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Sperm-borne sncRNAs: potential biomarkers for semen fertility?

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ABSTRACT

Semen infertility or sub-fertility, whether in humans or livestock species, remains a major concern for clinicians and technicians involved in reproduction. Indeed, they can cause tragedies in human relationships or have a dramatic overall negative impact on the sustainability of livestock breeding. Understanding and predicting semen fertility issues is therefore crucial and quality control procedures as well as biomarkers have been proposed to ensure sperm fertility. However, their predictive values appeared to be too limited and additional relevant biomarkers are still required to diagnose sub-fertility efficiently. During the last decade, the study of molecular mechanisms involved in spermatogenesis and sperm maturation highlighted the regulatory role of a variety of small non-coding RNAs (sncRNAs) and led to the discovery that sperm sncRNAs comprise both remnants from spermatogenesis and post-testicular sncRNAs acquired through interactions with extracellular vesicles along epididymis. This has led to the hypothesis that sncRNAs may be a source of relevant biomarkers, associated either with sperm functionality or embryo development. This review aims at providing a synthetic overview of the current state of knowledge regarding implication of sncRNA in spermatogenesis defects and their putative roles in sperm maturation and embryo development, as well as exploring their use as fertility biomarkers.

Keywords: epididymosomes, epigenetic, fertility, fertility prediction, non-genetic sperm legacy, semen, sncRNAs, sperm-born sncRNAs.

Introduction

A fully mature sperm is the result of multiple processes, starting before birth in the testis, followed by several maturation steps during the epididymis transit or following interactions with seminal plasma and ending with final modifications in the female tract. Multiple hormones and intermediates are required to achieve all these steps, leading to a sperm capable of fertilising the oocyte and potentially driving the embryo early development. Disruption of any of these processes can lead to sperm abnormalities and potentially reduce semen fertility. In humans, about 50% of infertility cases are related to men issues, with no clear aetiology for 30–50% of these cases. (Hamada *et al.* 2012). Identification of the disturbance is the first step towards understanding the biological mechanisms involved and potential treatment.

In livestock farming, since sub-fertile males or ejaculates decrease the breeding efficiency and induce economic losses, several semen quality control procedures have been proposed to ensure semen fertility (Vincent *et al.* 2014) and biomarkers have been searched for to establish effective fertility predictors. For instance, sperm characteristics, functionality and physiology have been assessed using computer analysis semen assessment (CASA) (Amann and Waberski 2014) or flow cytometry (e.g. membrane integrity, mitochondrial potential, oxidation sensitivity, acrosome statue, DNA compaction and fragmentation).

Due to the multifactorial nature of semen fertility, however, individual biomarkers remain insufficient to achieve reliable predictions. Even the combination of these biomarkers by 'stepwise' or 'logistic' regression approaches have failed to explain more than 40% of the variability in fertility (Sellem *et al.* 2015). This has led to the hypothesis that additional

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biomarkers are required to reach good fertility predictions, which should be unrelated to biological processes already included in routine semen quality assessment protocols. In this respect, the study of molecular mechanisms involved in spermatogenesis and sperm maturation have highlighted the role of a variety of sncRNAs (de Mateo and Sassone-Corsi 2014). In addition, the discovery that mature sperm carry thousands of sncRNAs, which comprise both remnants from spermatogenesis and post-testicular sncRNAs acquired through interactions with extracellular vesicles along epididymis, has raised many questions about their putative role. Though their functional significance is still a matter of debate, growing evidence suggests that sperm RNAs are thus delivered to the oocyte at fertilisation (Sendler *et al.* 2013), providing resources for embryo development (Shi *et al.* 2020), and being involved in paternal epigenetic transgenerational inheritance (Sharma 2019; Le Blevec *et al.* 2020). Given their suggested roles both in spermatogenesis and embryo development, sncRNAs have gained interest as relevant biomarkers of semen fertility.

This review aims at providing a synthetic overview of the current state of knowledge regarding implication of sncRNA in spermatogenesis defects and their putative roles in sperm maturation and embryo development, as well as exploring their use as fertility biomarkers.

