The contribution of epididymosomes to the sperm small RNA profile

Natalie A Trigg^{1,2}, Andrew L Eamens^{1,2} and Brett Nixon^{1,2}

¹Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, The University of Newcastle, Callaghan, New South Wales, Australia and ²Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia

Correspondence should be addressed to B Nixon; Email: Brett.Nixon@newcastle.edu.au

Abstract

It is now well established that mature spermatozoa harbour a rich and diverse profile of small non-protein-coding regulatory RNAs (sRNAs). There is also growing appreciation that this sRNA profile displays considerable plasticity, being altered in response to paternal exposure to a variety of environmental stressors. Coupled with evidence that upon delivery to the oocyte at the moment of fertilisation, sperm-borne sRNAs are able to influence both early embryonic development and the subsequent health of the offspring, there is now interest in both the timing and degree of change in the composition of the sRNA cargo of sperm. Models in which such epigenetic changes are linked to the spermatogenic cycle are seemingly incompatible with the lack of overt phenotypic changes in the spermatozoa of affected males. Rather, there is mounting consensus that such changes are imposed on sperm during their transit and storage within the epididymis, a protracted developmental window that takes place over several weeks. Notably, since spermatozoa are rendered transcriptionally and translationally silent during their development in the testes, it is most likely that the epididymis-documented alterations to the sperm sRNA profile are driven extrinsically, with a leading candidate being epididymosomes: small membrane enclosed extracellular vesicles that encapsulate a complex macromolecular cargo of proteins and RNAs, including the sRNAs. Here, we review the role of epididymosome–sperm communication in contributing to the establishment of the sperm sRNA profile during their epididymal transit.

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Introduction

The volume of studies reporting the inheritance of non-genome-derived information have increased over the past decade. This so-called epigenetic mode of inheritance challenges long held paradigms of classic Mendelian genetics by offering a rational mechanism for the transmission of environmental information to the offspring, which can conceivably lead to novel phenotypic outcomes. Mounting evidence for the existence of such alternate routes of inheritance includes examples from epidemiological analysis of humans and studies performed in model species ranging from worms to mammals (Kaati *et al.* 2007, Dunn & Bale 2009, Lim & Brunet 2013). Collectively, these studies provide an important precedence for the potential transmission of paternal traits acquired by environmental conditions to the offspring, suggesting the existence of additional ‘information carriers’ beyond that of an individual’s genome. Among the information carriers potentially responsible for relaying non-genetic information between generations, considerable attention has focused on small non-protein-coding regulatory RNAs (sRNAs). Accordingly, mature spermatozoa harbour

a rich and diverse sRNA profile, the foundations of which are established during the testicular phases of their development. Notably, however, recent evidence has shown that the sperm sRNA profile displays considerable plasticity, being substantially remodelled as the cells traverse the epididymis, an integral part of the extra-testicular male reproductive tract (Nixon *et al.* 2015). Importantly, alteration of the sperm sRNA profile during epididymal transit has recently been shown to be essential for the production of functionally mature spermatozoa capable of supporting not only fertilisation, but also normal embryonic development (Conine *et al.* 2018). Moreover, the sRNA profile of sperm has also been demonstrated to be altered in response to a male’s exposure to a number of environmental insults, with such alterations having been linked with significant post-fertilisation consequences, commonly converging on the transmission of altered behavioural and metabolic phenotypes to the offspring (Fullston *et al.* 2013, Rodgers *et al.* 2013, Short *et al.* 2016). The demonstration that such phenotypic changes can be recapitulated via the direct microinjection of sRNAs harvested from the sperm of insult-exposed males into otherwise ‘normal’ fertilised oocytes, effectively

eliminates the contribution of germ-line-independent paternal factors, and further, implicates sRNAs as the causative agents (Gapp *et al.* 2014, Grandjean *et al.* 2015, Rodgers *et al.* 2015, Chen *et al.* 2016a). Nevertheless, while the list of environmental insults that influence the composition of the sRNA cargo of sperm continues to grow, the mechanism(s) responsible for mediating these changes to the sperm epigenetic landscape remains uncertain. Among the plausible scenarios, there is mounting consensus that such changes are imposed on sperm during their transit and storage within the epididymis. Notably, since spermatozoa are rendered transcriptionally and translationally silent before departing the testes (Kierszenbaum & Tres 1975, Steger 1999), it is most likely that any alterations to the sperm sRNA profile in the epididymis are driven extrinsically, with a leading candidate being epididymosomes: small membrane enclosed extracellular vesicles that encapsulate a complex macromolecular cargo of RNA and protein. Indeed, there is recent evidence that chronic exposure of a male to insults such as alcohol elicits a change in their epididymosome sRNA cargo, a change that closely parallels the altered sRNA profile of exposed spermatozoa (Rompala *et al.* 2018). Given the hypothesized contribution of the sperm sRNA profile to the offspring, important emphasis should be placed on documenting the contribution of epididymosome-mediated communication with the sperm as these cells transit the epididymis. Hence, we discuss here the role of epididymosomes in modulating the sperm sRNA profile during epididymal transit, paying particular attention to their contribution to the sRNA profile of sperm, a profile that is altered by paternal exposure to stress. Further, we also discuss the relevance of an altered sperm sRNA profile on the subsequent development of the embryo and the health of offspring.

The action of small RNA species documented in spermatozoa

It is becoming increasingly accepted that spermatozoa are more than mere vectors for conveying the paternal haploid genome to the oocyte. Indeed, beyond the paternal genome, spermatozoa harbour non-genetic information that can be propagated to the ensuing embryo. This epigenetic information includes a diverse RNA population (Chen *et al.* 2016b), and from this global RNA pool, sRNAs command considerable attention.

