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human reproduction update

All you need to know about sperm RNAs

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BACKGROUND: Spermatogenesis generates a small and highly specialised type of cell that is apparently incapable of transcription and translation. For many years, this dogma was supported by the assumption that (i) the compact sperm nucleus, resulting from the substitution of histones by protamine during spermatogenesis, renders the genome inaccessible to the transcriptional machinery; and (ii) the loss of most organelles, including endoplasmic reticulum and ribosomes, limits or prevents translational activity. Despite these observations, several types of coding and non-coding RNAs have been identified in human sperm. Their functional roles, particularly during fertilisation and embryonic development, are only now becoming apparent.

OBJECTIVE AND RATIONALE: This review aimed to summarise current knowledge of the origin, types and functional roles of sperm RNAs, and to evaluate the clinical benefits of employing these transcripts as biomarkers of male fertility and reproductive outcomes. The possible contribution of sperm RNAs to intergenerational or transgenerational phenotypic inheritance is also addressed.

SEARCH METHODS: A comprehensive literature search on PubMed was conducted using the search terms 'sperm' AND 'RNA'. Searches focussed upon articles written in English and published prior to August 2020.

OUTCOMES: The development of more sensitive and accurate RNA technologies, including RNA sequencing, has enabled the identification and characterisation of numerous transcripts in human sperm. Though a majority of these RNAs likely arise during spermatogenesis, other data support an epididymal origin of RNA transmitted to maturing sperm by extracellular vesicles. A minority may also be synthesised by *de novo* transcription in mature sperm, since a small portion of the sperm genome remains packed by histones. This complex RNA

population has important roles in paternal chromatin packaging, sperm maturation and capacitation, fertilisation, early embryogenesis and developmental maintenance. In recent years, additional lines of evidence from animal models support a role for sperm RNAs in intergenerational or transgenerational inheritance, modulating both the genotype and phenotype of progeny. Importantly, several reports indicate that the sperm RNA content of fertile and infertile men differs considerably and is strongly modulated by the environment, lifestyle and pathological states.

WIDER IMPLICATIONS: Transcriptional profiling has considerable potential for the discovery of fertility biomarkers. Understanding the role of sperm transcripts and comparing the sperm RNA fingerprint of fertile and infertile men could help to elucidate the regulatory pathways contributing to male factor infertility. Such data might also provide a molecular explanation for several causes of idiopathic male fertility. Ultimately, transcriptional profiling may be employed to optimise ART procedures and overcome some of the underlying causes of male infertility, ensuring the birth of healthy children.

Key words: spermatozoa / RNA / transcriptome / male infertility / fertilisation / embryo development / epigenetics

Introduction

The spermatozoon is a highly differentiated cell characterised by its small size, lack of most typical organelles, minimal cytoplasm, compact nucleus and high motility. During spermatogenesis, most of the cytoplasm of round spermatids is depleted and the cytoplasmic droplet is phagocytosed by Sertoli cells (Santiago *et al.*, 2020). At this point, much of the RNA content and components required for translational activity (e.g. ribosomes and endoplasmic reticulum) are removed with the residual body (Ren *et al.*, 2017). The failure to identify intact 28S and 18S ribosomal RNAs (rRNA) and hence, insufficient 80S (cytoplasmic) rRNA, also questions the existence of cytoplasmic messenger RNA (mRNA) translation (Cappallo-Obermann *et al.*, 2011). Furthermore, the progressive replacement of histone by transition proteins (TPs) and subsequently by more basic protamines (PRMs) results in a highly condensed nucleus that progressively shuts down gene expression, leading to the assumption that transcription ceases at the round spermatid stage (Miller *et al.*, 2010; Ren *et al.*, 2017). For several years, these data supported the concept that sperm are 'quiescent' cells, transcriptionally inactive and devoid of translational activity, questioning the biological relevance of sperm RNA.

Sperm RNAs were first identified in human, mouse and rat sperm during the 1980s (Pessot *et al.*, 1989), with an increasing number of reports in the last 40 years describing several RNAs in the male gamete. The first attempt to characterise the complex population of human sperm RNAs was performed by Miller *et al.* (1999) using cDNA cloning and sequencing. Subsequently, several research groups identified sperm-specific RNAs using reverse transcription-PCR (RT-PCR) and *in situ* hybridisation (ISH). However, these methodologies seemingly explored only a minority of all potential sperm transcripts. The first global sperm transcriptome obtained using microarrays revealed that human sperm contain around 3000–7000 different coding transcripts (Ostermeier *et al.*, 2002). Since then, the employment of innovative and more sensitive techniques, such as RNA sequencing (RNA-seq), has facilitated the identification, quantification and characterisation of both known and unknown RNAs (Krawetz *et al.*, 2011; Sendler *et al.*, 2013). Thus, human sperm possess both coding and non-coding RNAs: mRNA, rRNA, miRNA (micro), piRNA (Piwi-interacting), lncRNA (long non-coding), siRNA (small interfering), tsRNA (transfer (t)RNA-derived small RNAs) and others (Krawetz *et al.*, 2011; Sendler *et al.*, 2013); all of these enhance the interest in sperm RNA carriage.

The comparison of sperm transcript profiles between fertile and infertile patients is highly relevant for the identification of novel biomarkers of male fertility. Similar studies may identify the mechanisms by which various environmental conditions and divergent lifestyles adversely affects spermatogenesis. Environmental (pollutants, toxins) and lifestyle (stress, cigarettes, drugs, alcohol, diet) factors represent potential causes of the reported decline in sperm quality, since both sperm RNA and protein content is strongly affected by these risk factors. Furthermore, emerging evidence suggests that the inheritance of certain traits may be associated with the sperm epitranscriptome. Herein, we provide a detailed review of contemporary literature concerning the origin, types and functional roles of sperm RNAs. Since these transcripts also provide important information concerning the developmental history of each sperm cell, we further explore the potential of employing sperm RNAs as biomarkers of male fertility and pregnancy outcomes after ART.

Origin of sperm RNAs

Testicular origin

The assumption that sperm RNAs are merely remnants of spermatogenesis that escape degradation but are not selectively retained is supported by the presence of a large subset of testis RNA (~80%) in human sperm (Fig. 1) (Wang *et al.*, 2004). Nevertheless, if there is no retention of specific transcripts, why some cytoplasmic mRNAs are retained in sperm whilst large subunits of cytoplasmic rRNAs are removed or degraded remains unresolved. Do these retained transcripts have a function in mature sperm? In 1976, Betlach and Erickson reported the presence of 28S and 18S rRNAs in murine epididymal sperm, suggesting that stored mRNAs could be translated (Miller and Ostermeier, 2006; Betlach and Erickson, 1976). Later, Gur and Breitbart (2006) demonstrated the *de novo* translation of certain human and bovine cytoplasmic sperm mRNAs during capacitation, using the uptake of ^{35}S-methionine-^{35}S-lysine and BODYPY lysine tRNAs in the presence or absence of translation inhibitors. Interestingly, the same authors demonstrated that protein synthesis was attenuated by D-chloramphenicol, a selective inhibitor of mitochondrial translation (Gur and Breitbart, 2006). Proteomic analyses further revealed the reduced expression of 44 proteins in D-chloramphenicol-treated sperm (Zhao *et al.*, 2009), supporting a potential role for mitochondrial ribosomes in *de novo* sperm translation. These data were interpreted as evidence that mRNAs contribute to specialised processes, including capacitation and sperm–oocyte interaction (Zhao *et al.*, 2009), though

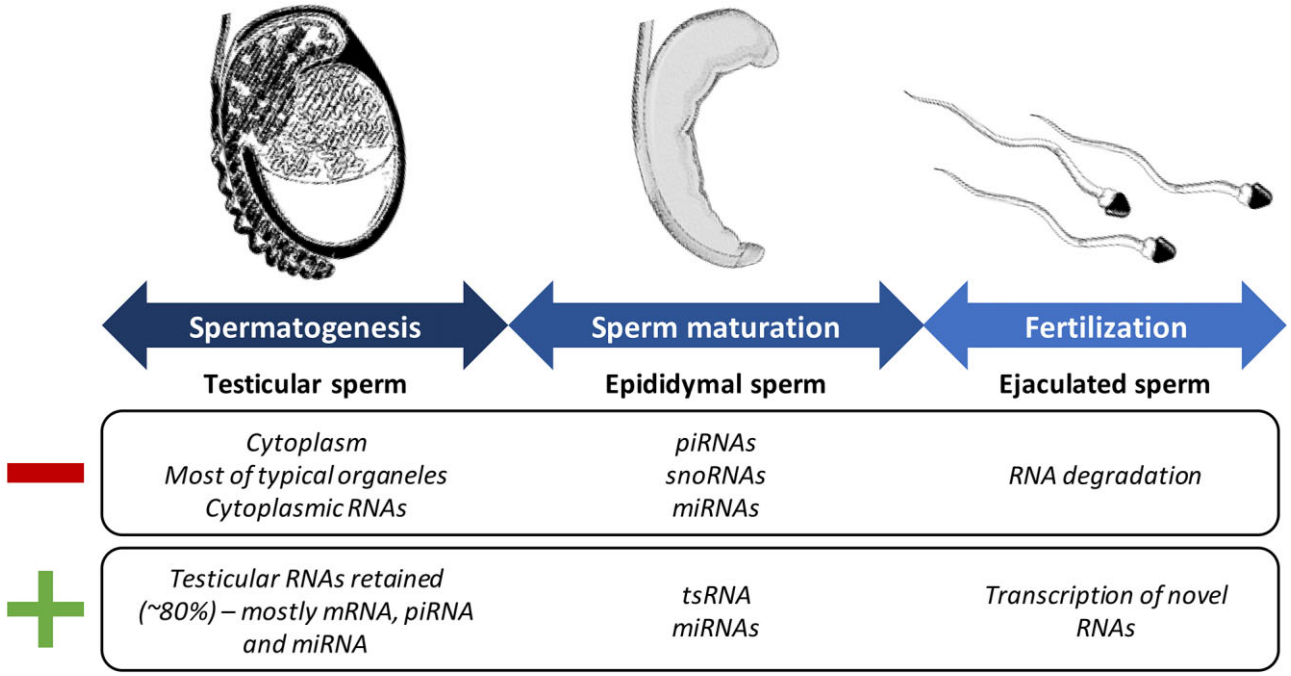

Figure 1. Origin of sperm RNA and its dynamics through the male reproductive tract. During spermatogenesis, testicular sperm lose most of their cytoplasm and typical organelles, and consequently, several cytoplasmic RNAs. The majority of RNAs remaining in testicular sperm are untranslated RNAs reminiscent of spermatogenesis (~80%). After leaving the testis, sperm maturate in the epididymis, acquiring motility during epididymal transit. Here, sperm are exposed to epididymal fluid and extracellular vesicles (epididymosomes), while losing most of their piRNA and snoRNA content and gaining several tsRNAs. The miRNA content of epididymal sperm is likewise strongly modulated during its journey through the different segments of the epididymis.

the evidence for *de novo* translation in human sperm remains unsubstantiated.