Discovery, biogenesis and function of the main sncRNA classes

SncRNAs are a broad and heterogeneous family of 18–200 nucleotides-long RNAs, having mainly a regulatory function through either RNA interference, RNA modification or spliceosome regulation. Next-generation sequencing (NGS) has led to the discovery and quantification of a diversity of sncRNA classes (Hombach and Kretz 2016; Wei *et al.* 2017), including microRNAs (miRNAs), small nuclear RNAs (snRNAs), piwi-interacting RNAs (piRNAs), rRNA-derived small RNAs (rsRNAs) and tRNA-derived small RNAs (tsRNAs). Overall, regulatory sncRNAs are expressed at specific development stages or under particular stimuli and are involved in a growing number of important biological processes. sncRNAs which repress expression of messenger RNA (mRNA) at the post-transcriptional level provide a rapid and adaptive mechanism to modulate gene expression. Other sncRNAs such as piRNAs can modulate gene expression at the epigenetic level, being likely involved in establishing and maintaining long-term patterns of RNA expression. Furthermore, snRNAs can influence alternative splicing, guide specific proteins to apply RNA modifications at a given location and may be involved in translational regulation due to their essential role for the spliceosome and the ribosome function (Karijolich and Yu 2010; Jia *et al.* 2012).

Advances in high-throughput sequencing technologies (NGS), which facilitated genome-wide determination and comparison of DNA and RNA sequences, have led to the discovery of a wealth of sncRNA variants produced by RNA editing. RNA editing is a core co- or post-transcriptional enzymatic modification process, through which a primary RNA sequence is altered by single-nucleotide substitutions, insertions, or deletions (Gott and Emeson 2000). In humans, the adenosine-to-inosine (A-to-I) RNA editing, mediated by adenosine deaminase acting on RNA (ADAR) family of enzymes, and the cytosine-to-uracil (C-to-U) RNA editing mediated by Apolipoprotein B mRNA Editing Complexes (APOBECs) are considered the canonical types, with the A-to-I type being the most prevalent form, accounting for millions of edits in both coding and noncoding transcripts (Zinshteyn and Nishikura 2009; Savva *et al.* 2012; Picardi *et al.* 2016). sncRNAs have been recognised as major targets for RNA editing enzymes and single-nucleotide changes through editing are thought to impact their biogenesis as well as their target specificity (Paris *et al.* 2012; Yang *et al.* 2015; Penzo *et al.* 2016; Zheng *et al.* 2016; Li *et al.* 2018*a*), but the large number of identified editing sites may also merely represent, to some extent, neutral transcriptional noise (Gommans *et al.* 2009).

miRNAs

miRNAs are a class of non-coding RNAs with an average of 22 nucleotides in length, discovered in the 1990s through the interaction of Lin-4 and Lin-14 in *Caenorhabditis elegans* and then the discovery of Let-7 in multiple species (Wightman *et al.* 1993). Most miRNAs are transcribed by RNA polymerase II in long precursors called pri-miRNA (Hammond 2015). In human or bull, almost half of miRNA coding sequences are intergenic, while the others are mainly intronic or located inside UTR regions (Galatenko *et al.* 2018; Sellem *et al.* 2020). They are separated from each other or organised within 'miRNA clusters' (ex: miR-323, -758, -329b and -329a on bovine chromosome 21). These sequences are then processed by DROSHA coupled to DGCR8, resulting in shorter sequences called pre-miRNA (Lee *et al.* 2003). The pre-miRNA are exported from nucleus to cytoplasm by Exportin 5 and processed by DICER to cleave the secondary structure (stem loop) and form mature miRNAs (Hutvágner *et al.* 2001) (Fig. 1).

The mature 5′ stand interacts with an Argonaut protein (Ago2) to form the RISC complex (RNA-induced silencing complex). The second strand is cleaved or can also interact with Ago2.

In most cases, the RISC complex interact with the 3′ untranslated region (3′ UTR) of target mRNAs to suppress expression through mRNA degradation or translational repression (O'Brien *et al.* 2018). However, interaction of miRNAs with 5′ UTR of coding sequences have also been reported. Advances in high-throughput sequencing

