Sperm and seminal plasma RNAs: what roles do they play beyond fertilization?

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Abstract

The paternal contribution to the new individual is not just limited to half the diploid genome. Recent findings have shown that sperm delivers to the oocyte several components, including a complex population of RNAs, which may influence early embryo development and the long-term phenotype of the offspring. Although the majority of sperm RNAs may only represent spermatogenic leftovers with no further function, the male gamete provides a specific set of RNAs to the oocyte that is able to modulate gene expression in the preimplantation embryo. Those sperm transcripts include coding and non-coding RNAs that might either be translated by the oocyte machinery or directly regulate embryo gene expression at the transcriptional or post-transcriptional level. Interestingly, some sperm RNAs seem to be acquired during post-testicular maturation through active communication between sperm and epididymal and seminal exosomes released by the epididymis and the male accessory sex glands, respectively. Exosomes contained in the seminal plasma seem to not only interact with the spermatozoa but also with cells from the female reproductive tract, modulating their gene expression and influencing female immune response triggered by the semen. This review also considers the findings that indicate the role of semen RNAs in preimplantation embryo development and offspring phenotypes. In this regard, different studies supporting the hypothesis of paternal epigenetic inheritance of altered metabolic phenotypes associated with environmental exposures are discussed. Lastly, potential mechanisms that could explain the impact of semen RNAs to both early embryogenesis and paternal epigenetic inheritance are suggested.

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Introduction

Mature mammalian spermatozoa are thought to be transcriptionally and translationally inert, at least at the nuclear level. This general shutdown is the result of the events taking place during the last steps of the spermatogenesis, which include the reorganization and condensation of the chromatin through the progressive replacement of histones by transition proteins and then protamines, and the removal of most of the sperm cytoplasm (Oliva 2006). Despite the fact that transcriptional and translational processes are blocked and most of the RNAs are lost during cytoplasmic extrusion, a small but complex population of RNAs is non-randomly preserved in mature sperm (Jodar *et al.* 2013, Sendler *et al.* 2013, Gòdia *et al.* 2018a).

Infertility is a growing concern in human and animal reproductive health. The study of the sperm RNA profiles in both humans and domestic animals is currently providing the framework for the discovery of novel clinical biomarkers and potential diagnostic tools for fertility evaluation. For example, comparative analysis on human sperm RNA profiles has helped to delineate

possible aberrant mechanisms underlying male infertility, revealing sperm molecular disturbances occurring during either spermatogenesis or sperm maturation (Platts *et al.* 2007, Jodar *et al.* 2012, Montjean *et al.* 2012). Moreover, human sperm RNAs might predict the success of specific assisted reproductive technologies (ART). This relies on the capacity of a set of 648 sperm RNA elements (SREs) to discern idiopathic infertile patient groups according to the likelihood of achieving pregnancy using less invasive ART, such as intrauterine insemination (IUI) (Jodar *et al.* 2015). Male subfertility also has a high economic impact in the livestock industry since the animals selected on the basis of their pedigree or meat quality have a high variability in their ability to withstand cryopreservation (Vilagran *et al.* 2015) and in their breeding ability (Colenbrander *et al.* 2003, Khatun *et al.* 2013). This can have a dramatic impact on the general practice for using a single male to inseminate hundreds of females. Therefore, sperm RNA is emerging as a valuable tool that may help in the prediction of animal fertility and in the selection of the best male for future breeding, improving the cost-effectiveness of the

livestock production (Fagerlind *et al.* 2015, Card *et al.* 2017, Suliman *et al.* 2018).

This review is focused on the composition of sperm RNAs in mammals, as well as on the impact of the secretions from the epididymis and the accessory sex glands on sperm RNA profiles. Although the most conservative perspective supports the view that sperm RNAs are just spermatogenic leftovers with no biological function in post-testicular events, growing evidence suggests that specific sperm RNAs might also be crucial beyond fertilization. Therefore, this review will discuss the most recent findings on the potential roles of semen RNA payload beyond fertilization.

