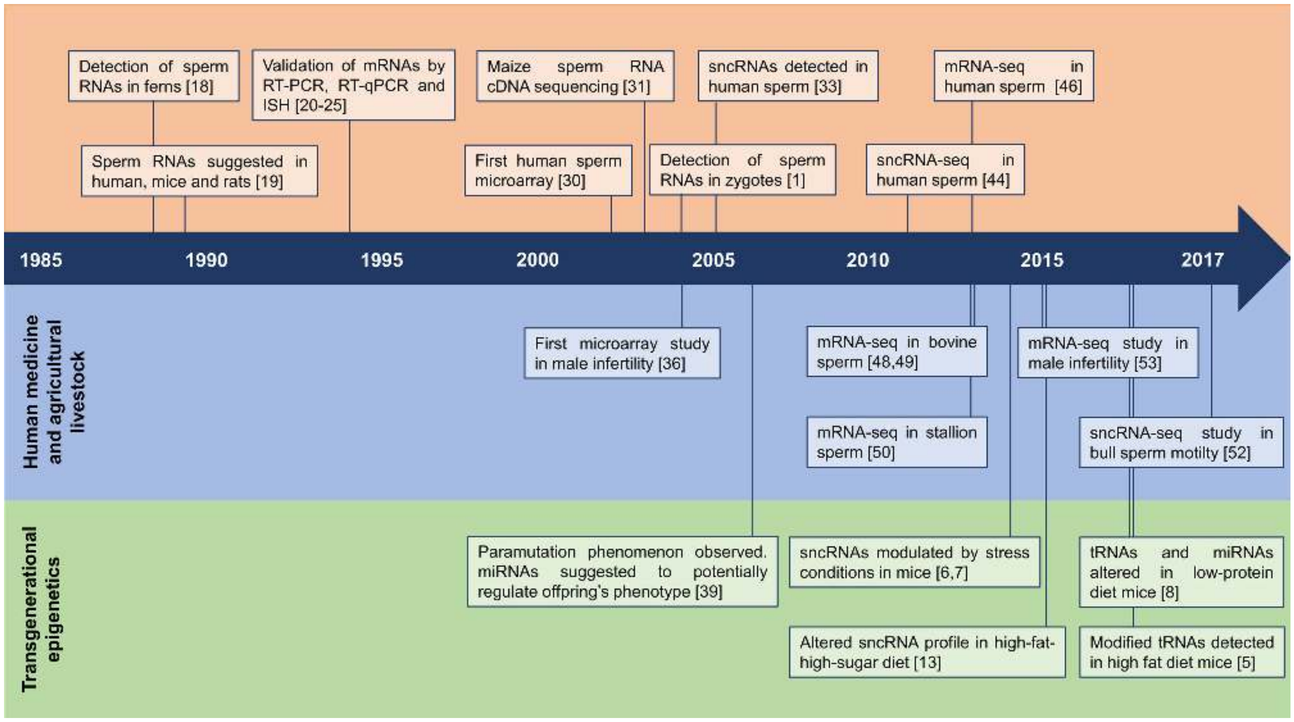

Figure 1. Three decades of sperm RNAs summarized with key studies. Sperm RNAs were first detected in ferns [18], and subsequently in mammals [19]. In the 1990s, several independent studies [20–25] validated the presence of mRNAs in sperm with different approaches including RT-PCR, RT-qPCR, and ISH. In the 2000s, with novel technologies, microarray allowed the first human sperm transcriptome profile [30], a technique posteriorly used for studying male infertility [36]. The first species to be sequenced (by sperm RNA cloning and cDNA sequencing) was in maize [31]. In 2004, sperm RNAs were shown to be delivered to the zygote [1]. Small noncoding RNAs were firstly detected in human sperm by microarray [33] and corroborated, a few years later, with sncRNA-seq [44]. With the boost of NGS techniques, mRNA-seq studies were carried in sperm RNAs in human [46], and agricultural species as bovine [48, 49] and stallion [50]. NGS has also been used to identify biomarkers related to male infertility in humans [53] or sperm quality (motility) in bulls [52]. Relevant transgenerational epigenetics studies start with the work from Rassoulzadegan and colleagues [39], where miRNAs were suggested to modify offspring phenotype. Several studies in mice have followed, where parents have been set in extreme environmental conditions, including stress [6, 7] and diet [5, 8, 13], and sncRNAs profiles have been studied. ISH (in situ hybridization); sncRNAs (small noncoding RNAs); miRNAs (micro RNAs); tRNAs (transfer RNAs).

by RNase colloidal gold. This provided an estimate of 70 and 100 fg RNA per cell, respectively, with the majority primarily localized within the nucleus [19]. RT-PCR and in situ hybridization (ISH) reconfirmed these observations in human sperm with the detection of *MYC* [20]. However, the field was still not convinced attributing these observations to spurious hybridization or priming events. Several independent studies unknown to each other were undertaken in the 1990s that addressed the issue of the presence of RNAs in sperm using various techniques. These included RT-PCR, ISH and RT-qPCR, following the exclusion of samples with genomic DNA or contamination by somatic cell RNAs. Detection of *PRM2*, *E2R* and *ACTB* by RT-PCR [21], *ITGB1* via PCR [22], and *PRM1*, *PRM2*, and *TNP2* in humans [23] and mice [24] by ISH, as well as *LGC1* by ISH and RT-PCR in lily plants was observed [25]. It was not until 1999, when Miller, Krawetz and colleagues attempted to characterize human sperm RNA transcripts at the sequence level [26]. A group of translationally quiescent RNAs, the majority of which were derived from repetitive elements with the near or complete absence of 28S and 18S rRNAs were revealed [26]. This was a curious observation in a transcriptionally inactive sperm cell [16, 27]. Translation of nuclear encoded RNAs by mitochondrial ribosomes in mature sperm during sperm capacitation has been proposed [28, 29], but these observations still await independent confirmation.