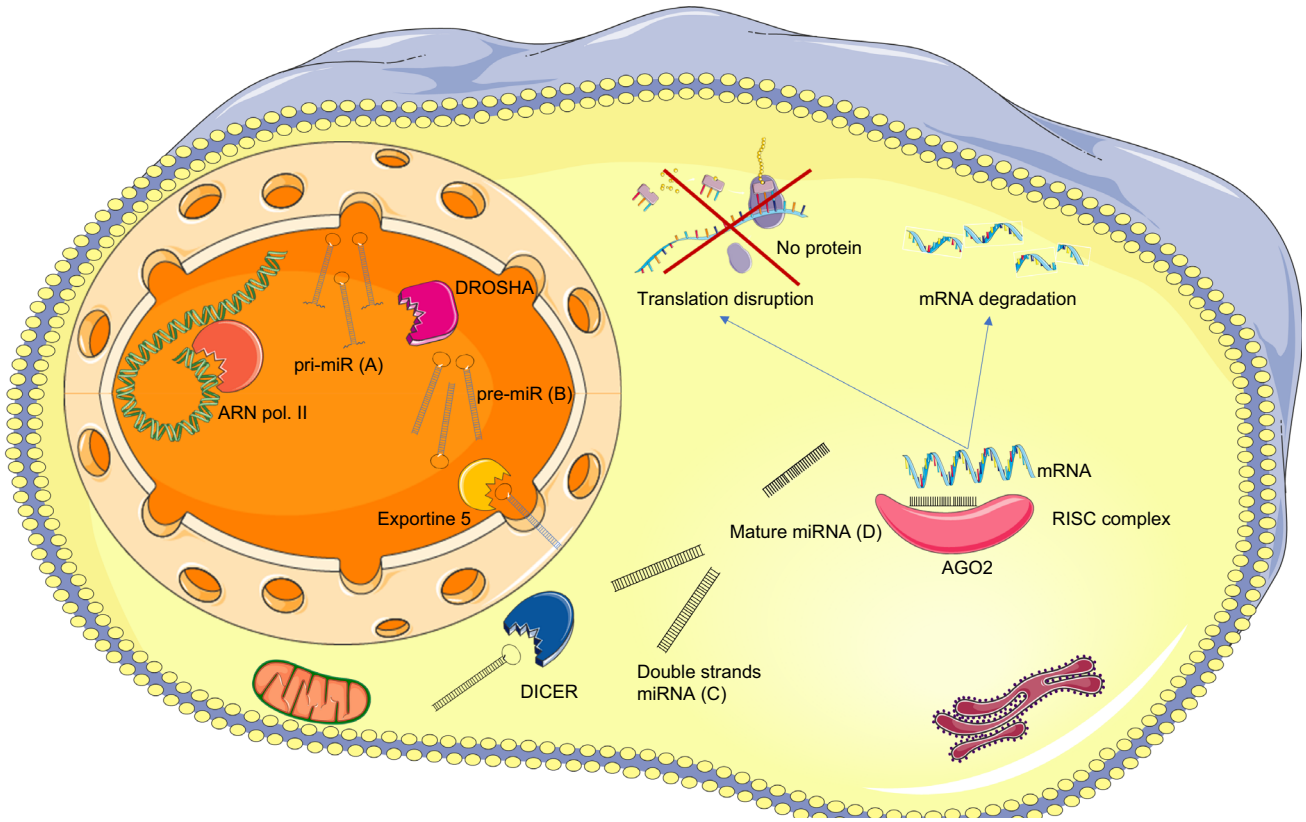

Fig. 1. miRNAs biogenesis, maturation and function. Different forms of miRNAs exist, from precursor to mature strand. The native and newly transcribed form, called pri-miRNA (A), presents typical stem-loop structure. pri-miRNA will be processed by enzymes such as DROSHA or DGCR8 to form a shorter sequence called pre-miRNA (B). Exported in the cytoplasm, the sequence will be matured by DICER, forming a double strand (C) sequence. The strand separation will lead to the formation of mature 5p and/or 3p miRNA (D). Captured by AGO2, this complex, called RISC, manages the repression of protein formation due to the cleavage of the complementary mRNA or the translation repression. @Allice.

technologies have led to the discovery of isomiRs, which are miRNA variants produced by editing, including substitutions (Li *et al.* 2018*a*), 3′ or 5′ additions (Katoh *et al.* 2009; Tan *et al.* 2014) and deletions (Lee *et al.* 2019). These isomiRs target distinct sets of mRNA, have been described in multiple species and are expressed constitutively in several tissues in a tissue-specific manner (Fernandez-Valverde *et al.* 2010; Telonis *et al.* 2017) as well as according to sex and health status (Loher *et al.* 2014; Telonis *et al.* 2015*b*), suggesting distinct functional roles (Tan *et al.* 2014; Tan and Dibb 2015).