Sperm-borne RNAs

Sperm RNA isolation is a challenging process that requires careful consideration of the following points: (i) one spermatozoon contains an exceptionally low quantity of RNA, which ranges from 10–50 fg in human sperm (Goodrich *et al.* 2013, Jodar *et al.* 2013), approximately 100 fg in mice and rat sperm (Zhang *et al.* 2017), 20 fg in stallion sperm (Das *et al.* 2010), 20–30 fg in bull sperm (Selvaraju *et al.* 2017) and 1–8 fg in boar sperm (Gòdia *et al.* 2018b, Kasimanickam *et al.* 2019); (ii) the ejaculate frequently contains non-sperm contaminant cells with ~100 times more RNA than sperm cells, which could mask the acquisition of accurate sperm RNA profiles by deep sequencing technologies and (iii) complete lysis of sperm is necessary to fully release the high proportion of RNAs embedded in the sperm nuclei (Johnson *et al.* 2015). However, the elevated level of disulfide bridges between protamines, which are the main DNA packaging proteins of the sperm nucleus, hinders the complete release of nucleic acids from sperm (Oliva 2006, Goodrich *et al.* 2013, Jodar & Oliva 2014). Therefore, the addition of reducing agents such as beta-mercaptoethanol to lysis buffers is critical to isolate high-quality sperm RNA (Goodrich *et al.* 2013) and (iv) the sperm RNAs show an inherent high biological fragmentation that prevents the use of standard controls (RIN value, for example) for assessing sperm RNA quality (Johnson *et al.* 2011, Jodar *et al.* 2013, Sendler *et al.* 2013). Despite these limitations, several studies have contributed to the description of the landscape of spermatozoal RNAs revealing a complex and unique RNA population in the male gamete. This includes coding RNAs, long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs) (Krawetz *et al.* 2011, Das *et al.* 2013, Jodar *et al.* 2013, 2015, Sendler *et al.* 2013, Pantano *et al.* 2015, Selvaraju *et al.* 2017, Zhang *et al.* 2017); all of them localized mainly in the sperm head (Johnson *et al.* 2015).

The use of RNA-sequencing technology (RNA-seq) in the study of spermatozoa has revealed that ribosomal RNAs (rRNAs) are the most abundant RNAs contained in sperm from human, pig, stallion and bull (around

80% of total RNA), while in mice these rRNAs represent only 30% of total RNA (Das *et al.* 2013, Jodar *et al.* 2013, Johnson *et al.* 2015, Selvaraju *et al.* 2017, Gòdia *et al.* 2018a). rRNAs appear selectively cleaved in all these species, most probably to ensure the translational shutdown observed in mature sperm (Johnson *et al.* 2011, Das *et al.* 2013, Selvaraju *et al.* 2017). Similarly, a substantial proportion of fragmented coding RNAs have been detected, which suggests their presence in the mature sperm as simply persistent spermatogenic leftovers. However, the spermatozoon also contains intact transcripts, which may be selectively protected from degradation and it could be indicative of a crucial role beyond fertilization (Sendler *et al.* 2013, Selvaraju *et al.* 2017). Indeed, the most intact transcripts reveal ontologies related to mature spermatids, male infertility and early embryogenesis (Sendler *et al.* 2013). In this regard, a single reliable cross-species analysis conducted using a standardized sperm RNA isolation protocol has revealed a set of conserved coding sperm RNAs in four different mammalian species (that is human, mice, rat and rabbit), functionally related to developmental processes (Schuster *et al.* 2016).

The sperm cell also contains a large proportion of non-coding RNAs with potentially important biological functions (ENCODE Project Consortium 2012, Jodar *et al.* 2013). It is interesting to note, whereas some of the sperm long non-coding RNAs (>200 nt) are also annotated in other cell types, a large number of sperm-specific non-coding RNAs have been identified, including intronic retained regions and short expression regions overlapping coding, intergenic or untranslated region (UTR) sequences (Fig. 1) (Jodar *et al.* 2013, Sendler *et al.* 2013, Selvaraju *et al.* 2017). Around 880 sperm lncRNAs seem to be conserved between human and mouse, but their roles in sperm function are unknown (Zhang *et al.* 2017).

Furthermore, 1% of sperm RNAs correspond to sncRNAs including repeat-associated small RNAs, micro(mi)RNA, tRNA-derived sncRNA (tRNA fragments), Piwi-interacting(piwi)RNA, small nucleolar RNAs, small nuclear RNAs, mitochondrial-derived small RNAs and YRNAs (Krawetz *et al.* 2011, Pantano *et al.* 2015, Schuster *et al.* 2016). Each subtype of sncRNAs differs in their biogenesis, length, roles and mechanisms to accomplish their likely biological function (Jodar *et al.* 2013, Gòdia *et al.* 2018b, Jodar & Anton 2018). Of note, the interspecies comparison has revealed that miRNAs are the most conserved sncRNA population across species, with at least 67 miRNAs commonly identified in the male gamete from human, mice, rat and rabbit (Schuster *et al.* 2016).