The structural functions of sperm RNAs might also explain their retention. During spermatogenesis a small portion of sperm DNA remains packaged by histones (Miller *et al.*, 2010). This histone-bound compartment seems to be associated with the nuclear envelope or matrix, probably on the nuclear periphery, creating a nucleohistone shell (Miller and Ostermeier, 2006). Since RNA is also a component of the nuclear envelope, these RNAs can be involved in its stabilisation and histone-bound DNA interaction. Additional evidence suggests that some of these passively retained RNAs, required for normal embryonic development, are delivered to the oocyte upon fertilisation (Jodar *et al.*, 2013; Ntostis *et al.*, 2017).

Epididymal origin

Sperm cells are morphologically mature but functionally inactive when they are released from the testis, but become function during epididymal transit upon exposure to extracellular vesicles, RNAs, proteins, ions and nutrients (Sullivan, 2016). This long-convoluted tubule is highly regionalised, with distinct profiles of both gene and protein expression within the different segments, providing a dynamic intraluminal environment to promote sperm maturation (Nixon *et al.*, 2015a). Jodar *et al.* (2016) reported the presence of 14 seminal fluid transcripts in human sperm (~6%), which were absent in the testis. These include transcripts encoding proteins secreted by epididymis (e.g. *SEMG1* and *SEMG2* transcripts) supporting the hypothesis that a subset of sperm RNAs may have an epididymal origin (Lalancette *et al.*, 2009). Epididymosomes are highly heterogeneous vesicles (40–100 nm) secreted by epididymal epithelial cells and able to transfer their contents (RNA, DNA, proteins and lipids) into surrounding cells (reviewed in da Silveira *et al.*, 2018). The regulatory RNA content (miRNA, tRNA, lncRNA, piRNA) of epididymosomes considerably varies within the different sections of the epididymis, with those released in the caput containing distinct RNAs from those released in the corpus or cauda (Reilly *et al.*, 2016; Chu *et al.*, 2019).

During sperm maturation in mouse, the profile of small RNAs in caput and cauda sperm is also dynamic (Nixon *et al.*, 2015b; Sharma *et al.*, 2018). When immature sperm enter the epididymis, most of the small non-coding RNA (sncRNA) content corresponds to piRNA (Sharma *et al.*, 2018). An enrichment of tsRNAs (Peng *et al.*, 2012; Sharma *et al.*, 2018) and rsRNA-28S (Chu *et al.*, 2017) is observed during caput to cauda transit (Fig. 1). Furthermore, despite a low abundance in epididymal sperm, small nucleolar RNAs (snoRNAs) seem to be enriched only in caput sperm while miRNA profiles also undergo substantial modifications during the caput to cauda transit (Chu *et al.*, 2019) (Fig. 1). In the past decade, several studies have indicated that small RNAs are trafficked to mammalian sperm during post-testicular maturation in the epididymis through epididymosomes (Peng *et al.*, 2012; Reilly *et al.*, 2016; Conine *et al.*, 2018; Sharma *et al.*, 2018;

Sciamanna et al., 2019). Nixon et al. (2015b) produced the first miRNA signature of maturing mouse sperm by next-generation sequencing and reported the loss of 113 miRNAs and the acquisition of 115 miRNAs between the caput and cauda epididymal sperm. Moreover, several miRNAs that are relatively rare in testicular sperm seem to be acquired upon entry into the epididymis (miR-21a, miR-29c, miR-199a, miR-200b/c, miR-10a/b) and dramatically increase during epididymal maturation (Sharma et al., 2018). Interestingly, epididymosomes are also likely to deliver tsRNAs into sperm (Sharma et al., 2016; Chen et al., 2020). Individually and collectively, these data suggest that a fraction of sperm RNAs are gained upon entry into the epididymis, supporting the existence of a soma-to-sperm RNA delivery mechanism mediated by extracellular vesicles during epididymal sperm maturation.

Transcription in mature sperm

Several RNA species exclusively present in sperm samples may contribute to fertilisation and/or post-fertilisation events. The majority of these transcripts derive from genomic regions packaged by histones, particularly those enriched in H3K4me3, suggesting that sperm chromatin is structured to enable *de novo* transcription (Sendler et al., 2013). The existence of sperm chromatin domains in a transcriptionally 'ready' state supports the contention that active genes in the paternal pronucleus of pre-cleavage stage zygotes predominantly reside in regions originally packaged by histones and thus possibly in a more open chromatin configuration. Indeed, distinct chromatin domains in the sperm nucleus may be related to sperm RNA carriage and gene expression in the zygote. In human sperm, distinctly modified histones seem to remain bound to the promoters of miRNA clusters (*let7e*, *mir-17*, *mir-15a*, *mir-96*, *mir-135b* and *mir-10a/b*) and genes associated with embryonic development (e.g. Homeobox (*HOX*) gene cluster) (Hammoud et al., 2009). Higher levels of H3K4me3 are usually associated with genes coding developmental factors such as *EVX1/2*, *ID1*, *STAT3*, *SOX7/9*, *KLF5* and *FGF9*, suggesting these are sites marked for transcription following fertilisation (Hammoud et al., 2009). In bull sperm, histones remain associated with repetitive genomic elements, such as centromeres, and with spermatogenesis-associated genes, but not with genes involved in early embryonic development (Sillaste et al., 2017). Histone-packaged genes include genes associated with the cGMP-dependent protein kinase G pathway, which is required for pre-fertilisation events, suggesting that genes needed for sperm development, maturation and fertilisation remain associated with histones (Sillaste et al., 2017). The full identification and characterisation of DNA sequences in mature sperm that are histone bound can be helpful to establish a connection between those sequences and the sperm RNA population.

The possible transcription of histone bound DNA is further evidenced by the observation that sperm contain RNA polymerase (Hecht and Williams, 1978). Hecht and Williams (1978) reported the apparent presence of RNA synthesis in the head and tail of bovine sperm, possibly resulting from the activation of a typically silent RNA polymerase. The relative abundance of transcription factors in sperm and the potential for RNA conversion into DNA by reverse transcriptase in mature mouse spermatozoa (Giordano et al., 2000) further supports the concept that sperm may be transcriptionally active. Conversely, Grunewald et al. (2005) reported a lack of *de novo* synthesis of RNA in mature human sperm, since these cells were unable to incorporate radio-labelled uridine triphosphate into RNA *in vitro*. Additional investigations are clearly warranted to resolve this persistent controversy.

Types and roles of sperm RNAs

Colloidal gold stain and electron microscopy provided the first evidence for RNA in the sperm nucleus of the fern *Scolopendrion*, and this observation was later extended to rat and human sperm (Pessot et al., 1989; Concha et al., 1993). Techniques including target-directed RT-PCR, microarrays and, more recently, RNA-seq have expanded the list of sperm RNA species, although it is noteworthy that such methodologies require a pure population of RNAs. The various methods used to purify and detect low abundant sperm RNAs have often resulted in the contamination of sperm RNA with the RNA from somatic cells. Furthermore, a single sperm contains only ~50 fg of long RNAs and ~0.3 fg of sncRNA (~200 times less than other cell types) making sperm purification crucial for the unambiguous identification of sperm transcripts (Goodrich et al., 2013). Thus, several protocols to isolate and extract high-quality sperm RNAs were developed (Goodrich et al., 2007, 2013; Mao et al., 2013; Barragán et al., 2015; Georgiadis et al., 2015; Schuster et al., 2016; El Fekih et al., 2017; Bianchi et al., 2018). In 2013, Jodar et al. (2013) reported a complex and heterogeneous population of RNAs in sperm, that comprises rRNA, mRNA and both sncRNA and lncRNA. However, the functional significance and individual importance of most sperm RNAs remains to be elucidated. The characteristics and roles of the most studied RNA classes in sperm are summarised in Table I.

Coding RNAs

The heterogeneous population of mRNAs was first investigated using several target-independent strategies, such as cloning and sequencing of randomly primed amplicons (Miller et al., 1994, 1999). Several individual transcripts were identified in human sperm using RT-PCR, including those encoding PRMs (Miller et al., 1994; Wykes, 1997; Carreau et al., 2007), TPs (Wykes, 1997), receptors (Roudebush et al., 2000; Sachdeva et al., 2000; Januchowski et al., 2004; Carreau and Galeraud-Denis, 2007) and many others (Lambard et al., 2004; Jedrzejczak et al., 2006; Carreau and Galeraud-Denis, 2007; Li et al., 2007). Additionally, the accumulation within the sperm nucleus of mRNAs encoding type II GnRH receptor (Wang et al., 2002), transcription factors NFkB, HOX2A and ICSBP, protein kinase JNK2, growth factor HBEGF and receptors RXRb and ErbB3 was demonstrated by ISH (Dadoune et al., 2005). The presence of *c-myc* mRNA in the midpiece and tail of human sperm was also reported (Kumar et al., 1993). Some of these mRNAs were found to be differentially expressed in several subfertile patients, including PRM transcripts that are strongly associated with altered semen parameters, sperm fertilisation and embryo quality (Lambard et al., 2004; Oliva, 2006; Steger et al., 2008; Jodar et al., 2012; Bansal et al., 2015; Savadi-Shiraz et al., 2015; Hamad, 2019) (Table II). Additionally, the levels of PRM2 mRNA (Nazmara et al., 2020) were also diminished in the sperm of heroin addicts, accompanied by a decrease in the levels of miR-125b-5p (Nazmara et al., 2021), aminopeptidase N and endopeptidase

Table I Summary of the types, characteristics and roles of the most studied RNA classes in sperm.

	mRNA	lncRNA	miRNA	piRNA	tsRNA
Length	**Variable**	**200–10 000 nt**	**20–24 nt**	**24–30 nt**	**18–40 nt**
Strand	Single-stranded				
Function	Chromatin remodelling; *de novo* translation	Heterochromatin formation; imprinting; DNA methylation; transcriptional and post-transcriptional regulation	Cytosolic post-transcriptional silencing; translational control; RNA degradation	Repression of retrotransposon; cytosolic post-transcriptional regulation; translational control and RNA degradation	mRNA stability, regulation, and silencing; protein binding; RNA modification, stress response
Expression	Expressed in all tissues	Expressed in all tissues	Expressed in most tissues	Mainly expressed in germ cells (spermatocytes, spermatids)	Highly expressed in sperm
Main location in sperm	Head, midpiece and tail	Head (nuclear or peri-nuclear compartment)	Head (nuclear or peri-nuclear compartment); tail	Tail	Head
Function in sperm	Fertilisation; delivery of sperm RNAs to the oocyte/early embryo development	Transgenerational inheritance; gene expression regulation/ histone modification; early embryo development	Transcriptional gene silencing; early embryonic histone replacement; early embryogenesis; epigenetic modification; sperm maturation	Protection from transposons; genome recognition and consolidation-confrontation; epigenetic modification	Epigenetic modification and transgenerational inheritance, early embryo development

neutral N mRNA and protein (Rezaei-Mojaz *et al.*, 2020). These heroin-induced alterations in sperm RNA content and semen parameters may directly affect the success of fertilisation and embryogenesis and/or can be retained for carrying epigenetic information to the zygote (Rezaei-Mojaz *et al.*, 2020).