Since they regulate almost 60% of genes (Friedman *et al.* 2009), miRNAs are involved in a variety of biological processes and are critical for normal animal development, as exemplified by the constitutive or tissue-specific DICER knock-out in mice, leading to embryogenic lethality (Bernstein *et al.* 2003) or impairment of several tissues such as skin, heart, lungs, muscle (Andl *et al.* 2006; Krill *et al.* 2013). Likewise, aberrant expression of miRNAs is associated with many human diseases and miRNAs have been reported as potential biomarkers for a variety of diseases (Paul *et al.* 2018).

piRNAs

PIWI proteins, a subfamily of Argonaut proteins, were discovered in the late 1990s in *Drosophila melanogaster* and then in other species (Weick and Miska 2014; Russell *et al.* 2016). They associate with a particular class of 26–33 nt sncRNAs called piRNAs. piRNAs are produced from long precursor transcripts, which are exported into the cytoplasm and cut every 25–35 nucleotides at an uracil by the endonuclease ZUC (in association with cofactors such as VRET, MINO or GASZ). This biogenesis pathway generates piRNAs having a 5′1U structure, called primary piRNAs' (Czech *et al.* 2018). Primary piRNA can enter the 'ping-pong cycle', that is initiated after interaction with argonaut protein AUB. The piRNA-AUB complexes cut active transposon transcripts at the nucleotide that pairs with the 10th nucleotide of piRNA, producing 'secondary piRNA' in sense orientation to transposons and having no 1U bias but Adenosine at position 10. Secondary piRNA are loaded into Ago3 and Ago3-piRNA complexes in turn recognise and cleave precursor transcripts to generate more primary piRNAs and start yet another cycle (Czech and Hannon 2016) (Fig. 2).

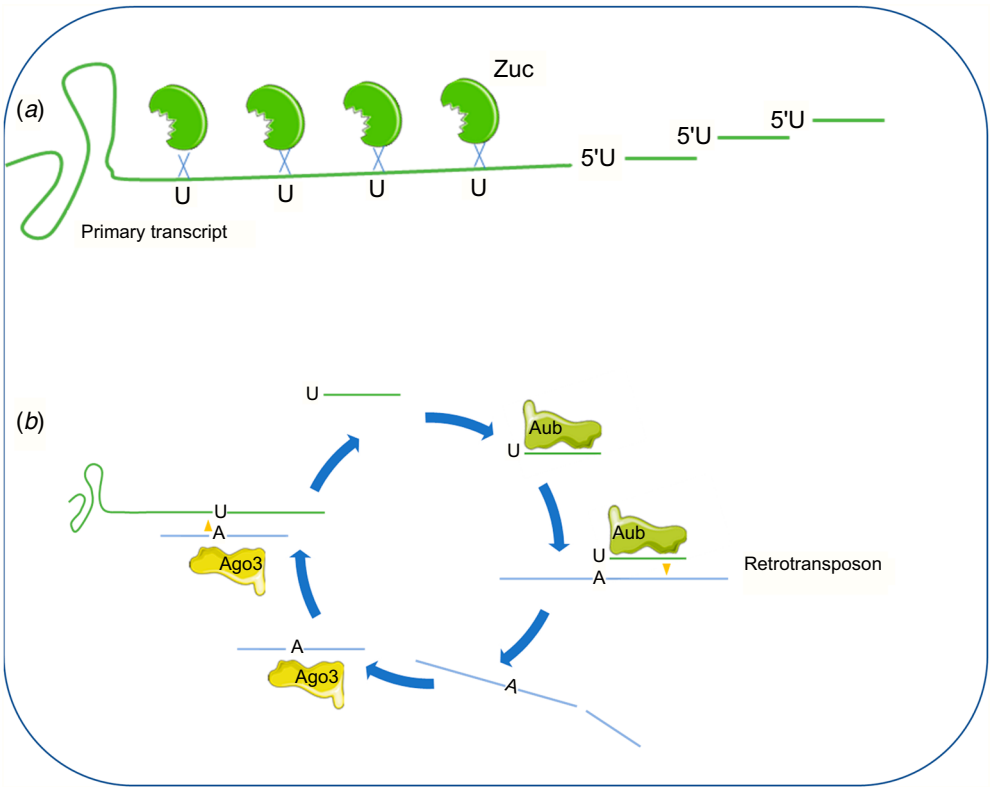

Fig. 2. piRNA biogenesis. The biogenesis of piRNAs involves two distinct pathways: The production of so-called 'primary' piRNAs, having a 5′ 1U bias (*a*). This pathway mainly involves ZUC protein which cleaves every 25–35 nucleotides the long primary transcript of piRNAs. The 'secondary' piRNAs are generated during the 'ping-pong' cycle and exhibiting an A at position 10 (*b*). This second pathway involves mainly AGO3 and AUB which generate primary and secondary piRNA sequences by cleaving either transposons or long primary transcripts of piRNAs. @Allice.