In spermatozoa harvested from the proximal caput segment of the epididymis, miRNAs are the most abundant sRNA species (Hutcheon *et al.* 2017), accounting for approximately half of the global sRNA population. miRNA production begins with the transcription and folding of the primary-miRNA (pri-miRNA) transcript (Ambros *et al.* 2003). Next, the pri-miRNA is processed to yield a mature miRNA duplex via the action of two

RNAse III-like endonucleases, DROSHA and DICER, and their partnering double-stranded RNA (dsRNA)-binding (DRB) proteins, DGCR8 (DiGeorge syndrome critical region 8) and TRBP (transactivation-responsive RNA-binding protein) (Fig. 1A) (Lee *et al.* 2003, Haase *et al.* 2005, Ha & Kim 2014). The miRNA/miRNA* duplex is subsequently loaded into the endonuclease, ARGONAUTE 2 (AGO2). While either strand of the miRNA/miRNA* duplex has the potential to act as a functional sRNA, only one strand is retained by AGO2 to form an activated miRNA-induced silencing complex, miRISC (Hu *et al.* 2009, O'Brien *et al.* 2018). miRNAs direct miRISC to regulate target gene expression by binding to lowly complementary target sequences, and in mammals, miRNA target sequences are almost exclusively harboured in the 3' untranslated region (3' UTR) of the targeted transcript (Lee *et al.* 1993, Bartel 2009). Once miRISC is bound to the 3' UTR of the targeted transcript, miRISC interferes with gene expression via blocking translation (Ha & Kim 2014) (Fig. 1A).

Another developmentally important sRNA species harboured by spermatozoa are the endogenous siRNAs (endo-siRNAs) (Song *et al.* 2011). Double-stranded RNA transcripts of perfect complementarity serve as precursor substrates for endo-siRNA production. These substrates are exported to the cytoplasm where they are processed by DICER to produce a mature endo-siRNA (Song *et al.* 2011). Once liberated from its precursor, the mature endo-siRNA is loaded into AGO2 to form an active siRISC to target mRNAs that harbour highly complementary sequences for endonucleolytic cleavage (Czech *et al.* 2008, Kawamura *et al.* 2008) (Fig. 1B). Research performed to date in plants, yeast and more recently flies, has revealed that this class of regulatory sRNA directs an additional function as an epigenetic regulator, via either RNA-directed DNA methylation (RdDM), or chromatin modification (Fagegaltier *et al.* 2009, Verdel *et al.* 2009). However, whether spermatozoa harboured endo-siRNAs are involved in an analogous pathway in animals remains to be determined. Nevertheless, sperm-borne endo-siRNA-directed gene expression regulation has been linked to critical early embryo development events in mice (Yuan *et al.* 2016).

Interestingly, distinct to the predominance of the miRNA class of sRNA in caput sperm, the most abundant sRNA class harboured by mature cauda sperm, are the tRFs, or tRNA-derived fragments (Sharma *et al.* 2016, Hutcheon *et al.* 2017). Transcription and processing of a precursor tRNA (pre-tRNA) transcript occurs within the nucleus to produce a mature tRNA (Fig. 1C). Once exported to the cytoplasm, the tRNA is able to fulfil its primary function in protein translation on the ribosomes. However, beyond their fundamental role as a linker molecule in translation, the specific fragmentation of mature tRNA transcripts by the endonucleases angiogenin (ANG) and DICER to produce tRFs, affords

Figure 1 The production and function of small RNA species harboured by mouse spermatozoa. Mouse spermatozoa harbour a diverse repertoire of sRNAs, including miRNA, endo-siRNA, tRF and piRNA classes (Hutcheon *et al.* 2017). The individual steps involved in the production, and subsequent action, of each class of sRNA that accumulates in mouse spermatozoa is distinct. (A) *miRNA*: MIR genes are transcribed by RNA polymerase (Pol) II to form a primary-miRNA (pri-miRNA) and the pri-miRNA is processed by DROSHA and DGCR8 to generate the precursor miRNA (pre-miRNA) (Lee *et al.* 2003). Post pre-miRNA nuclear export by exportin-5 (XPO5) (Yi *et al.* 2003), the pre-miRNA is processed by DICER and TRBP to generate the miRNA/miRNA* duplex (Haase *et al.* 2005), and either duplex strand is loaded into ARGONAUTE2 (AGO2) to form miRISC. Together with protein, GW182, miRISC represses miRNA target transcript translation (reviewed in Ha & Kim 2014). (B) *Endo-siRNA*: perfectly dsRNA molecules serve as endo-siRNA precursors (pre-endo-siRNA), and post nuclear export, the pre-endo-siRNA is processed by DICER to produce a siRNA/siRNA* duplex. Either duplex strand is loaded by AGO2 to form siRISC, and siRISC regulates siRNA target gene expression via AGO2-catalysed mRNA cleavage (Song *et al.* 2011). (C) *tRF*: precursor tRNAs (pre-tRNA) are transcribed from tRNA genes by RNA Pol III, and are subsequently processed by RNase P and RNase Z prior to nuclear export by exportin-T (XPOT). Mature tRFs are generated directly by the action of angiogenin (ANG), or via further DICER processing (Cole *et al.* 2009, Fu *et al.* 2009). The resulting tRF is loaded into AGO1, AGO3 or AGO4 to direct target gene translational repression. (D) *piRNA*: intergenic region-derived transcripts are loaded into PIWI proteins based on their 5' terminal nucleotide (Ishizu *et al.* 2012), and subsequently undergo 3' to 5' exonucleolytic trimming and 2'-O-methylation, to form mature primary piRNAs (Kirino & Mourelatos 2007). The primary piRNA is then either; (1) loaded directly into RISC to target transcripts for cleavage-mediated silencing, or; (2) used by other PIWI proteins (PIWI2/PIWI1) to direct cleavage-mediated silencing of transposable element (TE)-derived transcripts in the secondary piRNA production pathway, the piRNA 'ping-pong' pathway (Iwasaki *et al.* 2015).

a new regulatory role to these once thought static contributors to gene expression (Cole *et al.* 2009, Fu *et al.* 2009, Lee *et al.* 2009). Post production, mature tRFs are loaded into AGO-catalysed RISC and thereafter direct either transcriptional or translational repression, by targeting the promoter region, or the 5' UTR, of a targeted gene/target gene transcript. Such activity has, in turn, been linked to either transcriptional or translational repression of maternal transcripts in the embryo post fertilisation (Chen *et al.* 2016a, Schuster *et al.* 2016a).

Genomic integrity is of particular importance in the gamete destined to form a new organism. In terms of transposon or retrotransposon activity, the genome is afforded protection by an additional species of sRNA, the piRNAs. The piRNA production pathway occurs via a DICER-independent mechanism. Namely, nascent

single-stranded RNAs transcribed from intergenic regions of the genome are loaded into PIWI (P-element induced wimpy testes) proteins (Ishizu *et al.* 2012), with the PIWI-loaded transcript subsequently undergoing 3' to 5' exonucleolytic trimming and 2'-O-methylation at the 3' terminus to form a mature, primary piRNA (Kirino & Mourelatos 2007). Primary piRNAs can on occasion, trigger the generation of secondary piRNA sRNAs via a production pathway known as the ping-pong cycle (Iwasaki *et al.* 2015) (Fig. 1D). The main function of piRNAs to mediate suppression of retrotransposon activity is particularly important in the context of spermatogenesis and is also of central relevance during early embryogenesis (Carmell *et al.* 2007). However, the role of piRNAs extends beyond this canonical regulatory function to gene expression regulation via modulation

of the epigenetic status of protein-coding genes (Aravin *et al.* 2008, Thomson & Lin 2009).