The overall complexity of the coding transcripts of human sperm was first unveiled by microarrays. Comparison of testis-derived RNAs with the RNAs isolated from a pool of nine ejaculated sperm samples identified 7157 and 3281 transcripts, respectively (Ostermeier *et al.*, 2002). The same authors described 2780 transcripts in a single sperm sample, all present in the RNA derived from testis, which highlighted the potential of sperm RNAs to monitor past events of gene expression during spermatogenesis (Ostermeier *et al.*, 2002). By profiling sperm RNAs after different cycles of freezing and thawing (Ostermeier *et al.*, 2005a), it was demonstrated that sperm contain a stable set of full-length transcripts (n = 36), some of which may be implicated in male fertility regulation. Later, a set of 430 stable RNAs in sperm samples from fertile donors was identified, being the starting point for the development of an objective marker of normal male fertility (Lalancette *et al.*, 2009). Serial analysis of gene expression (SAGE) in human sperm identified 2712 unique transcripts from ten pooled sperm samples and 2459 in one individual ejaculated sample, and only 564 overlapped unique tags, providing evidence of inter-sample variation (Zhao *et al.*, 2006). These technologies were adopted in clinics to assess altered mRNA profiles in asthenozoospermic (Jodar *et al.*, 2012; Bansal *et al.*, 2015; Caballero-Campo *et al.*, 2020), teratozoospermic (Platts *et al.*, 2007), oligozoospermic (Montjean *et al.*, 2012), idiopathic infertile (Garrido *et al.*, 2009; García-Herrero *et al.*, 2010a; Bansal *et al.*, 2015) and cryptorchids (Nguyen *et al.*, 2009) males, highlighting the potential of mRNA profiles as biomarkers of male fertility (Table II).

Mature sperm have RNA isoforms distinct from those found in testis, suggesting the synthesis of transcript variants through alternative splicing or the use of alternative promotors during the final stages of spermatogenesis. Those sperm-specific transcripts include the sperm-specific isoform of pyruvate kinase isozymes M1/M2 (PKM2) which regulates glucose metabolism (Sendler *et al.*, 2013). Almost 25% of sperm transcripts show alternative polyadenylation sites which maintain the integrity of the coding region. However, these mRNAs have short 3′ untranslated regions (UTRs), which is common in testes and may be important for modulating the stability, location and/or transport of the transcripts (Jodar *et al.*, 2013). This structural feature also controls the binding of regulatory proteins and miRNAs to the UTR, modulating translation in sperm (Jodar *et al.*, 2013).

Usually, most coding RNAs present in human sperm are fragmented (Jodar *et al.*, 2013; Sendler *et al.*, 2013); nevertheless, some of the mRNAs present are intact, increasing the opportunity for regulatory interactions (Jodar *et al.*, 2013). The ontological analysis of the minor fraction of intact transcripts revealed enrichment of male infertility-associated genes, as well as genes involved in fertilisation and early embryo development (Sendler *et al.*, 2013). Despite most transcripts being involved in sperm physiology, fertilisation or early embryogenesis

Table II Studies investigating sperm RNA as biomarkers of men's fertility and health and RNAs differentially expressed in each condition studied.

Study	Condition (vs control)	RNA type	Method	Differentially expressed RNAs
(Jedrzejczak *et al.*, 2006)	Infertile men	mRNA	RT-qPCR	*CCR5* and *P450arom* mRNA (down)
(Avendano *et al.*, 2009)	Infertile men	mRNA	RT-qPCR	*PSG1* and *HLA-E* mRNA (down)
(Garrido *et al.*, 2009)	Idiopathic infertile men	mRNA	Microarray RT-qPCR	*TRY1*, *GGT1* and *CAB39L* mRNA (down)
(Depa-Martynow *et al.*, 2012)	Idiopathic or mild male factor infertile men	mRNA	RT-qPCR	*PRM1/PMR2* mRNA (down)
(Rogenhofer *et al.*, 2013)	Subfertile men	mRNA	RT-qPCR	*PRM1/PRM2* mRNA (down)
(Bansal *et al.*, 2015)	Idiopathic infertile and asthenozoospermic male	mRNA and lincRNAs	Microarray	Asthenozoospermic: *RPL24, RPL4, RPL9, RPL18, RPL11, RPL28, RPL35 and RPS16, CANX, NONO, RHOA, OAZ1, FAU, SLC25A3, HNRNPM, C1D, PRPF8, HTN3, CERCAM, GDI2, PARK7* (up); *RPS13, RPL27, RPS24, RPS11, RPS5, RPS27A, RPL30, RPL34, RPS25, DAD1, ILF2, SRSF9, HSP90AB1, EIF4G2, HNRNPC, SMARCAD1, HINT1, KIFAP3* (down) Idiopathic infertility: *RPS25, RPS11, RPS13, RPL30, RPL34, RPL27, RPS5, HINT1, HSP90AB1, SRSF9, EIF4G2, ILF2, RPL9, OAZ1, RPL18, RPL35, FAU, CAPNS1, FAM153C, ARF1, CFL1, RPL19, USP22* (up); *DAD1, ZNF90, SMNDC1, c14orf126, HNRNPK* (down)
(Hamad, 2019)	Infertile couples	mRNA	RT-qPCR	*PRM1, PMR2, H2A, H2B* mRNA (down)
(Hazem *et al.*, 2020)	Infertile men	mRNA	RT-qPCR	*CBR1* and CBR2 mRNA (down)
(Liu *et al.*, 2010)	Asthenozoospermic men	mRNA	RT-qPCR	*VDAC2* mRNA (up)
(Jodar *et al.*, 2012)	Asthenozoospermic men	mRNA	Microarray	*HNRPA3, MT-ND2, ANXA2, LOC100289246, FLJ45445, ANKRD9, OAZ3, C1orf148, FLJ45340, C7orf30, MT-ND3, LOC100132147, GABRB1, TBC1D3, LOC474358, BRD2, PRM1* and *PRM2* (down); *FLJ32605, DKFZP434B061* (up)
			RT-qPCR	**Validated by PCR:** *ANXA2, BRD2* and *OAZ3* mRNA (up)

Continued

Table II Continued

Study	Condition (vs control)	RNA type	Method	Differentially expressed RNAs
(Chen *et al.*, 2012)	Asthenozoospermic and oligoasthenozoospermic men	mRNA	RT-qPCR	*NRF2* mRNA (down)
(Pelloni *et al.*, 2018)	Asthenozoospermic men	mRNA	RT-qPCR	*ROPN1* and *CABYR* mRNA (down)
(Caballero-Campo *et al.*, 2020)	Asthenozoospermic high (A-F1) and low (A-F2) motile fraction vs normozoospermic high (N-F1) and low (N-F2) motile fraction	mRNA	Microarray	A–F1 vs N–F1: 116 up- and 1747 down-regulated genes A–F2 vs N–F2: 17 up- and 190 down-regulated genes N–F1 vs N–F2: 7 up- and 54 down-regulated genes A–F1 vs A–F2: 42 up- and 866 down- regulated genes
(Zheng *et al.*, 2011)	Oligoasthenozoospermic men	mRNA	RT-qPCR	*BDNF* mRNA (down)
(Steger *et al.*, 2008)	Oligozoospermic men	mRNA	RT-qPCR	*PRM1* mRNA (down) and *Bcl2* mRNA (up)
(Montjean *et al.*, 2012)	Oligozoospermic men	mRNA	Microarray RT-qPCR	157 transcripts up- or down-regulated. **Validated by PCR:** *TPD52L3*, *PRM2*, *JMJD1A and NIPBL* (down)
(Savadi-Shiraz *et al.*, 2015)	Teratozoospermic men	mRNA	RT-qPCR	*PRM1* and *PRM2* (down); *TNP2* (up)
(Giebler *et al.*, 2018)	Normozoospermic vs non-normozoospermic men	mRNA	RT-qPCR	*PIWI-LIKE 1* (up) and 2 (down) mRNA
(Ferlin *et al.*, 2010)	Varicocele patients	mRNA	RT-qPCR	Varicocele and oligozoospermia: *HSPA4*, *HSF1*, *HSF2* mRNA (up); Varicocele: *HSFY* (up)
(Linschooten *et al.*, 2009)	Smokers	mRNA	Microarray	781 genes differentially expressed
(Depa-Martynów *et al.*, 2007)	Patients in which IVF failed	mRNA	RT-qPCR	*Fertilin β*, *PRM1*, *PRM2* (down)
(García-Herrero *et al.*, 2010b)	Sperm samples with which pregnancy was not achieved with IUI (NP)	mRNA	Microarray	741 transcripts absent and 976 transcripts exclusively expressed in group NP
(García-Herrero *et al.*, 2010a)	Sperm samples with which pregnancy was not achieved with ICSI (NP)	mRNA	Microarray RT-qPCR	44 sequences down-regulated and 5 up-regulated in group NP **Validated by PCR:** *IFI30*, *S100A6*, *CTSZ* (down) and *COX7B2* (up)
(Bonache *et al.*, 2012)	Normozoospermic donors used for therapeutic IUI vs Normozoospermic general donors	mRNA	TaqMan Arrays RT-qPCR	*EIF5A*, *RPL13*, *RPL23A*, *RPS27A*, *RPS3*, *RPS8* and *TOMM7* mRNA **Validated by PCR:** *EIF5A*, *RPL13*, *RPL23A* and *RPS27A* mRNA
(Marczylo *et al.*, 2012)	Smokers vs non-smokers	miRNA	Microarray	