The piRNA pathway provides a powerful system to prevent transposon expression and propagation, through both direct slicing and transcriptional silencing via interaction with two other epigenetic mechanisms: piRNAs can recruit DNMT3L, 3A and 3B, to locally induce DNA methylation and block their activity (LINE or LTR; Aravin *et al.* 2008; Pillai and Chuma 2012; Weick and Miska 2014) or interact with factors leading to post translational repressive modifications on histones aiming to locally close the chromatin, resulting in retrotransposons silencing (Czech *et al.* 2018). Moreover, analysis of pachytene piRNAs in elongating spermatids showed a catalytic activity, leading to the concerted degradation of the bulk of cellular mRNAs, illustrating a role in gene expression regulation (Gou *et al.* 2014).

tRNAs derived fragments (tsRNAs)

Transfer RNAs are a conserved and highly abundant RNA class with a well-defined role in protein translation. They are transcribed by RNA Pol III as a premature tRNA transcript, which undergoes processing by two endonucleases, namely RNase P (5′ trimming) and RNaze Z (3′ trimming). High-throughput sequencing has led to the discovery of a variety of abundant RNA fragments related to tRNAs, with a diversity of size suggesting the existence of multiple biogenesis mechanisms (Keam and Hutvagner 2015; Chen *et al.* 2021). Indeed, tsRNAs can be broadly classified into two main groups: tRNA-halves (5′ and 3′ tRHs) and tRNA-derived fragments (tRF5s, i-tRFs and tRF3s). tRNA-halves are 35–45 nt RNA species produced by ribonucleolytic cleavage of mature tRNAs by Angiogenin. They are referred to 5′-halves or 3′-halves according to their mapping on the tRNA sequence: 5′-tRHs usually start at the first nucleotide of the tRNAs and typically terminate around the anticodon loop of the tRNA, while 3′-tRHs usually encompass the anticodon region and they may extend into the CCA region of the mature tRNA (Fig. 3*a*). tRFs ranging from 18 to 35 nt can be processed from either the 5′ or the 3′ arm of the mature tRNA, resulting from a cleavage by DICER, RNase Z or other RNAses in the TψC-arm of mature tRNA or a cleavage in the D-arm, respectively. Internal tRFs (i-tRFs) are produced from a combination of cleavages in the anticodon loop and either D-arm or TψC-arm (Fig. 3*a*).