Seminal plasma RNAs

Seminal plasma is a mixture of secretions from testes, epididymides and other accessory sex glands and its

Figure 1 Sperm-specific long non-coding (lnc) RNAs. The sequence reads obtained from sperm RNA-seq data (green) corresponding to three sperm-specific lncRNAs are shown. The first and second boxes show two sperm-specific lncRNAs, the first one located in the seventh exon (blue boxed of the gene representation) of the *ANKRD30A* gene and the second one located in the sixth intron (blue line between two boxes of the gene representation) of the *TRIM66* gene. In both examples, the levels of the specific exonic or intronic sperm RNAs are enhanced, whereas the coding regions of the corresponding transcripts are absent in sperm (note y-axis). The third example corresponds to a sperm-specific lncRNAs located in an intergenic region from chromosome 2 not expressed in other tissues.

composition varies among animal species. For example, human and mouse seminal plasma contains secretions mainly from seminal vesicles (70–80% of the volume), and less from the prostate (20%), epididymides – testes (5%) and bulbourethral glands (<1%) (Mcgraw *et al.* 2015). In contrast, the major contributors to boar seminal plasma are the prostate and the bulbourethral glands (Mcgraw *et al.* 2015). Secretions from the epididymides and accessory sex glands contain energy substrates, ions, proteins, RNAs and lipids, among others components, which could be found freely dissolved and in solution or encapsulated in extracellular vesicles or exosomes (Vojtech *et al.* 2014). There is increasing evidence showing that exosomes detected in different body fluids participate in intercellular communication, through the selective incorporation of their cargo into the target cell (Keerthikumar *et al.* 2016). Therefore, it seems obvious to think that the exosomes released by epididymides and accessory sex glands could play a role in the communication between seminal plasma and sperm. This functional involvement has been proposed for exosomes released by the epididymides (epididymosomes), which are capable of modifying the lipid composition of the sperm membrane, thus contributing both to the acquisition of sperm motility potential and to the ability to penetrate the zona pellucida (Sullivan & Saez 2013). Additionally, many studies have shown the role of prostate-derived exosomes (prostatosomes) in the stimulation of sperm motility while avoiding at the same time premature capacitation and spontaneous acrosome reaction (Aalberts *et al.* 2014). In addition, prostatosomes have been found to be involved in the protection of the sperm against female immune response triggered in the female genital tract after contact with the semen (Aalberts *et al.* 2014).

As indicated above, transcripts retained in mature mammalian sperm are mainly remnants of untranslated mRNAs transcribed during the massive wave of

transcription that takes place during the round spermatid stage before DNA compaction commences (Sassone-Corsi 2002, Jodar *et al.* 2016). However, testicular and ejaculated sperm show distinct RNA profiles, since the germ cell RNA signature is subject to modification during sperm post-testicular maturation. It is well known that the mouse sperm sncRNAs profile is remodeled during epididymal transit with loss of piRNAs, a gain of tRNAs fragments and the acquisition and loss of specific sets of miRNAs (Nixon *et al.* 2015a, Conine *et al.* 2018, Sharma *et al.* 2018). Taking into consideration that the spermatozoon is a transcriptionally quiescent cell, the acquisition of new RNAs during the post-testicular maturation is ascribed to the active communication between the sperm and the epididymal fluid, most likely through the incorporation of RNAs contained in the epididymosomes (Belleannée *et al.* 2013, Sullivan & Saez 2013, Sharma *et al.* 2016). It is interesting to note that not all the sncRNAs detected in sperm seem to have a testicular or epididymal origin, suggesting that the sperm could also incorporate exosomes released from other tissues (Nixon *et al.* 2015b). Although this hypothesis requires further experimental validation, a recent *in silico* analysis of the expression of human sperm proteins in tissues from the male reproductive tract and peripheral organs, at both transcriptomic and proteomic levels, supported a putative extra-testicular and extra-epididymal origin for some sperm proteins (Castillo *et al.* 2018).

However, since seminal plasma also interacts with the female reproductive tract, additional functions for its RNA have been proposed in addition to the regulation of sperm physiology. Seminal plasma is able to modulate ovulation in mammals including rabbits, cats and camels (Mcgraw *et al.* 2015), as well as the female immune response to the developing embryo (Robertson 2005, Watkins *et al.* 2018). Of note, exosomes contained in seminal plasma have

been reported to increase the expression of immune- and inflammatory response-related genes in porcine endometrial tissue, reaching similar levels as those observed in the endometrium from naturally mated pigs (Bai *et al.* 2018). This suggests that communication between seminal plasma and the female reproductive tract could also be through the interaction with seminal exosomes.