Spermatozoa harbour a diverse small RNA profile that is potentially delivered to the oocyte at fertilisation

Due to the transcriptionally and translationally inert state of spermatozoa, the RNA transcripts identified in this cell type were originally thought to be inconsequential remnants of untranslated mRNA stores generated during spermatogenesis. However, it is now understood that sperm sRNAs actively contribute to sperm function, early embryo development and transgenerational epigenetic inheritance. Therefore, sRNAs may potentially hold diagnostic value for assessment of the fertility status of a male (Salas-Huetos *et al.* 2016). This potential has served as a catalyst for several recent studies that utilised either a microarray or high-throughput sequencing approach to catalogue the RNA profile of spermatozoa. Such an approach not only confirmed a complex protein-coding RNA profile for sperm (i.e. mRNA transcripts; Miller & Ostermeier 2006), but additionally documented, numerous species of non-protein-coding RNA, including numerous classes of sRNA (Ostermeier *et al.* 2005, Krawetz *et al.* 2011). Indeed, as documented earlier, the sRNAs that accumulate in mature spermatozoa include an abundance of miRNAs, endo-siRNAs, tRFs and piRNAs, as well as numerous additional sRNA molecules derived from the snRNA, snoRNA and rRNA molecules (Krawetz *et al.* 2011, Song *et al.* 2011, Peng *et al.* 2012). In recognition of increased interest in sperm-borne RNAs, a comprehensive database featuring expression data for both the large (>200 nt) and small (<200 nt) RNAs identified in the spermatozoa of several model species has been curated (SpermBase; Schuster *et al.* 2016b). The searchable SpermBase repository contains data for mouse, rat, rabbit and human spermatozoa, making it a particularly valuable resource for assessment of cross-species conservation of sperm RNA profiles.

While the biological significance of each of the thousands of distinct sperm-borne RNA species delivered to the oocyte remains to be determined, there is nonetheless growing evidence that highlights the importance of sRNAs in influencing the trajectory of early embryo development and the subsequent later life health of offspring (Chen *et al.* 2016b, Guo *et al.* 2017). One notable example is miRNA, *miR-34c*, which ranks amongst the most abundant miRNAs in human spermatozoa, and a miRNA that is also present in mouse spermatozoa and zygotes, but not in unfertilised female gametes in either of these species (Liu *et al.* 2012). Detailed analysis of *miR-34c* revealed that this miRNA may direct an essential role in regulating the first embryonic cleavage event following fertilisation (Liu *et al.* 2012). However, subsequent studies have shown

that the sperm of mice harbouring a knockout mutation in the *miR-34c* encoding sequence retain their ability to fertilise oocytes and support normal embryonic development, thus casting doubt on the 'actual' biological role of this miRNA in both sperm development and oocyte fertilisation (Yuan *et al.* 2015). Subsequent studies have expanded on this theme, utilising strategies such as the germline-specific ablation of the DICER-encoding gene, the endonuclease responsible for miRNA, endo-siRNA and tRF production (Song & Rossi 2017). This conditional knockout mouse model produced spermatozoa partially deficient in miRNA and endo-siRNA production, and which additionally failed to support normal preimplantation embryonic development (Yuan *et al.* 2016). However, the same study demonstrated that this detrimental phenotype could be rescued via microinjecting fertilised oocytes with the total RNA pool extracted from the spermatozoa of wild-type males (Yuan *et al.* 2016).

The small RNA profile of spermatozoa is markedly altered during epididymal transit

Spermatozoa leave the testis harbouring a defined sRNA profile consisting of thousands of sequences mapping to all the major sRNA classes (Jodar *et al.* 2013, Sharma *et al.* 2016). Notably however, the sRNA landscape of spermatozoa is by no means static. Instead, the sperm sRNA profile undergoes considerable remodelling during the post-testicular maturation of these cells within the epididymis (Fig. 2A). In tracking the spatiotemporal distribution of the major classes of sperm sRNA, it has been shown that a proportion of sRNAs are maintained at their post-testicular levels as sperm transit the epididymis, while the composition of the remainder of the sRNA pool is altered (Nixon *et al.* 2015, Sharma *et al.* 2016). Illustrative of this remodelling, miRNAs account for approximately half of the global sRNA population of immature spermatozoa that are delivered into the caput epididymis. By comparison, the contribution of miRNAs amounts to only ~16% of the global sRNA population of mature spermatozoa isolated from the distal cauda segment of the epididymis. Coincident with the reduction in miRNA abundance, tRF accumulation returns a reciprocal abundance trend, with the tRF class accounting for 65% of the total sRNA population of cauda sperm: equating to an almost three-fold elevation in abundance compared to caput spermatozoa (24%) (Peng *et al.* 2012, Sharma *et al.* 2016, Hutcheon *et al.* 2017). Further, the abundance of each of the other sRNA classes is also altered during epididymal transit, with notable decreases in abundance to the snoRNA, snRNA and rRNA sRNA classes, and a substantial enrichment to the piRNA sRNA class in cauda sperm (Hutcheon *et al.* 2017) (Fig. 2A).