Microarray analysis that followed provided the first characterization of sperm mRNAs from healthy human donors [30]. A set of transcripts shared between sperm samples functionally enriched for spermatogenesis and early development were resolved, suggesting that the retention and presence of certain RNAs was not stochastic [30]. In maize (*Zea mays*), a set of sperm RNA transcripts were identified by cDNA sequencing with some derived by selective partitioning [31]. Delivery of human sperm RNAs to the oocyte was validated in the hamster penetration assay opening the door to a role in early embryonic development, and the male assuming a greater role in the birth of a healthy child [1]. Antisense and micro RNAs (miRNAs) were also identified amongst this population, and thus inferred to be delivered to the oocyte upon fertilization. This cemented the foundation for the beginnings of mechanistic proposals regulating parental gene activity through targeting paternal or maternal RNAs, thereby regulating early genomic events in the embryo [32, 33]. Characterization of the human sperm transcriptome by serial analysis of gene expression, i.e., tag sequencing [34], identified a series of highly abundant transcripts in fertile donors, enriched for roles related to spermatogenesis, sperm function, fertility, and conception [35], in accordance with previous microarray studies [30]. This further encouraged interest to assign potential roles for sperm RNAs. Microarray analysis was beginning to lay a path towards developing diagnostic strategies to understand male infertility [3, 4, 36].

Advances continued over the years and in 2005, shortly after the identification of miRNAs in human sperm by microarrays [33], miRNAs were observed in mice using a microarray and RT-qPCR approach [37]. Other small noncoding RNAs (snc RNAs) like P element-induced wimpy testes (Piwi)-interacting RNAs (piRNAs) were subsequently detected in mouse testis by pyrosequencing [38] but left their description in sperm wanting. The low abundance of paternal miRNAs in zygotes led to the assumption they possessed a limited role, if any, in fertilization [37]. Yet, Rassoulzadegan and colleagues provided the first study that suggested that miRNAs could influence offspring phenotype, a phenomenon known as paramutation [39]. As with most studies in this field, this initial foray into sperm RNA-mediated transgenerational epigenetics was controversial and met with skepticism by others, but this concept has also withstood the test of time.

In recent years, several compelling independent studies have corroborated the initial findings of Rassoulzadegan and colleagues that epigenetic modifications can be transmitted from father to offspring via paternal RNAs [reviewed in 13, 40, 41, 42]. Various components or stressors including mental and physical stress, induced by the physical environment, toxins, or diet that together constitute the allostatic load experienced by the father have been individually well-studied in mice and rats [5, 40, 43]. Both miRNAs [7] and piRNAs [6] have been implicated in paternal stress studies and have provided a framework. Similarly, paternal diet can coincide with alterations in the abundance of miRNAs and transfer RNAs (tRNAs) leading to transgenerational effects whether they are subject to a high-fat-high-sugar diet [13], a high-fat diet [5], or a low-protein diet [8]. Still, the results of these studies, especially in humans, require further study (see "RNAs and transgenerational epigenetic inheritance").

Next Generation Sequencing (NGS) provided a boost to RNA-seq studies early in the first decade of the millennium. The first sncRNA-seq study performed in human sperm showed the presence of several sncRNAs including miRNAs, piRNAs, and repeat-associated small RNAs [44]. Spurred on by this work, sncRNA-seq in mouse sperm followed, that also reported a highly enriched fraction of tRNAs [45]. Several sperm RNA-seq studies have now characterized the population of transcripts found in sperm including human [46], mouse [47], and agricultural livestock species such as bovine [48, 49] and stallion [50]. Moreover, an in-depth study in mature human sperm has shown that the rRNAs that remain, while abundant, are fragmented [51]. The continually decreasing cost of sequencing has enabled the RNA-seq approach to become widely adopted and is now a cornerstone of medical and agricultural research [52, 53] and is becoming the diagnostic standard. Nevertheless, sperm NGS techniques present several intricacies in comparison to somatic or other germinal cells. Hence, experimental design and analyses are crucial for reliable interpretation that yields meaningful results.

Methods, techniques and caveats when considering sperm RNA

The unique characteristics of sperm cells compared to somatic cells necessitate careful consideration as a new study is conceived or secondary analysis of a publicly available dataset is undertaken. The low concentration of sperm RNAs within each cell must be considered along with the technology that has been employed. Human sperm carries approximately 50 fg of long RNA (>200 nt) and 0.3 fg of sncRNAs (<200 nt) per sperm cell, which is very similar to early estimates [19]. However, this is dwarfed in comparison to somatic cells that contain 10 pg of long RNA and 1–3 pg of sncRNAs [54–56], and necessitates the use of highly purified samples to eliminate maturing cells, somatic cells, and genomic DNA that can obscure the results. Purification protocols have been developed to separate mature spermatozoa from seminal plasma, as well as immature sperm cells, leukocytes, epithelial cells, and bacteria. Three different approaches have generally been employed. The first, swim-up or sperm migration, in which 0.5–1 ml of semen is placed in a 45° angled centrifuge tube under a medium salt solution and incubated at 37°C for 60 min [57]. The sperm cells swim out of the semen to the medium, where they are aspirated using a sterile pipette. This approach selects motile sperm, however, the number of spermatozoa recovered can be low [57]. The second, density gradient centrifugation, uses an isotonic salt solution with saline-coated silica particles, such as PureSperm (Nidacon), to separate spermatozoa according to density [58]. In most mammalian studies, the starting concentration used is 50% [54]. As with the swim-up method, this approach favors motile and morphologically normal spermatozoa [58], but the number of spermatozoa recovered is significantly higher by this method [59]. The third method employs a somatic cell lysis buffer that typically contains 0.1% SDS and 0.5% Triton (X-100). This effectively lyses somatic cells, and leaves the sperm head intact [30, 60]. While this method is effective in eliminating somatic cells, this treatment has been proven to compromise the midpiece, and can solubilize sperm-membrane structures, with preferential loss of mitochondrial RNAs [30, 58, 60]. Although a higher recovery rate was observed, RNA yield was significantly lower than density gradient purification [58]. Optical microscopy to visually confirm the lack of immature sperm, somatic, or bacterial cells is typically used as an initial screen.

Species-specific protocol optimization of sperm RNA extraction has proven useful once RNase-free reagents have been confirmed as RNase free. Extraction methods generally include a homogenization and cell disruption using a chaotropic and reducing agent, e.g., TRIzol and TCEP (Tris (2-carboxyethyl) phosphine hydrochloride), typically followed by commercial RNA isolation protocols [58, 61, 62]. A final DNAse treatment step is requisite for eliminating residual genomic DNA. Several strategies to confirm spermatozoal recovery have been employed. For example, following sperm RNA extraction, the absence of intact rRNAs 18S and 28S as assessed by a Bioanalyzer trace would be consistent with their targeted removal during spermatogenesis [51]. The presence of transcripts from *CD45/PTPRC* (expressed in most somatic cells) is typically used as a somatic marker. RT-PCR and/or RT-qPCR quantitation can simultaneously assess the presence of genomic DNA when intron spanning primers are used. The ultimate test for sperm RNA purity is by comparative sequence analysis [63].