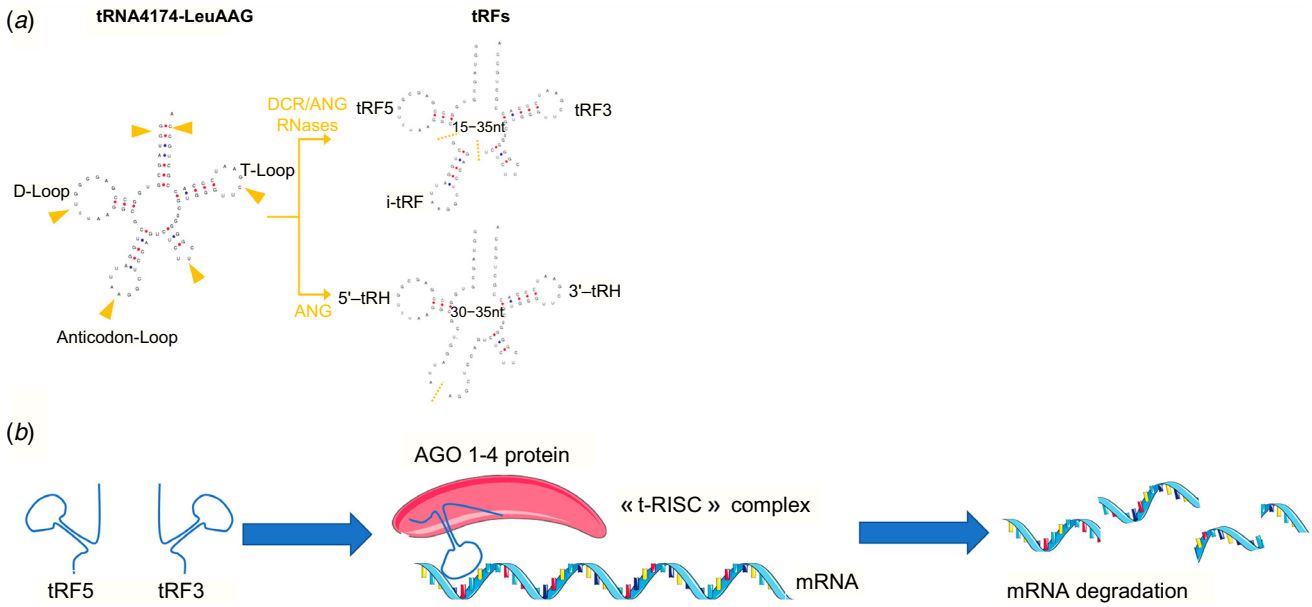

Fig. 3. Specific cleavages of tRNA into tRFs (*a*). The cloverleaf structure of a tRNA typically contains a D-Loop, an Anticodon-Loop, a variable loop, a T-loop, and an amino acid acceptor stem. Several endonucleases can cleave tRNAs at specific sites, generating tRFs of different categories: 5′ or 3 tRH, tRF3s, tRF5s and i-tRFs. (*b*) Regulation of genome expression through tRFs. tRF3 and tRF5 by complexing Argonaut proteins, are able to target complementary mRNAs, leading to repress genome expression due to mRNA degradation or translation disturbance. @Allice.

Until recently, tsRNA were considered as aberrant degradation products of endonuclease activity and their functional potential remained overlooked until recently. Intriguingly, a growing amount of evidence suggests that tRF may harbour unanticipated biological activity and dynamically impact genome expression (Guzzi and Bellodi 2020). For instance, the processing of mature tRNA into tRFs appears to be site-specific and restricted to specific isotypes of tRNAs, generating tRFs with specific lengths among different cell types. In addition, no correlation was observed between tRFs expression and abundance of their precursor tRNAs (Telonis *et al.* 2015*a*). Multiple studies have shown that tRFs can repress the expression of endogenous targets and have a role in regulating the simplest to complex biological processes. In particular, tRF5s and tRF3s have been shown to target the 3′UTR of specific mRNAs and regulate gene expression post-transcriptionally (Maute *et al.* 2013; Deng *et al.* 2015). This regulation seems to be Dicer-independent and Argonaute-dependent (Kuscu *et al.* 2018; Chen *et al.* 2021). tRF5s and tRF3s interact with AGO1, AGO3 and AGO4 to form AGO complexes (Fig. 3*b*) which regulate target RNA expression based on seed sequences complementary to target mRNAs (Kumar *et al.* 2016). Accumulation of tRNA-halves has been associated with oxidative and metabolic stress (Emara *et al.* 2010), leading to translational repression (Ivanov *et al.* 2011). Abnormal tRFs expression levels in disease conditions have been observed, including cancer and neurological disorders, suggesting that tRFs could be relevant biomarkers (Raina and Ibba 2014; Krishna *et al.* 2021). Moreover, in mice embryos, tRF3 are involved in retrotransposons repression during the epigenetic reprogramming (Schorn *et al.* 2017).

rRNAs derived fragments (rsRNAs)

rRNAs are the most abundant RNA molecules in eukaryotic cells, including four rRNAs (5S, 5.8S, 18S, and 28S) being encoded by the nuclear genome and two (12S and 16S) by the mitochondrial genome. Similar to tsRNAs, short fragments derived from rRNAs have been discovered in several species, which are produced non-randomly by at least four biogenesis pathways (Lambert *et al.* 2019). All six rRNAs produce rRFs with unique features, from the 5′-end (rRF5s), the interior (i-rRFs), and the 3′-end (rRF3s) of the parental rRNA. Expression of rRFs appears to be influenced by multiple factors, including tissue, health status and sex (Chu *et al.* 2017; Locati *et al.* 2018; Cherlin *et al.* 2020). Similar to tRF, several rRFs have been shown to interact with AGO proteins suggesting a role in gene silencing (Guan and Grigoriev 2020). For instance, change in expression of several key metabolic enzymes was observed in mouse hepatoma cells after overexpression or inhibition of several rRFs, showing that rRFs can regulate metabolic processes in a similar manner to miRNAs (Wei *et al.* 2013). rRFs have also been shown to act as precursors for the production of miRNAs or piRNAs (Chak *et al.* 2015; Locati *et al.* 2018).