A more comprehensive study of the content of exosomes contained in the ejaculate is crucial to unraveling their potential functions in mammalian reproduction. To the best of our knowledge, only two studies have assessed the small RNA and long RNA content of ejaculated-derived exosomes by high-throughput technologies, respectively (Vojtech *et al.* 2014, Johnson *et al.* 2015). One the one hand, the assessment of small RNA profile has revealed that ejaculated-derived exosomes selectively retain several types of sncRNAs such as, from more to less abundance, miRNA, YRNAs, rRNA, tRNAs fragments, protein-coding derived sncRNAs and piRNAs (Vojtech *et al.* 2014). On the other hand, transcripts encoding for the sperm-specific protamines were detected among the 100 most abundant transcripts in the long RNA fraction from ejaculate-derived exosomes suggesting that sample contamination with sperm RNA remnants may have occurred (Johnson *et al.* 2015). This fact exposes technical limitations on exosome isolation strategies based only on differential centrifugation. The design of improved experimental approaches to obtain pure exosome populations from semen are needed to better characterize the RNA profile of exosomes released by epididymides and the accessory sex glands (Li *et al.* 2017).

The role of semen RNAs beyond fertilization

It is now broadly accepted that the male gamete is more than a vehicle for paternal DNA. The sperms' haploid genome includes DNA associated with different epigenetic marks and a complex population of proteins and RNAs that could be crucial for early embryogenesis and the future health of the offspring (Hammoud *et al.* 2009, Amaral *et al.* 2014, Carrell *et al.* 2016, Castillo *et al.* 2018). Additionally, although seminal plasma is not mandatory for successful reproduction, as reflected by positive outcomes of ART using testicular, epididymal and washed-ejaculated sperm, this fluid may also play a role in the embryo development and long-term health of the progeny (Robertson & Sharkey 2016, Watkins *et al.* 2018). Specifically, it has been observed that the excision of the seminal vesicle glands in mice, which are the major contributors to the seminal plasma (~70% of volume), results in low pregnancy rates, placental hypertrophy and metabolic alterations in the offspring (Bromfield *et al.* 2014).

Semen RNAs and early embryogenesis

A critical role for sperm-borne RNAs on early embryogenesis is supported by studies conducted in mice (Table 1). In 2016, Yuan and colleagues observed that the reduction in the developmental potential of embryos generated with sperm containing disturbed sncRNAs profiles from testicular conditional knockouts of DICER and DROSHA were rescued by the injection of wild-type sperm RNAs (Yuan *et al.* 2016). Later on, Guo and colleagues observed reduced percentages of embryos reaching blastocyst stage and a lower ratio of live births when mature oocytes were injected with sperm containing 90% less RNA than controls (Guo *et al.* 2017). These negative outcomes were partially rescued when total RNA from the wild-type sperm was injected into embryos derived from *in vitro* ART with RNA-deficient sperm (Guo *et al.* 2017).

Additionally, the importance of seminal plasma-derived sperm RNAs on early embryogenesis has also been demonstrated, at least in the mouse (Conine *et al.* 2018). Specifically, in comparison with embryos generated with testicular or cauda-epididymal sperm, mouse embryos generated with caput-epididymal sperm displayed reduced implantation rates and alterations on post-implantation development. Interestingly, these preimplantation molecular defects were completely rescued by the injection of sncRNAs with miRNAs-like sizes from cauda-derived epididymosomes (Conine *et al.* 2018). Therefore, in the mouse at least, epididymal sncRNAs seem to have roles in embryo implantation and post-implantation events. In addition, 100 miRNAs were underexpressed in epididymal caput sperm compared to the germ cells recovered from the epididymal cauda, suggesting differential regulation of their expression as the potential cause of the implantation defects observed on embryos generated with caput-epididymal sperm (Conine *et al.* 2018).

However, the role of the paternal RNAs in early embryogenesis seems to be not only restricted to the sperm RNAs delivered to the oocyte. Some reports suggest that sncRNAs encapsulated into the exosomes contained in semen could be delivered to acceptor cells from the female reproductive tract modulating their gene expression (Robertson & Sharkey 2016, Bai *et al.* 2018). This modulation may contribute to the regulation of implantation or immune processes, both crucial for a successful pregnancy (Robertson & Sharkey 2016). Although the ability of exosomal miRNAs to regulate gene expression in distant tissues has also been demonstrated in other systems (Thomou *et al.* 2017, Castaño *et al.* 2018), the regulatory effects of specific paternal exosomal RNAs on the female tract cells need to be experimentally demonstrated.