Remodelling of the sperm sRNA profile during their epididymal transit has recently been linked to the

Figure 2 Epididymosomes modulate the sperm epigenome during epididymal transit. (A) The proportion of the three prominent small RNA (sRNA) classes implicated in transgenerational inheritance, microRNAs (miRNA), transfer RNA-derived fragments (tRFs) and PIWI interacting RNAs (piRNA). The line graphs depict the percentage of each of the sRNA classes contributing to the overall sRNA population in sperm (left) and epididymosomes (right) sampled from the caput, corpus and cauda mouse epididymis. (B) Venn diagrams illustrating the five most abundant miRNAs in sperm and epididymosomes sampled from the caput, corpus, and cauda segments of adult mouse epididymis. (C) Epididymosomes are released from the epididymal epithelium by apocrine secretion into the epididymal lumen. In the lumen, epididymosomes are able to, (i) interact with transiting spermatozoa and deliver cargo, including sRNA and (ii) effect a form of paracrine regulation by interacting with neighbouring epithelial cells. Epididymosomes play a role in modulating the sRNA profile of sperm, as they transit the epididymis. The population of sperm sRNA is subsequently delivered to the oocyte upon fertilisation and influences embryonic development and the health of the offspring. Figure adapted from [Nixon et al. \(2015\)](#), [Reilly et al. \(2016\)](#), [Hutcheon et al. \(2017\)](#) and [Zhou et al. \(2018\)](#).

functional transformation of the sperm cell ([Conine et al. 2018](#)). Specifically, embryos generated using intracytoplasmic sperm injection (ICSI) of immature caput sperm displayed signatures of aberrant pre-implantation signalling and, as a consequence, consistently failed to implant. Notably, no such defects were observed in embryos generated using either cauda epididymal spermatozoa or those sampled from the

testis. Furthermore, and of considerable interest, was the authors demonstration that these phenotypic aberrations were ameliorated via the co-injection of embryos with the sRNA cargo extracted from cauda epididymosomes ([Conine et al. 2018](#)). In contrast to this recent finding however, are the previous studies that reported that sperm isolated from the caput epididymis of mice ([Suganuma et al. 2005](#)), as well as sperm harvested from the human

testis (Greco *et al.* 2005, Weissman *et al.* 2008), are capable of supporting fertilisation and embryonic development following ICSI, despite harbouring contrasting sRNA profiles (Hutcheon *et al.* 2017, Sharma *et al.* 2018). At present, the reason(s) for these apparently contradictory results remain to be determined.

Whilst we clearly still have much to learn about the physiological importance of the reshaping of the sperm sRNA profile as these cells traverse the epididymis, we also remain unsure of the precise mechanisms responsible for driving these alterations. One plausible explanation for the apparent reduction in individual miRNAs, and potentially other classes of sRNAs, is that these transcripts are packaged within the cytoplasmic droplet (Nixon *et al.* 2015); a remnant of the germ cell cytoplasm, which remains adhered to the neck region of testicular spermatozoa, but thereafter, gradually migrates distally along the tail before being shed from mature spermatozoa. Thus, by the time murine spermatozoa reach the cauda epididymis, only approximately half the population still possess cytoplasmic droplets and their encapsulated cargo. More enigmatic however, are the mechanism(s) responsible for sRNA acquisition during epididymal transit, particularly given that spermatozoa are transcriptionally quiescent and therefore incapable of the *de novo* production of sRNA precursor molecules. Spermatozoa must therefore rely on an alternate extrinsic mechanism, with potentially important contributions being made by the epididymal luminal environment in which the sperm are bathed. One possible route for sperm sRNA acquisition is delivery via RNA-binding proteins (RBPs): a sRNA transport route documented in other tissues (Wang *et al.* 2010, Arroyo *et al.* 2011). Indeed, the RBP class of protein have previously been assigned numerous roles in spermatogenesis (Sutherland *et al.* 2015). An important advantage afforded by sRNA loading by RBPs, such as binding with either AGO2 or nucleophosmin1 (NPM1), is that such a protein-bound state would provide protection from luminal RNases (Wang *et al.* 2010, Hoy & Buck 2012). Currently however, little is known about the presence and/or abundance of RBPs in the epididymal lumen, or their potential for association with maturing spermatozoa. Rather, owing to pioneering studies conducted by Sullivan and colleagues, an alternative mode of sRNA delivery involving their encapsulation in extracellular vesicles, specifically epididymosomes, has attracted the balance of attention in this field (Belleannée *et al.* 2013, Sullivan & Saez 2013). Thus, while RBP-mediated sRNA transport remains a possibility within the epididymis, and worthy of further investigation, for the purpose of this review, here we will only focus on epididymosome-mediated inter-cellular communication, and bulk delivery of sRNA cargo to spermatozoa transiting the epididymis.

The contribution of epididymosomes to the altered small RNA profile of epididymal spermatozoa

Epididymosomes are broadly defined as membrane bound extracellular vesicles (EVs) that are produced within the principal cells of the epididymal epithelium (Fig. 2C). Noting that epididymosomes have a diameter in the range of between 50 and 150 nm (Frenette *et al.* 2010) and contain an abundance of proteomic cargo typical of small EV subtypes (Girouard *et al.* 2011, Nixon *et al.* 2018), these entities are generally classified as exosomes (Lötvall *et al.* 2014). However, epididymosomes do display some degree of heterogeneity, both in terms of their encapsulation of defined exosomal markers, such as CD9 and other tetraspanin partners, and also in their capacity to interact with target cells (Frenette *et al.* 2010), thus making definitive classification difficult (Sullivan 2015). Moreover, epididymosomes are delivered to the epididymal lumen via an apocrine secretory pathway that involves the formation of large blebs at the apical margin of the parent cell. After narrowing of the processes attaching these apical blebs to their parent cells, they eventually detach and subsequently fragment releasing their contents into the extracellular compartment (Hermo & Jacks 2002).

In the epididymis, bleb disintegration leads to the release of encapsulated epididymosomes, along with other cellular-derived content, into the epididymal lumen. Following their release, epididymosomes are able to interact with neighbouring epithelial cells to affect a form of paracrine regulation, thereby indirectly influencing sperm maturation via maintenance of the complex epididymal luminal milieu (Belleannée *et al.* 2013). Alternatively, epididymosomes are also brought into close apposition with luminal spermatozoa to exert a more direct influence on sperm maturation via delivery of fertility modulating cargo to these recipient cells (Sullivan & Saez 2013, Martin-DeLeon 2015). Since their initial discovery in the hamster (Yanagimachi *et al.* 1985), epididymosomes have been identified in the reproductive tract of a number of other species including the bull (Frenette & Sullivan 2001), mouse (Rejraji *et al.* 2006), ram (Ecroyd *et al.* 2004), cat (Rowlison *et al.* 2018) and human (Thimon *et al.* 2008). Furthermore, the interaction between epididymosomes and sperm is now a widely accepted means by which to deliver a complex proteomic and lipidomic payload to maturing spermatozoa (Frenette *et al.* 2002, Rejraji *et al.* 2006).