The construction of high-throughput sperm RNA sequencing libraries is confounded by several factors including selective fragmentation of coding transcripts [46] as well as residual fragmented rRNAs [51] and low total RNA yield [54]. While typical RNA-seq library construction protocols require 1 μg of total RNA, sperm only yields between 30 and 80 ng of total RNA per million cells [54–56]. In most tissue samples, poly(A^+) selection has been used to selectively enrich the population of mRNAs as compared to mitochondrial and rRNAs. RNA-seq library construction using poly(A^+) selection can result in 3' bias due to the exclusion of all short polyadenylated tailed transcripts, those lacking this modification, and non-polyadenylated transcripts or the majority of the other segments from fragmented transcripts [55]. A random amplification strategy can, somewhat, correct for potential 3' bias. Ribosomal RNA depletion or primer design can also be used to reduce the number of sequencing reads assigned to this biotype. Different commercial library preparation kits and methods have been tested in various species,

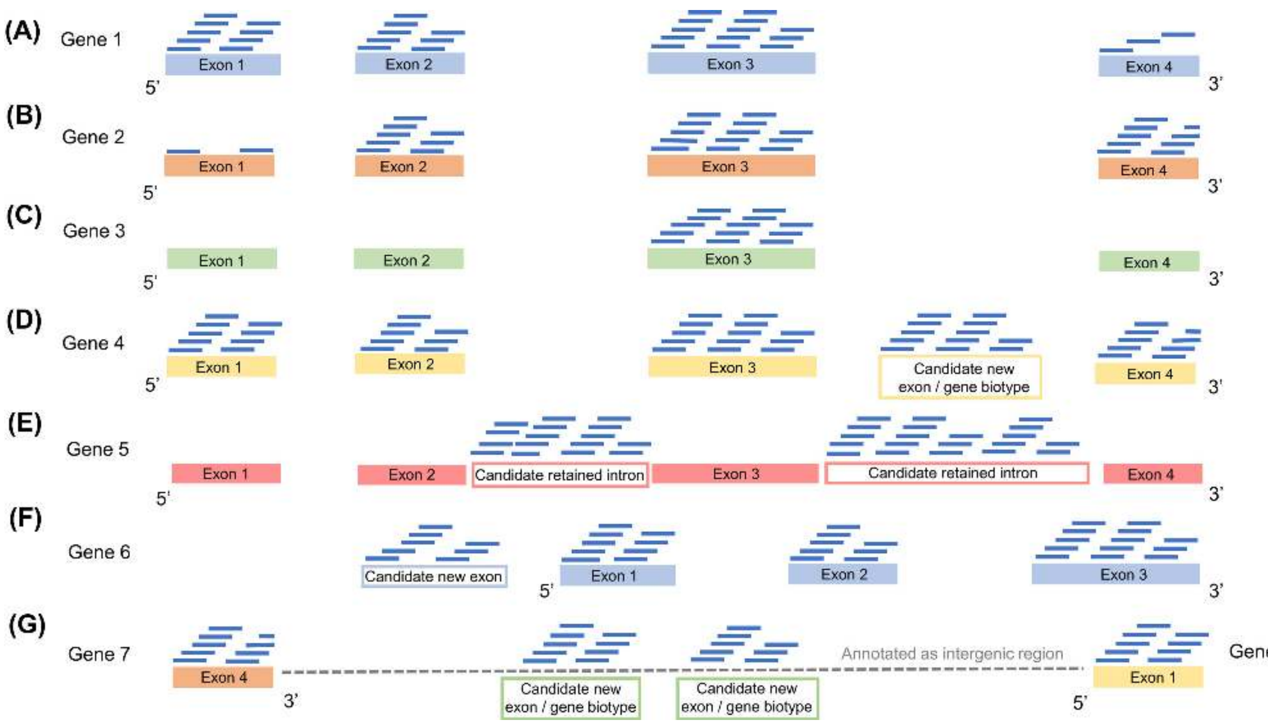

Figure 2. Defining sperm RNA Elements (REs) with the discovery analysis approach. Dark blue lines represent mapped sequencing reads. Dashed gray line corresponds to an annotated intergenic region. The approach also provides a reliable quantitation method of isoforms, where read abundance would vary as a function of transcript isoform. Since mature sperm are transcriptionally and translationally silent, the term "abundance" is preferred rather than "expression." (**A**) Four REs are detected, corresponding to the four annotated exons of Gene 1. Similar read abundance is observed in RE 1, 2, and 3, but lower abundance in RE 4, suggesting possible 3′ degradation of the transcript. (**B**) 5′—end degradation. (**C**) Only one RE is detected, corresponding to the annotated exon 3. (**D**) RE analysis detects all the annotated exons as well as an intronic RE that may correspond to an unannotated exon or other gene biotype. (**E**) Two REs are detected in intronic regions and may correspond to intronic retained elements. (**F**) RE-detected upstream of the 5′ UTR, but could also exist 3′. (**G**) Two REs are detected in an intergenic region, and may correspond to unannotated exons from nearby genes, exons from a new unannotated gene or other gene biotypes.

including human [64], bovine [48], and porcine (Gòdia M, Quoos Mayer F, Nafissi J, Catelló A, Rodríguez-Gil JE, Sánchez A, and Clop A, unpublished results), resulting in different outcomes with preferred protocols being developed. When comparing two studies, it is important to remember that the data obtained will vary in terms of the library preparation protocol [64]. In addition, quantification of sperm RNAs from sequencing data is dependent on the approach used to analyze the data and this can vary widely. Bioinformatics analysis can follow two different paths. The most commonly used method is "directed analysis." After read mapping, bioinformatic analyses are used to quantify the abundance of each of the annotated genes from the genome studied. This can be refined to quantify RNA levels for each of the annotated transcripts, enabling the detection of alternatively spliced isoforms, but this still remains a challenge when multiple isoforms are simultaneously expressed. Transcript quantitation is measured by FPKM (Fragments Per Kilobase of transcript per Million), when a paired-end sequencing approach is employed or RPKM (Reads Per Kilobase of transcript per Million), when single-end sequenced. These measurements consider the total number of reads mapped to the transcript after adjusting for the full length of the gene/transcript and applying a library size (sequencing depth) correction. This analytical strategy has been widely used in sperm RNA profiling as it provides a basic way forward to direct transcript comparison with other tissues and/or between species [46–50, 64]. However, as substantial portion of the sperm transcripts are fragmented [46], all exons may not be represented in an equivalent manner and depending on the question posed, this might obscure the results. "Discovery" strategies that utilize a biologically meaningful minimal unit of detection can provide an unbiased alternative. This approach considers sperm RNAs as a collection of sperm RNA elements (SREs) [53]. RNA elements are short-sized sequences, formed by a set number of reads and joined as a contig, independent of their annotation. Resultant REs are considered independent of each other and are annotated by genomic location as exonic, intronic, intergenic, close to exonic regions, or novel as existing within an unannotated region (Figure 2). This approach permits a comprehensive examination [53] and addresses low-quality genome annotations in some species.