Ejaculated sperm contains abundant sncRNAs

Several sncRNAs classes have been identified in ejaculated sperm, including miRNAs, piRNAs, tsRNAs, rsRNAs, snoRNAs or mtRNAs, with differences in terms of sequence diversity and expression levels according to species, sequencing technology and coverage, bioinformatic pipeline etc. Of note, even if most studies include technical steps to remove somatic cells (e.g. Percoll gradient or incubation in hypotonic somatic cell lysis buffer and washing steps), somatic cell contamination can't be ruled out and may at least in part explain differences observed between studies.

According to studies in humans (Hua *et al.* 2019; Nätt *et al.* 2019; Xu *et al.* 2020), bull (Fagerlind *et al.* 2015; Capra *et al.* 2017; Sellem *et al.* 2020; Sellem *et al.* 2021), swine (Li *et al.* 2018*b*; Gòdia *et al.* 2019; Alvarez-Rodriguez *et al.* 2020) and other species, about 85% of reads produced after NGS sequencing of ejaculated sperm small RNAs are annotated as sncRNAs, mainly piRNAs, rRFs, miRNAs and tRFs. For instance, 701 known miRNAs and about 2000 putative miRNA sequences have been identified in bovine, producing about 200 000 isomiRs, with 70 isomiRs per miRNA on average. (Sellem *et al.* 2020).

However, most publications agree on main sncRNAs classes and similarities were observed across species. For instance, based on SpermBase (www.spermbase.org), about 42% of human sperm miRNAs have been also identified in mouse. About 50% of rat sperm miRNAs were also described in humans and this inter species coverage reached 72% between rabbit and human sperm (Fig. 4).

Focusing on bovine, about 45% of sperm miRNAs were also described in mouse, including several key miRNAs involved in cell differentiation, proliferation, spermatogenesis, or embryo development (miR-10, miR-29, miR-34, miR-100, miR-148, miR-191) (Nixon *et al.* 2015; Chu *et al.* 2019). Likewise, human and bovine ejaculated sperm were shown to share a subset of their 20 most expressed miRNAs (miR-100, miR-34c, miR-191 and miR-30d) (Fagerlind *et al.* 2015; Capra *et al.* 2017; Hua *et al.* 2019; Sellem *et al.* 2020; Xu *et al.* 2020). Likewise, tsRNAs0 originating from glycine and glutamate isoacceptors are the most expressed tsRNAs in both human and bovine ejaculated sperm, accounting altogether for about 70% of tsRNAs expression (Hua *et al.* 2019; Sellem *et al.* 2020). Interestingly, while i-tRFs and tRF5s account altogether for about 75% of tRFs diversity in both species, they exhibit low expression levels. As a result, 5′-tRHs which represent only 10% of sequence diversity, account for a vast majority of tRFs expression in both species (52 and 82%, in bovine and human sperm, respectively).

These studies revealed another striking feature of sperm sncRNAome, namely the impressive diversity of isoforms (isomiRs, isopiRs, variants of tsRNAs and rsRNAs) produced

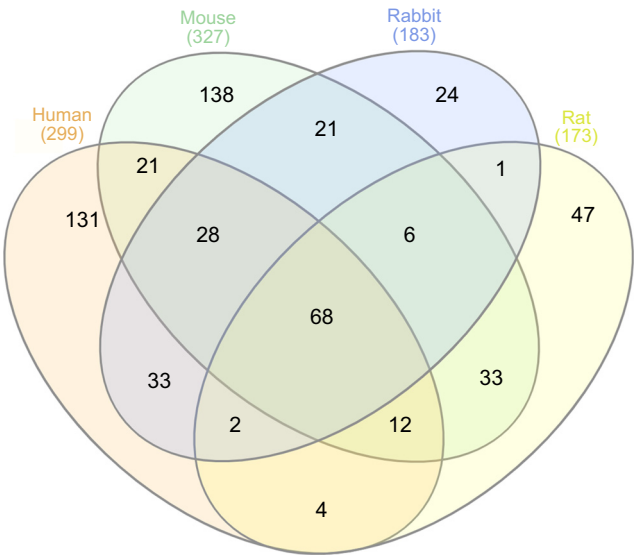

Fig. 4. Number of miRNAs shared among species according to SpermBase. Human, mouse, rabbit and rat sperm share several known miRNAs. While 42–43% of human sperm miRNA content is shared with mouse and rabbit, respectively, only 29% is shared with rat. Rat and mouse share a higher number of miRNAs with 69% of the rat miRNA content retrieved in mouse sperm. Diagram made using http://www.interactivenn.net/

by RNA editing, as for bovine where some miRNAs were composed by several thousand of isomiRs (Sellem *et al.* 2020).