Table 1 Summary of studies demonstrating the suggested role of paternal RNAs in early embryogenesis.

Study	Experiment	Sperm RNA profile	Altered early embryogenesis	Conclusion
Yuan <i>et al.</i> (2016)	Testicular conditional DICER knockout	15% miRNA upregulated and 32% miRNAs downregulated 2% endo-siRNAs upregulated and 6% endo-siRNAs downregulated	Embryos displayed significantly reduced developmental potential at 2 PN, 2-cell, 4-cell, morula and blastocyst	Developmental potential was rescued by the injection of sperm RNAs or sncRNAs from wild type reflecting the importance of paternal miRNAs and siRNAs in early embryo development
	Testicular conditional DROSHA knockout	19% miRNA upregulated and 37% miRNAs downregulated	Embryos displayed significantly reduced developmental potential at 4-cell, morula and blastocyst	
Guo <i>et al.</i> (2017)	Spermatozoa were treated with lysolecithin, pronase and RNases	Sperm RNAs was decreased by about 90%	Reduced blastocyst formation rate	Developmental potential was rescued by the injection of sperm RNAs from wild type reflecting the importance of sperm RNA including long and small in early embryo development
Conine <i>et al.</i> (2018)	ICSI using epididymal sperm from caput and epididymal sperm from cauda	High differences in sperm small RNA payload (piRNAs, tRNAs and miRNAs)	Multiple defects in post-implantation development from embryos derived from epididymal caput sperm	Developmental defects were rescued by the injection of RNAd contained in the epididymosomes isolated from the cauda epididymis reflecting the importance of sncRNAs gained during epididymal transit in early embryogenesis

WD, Western-like diet; HFD, high fat diet.

Semen RNA and paternal epigenetic inheritance

Several studies show that paternal preconception exposures, including paternal lifestyle, may be conditioning the health of future progeny. These include paternal age (Ramasamy *et al.* 2015), diet (Rando 2012, Schagdarsurengin & Steger 2016), stress and habits such as smoking and alcohol consumption (Finegersh *et al.* 2015, Esakky & Moley 2016). Interestingly, it has been proposed that alterations in gene expression during early embryo development can induce these long-lasting effects. Some of the known epigenetic factors able to modify gene expression are (i) DNA methylation and hydroxymethylation marks, (ii) post-translational modifications (PTMs) of histones, (iii) non-coding RNAs and (iv) proteins that could modulate gene expression by the direct regulation of transcription or through the modulation of other epigenetic marks. In the sperm cell, the aforementioned marks could act as potential carriers for the epigenetic information by which the male can influence progeny phenotypes (Zeybel *et al.* 2012, Vassoler *et al.* 2013, Castillo *et al.* 2018).

Growing evidence points to a role for sperm sncRNAs in the phenotypic variations reflective of paternal life experiences (Rodgers *et al.* 2013, Gapp *et al.* 2014, Grandjean *et al.* 2015, Chen *et al.* 2016a,b, Sharma *et al.* 2016, Jodar & Anton 2018). In particular, it has been shown that the injection of total or specific sperm miRNAs and tRNA fragments from males exposed to high-fat diet (HFD) or mental stress into zygotes that were not exposed to these stressors, achieves the recapitulation of those associated altered phenotypes (Rassoulzadegan

et al. 2006, Gapp *et al.* 2014, Grandjean *et al.* 2015, Chen *et al.* 2016b, Sharma *et al.* 2016). However, these microinjection experiments were unable to recapitulate all phenotypic traits observed in natural offspring from exposed fathers (Table 2). For example, the progeny of male mice reared under western diet-based chow (WD) exhibited increased body weight, altered fasting blood glucose and more hyperglycemia in response to both glucose and insulin injections. However, the microinjection of miR-19, a differentially expressed sperm miRNA in the WD group, into a wild-type zygote resulted in individuals with increased body weight but not glucose metabolic alterations (Grandjean *et al.* 2015). The partial recapitulation of the phenotypes by microinjection experiments (Table 2) suggests the involvement of additional sperm factors on the transmission of phenotypic alterations linked to paternal exposures. In this regard, long RNAs including coding mRNAs and lncRNAs have also been proposed as potential vehicles for paternal epigenetic inheritance. In fact, it has been observed that the injection of either the small or the long RNA fraction into wild-type oocytes recapitulates different hallmarks of the phenotype observed in offspring of males exposed to postnatal trauma (Gapp *et al.* 2018). Different RNA types may, therefore, be able to trigger specific phenotypic traits (Table 2).