The population of sperm RNAs

Mature spermatozoal RNAs represent a wide range of coding and noncoding RNAs, including long and short noncoding RNAs. This rich number and classes of RNAs are implicated in different roles, including spermatogenesis, regulation, fertilization, embryo development, and offspring phenotype [reviewed in 53, 55, 65]. Early microarray studies showed that a vast majority of transcripts were shared among healthy sperm donors, enriched in sequences reminiscent of past function [30], suggesting that their distribution among sperm was not stochastic. Human sperm RNA-seq enabled a more complete understanding of the unique features of the sperm transcript population [46]. Over 22,000 transcripts were detected with approximately 700 of which were moderate to high abundance [46]. These RNAs present a complex heterogeneous profile of intact and variously fragmented transcripts [46]. As shown in human [46] and other species including mouse [47], horse [50], and bovine [48, 49], a substantial proportion of RNAs are fragmented. Nevertheless, like sncRNAs, they might also function in sperm maturation and the initial events as part of and following fertilization. Integrative analysis between human RNA-seq datasets of testes, mature sperm, and seminal fluid has provided additional insight that highlights the importance of precise transcriptional regulation during spermatogenesis, to maintain the integrity of gamete generation and maturation [66]. The presence or absence of shared RNAs and corresponding proteins between the different tissue types is indicative of cross-talk between the cellular and extracellular environment from the prostate, epididymis, and seminal vesicles [66].

Long noncoding RNAs

Long noncoding RNAs (lncRNAs) can modulate both transcriptional and post-transcriptional processes [reviewed in 55], while often serving a nuclear structural role [67]. Approximately one-third of sperm RNAs are confined within the bounds of the perinuclear theca encapsulated nucleus [52]. The complex packaging of the paternal genome by protamines alongside nucleosome-bound DNA forms a unique chromatin structure. In part, chromatin remodeling has been attributed to lncRNAs that directly complex with chromatin [41] as a function of its affinity to the DNA or DNA-binding proteins. Interaction with target sites through long-range chromatin interactions exemplify a means of regulation often mediated by their 3D organization [reviewed in 68, 69]. Interestingly, this structure appears in close association with a repertoire of RNAs [67], positioned by chromosomal regions attached to the nuclear matrix by the matrix attachment regions [70].

Several classes of lncRNAs have been observed in human [46, 53] and mouse [71] sperm. This includes members of the RNA U small nuclear family. These components of the sperm spliceosome are intact and very abundant [46], perhaps suggestive of an early postfertilization role. Mouse sperm and testes are rich in lncRNAs, of which *Lnc2* and *Lnc3* are the most abundant as evidenced by fluoresce ISH RT-PCR, and RT-qPCR [71]. Interestingly, they appear enriched in sperm when compared with testes [71]. Long noncoding RNA profiles in both testes and sperm can be affected by environmental exposures, e.g., cadmium, which impacts spermatogenesis and male fertility [72]. This is consistent with the view that lncRNAs modulate target genes that play a regulatory role in spermatogenesis, fertility, and embryo development [71–74].

Other types of noncoding RNAs have been described in germinal cells. These include the intronic retained elements and the recently discovered circular RNAs (circRNAs). Intronic-retained elements up to full-length introns have been reported in sperm, with more than 200 distinct REs of this class identified [46]. They appear more abundant in sperm than in testes [reviewed in 55]. Although suggested as retained in mature sperm it has yet to be resolved as to how they evade degradation [reviewed in 55]. Circular RNA are stable 3' and 5' covalent linked noncoding RNAs formed from the same transcribed segment that are inherently resistant to exonuclease degradation [75] thus evading degradation. The function of circRNAs is origin dependent. Exonic circRNAs are primarily located in the cytoplasm, thought to act as "sponges," and counteract mRNA repression by miRNAs. Nuclear localized intronic circRNAs and exon-intron circRNAs are thought to regulate their parental genes through direct cis/trans interaction [reviewed in 75, 76]. They have recently been detected in testes and seminal plasma suggestive of a role in gamete generation [77]. Gene Ontology of testes circRNAs shows enrichment for genes related to spermatogenesis, sperm motility, and fertilization [77]. Their stability and presence in seminal plasma may provide a source of infertility biomarkers. Some of the sperm REs described in previous studies [46, 53, 56] warrant further consideration as they do possess characteristic circRNA signatures.

Small noncoding RNAs

Small noncoding RNAs encompass a variety of different RNA types that play crucial roles in the maintenance and function of the germline genome [reviewed in 42, 55, 78]. Major functions may be classified by RNA type and include regulation of gene expression by miRNAs [44], defense against transposable, repetitive elements or viruses by small interfering RNAs (siRNAs) [79] or piRNAs [80], and protein synthesis and signal modulation by tRNAs and their fragments [5, 45]. Together, they can play a role in genome structure and integrity. Characterization of the population of sncRNAs from human and mouse has revealed their diversity, and provided a glimpse into their roles in spermatogenesis, early embryo development, and how they may modify the genome to heritably affect offspring [44, 45].

Micro RNAs

The most well-characterized sncRNAs are miRNAs, contributing to approximately 7% of the total sncRNAs to the fertile human sperm profile [44]. They play a crucial role in spermatogenesis and fertility, and have been reported to modulate expression during the different stages of sperm maturation [reviewed in 81]. Micro RNAs are an integral part of the RNA-induced silencing complex (RISC) and together with the AGO proteins generally target the 3' UTRs of mRNAs using sequence complementary to repress mRNA expression through degradation or activation [reviewed in 55]. On one hand, miR-140, miR-21, miR-152, and miR-148a have been shown to repress expression of RNAs encoding epigenetic modifiers, e.g., including *DMNT3* and *RASGRP1*. This is consistent with their absence in mature sperm [46]. On the other hand, *DNMT1* transcripts are present in sperm [46], concordant with their epigenetic role suggesting they escape this form of repression. Specific paternal origin miRNAs such as miR-34c have also been described [44]. Attempts to study its role have led to different conclusions. While in vitro they appear required for the first cleavage following fertilization [82], in vivo, it was not essential for fertilization or embryo development but was crucial for spermatogenesis, as its absence disrupted spermatogenesis leading to murine infertility [83].