Continued

Table II Continued

Study	Condition (vs control)	RNA type	Method	Differentially expressed RNAs
				hsa-mir-365, hsa-mir-944, hsa-mir-1267, hsa-mir-340, hsa-mir-4513, hsa-let-7a-2-3p, hsa-mir-576-3p, hsa-mir-576-5p, hsa-mir-1246, hsa-mir-30c, hsa-mir-933, hsa-mir-7, hsa-mir-1285, hsa-mir-1270, hsa-mir-509-5p, hsa-mir-146b-3p, hsa-mir-3145-3p, hsa-mir-4748, hsa-mir-519d, hsa-mir-550a, hsa-mir-550b (up); hsa-mir-574-5p, hsa-mir-3145-5p, hsa-mir-146b-5p, hsa-mir-634, hsa-mir-129-3p, hsa-mir-652, hsa-mir-4723-5p (down)
			RT-qPCR	**Validated by PCR:** miR-340, miR-365 (up); miR-129-3p and miR-634 (down)
(Metzler-Guillemain *et al.*, 2015)	Smokers vs non-smokers	miRNA	Microarray	5 mRNA and 16 miRNAs up-regulated, and 10 mRNAs and 7 miRNAs down-regulated.
			RT-qPCR	**Validated by PCR:** hsa-miR-296-5p, hsa-miR-3940 and hsa-miR-520d-3p (down)
(Li *et al.*, 2012)	Men living in an environment contaminated with electronic waste vs Men living in a normal environment	miRNA	Microarray	73 significantly up-regulated and 109 down-regulated miRNAs **Validated by PCR:** hsa-miR-208a, hsa-miR-155, hsa-miR-222, hsa-miR-183, hsa-miR-205, hsa-miR-106a, hsa-miR-33b, hsa-miR-223, hsa-miR-10b (up); hsa-miR-363, hsa-let-7d (down)
(Abu-Halima *et al.*, 2013)	Asthenozoospermic and oligoasthenozoospermic men	miRNA	Microarray	Asthenozoospermic: 50 miRNAs up-regulated, and 27 miRNAs down-regulated
			RT-qPCR	Oligoasthenozoospermic: 42 miRNAs up-regulated, and 44 miRNAs down-regulated **Validated by PCR:** miR-34b, miR-122 and miR-1973 (asthenozoospermic); miR-34b, miR-34b*, miR-15b, miR-34c-5p, miR-122, miR-449a, miR-1973, miR-16, and miR-19a (oligoasthenozoospermic)
(Abu-Halima *et al.*, 2014)	Subfertile, non-obstructive azoospermia [NOA] patients	miRNA	RT-qPCR	hsa-miR-34b, hsa-miR-34b, hsa-miR-34c-5p and hsa-miR-122 (down); hsa-miR-429 (up)
(Said *et al.*, 2014)	Asthenozoospermic, teratozoospermia and infertile male	mRNA	RT-qPCR	P450arom mRNA (down)

Continued

Table II Continued

Study	Condition (vs control)	RNA type	Method	Differentially expressed RNAs
(Salas-Huetos *et al.*, 2016)	Infertile men	miRNA	RT-qPCR	45 up- and 12 down-expressed miRNAs: hsa-miR-518f-3p, hsa-miR-208a, hsa-miR-34a-5p, hsa-miR-636, hsa-miR-520d-3p, hsa-miR-708-5p, hsa-miR-483-5p, nhsa-miR-212-3p, hsa-miR-1254, hsa-miR-324-3p, hsa-miR-491-5p, hsa-miR-564 (down); hsa-miR-149-3p, hsa-miR-596, hsa-miR-346, hsa-miR-1296, hsa-miR-1298, hsa-miR-30d-3p, hsa-miR-935, hsa-miR-766-3p, hsa-miR-659-3p, hsa-let-7a-5p, hsa-miR-103a-3p, hsa-miR-193b-5p, hsa-miR-339-5p, hsa-miR-365a-3p, hsa-miR-942, hsa-miR-22-3p, hsa-let-7f-5p, hsa-miR-130b-5p hsa-miR-644a, hsa-miR-432-3p, hsa-miR-552, hsa-miR-487a, hsa-miR-526b-5p, hsa-miR-744-5p, hsa-miR-145-5p, hsa-miR-543, hsa-miR-222-5p, hsa-miR-517-5p, hsa-miR-340-3p, hsa-miR-296-5p, hsa-miR-518d-5p, hsa-miR-520, hsa-miR-93-3p, hsa-miR-132-5p, hsa-miR-520c-3p, hsa-miR-622, hsa-miR-573, hsa-miR-9-3p, hsa-miR-181a-2-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-28-5p, hsa-miR-331-3p, hsa-miR-15b-5p, hsa-miR-183-3p (up)
(Salas-Huetos *et al.*, 2015)	Infertile (asthenozoospermic, teratozoospermic, oligozoospermic) men	miRNA	TaqMan miRNA array	32 differentially expressed miRNAs in the asthenozoospermic group (26 up- and 6 down-regulated), 19 in the teratozoospermic group (11 up- and 8 down-regulated), and 18 in the oligozoospermic group (3 up-regulated and 15 down-regulated) in relation to fertile control group; **Validated by PCR:** hsa-miR-34b-3p (age); hsa-miR-629-3p (motility); hsa-miR-335-5p, hsa-miR-885-5p, and hsa-miR-152-3p (concentration)
(Muñoz *et al.*, 2015)	Oligozoospermic men	miRNA	RT-qPCR	miR-449a, miR-34c-5p, miR-34b and miR-122 (down) **Validated by PCR:** miR-34b and miR-122

Continued

Table II Continued

Study	Condition (vs control)	RNA type	Method	Differentially expressed RNAs
(Zhu et al., 2019)	Oligospermic men	miRNA	RT-qPCR	miR-122-5p (down)
(Wenhao et al., 2015)	Oligoasthenozoospermic men	miRNA	Microarray	32 miRNAs showed significant differences in expression between the OA and NC groups **Validated by PCR:** Hsa-miR-15a Hsa-miR-19b Hsa-miR-21 Hsa-miR-23a Hsa-miR-24 Hsa-miR-27a, Hsa-miR-142-5p (up); Hsa-miR-132, Hsa-miR-449a Hsa-miR-552 (down)
(Zhou et al., 2016)	Asthenozoospermic men	miRNA	RT-qPCR	miR-27a (up)
(Heidary et al., 2019)	Asthenozoospermic men	miRNA	RNA-seq	miR-206, miR-1-3p, miR-888-3p, miR-1299 (up); miR-625-3p, miR-7153-5p, miR-328-3p, miR-92b-3p, miR-27a-5p, miR-143-5p, miR-194-3p, miR-1273g-3p, miR-197-3p, miR-296-5p, miR-6510-3p, miR-3609, miR-4326, miR-26b-3p (down) **Validated by PCR:** miR-888-3p (up)
(Heidary et al., 2020)	Asthenozoospermic vs men	miRNA	RT-qPCR	miR-4485-3p (down)
(Cui et al., 2018)	Patients with idiopathic male infertility who had undergone first ICSI vs Fertile patients	piRNA	RT-qPCR	hsa-piR-31704, hsa-piR-39888 (down)
(Zhang et al., 2015)	Asthenozoospermic vs oligoasthenozoospermic men	lncRNA HOTAIR	RT-qPCR	HOTAIR (down)

(Table I), the roles of others, such as Type 2 Taste Receptors transcripts (Governini et al., 2020), were not clearly elucidated.

lncRNA

Long non-coding RNAs (lncRNAs) are large RNA transcripts (~200–10 000 nt) whose genes are dispersed throughout the genome and are usually classified according to their position in relation to coding genes (reviewed by Rinn and Chang, 2012). In somatic cells, lncRNAs seem to be involved in heterochromatin formation and imprinting, DNA methylation and transcriptional and post-transcriptional events (Rinn and Chang, 2012). In testis, they have an important role in spermatogenesis regulation, specifically the abundant antisense type (Liang et al., 2014) (Table I).

In sperm, most lncRNAs are enriched in the outer membrane and in the perinuclear/nuclear theca (Johnson et al., 2015). A specific class of lncRNAs derived from the reverse strand, natural antisense transcripts (NATs), is present in mature sperm (Ostermeier et al., 2005b; Sendler et al., 2013). NATs overlap with coding transcripts and are involved in gene silencing, selective transcript editing, promoter inactivation and epigenetic modification of the genome. In sperm, some NATs overlap genes associated with the first stages of embryogenesis, suggesting that they may have important roles in fertilisation and early embryo development (Ostermeier et al., 2005b). Additional lncRNAs described in human sperm include chromatin-associated RNA (CAR) and small-nuclear ILF3/NF30-associated RNA (snaR). CARs include transcripts of intergenic or intronic regions of the genome, which are associated with chromatin and may act by influencing genome architecture or regulating gene expression (Jodar et al., 2013). A significant number of unidentified CARs in sperm may be required for the packaging of the paternal genome (Jodar et al., 2013).

Despite being more abundant in sperm than in testes, the function of sperm lncRNAs is poorly understood. Evidence from mouse suggested a possible role of lncRNAs in intergenerational inheritance (Jiang et al., 2016). Sperm from diabetic mice showed altered lncRNA and mRNA expression profiles, compared with the control group (Jiang et al., 2016). These authors identified 4134 up-regulated and 3407 down-regulated lncRNAs in diabetic mice by microarray analysis,