Exosome-mediated cell-to-cell communication is well characterised in many biological systems. In comparison however, research into this vesicle-dependent mechanism of molecular transport in the male reproductive tract is relatively scarce. Nevertheless, the balance of evidence indicates that epididymosome–sperm interactions are likely to be selective, with epididymosome proteins

having been shown to be preferentially transferred to the post-acrosomal domain of the sperm head (Nixon *et al.* 2018) before putatively being distributed in a bidirectional manner to the anterior region of the head and mid-piece of the flagellum (Frenette *et al.* 2005). This selectivity raises the prospect that the composition of the sperm plasma membrane and/or the epididymosomes themselves may influence the efficacy of their union, with specialised membrane micro domains known as lipid rafts having been implicated in the coordination of initial epididymosome–sperm interactions (Girouard *et al.* 2009). Accordingly, these micro domains are highly enriched in cholesterol and sphingolipid (Harroun *et al.* 2008) and serve to compartmentalize GPI-anchored proteins such as P25b and SPAM1 (Griffiths *et al.* 2008), which themselves have been implicated in epididymosome–sperm adhesion. In this context, the proteolytic release of GPI-linked proteins from the sperm lipid rafts leads to a significant reduction in the efficacy of epididymosome cargo transfer to the sperm cells (Girouard *et al.* 2009). Moreover, it has been shown that extensive remodelling of the lipid membrane of both epididymosomes and spermatozoa occurs along the length of the epididymis, with the epididymosome lipid bilayer being characterised by progressive increases in rigidity, and the sperm plasma membrane displaying the opposing trend of increased fluidity (Rejraji *et al.* 2006). Such changes are believed to favour the transfer of lipids from epididymosomes to sperm, a finding supported by the uptake of labelled lipids (DiIc12) from bovine epididymosomes to spermatozoa *in vitro* (Caballero *et al.* 2013).

Despite this evidence, the absence of major epididymosome membrane-bound proteins in spermatozoa suggests that epididymosome–sperm interactions do not encompass a complete fusion event (Gatti *et al.* 2005). Similarly, it has been suggested that endocytic uptake, one of the principal routes for exosome internalisation in somatic cells (Mulcahy *et al.* 2014), is unlikely to occur in mature spermatozoa (Jones *et al.* 2013). This is because spermatozoa lack both the machinery needed to internalise exogenous molecules via endocytosis and the lysosomal organelles to which endocytosed cargo are typically targeted (Jones *et al.* 2013). Rather, epididymosome adherence may be followed by the creation of a transient fusion pore and subsequent release of the epididymosome once delivery of their cargo is complete. Accordingly, proteomic analyses of epididymosomes, and spermatozoa themselves, have identified a myriad of complementary trafficking proteins (including SNARE proteins, Ras-like proteins and dynamins) (Girouard *et al.* 2011, Nixon *et al.* 2018) that could regulate this form of intercellular communication. This model may, in part, account for why a portion of epididymosomes persist in seminal fluids rather than being completely absorbed by spermatozoa within the duct. While there is currently limited

functional evidence linking any of these trafficking proteins to a role in sperm–epididymosome interaction, immunoelectron microscopy analysis has confirmed the presence of stalk-like projections forming at the interface of epididymosome–spermatozoa contact (Nixon *et al.* 2018). Similar ultrastructural features are also witnessed between spermatozoa and oviductosomes (extracellular membrane vesicles released into the oviductal fluid) (Al-Dossary *et al.* 2015), raising the prospect of conserved mechanisms for facilitating cargo delivery between spermatozoa and the different populations of extracellular vesicles they encounter during their journey to the site of fertilisation. Consistent with these findings, the transfer of epididymosome proteins can be significantly inhibited by antibody masking of MFGE8, a protein that possesses a RGD recognition motif implicated in integrin/ligand interactions that proceed cellular fusion (Raymond *et al.* 2009, Nixon *et al.* 2018). Regardless of their mechanism of uptake, epididymosome interactions culminate in significant compositional modifications of the sperm membrane architecture and cytosolic domains (Griffiths *et al.* 2008, Schwarz *et al.* 2013). Further, the demonstrated ability of epididymosomes to encapsulate their ferried cargo within a protective lipid bilayer in the epididymal lumen identifies epididymosomes as an attractive vehicle to transport sRNA to transiting epididymal sperm.

Indeed, recent studies have begun to provide proof of principle that epididymosomes are capable of trafficking sRNA cargo to spermatozoa (Reilly *et al.* 2016), with deep sequencing analysis revealing a global increase in sRNA abundance in sperm following co-incubation with epididymosomes *in vitro* (Sharma *et al.* 2018). Hence, a role for epididymosomes in modulating the sRNA profile of sperm is possible (Eaton *et al.* 2015). Accordingly, analysis of epididymosomes sampled from specific segments along the length of the epididymis has revealed substantial modification to their sRNA cargo, many of which correspond with changes documented in spermatozoa, thereby providing correlative evidence to implicate these vesicles in influencing the epigenome of sperm during epididymal transit (Reilly *et al.* 2016, Hutcheon *et al.* 2017, Sharma *et al.* 2018) (Fig. 2B).

Analysis of the sRNA cargo of epididymosomes documented numerous sRNA species, including the miRNAs and tRFs as well as those sRNAs derived from snRNAs, snoRNAs and rRNAs (Hutcheon *et al.* 2017). Interestingly, the sRNAs encapsulated in epididymosomes are distinct to many of those harboured by their parent cells, a finding that indicates selective sRNA packaging by parent cells for subsequent release via epididymosomes (Belleannée *et al.* 2013, Reilly *et al.* 2016); a characteristic that is shared among exosomes released from non-reproductive tissues (Guduric-Fuchs *et al.* 2012). It is of particular interest to note here however, that mouse epididymosomes do not harbour substantial amounts of the piRNA sRNA

class (Hutcheon *et al.* 2017), and co-incubation of epididymosomes and spermatozoa *in vitro* does not result in substantial piRNA transfer (Sharma *et al.* 2018). Thus, it is unlikely that epididymosomes account for the elevated accumulation of this sRNA class documented in mature cauda spermatozoa (Fig. 2A). Rather, the identification of proteins involved in piRNA production in cauda spermatozoa invites speculation that sperm may harbour precursor piRNA transcripts that can be used as substrates by pathway machinery proteins for piRNA production (Hutcheon *et al.* 2017).