Small interfering RNAs

Small interfering RNAs, also known as RNA-mediated interference RNAs, are active mediators of transcriptional and post-transcriptional gene-silencing [reviewed in 84]. As with miRNAs, siRNAs act in concert with RISC and AGO proteins to target complementary RNAs for translational inhibition or degradation [reviewed in 84]. Small interfering RNAs are primarily known to function in the host defense against transposable elements (TE) and RNA viruses, and aid in the maintenance of heterochromatic DNA [reviewed in 84]. As shown in plants, somatic cell TE-derived siRNAs migrate into sperm cells contributing to TE silencing prior to fertilization [reviewed in 85]. Preliminary work on the role of siRNAs in fertilization using Dicer knockout mice presented an altered profile of siRNAs in spermatozoa (and miRNAs), where sperm microinjection in wild-type oocytes resulted in embryos with reduced developmental potential [79]. Other independent studies have attempted to discern the role between siRNAs and miRNAs in spermatogenesis. While miRNAs require DICER and DGCR8 proteins for maturation, siRNAs only require DICER [reviewed in 86]. Results have shown that a conditional *Dicer* knockout presents severe sperm morphological defects as compared to the conditional *Dgcr8* mice mutant, implicating siRNAs in mammalian spermatogenesis [86, 87].

piRNAs and TEs

Piwi-interacting RNAs are specialized RNAs that interact with Piwi proteins, a gonad type of AGO proteins, which mediate RNA silencing of TEs [reviewed in 88, 89]. Murine Piwi proteins MIWI, MILI, and MIWI2 are essential to spermatogenesis, and their absence is associated with male infertility [reviewed in 78, 88]. In humans, ~17% of the sncRNAs correspond to piRNAs [44]. Their presence still remains controversial, although piRNAs are now beginning to be considered by others [90]. Altered levels of PIWI-like 1 and 2 mRNAs have now been detected in men with decreased sperm count and motility by RT-qPCR [91]. The PIWI-like 1 RNA remains essentially intact in mature spermatozoa as determined by RNA-Seq [53, data not shown]. Given these independent observations, their presence will likely become accepted.

In murine spermatocytes and spermatids, the vast majority of piRNAs map to specific genomic regions [reviewed in 78], and ~17% map to repeat sequences such as DNA transposons, short interspersed nuclear elements (SINEs), and long interspersed nuclear elements (LINEs) [reviewed in 89]. This is not surprising since TEs constitute a large fraction of the eukaryotic genome. SINES, LINES, and long terminal repeats (LTRs) are the most abundant [44]. LINE1 is the most common and well-studied retrotransposon in germ cells [reviewed in 88]. Piwi-interacting RNA biogenesis is regulated by the PARN family proteins [92]. The role of one of its members, PNLDC1 has been examined in vivo [93]. In the corresponding mouse knockout model, LINE1 retrotransposon silencing was disrupted leading to aberrant piRNA biogenesis and spermatogenesis [93]. Studies in mutant Piwi proteins have shed light on their roles in spermatogenesis. *Miwi2* knockout mice showed spermatogenic arrest, increased LINE1 retrotransposon expression, and loss of methylation in testes, suggesting possible regulatory roles of Piwi proteins and piRNAs in methylation [80]. Moreover, *Mili* mice knockout mutants exhibited loss of DNA methylation of the LINE1 element and an increased expression of both LINE1 and *IAP* [94]. Interestingly, the piRNAs produced from lncRNAs have the capability to act as histone modifiers. For example, the piRNA sno75, derived from the lncRNA *GAS5*, has been shown to increase transcription of *TRAIL* by guiding H3K4 methylation and H3K27 demethylation [95]. Transposable elements can be regulated by DNA methylation [reviewed in 96], which may also be guided and modulated by RNAs [reviewed in 68]. This DNA methylation is maintained by DNMT1 [reviewed in 96], whose transcript is present in sperm [44]. It has also been suggested that piRNAs play a central role in the confrontation and consolidation when the sperm and oocyte meet to initially assess genetic compatibility through their interaction with repetitive elements [reviewed in 55] (see "Contributions from sperm to the oocyte and early development").

Transfer RNAs

Transfer RNAs and their fragments are some of the more abundant sperm sncRNAs [reviewed in 97]. Their abundance is directly linked to the general translational needs of a given cell type rather than to a specific gene, reflective of their metabolic state. The original description of tRNAs in mouse sperm showed an enrichment of 5' end of tRNAs [45]. While generally scarce in testis, their abundance increases as the maturing sperm exits from testis as it passes through the caput, corpus, and cauda [8]. This has supported the view that the majority of tRNA fragments in mature spermatozoa arise from trafficking via epididymosomes from the epididymis to the sperm [8], with some upregulated in mice fed a high-fat diet [5].

In addition to fragmentation that yields 5' enrichment of mouse sperm tRNA fragments [45], modifications have also been reported [5]. These include the incorporation of 5-methylcytidine (m^5C) in response to high- or low-fat diets [5], which may act to increase their stability. This chemical post-transcriptional modification may provide a mechanism to signal current metabolic state reflecting a change in environment [97]. It has also been argued that tRNA fragments may act to repress embryonic Mouse Endogenous RetroeLements (MERVLs). This is particularly attractive since some are in close proximity to genes expressed during the early stages of embryo development. It has thus been proposed that they regulate a significant proportion of the transcriptome in development [reviewed in 98], perhaps affecting placental size by altering metabolic pathways [8].