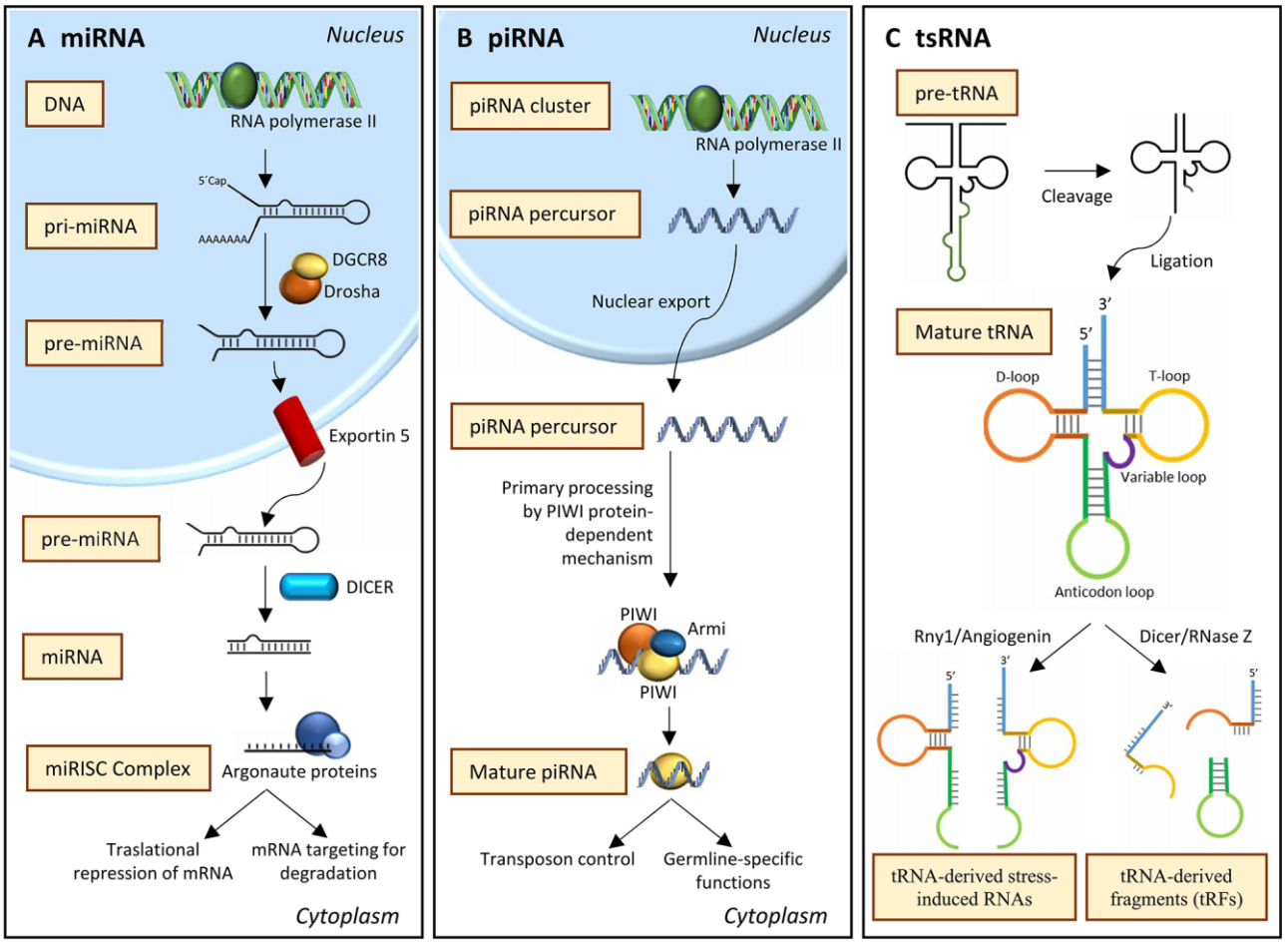

Figure 2. Synthesis of miRNA, piRNA and tsRNA and their main functions. (**A**) miRNA is transcribed in the nucleus from coding or non-coding genes by RNA polymerase II, as miRNA precursors (pri-miRNA) (Khawar *et al.*, 2019). Pri-mRNAs are further processed to an intermediate form by DROSHA (Ribonuclease 3) and DGCR8 (DiGeorge syndrome critical region 8), then transported to the cytoplasm via exportin-5′ and maturated by an RNase III endonuclease (DICER) to their functional form (20–24 nt) (Khawar *et al.*, 2019). One strand of miRNA is incorporated into an Argonaut containing ribonucleoprotein particles (RNP) leading to the formation of a miRNA-induced silencing complex (miRISC) (Khawar *et al.*, 2019). The other strand is usually degraded. miRNAs regulate gene expression by inhibiting or activating translation, or by targeting mRNA for degradation, through the binding of a 3′ UTR target sequence. (**B**) piRNAs are usually organised in the genome as clusters ranging up to 100 kb in size and, contrary to miRNAs, they are not associated with histones and exhibit less CpG enrichment. They typically have uracil at the first position and result from the processing of precursors to their mature form of 24–30 nucleotides (nt) by a PIWI protein-dependent mechanism (Krawetz *et al.*, 2011). piRNAs interact with the piwi-family proteins Piwi-like protein 1 (MIWI), Piwi-like protein 4 (MIWI2) and Piwi-like protein 2 (MILI). Some piRNAs regulate the expression of the associated protein-coding gene (Pantano *et al.*, 2015) and protect the genome from the adverse effects of invasive elements, such as transposons. (**C**) tsRNA results from the cleavage of transfer RNA (tRNA) or pre-tRNA by endonucleases (revised by Jin and Guo, 2019). Two main types of tsRNAs can be produced, according to their length and the region of tRNAs from which they are derived: (i) tRNA-derived stress-induced RNAs (tiRNAs), that are 5′- or 3′-tRNA halves of 30–40 nt, generated by tRNA cleavage by ribonuclease Rny1 and angiogenin near or in the anticodon loop; and (ii) tRNA-derived fragments (tRFs), which are 18–22 nt sequences generated from mature tRNAs or precursors by the nucleases Dicer or RNase Z (Jin and Guo, 2019).

but the exact functions of most of these transcripts are still unknown (Jiang *et al.*, 2016). Nevertheless, the lncRNAs Rpl31-ps7, AB352974, Rpl35a-ps7, Rn4.5s and XLOC_01985 were associated with diabetes-related phenotypes (Jiang *et al.*, 2016), being attractive biomarkers of diabetes-associated male infertility. The expression of the lncRNA HOTAIR is down-regulated in the sperm of patients with asthenozoospermia and oligoasthenozoospermia, which leads to lower levels of histone H4 acetylation of the *NRF2* gene promoter resulting in down-regulation of NRF2 expression (Zhang *et al.*, 2015) (Table II). Low levels of HOTAIR are also associated with a decline in semen parameters, especially poor motility and vitality, but not concentration. The identification of altered lncRNAs in the sperm of men with harmful lifestyles also supports the possible involvement of this RNA type in epigenetic inheritance, as previously described in mouse. Alcohol

consumption, a major sociodemographic and lifestyle factor, was strongly associated with an increase in the large RNAs of ejaculate sperm (Bianchi et al., 2019). Total sperm counts and sperm concentration were inversely associated with large RNA content, whilst sperm motility was inversely associated with both large and small RNA content (Bianchi et al., 2019). Cadmium also seems to modulate lncRNA profiles, impacting spermatogenesis and male fertility (Gao et al., 2017). Thus, lncRNAs may have a role in the regulation of gene expression by causing epigenetic modifications, including histone modifications (Zhang et al., 2015), as well as in early embryo development and, possibly, in transgenerational inheritance. However, compared to mRNA, miRNA and piRNA, information concerning the function of lncRNA in sperm is still scarce and this topic deserves further attention.

sncRNAs

The sequencing of sncRNAs (18–30 nt) in the sperm of normozoospermic men revealed significant complexity, including four major classes, repetitive elements, transcription start sites (TSS)/promoter-associated (quiescent (q)RNAs), miRNA and piRNA as well as other less studied classes (small nuclear (sn)RNA, snoRNA, tsRNA and YRNA) (Krawetz et al., 2011). Interestingly, 20–60% of the sequences mapped in this study were donor-specific, showing sample heterogeneity (Krawetz et al., 2011). Except for piRNAs that are more abundant in the sperm tail, small RNAs are usually enriched in the sperm nucleus (Peng et al., 2012).

It seems likely that sperm small RNAs may have roles in gene expression, chromatin remodelling and genome protection against transposition during spermatogenesis. Their roles in epigenetic inheritance were also illustrated by Mansuy and colleagues, whose studies indicated that traumatic stress in early life induced alterations in the production of several miRNAs and piRNAs in mouse sperm (Gapp et al., 2014). Such changes modified the progeny's behaviour, cognitive function, metabolism and stress-induced glucose release, and such features were transmitted to the subsequent generations (Gapp et al., 2014). Besides being risk factors for male infertility, data from animal models have confirmed that diet-induced obesity and metabolic disorders may also be passed to the offspring by paternal sncRNAs (recently reviewed by Nätt and Öst, 2020).

miRNA

The most well-characterised class of sncRNAs is miRNA, a family of short single-stranded RNAs that regulate gene expression by inhibiting/activating translation or by targeting mRNA for degradation (Fig. 2A, Table I). A single miRNA can target many mRNAs modulating the expression of thousands of proteins, and an individual mRNA can be targeted by several miRNAs. miRNAs were first identified in human sperm (Ostermeier et al., 2005b) and later in mouse and porcine sperm (Krawetz et al., 2011), being located mainly in the sperm tail (Schuster et al., 2016).

Most sperm miRNAs are also found in the testis and, most of their predicted 3′ UTR targets are absent in mature sperm (Krawetz et al., 2011). A large set of sperm miRNAs associated with TSS/promoters, histones and repetitive elements (Krawetz et al., 2011; Jodar et al., 2013), are possibly important in the regulation of early embryonic histone replacement, in the control of early embryonic expression and in epigenetic modification (Johnson et al., 2011a; Yuan et al., 2016). Studies from mouse showed that miRNAs regulate several stages of spermatogenesis (Luo et al., 2015) and deregulation of miRNA expression has been implicated in male fertility, leading to sperm abnormalities and spermatogenic arrest (Yao et al., 2015; Gao et al., 2019; Li et al., 2019). Recent studies revealed an altered miRNA profile in semen, seminal plasma and sperm of asthenozoospermic patients (Abu-Halima et al., 2013; Salas-Huetos et al., 2015; Zhou et al., 2016; Heidary et al., 2019), in accordance with data from animal models. Sperm produced by fertile men is enriched in miRNAs that regulate genes involved in cell differentiation, development, morphogenesis and embryogenesis (Salas-Huetos et al., 2014). The same authors reported that 736 miRNAs are differentially expressed between normozoospermic infertile patients and normozoospermic fertile individuals, possibly affecting reproduction (Salas-Huetos et al., 2016).

High levels of miR-27a and miR-27b were detected in asthenoteratozoospermic and asthenozoospermic patients, respectively (Zhou et al., 2015, 2016). These miRNA species negatively regulate the expression of cysteine-rich secretory protein 2 (CRISP2) (Zhou et al., 2015, 2016), a protein involved in sperm flagellar motility, the acrosome reaction, and gamete fusion. miR-27a overexpression and CRISP2 down-regulation were associated with low progressive sperm motility, abnormal morphology and infertility (Zhou et al., 2016). Studies of the microRNA profile in the sperm of men with several forms of infertility recognised specific miRNAs associated with sperm motility (has- miR-629-3p; down), concentration (has-miR-335-5p, has-miR-885-5p and has-miR-152-3p, down) and age (hsa-miR-34b-3p; up) (Salas-Huetos et al., 2015). miR-335-5p was previously described as being down-regulated in oligoasthenozoospermic patients (Abu-Halima et al., 2013), being a potential biomarker of human male infertility. Sperm miRNAs may also regulate the expression of some heat shock proteins (HSP40, HSP70 and HSP90) in oligoasthenozoospermic men (Tang et al., 2015), a mechanism that may be relevant for sperm vitality, motility, apoptosis and capacitation.

Lifestyle-associated alterations in semen parameters are possibly associated with sperm small RNA content. Cigarette smoking significantly affects sperm miRNA (Marczylo et al., 2012; Metzler-Guillemain et al., 2015) and mRNA profiles (Linschooten et al., 2009; Metzler-Guillemain et al., 2015; Hamad et al., 2019) (Table II). Metzler-Guillemain et al. (2015) showed that hsa-miR-296-5p, hsa-miR-3940 and hsa-miR-520d-3p were down-regulated in the sperm from smokers compared to non-smokers, with a consequent increase in the levels of six potential target mRNAs (B3GAT3, HNRNPL, OASL, ODZ3, CNGB1 and PKD2) in their sperm. However, since the sperm RNA content changes during epididymal transit, and several RNAs are transmitted to sperm through epididymosomes, these alterations may reflect the impact of smoking on other somatic cells/tissues rather than specific alterations in the spermatogenic process *per se*. The sperm of men living in environmentally polluted sites, including those contaminated with electronic waste, also present distinct miRNAs expression profiles, compared with men living in what the authors describe as a normal environment (Li et al., 2012). The recently completed randomised controlled clinical trial FERTINUTS reported the effect of higher nut consumption on conventional semen parameters, sperm DNA fragmentation (SDF), reactive oxygen species production, chromosome anomalies, total DNA methylation and miRNA expression (Salas-Huetos et al., 2018). Participants in the nut group showed a significant improvement in overall semen parameters, including sperm motility,

and a reduction in SDF and hsa-miR-34b-3p expression compared with the control group. Interestingly, this miRNA was already associated with sperm motility, since its down-regulation in asthenozoospermic men was reported (Abu-Halima *et al.*, 2013; Salas-Huetos *et al.*, 2015). These contradictory results in miR-34b-3p expression levels confound the establishment of a clear association with sperm motility, limiting its use as a biological marker.