Taken together, these data build a case for a role of epididymosomes in establishing the sperm sRNA profile during epididymal transit. However, as evidenced by work on the piRNA class of sRNA, the extent of this contribution potentially differs between each sRNA species. A recent perspective piece further extrapolated this role by suggesting that epididymal derived extracellular vesicles and their sRNA cargo are important in the transgenerational inheritance of environmental effects (Eaton *et al.* 2015). This role was suggested to involve epididymosomes facilitating communication between the exposed epididymal epithelium and the spermatozoa migrating through the epididymal lumen. Indeed, successive research has revealed that exposure to a number of environmental stressors can influence the sperm sRNA profile (Marczylo *et al.* 2012, Fullston *et al.* 2013, Gapp *et al.* 2014) and that epididymosomes are potential mediators of this altered sRNA accumulation profile (Sharma *et al.* 2016, Rompala *et al.* 2018).

The role of epididymosomes in mediating an altered small RNA profile in spermatozoa in response to the environment

It is now well established that the sRNA profile of sperm is altered in response to a changed environment. One of the first studies to examine this phenomenon utilised a high-throughput sequencing approach to reveal an increased accumulation of a subset of miRNAs, in addition to an opposing reduced accumulation of the piRNA class of sRNA, in mouse spermatozoa post-exposure to early life trauma in the form of unpredictable maternal separation in combination with unpredictable maternal stress (Gapp *et al.* 2014). Further, the offspring of exposed fathers displayed distinct behavioural phenotypes, including heightened fear and depressive-like behaviours. Via microinjection of the total RNA pool of sperm of traumatised mice into fertilised oocytes, the causal relationship of these responses was demonstrated. Such an approach successfully recapitulated the offspring phenotype; a demonstration that implicated sperm as the vector, and their RNA cargo as the causative agents, in this intergenerational inheritance model (Gapp *et al.* 2014). This demonstration also subsequently led to a series of

studies that further explored this paradigm using other stress models (Table 1). Collectively, from the studies summarised in Table 1, it is now apparent that an array of paternal stressors, ranging from dietary perturbation (e.g. high fat, low protein), chronic psychological stress, ethanol consumption, environmental pollution and cigarette smoke exposure, can each differentially alter the sRNA profile of sperm of exposed males (Li *et al.* 2012, Marczylo *et al.* 2012, Rodgers *et al.* 2013, Saavedra-Rodriguez & Feig 2013, Gapp *et al.* 2014, Metzler-Guillemain *et al.* 2015, Paris *et al.* 2015, de Castro Barbosa *et al.* 2016, Donkin *et al.* 2016, Fullston *et al.* 2016, Murashov *et al.* 2016, Rompala *et al.* 2016, Sharma *et al.* 2016, Short *et al.* 2016, 2017, Benito *et al.* 2018, Dickson *et al.* 2018, Ingerslev *et al.* 2018, Rompala *et al.* 2018). Further, some of these studies went on to demonstrate that an altered sperm sRNA profile contributed to altered phenotypic outcomes in the offspring post fertilisation. For example, independent work by de Castro Barbosa *et al.* (2016) and Fullston *et al.* (2016) has provided evidence that fathers exposed to a high-fat diet produce spermatozoa with altered sRNA profiles and that the resulting offspring of these males express overt metabolic disturbances. However, an altered sperm sRNA profile is not always associated with negative phenotypic outcomes, with exercise intervention in mice leading to an anxiolytic behavioural phenotype in offspring (Short *et al.* 2017).

Whilst the majority of these studies have focused on defining the role of sperm-borne sRNAs as ‘information carriers’ in intergenerational inheritance models, determining the mechanism(s) by which the sperm sRNA profile is altered under conditions of stress remains an important area of investigation. Interestingly, altered abundance of several sperm miRNA and tRF sRNAs, including *miR-375*, *miR-191-5p*, *miR-467e*, tRF-Gly-GCC and tRF-Glu-CTC, has been directly attributed to epididymosome-mediated transference mechanisms via *in vitro* co-culture experiments (bolded text in Table 1 and; Reilly *et al.* 2016, Sharma *et al.* 2018). However, more compelling causal links implicating epididymosomes in this phenomenon have been established in recent reports that utilised the mouse model to study chronic ethanol consumption. Specifically, the tRF cargo of epididymosomes from mice exposed to this insult demonstrated equivalent alterations to those observed in their spermatozoa (Rompala *et al.* 2018). By providing the first evidence of a concomitant modification of the sRNA cargo of both epididymosome and sperm in response to paternal insult (Rompala *et al.* 2018), this study builds on earlier reports alluding to the importance of epididymosomes in conveying an altered tRF profile to the spermatozoa of males placed on a low-protein diet (Sharma *et al.* 2016).

To begin to explore whether conditions of stress converge to elicit a common dysfunction in epididymosome trafficking, or alternatively, whether

Table 1 Small RNA present in mammalian cauda sperm and reported as significantly differentially accumulated in response to insult.