Contributions from sperm to the oocyte and early development

Upon fertilization, the spermatozoon and oocyte confront one another for the first time towards consolidation [2, 99]. During this initial event when the ooplasm is exposed to the paternal contribution, it is also potentially exposed to a host of retroviruses and retrotransposons that have the potential to alter the zygotic genome [99]. They can insert within active regions of the genome as it is rearranged and/or affect the expression of neighboring genes by functioning as an enhancer, altering splicing or polyadenylation [100]. LINE1 transcription affects embryo chromatin accessibility in mice, and may play a role in coordinating the activity of multiple genes throughout the genome adding to genomic stability for the embryo [98]. Yet, in contrast to MERVLs, sperm LINE1 elements do not contain genes implicated in embryonic genome activation (EGA) [98]. By a mechanism of RNA activity through maternal and paternal sncRNAs, such as miRNAs and piRNAs [2, 44, 55], parasite RNAs are degraded, leading to a consolidated state of genetic compatibility [99]. Embryo development goes forward if these checkpoints are passed. While the potential contribution of repetitive elements in early development remains unclear, embryonic arrest at the 2- or 4-cell stage in mice coincides with the disruption of LINE1-encoded reverse transcriptase [reviewed in 101].

In humans, the transition to an embryo requires approximately 3 days and comprises the migration and fusion of the germ cell pronuclei and several cleavage divisions [reviewed in 102]. The human embryo is relatively transcriptionally silent until day 3. Having reached the 4–8-cell stage, the major wave of human EGA occurs [reviewed in 102, 103]. Following EGA, the embryo continues development, including implantation of the blastocyst into the uterine wall at day 7 [reviewed in 102]. Although humans and mice morphologically share embryo similarities, key differences necessitate that cross-species extrapolations be considered with caution. In mouse embryos, EGA occurs 26–29 h postfertilization at or around the first cellular division so the transcriptionally silent period is substantially reduced [reviewed in 102]. Similarly, the timing of compaction is shorter in mice, and human embryos hold at least one extra round of cell division before implantation [reviewed in 102]. It is not surprising that marked differences between gene expression, genome instability, and epigenetic modifications between human and mouse embryos are apparent [reviewed in 102]. While the quantity of paternal RNAs delivered by sperm to the oocyte may seem small compared to the oocyte, it is sufficient to play a role in transgenerational inheritance altering offspring phenotype [99, 104].

The role of sperm RNAs in EGA in mice has recently been examined in silico providing key elements for testing [105]. This provided an RNA-seq survey of sperm, MII eggs, and zygotes, supporting a potential role of paternal RNA or proteins to interact with maternal cofactors contributing to EGA [105]. A sperm RNA corresponding to an intragenic LTR of *Hdac11*, present in sperm but absent in MII eggs and zygotes, suggested that the paternally derived *Hdac11* LTR or others might complement maternal cofactors or pathways and participate together in EGA [105], not unlike the well-known oocyte activator factor PLCZ [106].

The first days of embryo development are critical as specification begins. Perturbation may resolve as the habitual first trimester pregnancy loss, which occurs in 15–25% of pregnancies [107]. Although most of the efforts to understand possible causes have focused on the mother reflecting the direct relationship with the fetus, the possible role of the male contribution has only begun to be discussed [108]. The factors from which sperm may contribute to miscarriage have been the focus of various studies. The influence of DNA fragmentation, aneuploidy, and integrity, as well as sperm morphology have been considered to be correlated with Recurrent Miscarriage (RM), characterized as two or more consecutive failed pregnancies [107], yet, results remain controversial and inconsistent [109–112]. A recent study has focused on the possible role of sperm RNAs in RM etiology [108]. The ratio of protamine RNAs (*PRM1*/*PRM2*) was significantly different between spermatozoa when RM couples were compared to healthy donors suggesting that abnormal protamine packaging can negatively affect embryo development [108]. Whether this reflects histone retention and/or abnormal epigenetic marks [108, 113] remains to be resolved. Interestingly, alterations in the protamine transcript ratio have also been associated with reduced sperm quality, including low concentration, reduced motility, abnormalities, and increased DNA fragmentation [114].

RNAs and transgenerational epigenetic inheritance

The study of Rassoulzadegan and colleagues was the first report providing evidence of mammalian RNA-mediated epigenetic inheritance. Here, it was observed that wild-type mouse offspring of *Kit* mutant parents exhibited white patches characteristic of the *Kit* mutant phenotype coupled with altered RNA levels of *Kit* in sperm [39]. In the testis, a relatively dispersed population of RNAs was observed, including the identification of abnormal short mRNAs, derived from the wild-type allele responsible for the *Kit* mutant phenotype. These abundant RNAs were only found in the mature sperm of heterozygotic offspring [39], suggesting that the increase was responsible for the disruption of wild-type phenotype. To test this hypothesis, two miRNAs thought to target *Kit* mRNA, miR-221, and miR-222 were injected into 1-cell embryos. The resultant mice exhibited the same *Kit* mutant phenotype, leading to the conclusion that the presence of certain miRNAs early in embryogenesis will result in a stable, heritable change in gene expression and, correspondingly, phenotype [39]. This study also highlighted miRNAs as possible modifiers of the epigenome [39], that perhaps directed in some manner DNA methylation, the most widely recognized mechanism of epigenetic heritability [reviewed in 40].

Several sncRNAs can regulate DNA methylation and histone modifications [reviewed in 68] and, as above miRNAs, are known to influence DNA methyltransferases. CARM1 is an embryonic stem cell pluripotency factor involved in the H3 promoter methylation of two transcription factors, POU5F1 and SOX2, providing an active chromatin mark upon induction [reviewed in 55]. miR-181c is known to target *CARM1*, and its immature form, pri-miR-181c, is abundant in human sperm [46]. Upon delivery to the oocyte, pri-miR-181c is processed to its mature form that is coupled with a 70% decrease in these and 27 other *CARM1*-associated target genes by the morula stage [46]. Recent results from a paternal chronic stress study identified that zygotic microinjection of nine abundant miRNAs resulted in targeted degradation of maternal mRNA transcripts, including genes involved in chromatin remodeling [7]. These results infer a role for sperm miRNAs in ensuring or modifying cell specification.