The extra-testicular origin of some sperm sncRNAs is implicated in paternal epigenetic inheritance, which might be absorbed and incorporated into sperm via epididymosomes during sperm maturation or in semen

Table 2 Summary of studies assessing epigenetic inheritance of altered phenotypes associated with a paternal exposure by the male germline including study, species, paternal exposure and offspring phenotype (F1) observed after both natural mating and microinjection experiments.

Study	Species	Paternal exposure	Altered phenotypic traits in offspring (F1)	
			Natural mating or ICSI procedures with males under the paternal exposure	RNA injection experiments
Gapp <i>et al.</i> (2014)	Mice C57Bl/6	Traumatic stress in early life	Reduced avoidance and fear, altered response to aversive conditions, depressive-like behaviors, insulin hypersensitivity, hypermetabolism	<i>Sperm RNA (male under early traumatic stress) injection</i> : Comparable behavioral, metabolic and molecular effects
Grandjean <i>et al.</i> (2015)	Mice C57Bl/6	WD	Increased body weight, altered fasting blood glucose and higher glycemia in response to both glucose and insulin	<i>Sperm RNA (male under WD) injection</i> : Increased body weight and higher glycemia in response to both glucose and insulin <i>miR19b injection</i> : Increased body weight <i>miR29 injection</i> : –
Chen <i>et al.</i> (2016a,b)	Mice C57Bl/6	HFD	Impaired glucose tolerance and insulin resistance	<i>Sperm RNA (male under HFD) injection</i> : Impaired glucose tolerance <i>Small RNAs (15-25 nt, males under HFD) injection</i> : – <i>Small RNAs (30-40 nt, males under HFD) injection</i> : Impaired glucose tolerance <i>Small RNAs (>40 nt, males under HFD) injection</i> : – <i>Synthetic tRNA fragments</i> : –
Gapp <i>et al.</i> (2018)	Mice C57Bl/6	Traumatic stress in early life	Reduced avoidance and fear, altered response to aversive conditions, depressive-like behaviors, insulin hypersensitivity, hypermetabolism	<i>Sperm small RNA (male under early traumatic stress) injection</i> : Changes in locomotor activity not observed by natural mating <i>Sperm long RNA (male under early traumatic stress) injection</i> : altered response to aversive conditions, insulin hypersensitivity, hypermetabolism

(Chen *et al.* 2016b, Sharma *et al.* 2016). This strategy could permit the transfer of epigenetic information about the paternal environment to the new individual without the need to overcome the blood–testis barrier or be incorporated into the germ line during spermatogenesis per se.

Despite experimental evidence supporting the transmission of phenotypic alterations associated with paternal exposures, one of the major concerns surrounding an RNA-mediated epigenetic inheritance by the paternal germ line is the incomplete penetrance observed (Huypens *et al.* 2016). Indeed, several examples of this phenomenon have been reported in the literature showing that the response of male animals to environmental exposures varies widely even among the individual cohorts (Dias & Ressler 2014, Gapp *et al.* 2014, Huypens *et al.* 2016). For example, only approximately 15% of the female progeny of male mice raised under HFD exhibited extreme obesity, with approximately 65% showing similar body weights to that observed in the control group (Huypens *et al.* 2016). This incomplete penetrance could reflect the heterogeneity of sperm epigenetic marks, such as a different RNA content between sperm from the same ejaculate. Sperm single-cell RNA-seq data might clarify this hypothesis.

Mechanisms underlying semen RNA function in the early embryo

The molecular mechanisms underlying semen RNA function in the early embryo remain unclear. Nevertheless,

it is important to bear in mind that whereas changes at the (sperm) chromatin level may not be detected before the activation of the embryonic genome, alterations in the sperm RNA profile could induce preimplantation effects as early as the 1-cell zygote stage. Here, two potential modes of action for semen RNAs during preimplantation embryo development are proposed: (i) the translation of intact sperm-coding mRNAs using the zygote translational machinery provided by the oocyte and (ii) the regulation of embryo gene expression by sperm-borne non-coding RNAs (Miller 2015, Ntostis *et al.* 2017).

Intact sperm mRNAs could be translated in early embryogenesis using the zygote translational machinery provided by the oocyte

It is well known that the injection of phospholipase C α 1 (*Plcz1*) transcripts into a mouse oocyte induces its translation and, therefore, oocyte activation (Yoda *et al.* 2004). These findings have raised the possibility for the potential translation of sperm-borne coding mRNAs by the oocyte machinery (Ntostis *et al.* 2017). Supporting this hypothesis, we recently undertook an integrative analysis of transcriptomic and proteomic data from human sperm, oocytes and early embryos, which revealed 11 blastocyst proteins most likely originating from the translation of sperm RNAs during early embryogenesis (Table 3; Castillo *et al.* 2018). In particular, these 11 human blastocyst proteins were not detected in the proteomic profile of sperm, oocyte and

Table 3 Potential blastocyst proteins originated by the translation of sperm RNAs in early embryo.