Insult	Animal model	Increased accumulation	Decreased accumulation	ES	F1	Offspring phenotype	References
High-Fat Diet	Species: Mouse Strain: C57BL/6	<i>miR-30a-5p</i> [‡]	<i>miR-141-5p</i>	n/a	n/a	Metabolic and reproductive phenotype	Fullston <i>et al.</i> (2016)
		<i>miR-126a-3p</i> [‡]	<i>miR-143-3p</i> [‡]				
		<i>miR-126a-5p</i>	<i>miR-145a-5p</i>				
		<i>miR-133b-3p</i>	<i>miR-337-3p</i>				
		<i>miR-135b-5p</i>	<i>miR-367a-3p</i>				
		<i>miR-136-5p</i>	<i>miR-376b</i>				
			<i>miR-467</i>				
			<i>miR-466a-3p</i>				
			<i>miR-466b-3-3p</i>				
			<i>miR-669c</i>				
High-Fat Diet	Species: Rat Strain: Sprague-Dawley	<i>let-7c-5p</i> [‡]	tRNA1684-GluCTC.5	n/a	✓	Metabolic phenotype	de Castro Barbosa <i>et al.</i> (2016)
		<i>piR-025883</i>	tRNA11370-GluCTC.5				
Obesity	Species: Human	<i>piR-015935</i>	tRNA16347-GluCTC.5	n/a	n/a	n/a	Donkin <i>et al.</i> (2016)
		<i>piR-31115</i>	<i>piR-36246</i>				
Low protein diet	Species: Mouse Strain: FVB/NJ	<i>piR-31445</i>	<i>piR-45120</i>	n/a	n/a	n/a	Sharma <i>et al.</i> (2016)
		<i>piR-33044</i>	<i>piR-57942</i>				
Chronic Ethanol	Species: Mouse Species: C57BL/6	<i>piR-35407</i>		n/a	n/a	n/a	Rompala <i>et al.</i> (2018)
		<i>miR-103</i>	tRF-His-GTC [†]				
Chronic variable stress	Species: Mouse Strain: C57BL/6;129 F1 hybrid	tRF-Gly-TCC	tRF-Gly-GCC	✓	n/a	Same exposure previously shown to impart stress hyposensitivity (Rompala <i>et al.</i> 2016)	Rodgers <i>et al.</i> (2013)
		tRF-Gly-CCC					
		tRF-Gly-CTT					
		tRF-Lys-CTT [†]					
		<i>miR-10a</i> [‡]	<i>let-7c</i> [‡]				
		<i>miR-10b</i>	<i>let-7a</i> [‡]				
		<i>miR-99b</i> [‡]	<i>let-7b</i> [‡]				
		<i>miR-125a</i>	<i>let-7e</i> [‡]				
		<i>miR-196a</i> [‡]	<i>let-7f</i> [‡]				
		<i>miR-205</i> [‡]	<i>let-7b</i> [‡]				
Trauma – unpredictable maternal separation	Species: Mouse Strain: C57BL/6	<i>miR-3535</i>	<i>let-7b</i> [‡]	n/a	n/a	Behavioural phenotype – HPA stress axis dysregulation	Capp <i>et al.</i> (2014)
		<i>miR-29c</i> [‡]	<i>miR-16a</i>				
		<i>miR-30a</i> [‡]	tDR-Ser-ACA				
		<i>miR-30c</i> [‡]	tDR-His-GTC [‡]				
		<i>miR-32</i>	tDR-Tyr-GTA				
		<i>miR-193</i>	tDR-Pro-AGG				
		<i>let-7a-5p</i> [‡]	tDR-Ile-AAT				
		<i>let-7b-5p</i> [‡]	tDR-Lys-TTT				
		<i>let-7c-5p</i> [‡]	tDR-Ser-CCA				
		<i>let-7e-5p</i> [‡]	tDR-Ser-TCA				
Chronic variable stress	Species: Mouse Strain: C57BL/6;129 F1 hybrid	<i>let-7f-5p</i> [‡]		n/a	n/a	Behavioural phenotype – HPA stress axis dysregulation	Rodgers <i>et al.</i> (2013)
		<i>let-7g-5p</i>					
		<i>let-7i-5p</i>					
		<i>miR-9-5p</i>					
		<i>miR-10a-5p</i> [‡]					
		<i>miR-10b-5p</i> [‡]					
		<i>miR-15b-5p</i>					
		<i>miR-21a-5p</i>					
		<i>miR-26a-5p</i>					
		<i>miR-26b-5p</i> [‡]					

(Continued)

Table 1 Continued.

Insult	Animal model	Increased accumulation	Decreased accumulation	ES	F1	Offspring phenotype	References	
		<i>miR-30c-5p</i> [†] <i>miR-30b-5p</i> <i>miR-30d-5p</i> <i>miR-34b-3p</i> <i>miR99a-5p</i> <i>miR99b-5p</i> [†]	<i>miR-375-3p</i> <i>miR-375-5p</i> <i>miR-449a-5p</i> [†] <i>miR-465c-3p</i> <i>miR-466c-3p</i> <i>miR-497-5p</i> <i>miR-532-5p</i> [†] <i>miR-672-5p</i> <i>miR-192</i> <i>miR-350</i> <i>miR-449a</i> [†] <i>miR-467e</i>					
Stress-corticosterone administration	Species: Mouse Strain: C57BL/6	<i>miR-26b</i> [†] <i>miR-98</i> [‡] <i>miR-144</i> [‡] <i>miR-190b</i> [‡] –	–	n/a	n/a	Hyper anxiety-like behavioural phenotype	Short <i>et al.</i> (2016)	
Early life trauma	Species: Mouse Strain: CD-1	–	<i>miR-34c-5p</i> [†] <i>miR-449a</i> [†]	n/a	✓	Induced anxiety and sociability defects detected from previous study (Saavedra-Rodriguez & Feig (2013))	Dickson <i>et al.</i> (2018)	
Smoking	Species: Human	<i>let-7a-2-3p</i> <i>miR-7</i> <i>miR-30c</i> [†] <i>miR-146b-3p</i> <i>miR-340</i> <i>miR-365</i> <i>miR-509-5p</i> <i>miR-519d</i> <i>miR-550a</i> <i>miR-550b</i> <i>miR-576-3p</i> <i>miR-23b-3p</i> <i>miR-33b-3p</i> <i>miR-92a-2-3p</i> <i>miR-126-3p</i> [†] <i>miR-129-3p</i> [†] <i>miR-181d</i> <i>miR-186</i> <i>miR-221-3p</i> <i>miR-10b</i> [†] <i>miR-33b</i> <i>miR-106a</i> <i>miR-155</i> <i>miR-183</i> [†] <i>miR-30c</i> [†] <i>miR-30e</i> <i>miR-34c</i> [†] <i>miR-92a</i> <i>miR-98</i> [†] <i>miR-99b</i> [†] <i>miR-122</i> <i>miR-124</i> <i>miR-134a</i>	<i>miR-576-5p</i> <i>miR-933</i> <i>miR-944</i> <i>miR-1246</i> <i>miR-1267</i> <i>miR-1270</i> <i>miR-1285</i> <i>miR-3145-3p</i> <i>miR-4513</i> <i>miR-4748</i>	n/a	n/a	Not investigated	Marczylo <i>et al.</i> (2012)	
Smoking	Species: Human		<i>miR-26a-1</i> <i>miR-296-5p</i> <i>miR-520d-3p</i> <i>miR-3940</i>	n/a	n/a	Not investigated	Metzler-Guillemain <i>et al.</i> (2015)	
Environmental pollution	Species: Human		<i>let-7d</i> [†] <i>miR-363</i> [†]	n/a	n/a	Not investigated	Li <i>et al.</i> (2012)	
X-ray irradiation	Species: Mouse Strain: CD1		<i>miR-29c</i> [†] <i>miR-144</i> [†] <i>miR-181a</i> <i>miR-326</i> <i>miR-338</i> <i>miR-341</i> <i>miR-465a</i> <i>miR-500</i> <i>miR-503</i>	<i>miR-466h</i> <i>miR-466i</i> <i>miR-466q</i> <i>miR-471</i> <i>miR-532</i> [†] <i>miR-666</i> <i>miR-669d</i> <i>miR-669f</i>	n/a	n/a	Not investigated	Paris <i>et al.</i> (2015)