Effects of alterations in miRNAs and other sncRNAs described may be passed epigenetically to the next generation, affecting the phenotype of progeny independently of Mendelian inheritance (Sharma, 2019; Tyebji *et al.*, 2020). It was reported that the microinjection into naive one-cell embryos of miR-19b, found to be up-regulated in the sperm of male mice fed with a Western-like Diet, induced metabolic alterations in the progenies similar to that in the progenitors (Grandjean *et al.*, 2015), supporting the role of this miRNA in epigenetic inheritance (Table III).

Mature human sperm also contain intact miRNA-precursors (pri-miRNAs, 100–150 nt), of which the most abundant is pri-miRNA-181c. This miRNA precursor may be involved in early embryo development where its target mRNAs are diminished at the four- to eight-cell stage of human embryo development (Vassena *et al.*, 2011; Sendler *et al.*, 2013). miR-181c specifically targets the coactivator-associated arginine methyltransferase 1 (*CARM1*) gene which is an embryonic stem cell pluripotency factor responsible for the establishment of active chromatin marks in the promoters of the transcription factors POU5F1and SOX2. Since the overexpression of CARM1 in one blastomere contributes to the development of the inner cell mass (Torres-Padilla *et al.*, 2007) it was proposed that the delivery of sperm miR-34c and pri-miR-181c may result in CARM1 down-regulation, decreasing the pluripotency factors in one blastomere and pushing the other towards the trophectoderm lineage (Jodar *et al.*, 2013). Collectively, these findings support a role for miRNA in sperm maturation, fertilisation and early embryogenesis.

piRNA

Piwi-interacting RNAs (piRNAs) (Fig. 2B, Table I) are abundant in the mammalian male germline, especially in spermatocytes and spermatids and are also present in sperm (Krawetz *et al.*, 2011; Peng *et al.*, 2012; Pantano *et al.*, 2015; Cui *et al.*, 2018; Giebler *et al.*, 2018). In sperm, some piRNAs are processed from the antisense strands of pseudogenes located inside the clusters, which regulate the expression of their associated protein-coding genes (Pantano *et al.*, 2015). This type of sncRNA is highly enriched (~5-fold) in sperm tails (Sharma *et al.*, 2018).

Several lines of evidence suggests that piRNAs protect the germline genome from the adverse effects of transposons and are involved in genome recognition, confrontation and consolidation upon fertilisation (Bourc'his and Voinnet, 2010; Krawetz *et al.*, 2011). Following fertilisation and prior to embryonic development, the cytoplasmic and genomic compatibility between oocyte and sperm may be submitted to a checkpoint (confrontation phase) (Miller, 2015). Paternal RNAs with complementary maternal repetitive elements activate or suppress their partner and, once this compatibility is assured, the RNA-based information may be transferred to a chromatin state (consolidation process) (Miller, 2015). Consolidation failure may result in fertilisation failure or lead to incorrect embryo development or compromised offspring fertility. During early embryo development, when the genome undergoes extensive demethylation and remethylation, piRNA may also act to protect genomic integrity by binding to DNA and preventing the action of repetitive and transposable elements at specific stages of embryogenesis (Krawetz *et al.*, 2011). Other functions have been proposed for this class of small RNAs, including the regulation of RNA stability and epigenetic states (Gapp *et al.*, 2014; Grandjean *et al.*, 2015; de Castro Barbosa *et al.*, 2016; Donkin *et al.*, 2016; Tyebji *et al.*, 2020). In fact, young healthy untrained individuals subjected to 6 weeks of endurance training presented 5 piRNAs and 27 fragments of repetitive elements which were differentially expressed at the second time point compared to the first time point (Ingerslev *et al.*, 2018). A period of 3 months without exercise then reversed these acute changes in piRNAs expression. These findings suggest that a similar mechanism to the one described in mouse may be working in humans concerning the possible involvement of piRNAs in intergenerational transmission.

tsRNAs

tRNA-derived small RNA (tsRNA) is a novel class of regulatory sncRNAs resulting from the cleavage of tRNA or pre-tRNA by endonucleases or other uncharacterised proteins (reviewed by Jin and Guo, 2019) (Table I). According to the length and the region from which they are derived, tsRNAs can be classified as tRNA-derived stress-induced RNAs (tiRNAs) and tRNA-derived fragments (tRFs) (Jin and Guo, 2019) (Fig. 2C).

tsRNAs are more enriched than miRNAs in sperm and were identified by sequencing a 17–26 nt small RNA library; but the entire landscape of tsRNAs in mouse sperm was only revealed when the <40 nt fraction of sncRNAs was sequenced (Peng *et al.*, 2012). tsRNAs are frequently 5′ end fragments (29–34 nt) formed by cleavage between the D-loop and the anticodon loop (Peng *et al.*, 2012; Chen *et al.*, 2020). However, tsRNAs can also derive from the 3′ region (Krawetz *et al.*, 2011; Peng *et al.*, 2012). $tRNA^{Gly}$ species accounted for the majority of 5′ tRNA halves, followed by $tRNA^{Glu}$, $tRNA^{Val}$, $tRNA^{Met}$ and $tRNA^{Lys}$, and, compared with miRNAs, tsRNAs seem to be much more abundant in sperm heads than in the tail (Peng *et al.*, 2012; Schuster *et al.*, 2016; Chen *et al.*, 2016a; Hua *et al.*, 2019).

Despite their mechanisms of action being poorly understood, tsRNAs seem to share common features with other sncRNAs such as miRNAs and piRNAs and are thus involved in mRNA stability, regulation and silencing, protein binding, RNA modification, stress responses and cell proliferation (Chen *et al.*, 2020). The first role proposed for tsRNAs in sperm was intergenerational/transgenerational inheritance mediation of paternally acquired traits, acting as epigenetic factors that modulate offspring phenotypes (Sharma *et al.*, 2016; Yan and Zhai, 2016; Chen *et al.*, 2016a; Nätt *et al.*, 2019) (Table III). Sharma *et al.* (2016) showed the involvement of tsRNAs in paternal intergenerational metabolic response, since a paternal low protein diet led to increased levels of 5′ fragments of $tRNA^{Gly}$ and decreased levels of miRNA Let7c in mature mouse sperm. Chen *et al.* (2016a) demonstrated that a high-fat diet (HFD) increases tRFs in mature mouse sperm and induces insulin resistance and impaired glucose tolerance in the offspring. Moreover, the microinjection of purified tRFs from the sperm of mice raised on an HFD into control zygotes replicate the metabolic phenotypes in the offspring (Chen *et al.*, 2016a). An interesting follow-up study reported that the tRNA methyltransferase DNMT2, responsible for methylating the C38 position (m5C) of $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Val}$, is essential for sperm sncRNA-

Table III Studies with significance for the involvement of sperm small RNAs in paternal intergenerational and transgenerational transmission.

Stimuli studied	Organism	Method	Differentially expressed sncRNAs	Microinjection	Altered offspring phenotype and epigenome	Ref.
Chronic stress	Mouse	RNA-seq, RT-qPCR	UP: miRNAs miR-375-3p, miR-375-5p, miR-200b-3p, miR-672-5p and miR-466-5p DOWN: piRNA cluster 110	Yes	Depressive behaviour and metabolic alterations	(Gapp *et al.* (2014)
Early life stress	Mouse and human	Microarray; RT-qPCR	DOWN: miR-449a, miR-449b, miR-34b and miR-34c	No	Decrease in miR-449a and miR-34c levels in embryos at the two-cell, four-cell, eight-cell and in the sperm of adult offspring from stressed fathers, consistent with findings that these mice transmit stress phenotypes to their F2 offspring	(Dickson *et al.*, 2018)
Chronic ethanol consumption	Mouse	RNA-seq, RT-qPCR	UP: tDR Glu-CTC, tDR His-GTG, miR-10a, miR-99b DOWN: tDR Ser-AGA, tDR Pro-AGG	No		(Rompala *et al.*, 2018)
Salient olfactory experience	Mouse	RNA-seq	miRNA targeting processes like regulation of cell growth, cellular response to chemical and amino acid stimulus (not specified)	Yes	Behavioural sensitivity to odour A and increased representation of Odour A-related neuroanatomy in the adult olfactory system	(Aoued *et al.*, 2020)
Infection by *Toxoplasma gondii*	Mouse	RNA-seq	UP: miRNA count; 75 miRNAs DOWN: piRNA count; 35 miRNAs	Yes	Sexually dimorphic changes in the anxiety-like phenotype, learning and memory in the F1 as well as F2 generations	(Tyebji *et al.*, 2020)
Physical health	Human	RNA-seq	UP: piR-has-11690; piR-26632 DOWN: miR-3653-3p; piR-28160; piR-27503; piR-17444	No		(Ingerslev *et al.*, 2018)
Diet						
High fat	Mouse	Microarray, RT-qPCR	UP: miRNA-133b-3p, miRNA-196a-5p, miRNA-205-5p; DOWN: miRNA-340-5p	No	Increased body weight in females, impaired glucose tolerance and insulin resistance	(Fullston *et al.*, 2013)
Western-like	Mouse	RNA-seq, RT-qPCR	UP: miRNAs and piRNAs miRNA-19b DOWN: miRNAs and piRNAs	Yes	Increased body weight, impaired glucose tolerance	(Grandjean *et al.*, 2015)