Gene ID	Proteomic data	RNA-seq data (FPKM values)							
		Sperm	Oocyte	Zygote	2-Cell	4-Cell	8-Cell	Morula	Blastocyst
ANKRD12	Blastocyst	30.59	3.81	1.56	0.83	5.33	2.80	1.60	1.89
ARHGAP26	Blastocyst	26.51	9.67	7.21	5.08	2.34	1.32	0.51	0.92
ATP7B	Blastocyst	25.51	3.99	2.31	1.87	0.22	0.13	0.09	3.04
BBX	Blastocyst	65.19	0.34	0.16	0.07	1.56	2.37	1.49	2.09
CYP2R1	Blastocyst	25.68	2.11	2.81	0.72	1.01	2.38	0.53	0.11
FGD4	Blastocyst	60.71	7.02	4.61	3.28	1.04	0.94	1.36	2.24
KIAA0586	Blastocyst	25.25	9.76	8.05	5.19	1.67	2.46	0.93	4.02
RASSF8	Blastocyst	39.79	8.25	6.32	4.48	2.90	3.92	3.30	1.44
SIPA1L3	Blastocyst	25.48	2.50	0.43	0.58	0.12	0.32	0.11	0.20
SPIRE1	Blastocyst	50.52	7.53	5.23	3.29	0.59	0.51	0.39	4.50
ZNF646	Blastocyst	34.47	1.98	1.23	1.81	0.28	0.58	0.41	0.58

A total of 11 blastocyst proteins were classified as paternally derived due to their exclusively presence in blastocyst proteome and their corresponding RNAs detected solely in sperm RNA-seq dataset (values over 25FPKM).

any other embryo-related cells and fluids, such as the cumulus cells and the follicular and endometrial fluids. However, their corresponding RNAs were detected with high abundance in spermatozoa (>25 FPKM), very low abundance in the oocyte (<10 FPKM) and gradual decreasing levels in the different stages of the preimplantation embryo, even after the activation of the genome (Table 3). The fact that the transcription of the human embryo genome is shut down until it reaches the four-cell stage, combined with the absence of this set of proteins and their corresponding RNAs in the oocyte leads to the proposition that the translation of these 11 coding transcripts in the preimplantation embryo was accomplished after being delivered by sperm. Blastocyst proteins potentially derived from sperm RNAs include transcript factors, such as the BBX, the HMG-box containing (BBX) and the zinc finger protein 646 (ZNF646), as well as histone modifiers, such as the Ankyrin repeat domain 12 (ANKRD12), which might be able to regulate gene expression once the zygote genome is activated. Additionally, some of these sperm RNAs encode for proteins that regulate the activity of GTPases that may be essential for the cytoskeleton dynamics during early embryo development (Duquette & Vane 2014) including the Rho GTPase-activating protein 26 (ARHGAP26), the FYVE, RhoGEF And PH domain containing 4 (FGD4) and the signal induced proliferation associated 1 like 3 (SIPA1L3). Finally, the blastocyst centrosomal protein KIAA0586, specific depletion of which in mice leads to embryos with abnormal left-right axis patterning (Bangs et al. 2011), has also been predicted as paternally derived.

Similar studies in mice suggested that sperm mRNAs most likely translated in the zygote might complement maternal cofactors or pathways required for maternal RNA clearance during the egg-to-zygote transition, acting as a checkpoint to start the activation of the embryonic genome (Ntostis et al. 2017). Furthermore, other proteins crucial for murine early embryogenesis such as the Wnt family member 4 (WNT4) protein has been predicted to be paternally derived as a result of

sperm RNA translation in the zygote. (Fang et al. 2014). Further experimental data are required to validate the impact of these paternally derived RNAs and proteins during preimplantation embryogenesis.

Sperm-borne non-coding RNAs regulate gene expression in the early embryo

The participation of non-coding RNAs in the modulation of gene expression at either the transcriptional or post-transcriptional level is well established in different tissues and systems. For example, miRNAs and tRNA fragments are able to regulate mRNA degradation and/or translational repression in a sequence-specific manner through complementary sequences on either the 3'-UTR or 5'-UTR regions of transcripts, respectively (Schuster et al. 2016). Additionally, non-coding RNAs including sncRNAs and lncRNA show specific nuclear functions such as the regulation of both activation and inhibition of transcription, mRNA splicing, DNA methylation, histone modifications and chromatin conformation (Jodar et al. 2013, Liebers et al. 2014, Catalanotto et al. 2016).