Both multigenerational and transgenerational epigenetic inheritance imposed upon future generations have important ramifications for medicine and agriculture. The concept of fetal origins of adult health and disease [9] has given rise to numerous studies that illustrate the ability for the effects of an exacerbating input environment (allostatic overload [10]) in the father, to be inherited by later generations. While, male-mediated multigenerational inheritance only passes from the affected father to child in a transient manner, transgenerational inheritance extends, affecting the father's grandchildren and/or beyond. Unlike multigenerational transmission, transgenerational inheritance can represent a permanent change to the gene pool [115]. Mechanistically, these responses to environmental factors, including stress, toxins, and diet may initially be mediated by epigenetic effectors, including RNAs. The ability of nucleic acids, including sncRNAs, to distribute throughout the body encapsulated in vesicles provides the opportunity to influence sperm [reviewed in 115] that is normally protected from external effects by the blood–testes barrier. Data have also suggested that reverse transcription, by the LINE1 retrotransposon reverse transcriptase, might be a route for passing non-Mendelian traits to the offspring [101, 116]. The transmission of information from somatic to germ cells by exogenous RNA was suggested from a series of experiments in which mice xenografted with human A-375 melanoma cells stably expressed EGFP that was then distributed through the bloodstream. Interestingly, transcripts were also detected in mouse spermatozoa, with exosomes as the suggested mechanism of transport [117]. In this paradigm, spermatozoa would take up exogenous DNA or transcribe exogenous RNA for delivery to the oocyte at fertilization [94]. This sperm-mediated reverse transcriptase may act to expand the exogenous DNA or RNAs once in the sperm [116] providing a means to directly impact early embryo development.

Several compelling studies have exhibited the capacity for the environment experienced by the father to influence the offspring without the offspring ever being in contact with the environment [reviewed in 40, 42]. For example, both chronic and acute stress models have been used to explore how the paternal environment affects progeny. For example, chronic stress mouse models have identified increased levels of a group of sperm miRNAs [7] that act

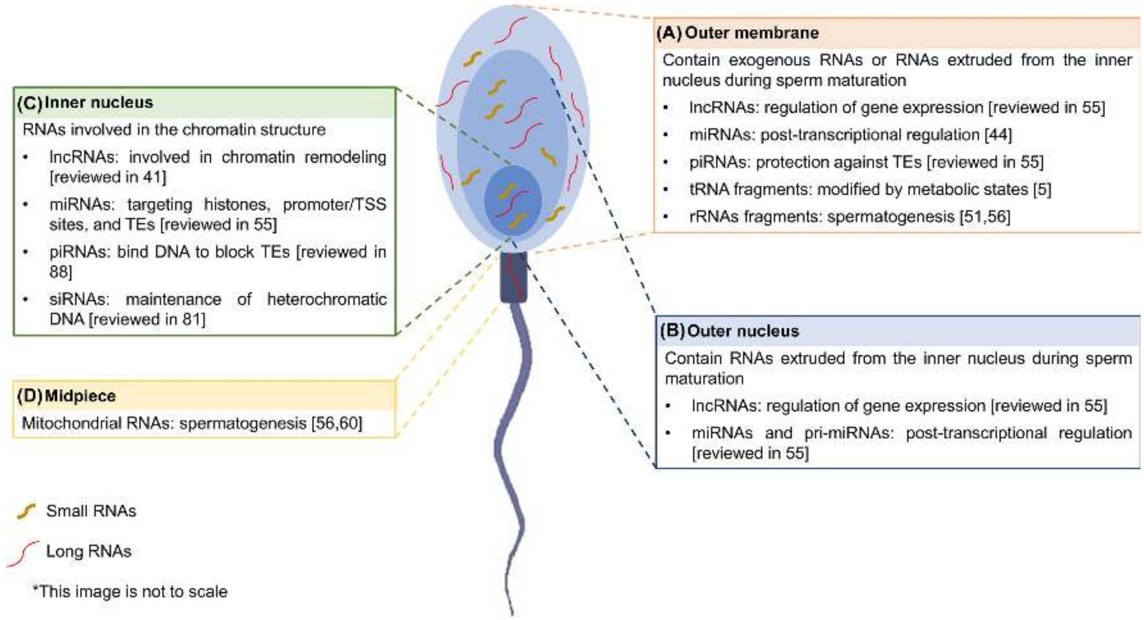

Figure 3. Distribution of sperm RNAs and their roles. (**A–B**) The outer nuclear layers indicated in light and medium blue contain ~2/3 of all sperm RNAs (including coding and noncoding), the majority of which are long RNAs (>200 nt). These RNAs include exogenous RNAs packaged within vesicles and RNAs extruded from the inner nucleus either bound to associated proteins or within vesicles during cytoplasmic extrusion. The remaining ~1/3 of total sperm RNAs reside within the dark blue inner nuclear fraction. (**C**) RNAs found within the inner nuclear fraction are likely directly associated with the DNA or chromatin bound proteins to influence chromatin structure and may provide an epigenetic signature. Examples include the chromatin associated RNAs, such as lncRNAs, piRNAs, miRNAs, and siRNAs. (**D**) Mitochondrial RNAs have been observed in the midpiece of the spermatozoa. lncRNAs (long noncoding RNAs); miRNAs (microRNAs); piRNAs (Piwi-interacting RNAs); pri-miRNAs (primary miRNAs); siRNAs (small interfering RNAs); TEs (transposable elements); tRNAs (transfer RNAs); TSS (transcript start site); rRNAs (ribosomal RNAs).

to specifically mark the response. Offspring developed a depressed hypothalamic-pituitary-adrenal (HPA) response in the absence of their father or shared paternal environment. This depressed HPA response was modulated by altered expression in the paraventricular nucleus of the hypothalamus [7]. Microinjection of the nine miRNAs into the wild-type oocyte resulted in zygotes expressing the same altered pattern of gene expression [7]. An acute stress mouse model resulted in depressive-like behaviors, exhibiting altered levels of both sperm miRNAs and piRNAs compared to controls [6]. Male offspring presented a similar depressive-like behavior as the father, in addition to altered levels of miRNAs within different brain regions and in serum. Interestingly, male offspring also exhibited altered glucose and insulin metabolic traits [6]. These results suggested that behavioral phenotypes could be transmitted down the male line, in the absence of paternal rearing, using an RNA mechanism [6, 7].

The impact of paternal diet has also drawn attention. For example, male mice subjected to a high-fat-high-sugar diet presented upregulated levels of miR-19b [13]. When miR-19b was microinjected into zygotes, offspring presented both obesity and reduced glucose tolerance, which persisted through several generations [13]. A low-protein diet in mice has been found to alter levels of sperm miRNAs, piRNAs, and several tRNAs [8]. In mice, alterations in miRNA levels as well as 5-methylcytidine (m^5C) and N^2-methylguanosine (m^2G) modified tRNAs in sperm have also been observed in response to a high-fat diet [5]. Microinjection of total sperm RNA from high-fat diet mice at fertilization resulted in offspring exhibiting impaired glucose tolerance, although insulin sensitivity was not observed [5]. This inferred that while modified sperm miRNA and tRNA may result in inherited metabolic traits, other layers of regulation may be involved in the inheritance of metabolic diseases such as diabetes. In humans, epidemiological studies on male-line transmission as exemplified in those of a Swedish Överkalix cohort [15, 118] span at least three generations. The impact of a grandfather's access to excess food during his childhood correlated with a higher incidence of both diabetes-related mortality and mortality risk ratios in his grandsons [15, 118], while fathers with low food access as a child correlated with a decreased mortality by cardiovascular disease in their sons [118]. These results suggested a role of paternal influence in transgenerational responses in humans. As measured by body mass index (BMI), male obesity has been shown to alter the human sperm methylome, with its reversal following bariatric surgery [119]. While alterations of sperm sncRNAs were also identified [119], their association was not well-defined. Nevertheless, these results identify potential alterations in epigenetic effectors that may be transferred to the oocyte at fertilization, to influence embryo development and potentially be inherited by offspring.