Continued

Table III Continued

Stimuli studied	Organism	Method	Differentially expressed sncRNAs	Microinjection	Altered offspring phenotype and epigenome	Ref.
High fat	Mouse	RNA-seq, MS, PCR	UP: miRNAs and tRF m5C and m2G DOWN: miRNAs and tRFs	Yes	Impaired glucose tolerance	(Chen *et al.*, 2016a,b)
Low protein	Mouse	RNA-seq, RT-qPCR	UP: miRNAs, piR-NAs and tRF-Gly-CCC, -TCC, -GCC, tRF-Lys-CTT, tRF-His-GTG DOWN: piRNAs, tRFs and miRNA-let7 family	Yes	Decreased expression of gene targets regulated by the endogenous retroelement MERVL in two-cell embryos. Low Protein RNAs could inhibit tRF-Gly-GCC targets in two-cell embryos.	(Sharma *et al.*, 2016)
High fat	Rat	RNS-seq and qPCR	UP: let7c-5p, piRNA-025883, piRNA-015935, tRF-Glu-CTC, -TTC DOWN: miRNA-293-5p, miRNA-880-3p, piRNA-036085	No	Decreased body weight, impaired glucose tolerance in females	(de Castro Barbosa *et al.*, 2016)
High fat	Mouse	RNA-seq, qPCR	UP: tsRNAs; DOWN: rsRNA-28S	Yes	Glucose intolerance	(Zhang *et al.*, 2018)
High sugar	Human	RNA-seq, qPCR	UP: tsRNA; DOWN: rsRNA	No		(Nätt *et al.*, 2019)
Obesity	Human	RNA-seq	tRF; piRNA; miRNA; snRNA	No		(Donkin *et al.*, 2016)

mediated transmission of HFD-induced metabolic disorders (Zhang et al., 2018). Mice lacking DNMT2 showed changes in tsRNAs and rsRNA-28S content in mature spermatozoa (Zhang et al. 2018). The observations that an HFD induces metabolic and pleasure seeking phenotypes in progeny, and that such effects were reproduced by the injection of total sperm RNA or tRF-enriched sperm RNA fraction, but not by the injection of the fraction of large sperm RNAs, corroborate this role of tRFs (Mashoodh and Ferguson-Smith, 2018). Rats exposed to an HFD also exhibited alterations in piRNA, miRNA and tRF profiles (de Castro Barbosa et al., 2016). Their progeny displayed a reduced body weight and pancreatic beta-cell mass compared with the control group, whilst adult female offspring were glucose intolerant and resistant to HFD-induced weight gain. Even more interestingly, the parents exposed to HFD and their F1 male progeny presented common DNA methylation and sncRNA expression signatures, indicating a possible intergenerational epigenetic inheritance (de Castro Barbosa et al., 2016). An important caveat in considering these effects in humans is that the direct relationships between sperm quality, sperm RNAs and intergenerational responses have almost exclusively relied on and been reported in animal models. However, it has been shown that human sperm can also present a rapid response to diet, both in terms of sperm motility and tsRNA content (Nätt et al., 2019). Healthy young men were exposed to a two-step diet intervention, 1 week of healthy diet followed by 1 week of additional sugar intake, and the authors investigated three ejaculates of the same men (before the intervention, after the first week and after the second week). After the first week, sperm showed an increase in motility and, after just one week of a high-sugar diet, the sperm of these individuals revealed an up-regulation of tsRNAs, especially nuclear internal T-loop tsRNA (nitRNA) (Nätt et al., 2019). This was the first controlled study in men reporting that the sncRNA repertoire of human sperm is rapidly and specifically modulated by dietary changes, although no evidence that these changes might affect their offspring exists.

Although their function in sperm remains poorly explored, new data points to a possible role of $tRNA^{Gln\text{-}TTG}$-derived small RNAs in regulating early cleavage events in preimplantation embryos (Chen et al., 2020). Using a porcine model, Chen et al. demonstrated that the microinjection of antisense $tRNA^{Gln\text{-}TTGs}$ into IVF oocytes resulted in a diminished number of two-cell, four-cell and blastocyst embryos, causally attributed to an aberrant first cell cleavage (Chen et al., 2020). Sperm Gln-TTGs may also act by regulating cell cycle-associated genes and transposable elements (Chen et al., 2020).

In summary, there is growing interest in tsRNAs, fuelled by evidence, at least from animal models, supporting their involvement in intergenerational or transgenerational inheritance. Additionally, emerging data indicate that these fragmented tRNAs are also involved in the regulation of early embryogenesis, which should be further explored.

rRNA

Nuclear and mitochondrial rRNAs are transcribed as long RNA precursors subsequently spliced into functional transcripts. These are recognised as one of the most abundant RNA classes in sperm, representing approximately three-quarters of all retained RNAs (Johnson et al., 2011b). Several ribosomal protein transcripts (5S, 5.8S, 18S, 28S and 45S) are differentially expressed in the sperm of asthenozoospermic and idiopathic infertile men compared with fertile patients (Bansal et al., 2015). The presence of these transcripts in sperm and the abnormal profiles observed in infertile patients points to a crucial role of ribosomes in the production of high-quality sperm. However, rRNA is usually highly fragmented into small RNAs (rsRNA), maintaining the translational quiescent state of sperm (Johnson et al., 2011b; Jodar et al., 2013). The 28S rRNA precursor-derived rsRNA (28S rsRNA) represents near 60% of all rsRNAs in mature sperm (Hua et al., 2019). However, in common with tsRNA, relatively little is known of the role of rsRNA in sperm physiology.

Transposable elements

A large proportion of human sncRNAs are repetitive elements, mainly represented by members of the short interspersed nuclear elements (SINE)/ALU and long interspersed nuclear elements (LINE) families of transposable elements (Krawetz et al., 2011). LINE1, for instance, is a transposable element with dynamic activity during mouse embryo development and cessation of its activity results in the arrest of embryonic development at the two- or four-cell stage (Beraldi et al., 2006). The possibility that transposable elements found in sperm modulate gene expression during early embryo development has not been confirmed. Thus, the function of transposable elements in the male germline and in early embryo development remains uncertain.

Endo-siRNA

The siRNA is a small double-stranded RNA of ~21 nt in length. Like miRNAs, siRNA seems to act with RISC complex and argonaut proteins to target complementary RNAs, thus mediating transcriptional and post-transcriptional gene silencing (Yuan et al., 2016). However, contrary to miRNAs (Fig. 2), endo-siRNA production bypasses DROSHA/DGCR8 processing, being instead directly cleaved by DICER from endogenous double-stranded RNA substrates (Yuan et al., 2016).

A study aiming to investigate the role of miRNAs and siRNAs in fertilisation and preimplantation embryonic development revealed that Dicer knockout mice have an altered set of sperm siRNAs and miRNAs, and the microinjection of their sperm into wild-type (WT) oocytes resulted in embryos with reduced developmental potential (Yuan et al., 2016). This subfertile phenotype can be rescued by injecting total or small RNAs derived from WT sperm into ICSI embryos (Yuan et al., 2016). These observations support a role for paternal miRNAs and endo-siRNAs in the control of post-transcriptional events upon fertilisation and early embryo development. However, whether this rescue was due to the role of endo-siRNAs or only due to miRNAs remains unclear.

Other sncRNAs

Other classes of sncRNAs present in human sperm include snRNAs, snoRNAs, YRNAs and quiescent (q)RNAs. The presence of snRNAs in sperm was first described in 1993 in rat (Concha et al., 1993). U1 and U2 snRNAs were detected in the sperm nucleus by ISH and, as components of the small ribonucleoprotein particle (RNP), these may be involved in the processing of nuclear pre-mRNAs (Concha et al., 1993). A minor portion of sncRNAs are YRNAs, small cytoplasmic RNAs (85-115 nt) associated with Ro protein, forming an RNP complex that is involved in quality control pathways for misfolded RNAs. In human sperm, specific YRNAs fragments are possibly involved in the first stages of embryo development to initiate DNA damage repair (Krawetz et al., 2011). qRNAs account for >10% of sncRNAs in

sperm and are located in histone-enriched TSS and promoter sequences, although their function remains unknown (Krawetz *et al.*, 2011).

A potential novel class of sncRNAs, including sperm RNAs (spR)-12 and -13 (~20 nt), identified in mouse sperm appears to be derived from additional processing of piRNAs (Kawano *et al.*, 2012). These are present in one-cell embryos and maintained until the blastocyst stage, having a potential role in the maintenance of genome integrity during early embryo development (Kawano *et al.*, 2012). Additional sncRNAs include NF90-associated RNAs (snaR; ~117 nt) that are relatively abundant in human sperm (*snaR-C3*, *-C4*, *-E*, *-F*, *-G1* and *-F*) (Sendler *et al.*, 2013). *snaR-G1* levels are higher in sperm than in testes and other somatic cells and are associated with the promoter region for the beta 1 subunit of the *hCG1*, an important hormone in implantation and embryo development (Parrott and Mathews, 2007).

Other sperm RNAs

In mature sperm, more than 65% of reads align to regions with unknown annotation or function (Sendler *et al.*, 2013). Non-coding RNAs located within the introns of coding mRNAs, i.e.intronic retained elements, are transcribed after gene expression and seem to have a role similar to miRNAs (Hill *et al.*, 2006). In sperm, 200 different non-coding transcripts are derived from full-length introns, but the mechanisms by which these transcripts escape degradation after alternative splicing remains unclear (Sendler *et al.*, 2013). The genes in the origin of these intronic elements do not group within a specific ontological category and are not correlated with a particular expression pattern during embryo development (Vassena *et al.*, 2011), and their function remains unclear.

Influence of sperm RNAs in pre-implantation embryo quality and embryonic development

DNA damage and sperm aneuploidies have been correlated with poor fertilisation and reduced rates of implantation and pregnancy. As described above, alterations in sperm mRNA content may indicate abnormalities in spermatogenesis and lead to abnormal embryo development and growth, especially those delivered to the oocyte upon fertilisation. Based on the zona-free hamster oocyte/human sperm penetration assays and from the comparison of sperm, oocyte and embryo RNA from mouse, it was found that some sperm transcripts that were not present in the unfertilised oocyte (sperm-specific transcripts) were introduced by the sperm upon fertilisation (Ostermeier *et al.*, 2004; Ntostis *et al.*, 2017). Furthermore, the possibility that a large subset of sperm RNAs are deeply embedded in the nuclear matrix, forming a complex with sperm DNA (Miller *et al.*, 2010), further supports this hypothesis. Most of the proteins encoded by those sperm-specific transcripts are associated with fertilisation. Those include transcripts encoding clusterin (CLU), calmegin (CLGN) and several heat shock response proteins involved in embryo development. Additionally, the injection of the sperm-borne factor l-phosphatidylinositol 4,5-biphosphate phosphodiesterase zeta (*PLCζ*) mRNA into mouse oocytes resulted in a functional calcium oscillator and oocyte activation, supporting its role in the control of cell cycle-dependent Ca^{2+} oscillations in early embryogenesis (Sone *et al.*, 2005). The integrator complex subunit l (*INTSl*), which is involved in the transcription and processing of the snRNAs U1 and U2, is also retained in human sperm (Vassena *et al.*, 2011). Microarray analysis showed that the *INTSl* mRNA levels increase immediately after fertilisation and before zygotic genome activation, and knockout of this gene in mouse is lethal for embryos at the blastocyst stage, supporting its potential role at the initial steps of embryogenesis (Vassena *et al.*, 2011).