Although the quantity of sperm RNAs released into the oocyte is minimal compared to the oocyte reservoir, small biases in particular ncRNAs could impact on the expression of several genes at the same time in a cascade-like fashion, at transcriptional and/or post-transcriptional level (Chen et al. 2016a). Supporting this hypothesis, it has been observed that the injection of tRNA-Gly fragments (one of the upregulated sperm sncRNAs by a low-protein diet) into zygotes represses MERVL-regulated transcripts in two-cell embryos and slows down the development of the embryo compared to controls (Sharma et al. 2016). At this point, however, it is not possible to discern if the altered metabolic phenotype associated with paternal diet is the result of altered preimplantation growth kinetics or the regulation of MERVL targets.

Additionally, single-cell RNA-seq from embryos generated using sperm from the epididymal caput or cauda revealed differences in the expression of RNA-

Early stages of embryogenesis may not be under the sole control of the oocyte cytoplasm, since sperm also contribute, although to a lesser extent, components such as RNAs. Research in the reproductive field has been focused mainly on the biology of the gametes. However, growing evidence shows that seminal plasma and its constituent RNAs (including RNAs of epididymal origin) is not just a transport medium for sperm, but is probably also important for sperm function, fertilization, early embryogenesis and even for the modulation of the offspring phenotype (at least in the murine model).

era of ART is that the RNA molecule is labile and easily degraded. For this reason, the acquisition and use of RNAs from secretions of the accessory sex glands could be a good strategy to preserve the sperm transcriptomic profile under optimal conditions for subsequent support of early embryogenesis. However, sperm are susceptible to damage during their long journey to reach the oocyte, suggesting that additional steps to guarantee the stability of the RNAs may also be required.

RNA modifications such as the addition of a methylcytidine (m5C) or N2-methylguanosine (m2G) contribute to tRNA stability (Tuorto *et al.* 2015). The participation of these RNA modifications in the paternal epigenetic inheritance was proposed when the injection of synthetic tRNAs fragments into normal oocytes was not able to recapitulate the altered metabolic phenotype in the same way as native sperm tRNAs fragments (Table 2; Chen *et al.* 2016b). Whereas sperm tRNAs fragments harbor several modifications, the absence of such modifications in the synthetic tRNAs was associated with their rapid degradation upon the sperm-oocyte fusion.

There are other hypothetical mechanisms that could explain the stability of paternal RNAs during early embryogenesis, but none of them have been experimentally tested so far. One mechanism is associated with the reported ability of sperm to reverse-transcribe exogenous RNAs to cDNA by reverse transcriptase (RT) activity encoded by LINE-1 retrotransposons (Spadafora 2017). cDNA molecules show higher stability than RNAs and could arrive in the oocyte under optimal

conditions to carry out their functions. Another potential mechanism could involve the participation of circRNAs, a new type of ncRNAs which is more stable than the linear RNAs (Szabo & Salzman 2016). Although natural circRNAs are not translated, several regulatory roles at both transcriptional and post-transcriptional levels have been ascribed to them, such as the modulation of the RNA polymerase II activity, the regulation of alternative splicing or the sponging of miRNAs and other factors such as RNA-binding proteins or ribonucleoproteins complex (RNPs) (Memczak *et al.* 2013, 2015). The potential regulatory functions ascribed to circRNAs in conjunction with their remarkable stability with half-lives exceeding 48 h (Jeck *et al.* 2013) suggests that this new type of non-coding RNAs could be self-protecting themselves or protecting other sperm RNAs from degradation during their long sperm-borne journey to the oocyte.

Conclusions

The majority of the paternal RNAs are unlikely to have a post-testicular role, and probably only represent the residue from spermatogenesis and sperm maturation, making them potentially useful as biomarkers of male fertility. However, there is a subset of specific sperm RNAs that could play a role in early embryogenesis, as demonstrated by microinjection experiments. These paternal RNAs may regulate events both prior to the synthesis of embryonic gene products and beyond zygotic genome activation. Additionally, it has been proposed that RNAs from seminal exosomes could regulate gene expression in cells within female genital tract, probably by modulating critical events for the pregnancy success, including the female immune response and embryo implantation (Fig. 2). However, further experimental data are required to validate these hypotheses.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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