Long term exercise	Species: Mouse Strain: C57BL/6j	miR-145	miR-669g	n/a	n/a	More susceptible to effects of high fat diet	Murashov et al. (2016)
		miR-147	miR-701				
		miR-183 [†]	miR-713				
		miR-196a [†]	miR-714				
		miR-346	miR-762 [†]				
Exercise	Species: Mouse Strain: C57BL/6	miR-21	miR-221	n/a	n/a	Anxiolytic behavioural phenotype	Short et al. (2017)
		miR-431					
		miR-483-3p	miR-133a-1				
		miR-19b-2	miR-133a-2				
		miR-190b [†]	miR-455				
Endurance training	Species: Human	miR-143-3p [†]	miR-223-3p	n/a	n/a	Not investigated	Ingerslev et al. (2018)
		piR-11690	miR-3653-3p				
		piR-26632	piR-17444				
		tDR-lys-CIT.4 [†]	piR-1834				
		miR-132-3p	—				
Environment enrichment	Species: Mouse Strain: C57BL/6	miR-132-5p		n/a	n/a	Improved cognition	Benito et al. (2018)
		miR-212-3p					
		miR-212-5p					

Underlined sRNA species signifies altered accumulation of sRNA was validated by a second technique (commonly real time-quantitative PCR). Bold sRNA indicate species shown to be transferred from epididymosomes to sperm via *in vitro* co-incubation experiments. ES column: studies that found a parallel change in the sRNA cargo of isolated epididymosomes (ES) under conditions of stress (✓, yes; ×, no; n/a, not investigated). F1 column: studies that examined the sperm sRNA profile of offspring from exposed fathers (×, found no parallel change in offspring sperm sRNA profile; ✓, found similar alteration in offspring sperm sRNA profile to that established in fathers; n/a, did not investigate). Note: threshold of significance varied between studies included in this analysis (*P*-value cut off range <0.1–0.05).

[†]Denotes sRNA species reported in more than one of the studies summarised.

each stress drives unique alterations to the composition of the total sperm sRNA population, we surveyed the curated lists of sRNA profiles documented across multiple stress models (Table 1). Perhaps expectedly, this analysis revealed that each stress model displayed a divergent combination of changes to the sRNA profile of stressed sperm. Notably however, this analysis also identified a specific subset of sRNA species that have been reported to differentially accumulate in response to more than one environmental insult. Illustrative of this, among the 17 studies summarised in Table 1, 30 sRNAs, including 28 miRNAs repeatedly returned an altered change in abundance post-exposure of sperm to multiple environmental insults (denoted by [†] in Table 1). As a cautionary note, we emphasise that each miRNA has the ability to modulate the abundance of many numerous, functionally unrelated protein-coding transcripts, and thus, it is important to not over-interpret the significance of similar changes in sRNA abundance (i.e. the same sRNA and a matching abundance trend) post exposure to multiple stressors. Further, we acknowledge the variations in documented sperm sRNA changes arising from the application of similar stress exposure models in independent laboratories. The extent to which such variability is attributed to differences in experimental design, methodologies and/or model species/strains employed remains to be determined. Notwithstanding these caveats, our interrogation of the available literature provides preliminary evidence to suggest that each imposed stress results in a unique signature of altered sRNA abundance in exposed sperm. Considering this information, it is tempting to speculate that rather than simply being a legacy of reproductive tract dysfunction, these changes may hold some adaptive value (Rechavi & Lev 2017). Indeed, this hypothesis has recently been promulgated on the basis of studies in invertebrate models, where it is suggested that males are able to transmit an adaptive advantage to subsequent generations (Rechavi et al. 2011, Wang et al. 2017). However, whether this paradigm is applicable to complex organisms, such as vertebrates, remains to be elucidated.

While epididymosomes potentially mediate the transfer of sRNA from the epididymal soma to maturing spermatozoa, these vesicles and their harboured cargo are produced within the epididymal epithelial cells. Therefore, under stress conditions, the physiology of the epididymal epithelial cells is likely altered; a physiological change that could initiate a series of biochemical events which result in an altered sRNA population being produced and packaged into the epididymosomes generated by stressed cells. Currently, this is not an active area of research focus within this field, however, with each individual insult demonstrated to elicit a unique change to the sRNA profile of sperm (Table 1), it is likely that the production and packaging of sRNAs into epididymosomes within the stressed

epididymal epithelial cell, differs with each individual insult. To date, this paradigm has been considered in the context of chronic stress, where it has been suggested that exposure to elevated corticosterone modulates the expression of the glucocorticoid receptor, and this in turn promotes aberrant epididymal epithelial cell signalling (Pang *et al.* 2017). The dysregulation of intracellular signalling is, in part reflected by an altered miRNA production profile and packaging into epididymosomes, thus putatively driving equivalent changes to the sperm sRNA profile (Pang *et al.* 2017). Whilst plausible, this proposed model obviously requires experimental validation.

Concluding remarks

The sRNA profile of spermatozoa is now recognised as an important epigenetic contributor to early embryo development and subsequent offspring health. Indeed, the specific reshaping of this profile that occurs during epididymal transit is required to produce a mature spermatozoon that is not only capable of initiating fertilisation but also supporting early embryonic development and the generation of healthy offspring. However, the plasticity of the sRNA profile under conditions of stress and the consequences of an altered sRNA profile are becoming increasingly apparent. This therefore places heightened emphasis on the need to understand the mechanisms responsible for altering the sRNA landscape of sperm of stressed males. Among the most attractive mechanisms for modulating the global sRNA landscape of sperm under normal physiological conditions, and those encountered during episodes of stress, are epididymosomes. These specialised extracellular vesicles have been implicated in a novel form of intercellular communication, which is capable of dramatically altering the proteomic, lipidomic and epigenetic landscape of the maturing spermatozoon. What remain less certain are the underlying biological processes that drive alterations of the sRNA cargo of epididymosomes. Further research in this field promises to advance our currently limited understanding of the legacy of exposure to environmental insult on future generations and to inform rational therapeutic intervention strategies.

Declaration of interest

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

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