Conversely, the evidence that non-coding RNAs appear during the final stages of spermatogenesis or are acquired during epididymal transit suggests that they may be involved in post-fertilisation events (Ostermeier *et al.*, 2004). The most abundant miRNA in human sperm, miR-34c, has been described as essential for the first cellular division in mouse zygotes (Liu *et al.*, 2012). This sncRNA was correlated with ICSI success, since patients with higher levels of sperm miR-34c exhibited higher numbers of good quality embryos and increased rates of implantation, pregnancy and live births (Cui *et al.*, 2015). Recently, it was reported that in bulls, sperm-borne miR-216b regulates cell proliferation and the first cleavage during early embryo development, and determines blastocyst quality, specifically through *K-RAS* levels modulation (Alves *et al.*, 2019). Mouse embryos generated by ICSI using sperm from caput epididymis presented abnormal gene regulation likely associated with implantation failure (Conine *et al.*, 2018). Moreover, the microinjection into the caput-sperm-derived embryos of miRNAs purified from cauda epididymosomes normally gained during epididymal transit, rescued these preimplantation molecular defects and suppressed the observed post-implantation embryonic lethality (Conine *et al.*, 2018). These experimental data strongly suggests that a fraction of sperm RNAs crucial for embryogenesis is gained upon sperm entry into the epididymis. Moreover, since the sperm-mediated delivery of paternal mRNAs and small RNAs is seemingly essential for correct embryological development in mice, evaluation of these may contribute to fertility diagnosis and the prediction of ART outcomes (Conine *et al.*, 2018; Bashiri *et al.*, 2021). Considering the apparent important role of these acquired RNAs, it is important to explain how it is possible to achieve live healthy progeny by ICSI using testicular sperm or caput epididymal sperm. In humans, the origin of the sperm used in ICSI does not appear to have a major influence on early-life outcomes for the offspring, thus questioning the genuine relevance of sperm RNAs for human implantation, embryo quality and development. Indeed, a recent meta-analysis confirmed that clinical pregnancy, live birth and miscarriage rates do not differ between the use of testicular sperm obtained by testicular sperm extraction and ejaculated sperm in patients with azoospermia factor c (AZFc) microdeletions (Zhou *et al.*, 2021). Similarly, in patients with obstructive azoospermia, percutaneous epididymal sperm aspiration followed by ICSI revealed no differences in the rate of clinical pregnancy and miscarriage compared with testicular sperm aspiration (Shih *et al.*, 2019). According to these data, we hypothesise that human testicular sperm contain sufficient amounts of those RNAs required to promote fertilisation, normal development and implantation success or that the protein levels encoded by the mRNAs or regulated by sncRNAs are sufficient to compensate for the lack of transcripts in immature sperm. Alternatively, differences between human and rodent epididymal differentiation may explain these conflicting observations (Nätt and Öst, 2020). Further research in this field should help to clarify this issue, as well as help us understand possible longer-term effects, such as possible intergenerational/transgenerational passage of male subfertility acquired and potentially transmitted through epigenetic processes. Several meta-analyses indicate that children conceived by ICSI have a higher risks of

congenital malformations (Catford et al., 2018; Chen et al., 2018; Giorgione et al., 2018) and poor semen quality (Belva et al., 2016) compared with spontaneously conceived offspring, supporting a crucial role of at least some of these epididymal-acquired RNAs.

Sperm RNA profiles could also predict the *in vivo* reproductive fitness of men with normal semen parameters (Bonache et al., 2012). Significant differences have been reported between the sperm RNA profiles of men whose sperm achieved pregnancy through IUI (García-Herrero et al., 2010b) or ICSI cycles (García-Herrero et al., 2011) and the profiles of men whose sperm did not achieve pregnancy (Table II). The investigation of the relationship between sperm piRNAs levels and semen parameters and the evaluation of their roles in fertilisation and embryo development after ICSI, revealed reduced levels of piR-31704 and piR-39888 in a male factor infertility group compared with the control group (Cui et al., 2018). Interestingly, the same authors demonstrated a significant increase in the levels of piR-31704, piR-39888 and piR-40349 in sperm from a group with higher fertilisation rates, but no correlation with rates of embryo early cleavage, day 3 good-quality embryos and pregnancy. Similarly, 30% of idiopathic infertile couples presented an incomplete set of required sperm RNA elements and a reduced probability of achieving a live birth by timed intercourse or IUI, but not by ICSI (Jodar et al., 2015). Differentially expressed sncRNAs, most of them tRFs, rsRNAs and miRNAs, were also significantly associated with sperm quality according to embryo quality, despite these sperm samples being considered normal using basic semen analysis (Hua et al., 2019). The analysis of sperm RNA profiles by RNA-seq from patients with normal sperm parameters but treated with ART revealed that sperm samples with higher hsa-mir-191 expression caused improved fertilisation rates and higher-quality embryos (Xu et al., 2020). Together, these studies point to the potential of sperm RNAs as prognostic markers to predict birth outcomes after fertility treatments for some groups of men. The use of microarrays, RNA-seq or a panel of differentially expressed RNAs as clinical diagnostic tools, particularly to establish the sperm transcript profile of patients undergoing ART, could enable the identification of specific paternal factors and pathways negatively affecting fertility outcomes. If sperm can provide information equivalent to invasive testicular biopsies regarding the mechanisms underlying subfertility or infertility, then a non-invasively obtained semen sample is a much more appealing and acceptable option.

Global summary and final considerations

The male gamete is far from being a quiescent cell, the solitary role of which is to deliver the paternal genome to the oocyte. The constant development of more sensitive and accurate RNA technologies has allowed the identification and characterisation of diverse transcripts in human sperm. This complex RNA population, comprising coding and non-coding RNAs, reflects not only the past course of spermatogenesis, but may also influence fertilisation, early embryogenesis and developmental maintenance. Other predicted functions of these transcripts include paternal chromatin packaging, sperm maturation and capacitation as well as intergenerational or transgenerational inheritance, possibly modulating the genotype and phenotype of the progeny.

Emergent data from animal models have revealed sperm RNA content remodelling during post-testicular maturation in the epididymis, which can be modulated by environmental changes and thus, may be involved in the transmission of epigenetic information to the next generation. The epididymosomes-secreted sncRNAs seem to be crucial for fertilisation and embryo development, possibly providing important diagnostic markers for some male reproductive disorders. Nevertheless, further investigation is needed to unravel the underlying mechanisms between changes in epididymal-secreted sncRNAs and male subfertility. The recognition of which specific RNAs are markers of past gene activity or have an active role post-fertilisation remains crucial.

Transcriptional profiling has considerable potential for the discovery of fertility biomarkers in human medicine. The RNA fingerprint of sperm from normal fertile men could help to elucidate the underlying causes of idiopathic male fertility. Studies have emerged that attempt to define a panel of male (in)fertility biomarkers with improved discriminatory power compared with individual biomarkers (Abu-Halima et al., 2014; Corral-Vazquez et al., 2019; Hua et al., 2019). Several miRNA pairs in human sperm have been correlated with infertility conditions, suggesting their potential use as fertility biomarkers: hsa-miR-942-5p/hsa-miR-1208 (asthenozoospermia), hsa-miR-296-5p/hsa-miR-328-3p (teratozoospermia), hsa-miR-139-5p/hsa-miR-1260a (oligozoospermia) and hsa-miR-34b-3p/hsa-miR-93-3p (idiopathic infertility) (Corral-Vazquez et al., 2019). Also, a set of five miRNAs that discriminate individuals with subfertility and non-obstructive azoospermia from control subjects was validated, with an accuracy of 98.65% and 99.91% respectively (Abu-Halima et al., 2014) (Table II). Furthermore, the recognition of the RNA profile in different germ cell subtypes and distinct stages of spermatogenesis (Luo et al., 2015; Jan et al., 2017), in combination with the profiles obtained from mature sperm, may reveal the association between spermatogenic perturbations and some forms of male infertility. One possibility may be to trace the cause of teratozoospermia to the pachytene spermatocyte, based on sperm RNA profiling, avoiding testicular biopsies (Platts et al., 2007). Additionally, some sperm RNAs have been linked or associated with developmental effects and/or neonatal lethality suggesting that RNA content may be indicative of the success of live birth (Burl et al., 2018). Evidence suggests that mature sperm retain the dysregulated miRNA pattern observed in the developing germ cells due to partial spermatogenic failure (Muñoz et al., 2015). We advocate that the RNA profiling of individual sperm samples should be undertaken, since it has the potential to identify the origin, establish a prognosis, and determine the treatment pathway of several types of idiopathic infertility and would perhaps offer a molecular explanation for the impact of a growing number of lifestyle and environmental factors on male fertility.

It is now more widely accepted that sperm RNA can mediate information transmission to the offspring in mammals and any perturbation will not only compromise sperm function but could also be transmitted to the next generations (Sciamanna et al., 2019; Sharma, 2019). Over the past decade, several papers have been published suggesting that chronic (Gapp et al., 2014) and early life stress (Dickson et al., 2018), cigarette smoke exposure (Marczylo et al., 2012), chronic alcohol consumption (Rompala et al., 2018) and diet (Grandjean et al., 2015; de Castro Barbosa et al., 2016; Donkin et al., 2016; Sharma et al., 2016; Chen et al., 2016a; Nätt et al., 2019) may change paternal sperm RNA content to influence the health of their progeny (Table III). The use of ART in the treatment of male infertility may perpetuate this problem, since the use of sperm from infertile men with previous epigenetics

marks could possibly increase the risk of passing undesirable epigenetic influences to the next generation (Ge *et al.*, 2019). Whether these marks are pre-existent or induced by the ART procedure (semen processing, cryopreservation, etc.) itself is unknown. The damage caused to sperm during ART procedures may induce modifications of sperm RNA content, leading to altered semen parameters, such as motility and fertility. Sperm cryopreservation is commonly applied in ART and it is known that cryopreserved semen samples present altered protein (Wang *et al.*, 2014; Bogle *et al.*, 2017) and RNA profiles (Ostermeier *et al.*, 2005a; García-Herrero *et al.*, 2011; Valcarce *et al.*, 2013) compared with fresh samples, and this may affect ART outcomes and/or pass epigenetic marks to the offspring. A systematic review and meta-analysis showed that ICSI performed with fresh and frozen semen obtained from men with non-obstructive azoospermia presented similar fertilisation, implantation and clinical pregnancy rates, embryo quality and live birth rates (Yu *et al.*, 2018). Nevertheless, the adverse perinatal outcomes observed in the offspring born from ART compared to or naturally conceived offspring may be associated with altered epigenetic profiles (Berntsen *et al.*, 2019). When considering the general roles of sperm RNAs in epigenetic inheritance, it must be remembered that depending on when in life the exposure to an environmental challenge occurred (prenatal, postnatal, adolescence/adulthood), different populations of germ cells can be affected. Despite being probably more vulnerable during early development, when rapid cell division and reprogramming occurs, the sperm epigenome also changes throughout adult life, with constant exposure to environmental insults. It remains uncertain whether one epigenetic mark is more potent at responding to environmental insults than another and whether this depends on the type of environmental change. Even so, crosstalk between different epigenetic marks is more likely to transmit paternal environmental information to the progeny. The potential contribution of other sperm sncRNAs and lncRNAs in shaping offspring phenotypes remains unresolved. Finally, there is scope to determine whether results using rodent models are directly relevant to human epigenetics.

Data availability

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Authors' roles

J.S. and J.V.S. made substantial contributions to the article conception and design. J.S. performed the literature search and drafted the manuscript. J.V.S., J.H., M.F. and M.A.S.S. critically revised the work. All authors read and approved the final version of the manuscript to be published.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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