Challenges and questions that remain

The low viability of parthenogenic mice [120] from two maternal genomes sheds light on the importance of sperm beyond providing genomic information for successful zygote and embryo formation. The role of sperm RNAs is indicative of the intricate regulatory mechanisms of spermatogenesis, fertilization, and embryogenesis. Different classes of sperm RNAs have been found to act with different *modus operandi* to maintain the integrity of the genome and regulate gene expression, and chromatin state (Figure 3).

Currently, 10–15% of reproductive aged couples are affected by infertility [reviewed in 55]. Existing diagnostics rely on observable semen parameters [reviewed in 65] leaving many cases of male infertility unexplained. Differential RNA profiles exhibited by infertile men have been identified [reviewed in 4, 53, 55], and altered presence and/or abundance of RNAs are being used as molecular biomarkers to address reproductive health concerns. This includes the 648 sperm REs that appear essential for natural conception [53]. Circular RNAs which can be stable at room temperature for several hours [reviewed in 75] may offer yet another avenue. Factors such as advanced paternal age and increased BMI have been amplified in recent years [reviewed in 121, 122], and are increasingly linked to reproductive success. Standard semen parameters decline with advanced age [123] and increased BMI [124]. Similarly, both paternal age and BMI impact the potential for successful live birth [125, 126]. It is likely that these factors, among others, correlate with reproductive potential through the alteration of sperm RNAs. While diet modulates RNA abundance in sperm [5], these alterations have not been assessed in terms of reproductive potential, although work on this subject should be forthcoming.

Sperm RNAs used as biomarkers are also being developed in livestock, both as a model system and for enhanced breeding. In agriculture, identification of genetic markers of sperm quality, fertility, and those minimizing frozen-thawed cryo-damage is presently being pursued to optimize animal selection [50, 127–129]. Bovine is a well-studied agricultural species due to its high economic impact. The corresponding sperm microarray studies have focused on sperm quality [127], fertility [130], and cryo-damage [131], while NGS technology is beginning to be applied to sperm motility [52]. Other agricultural species including stallion [50], porcine [128], and chicken [129] are still focused on the fundamental profiling of sperm RNAs. As the cost of sequencing decreases the potential impact for agriculture and novel studies continues to grow.

Research focusing on the concept of health maintenance and lifestyle continues to move forward. Given the comparatively long-generation times between humans as compared to mice [132], the majority of human studies addressing epigenetic inheritance are epidemiologically based [15, 118]. Their significance is highlighted by the observation that access to food during a male's slow growth period correlates with the risk of diabetes and cardiovascular-related mortality, as well as overall mortality risk, in sons and grandsons. The age of onset of smoking of a father has also been suggested to be correlated with the son's BMI [15]. These studies while limited have identified that responses to the environment appear to reflect sex-specific transfer between generations. One must also consider that unlike females, males experience continual gamete (sperm) renewal, which can act to "wash out," or reset the effect of a given exposure (e.g., diet, exercise, stress level). This capability affords males the unique possibility to marginalize the transfer of a potentially harmful effect to their children [reviewed in 121]. In this model, alterations in sperm methylation or sncRNAs resultant from a lifestyle choice like diet may be at least partially restored by a simple change in caloric intake.

The relationship between the father's diet and smoking status during his prepubescent, slow growth period, on the health of his children [15, 118] suggests there are critical times of exposure. Epidemiological studies in human suggest that a critical time of exposure for males occurs prior to the onset of puberty, between 9 and 12 years of age [118]. Mouse models and human studies [reviewed in 133] have highlighted the impact of in utero environment as a time of relative phenotypic plasticity [reviewed in 134], and suggest this as a critical time of exposure for male-line epigenetic inheritance [134, 135].

The focus of most studies surrounds a single system input, but this does not reflect the intricate network of inputs that constitute allostatic load. For example, the common functional measure of obesity, BMI, will be influenced by multiple factors, including diet and exercise. While animal models provide a means to better control for these cofounding factors [132], this is rarely possible in human studies. A limited number of studies have begun to examine the relationship between age and diet with respect to this paradigm. On one hand, mouse model studies have identified that undernourishment of males in utero can lead to inheritance of altered DNA methylation via sperm [134], yet this effect was not identified if the period of undernourishment followed birth [135]. On the other hand, human epidemiological studies [15, 118] noted that the age at which a male experiences malnutrition impacts the health of multiple generations. This may be in part due to age of exposure impacting the ability for the alteration to be "washed out."

The fertilizing spermatozoon is essentially a dynamic single-cell system that contains half of the information for the next generation in which a single cell is selected in some manner from millions. The field continues to seek to understand how specific paternal components and their respective mechanisms influence reproductive health. The potential importance of sperm RNAs in the maintenance and transference of biological information between generations underscores the necessity of achieving an understanding. Perhaps, this provides a mechanism to assess specific fitness of a new trait prior to adaptive selection, and in part, provide one of the components of genetic resilience [11, 136]. For now, we are hindered by the lack of a comprehensive understanding of how these epigenetic effectors are modulated.

Four fundamental questions remain with respect to paternal RNAs: (1) Do sperm RNAs directly interact with other epigenetic effectors, perhaps directly with the genome (chromatin) or other RNAs or structures? (2) What is the role of RNAs in transmitting specific phenotypes? (3) What is the corresponding mechanism by which this is achieved? (4) How may we modify the future-past in a controlled manner? Addressing each of these questions in this unique human single-cell system will require the development of new molecular and computational tools. The answers will likely revolutionize our understanding of reproduction and health, as the totality of the male contribution reflective of his past experiences that impacts the future birth and life course of his child, becomes appreciated.

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