Since ejaculated sperm are transcriptionally silent, the sperm sncRNAome was initially considered as a legacy of spermatogenesis. However, several studies in mice (Sharma *et al.* 2016; Sharma *et al.* 2018; Chu *et al.* 2019) and bulls (Sellem *et al.* 2021) have shown a remarkable level of sncRNAome plasticity resulting from the combination of multiple factors, including interaction with epididymosomes. Indeed, beyond spermatogenesis, spermatozoa undergo several functional maturation steps to acquire their fertilising capacity. In particular, during epididymal transit, the sperm membrane is remodelled, with sequential attachment and shedding of various molecules provided by the epididymal lumen fluid (Zhou *et al.* 2018) and extracellular vesicles such as epididymosomes (Rejraji *et al.* 2006; Sullivan *et al.* 2007; Girouard *et al.* 2011; Rowlison *et al.* 2018; Nixon *et al.* 2019*b*). Interestingly, sncRNA cargo are delivered to sperm by epididymosomes (Belleannee *et al.* 2013; Vojtech *et al.* 2014; Reilly *et al.* 2016; Sharma *et al.* 2018), whose contents vary along the epididymis whether in mice, humans or bulls (Nixon *et al.* 2015; Russell *et al.* 2016; Chu *et al.* 2019; Nixon *et al.* 2019*a*; Trigg *et al.* 2019; Sellem *et al.* 2021). As a result, the payload of sperm sncRNAs is dramatically remodelled as sperm mature along the epididymis from caput to the cauda.

One common finding across species is the observed enrichment of piRNAs within testis parenchyma relative to

the sperm fraction isolated from epididymis or ejaculated sperm. Conversely, an increase in miRNA, tsRNA and rsRNA content is observed from testis to ejaculated sperm, with distinct trends according to species. For instance, expression of rsRNA was shown to increase continuously in mouse (Chu *et al.* 2019), whereas a particular expression profile peaking at epididymis corpus was observed in bovine sperm (Sellem *et al.* 2021). As a result, rsRNA are the most expressed sncRNA species in mouse cauda sperm, whereas miRNA and tsRNA are the most prevalent sncRNA species in bovine cauda sperm.

Unfortunately, data unavailability, methodological differences as well as the plethora of isoforms produced through RNA editing mechanisms preclude a detailed comparison between species. Whether observed differences represent species specificities or technical artefacts warrants further study.

It can be assumed that sncRNA acquired during epididymal transit or later may be related to sperm functionality, fertilising capacity and embryo development, while the others can be considered as remnant from spermatogenesis, maybe related for instance to sperm concentration or morphology. In this respect, sncRNA may represent relevant biomarkers associated with semen fertility.

Deregulation of sncRNAs related to spermatogenesis impairs fertility

Spermatogenesis is a tightly regulated stepwise process involving thousands of genes (Chalmel and Rolland 2015) as well as sncRNAs, especially microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) as illustrated by the high concentration of DICER, AGO proteins and miRNAs in the chromatoid body in male germ cells (Kotaja *et al.* 2006; de Mateo and Sassone-Corsi 2014). Direct evidence for the involvement of sncRNA in spermatogenesis regulation has been provided in mice by several selective knock-out of genes involved in the sncRNA biogenesis pathway. For instance, the selective knock-out of Dicer1 in the male germ cell leads to a decrease in testis volume and sperm number as well as multiple cumulative defects at the spermatogenesis and spermiogenesis stages, leading to morphological abnormalities and infertility (Korhonen *et al.* 2011; Romero *et al.* 2011). Likewise, piRNAs are known to play roles in spermatogenesis, as evidenced by the mitosis, meiosis, chromatin compaction, flagella elongation and fertility defects in mutants lacking Piwi (Weick and Miska 2014). Knock-out of other key genes belonging to the piRNA pathway (Miwi, Mili, Tdrd1, Tdrd9, Mvh, Maelstrom, Pld6 or SPOCD1) results also in major spermatogenesis defects during meiosis or spermiogenesis (Aravin *et al.* 2008; Pillai and Chuma 2012; Chuma and Nakano 2013; de Mateo and Sassone-Corsi 2014; Russell *et al.* 2016) leading to sterility.

Many studies in mouse have now identified key sncRNAs regulating spermatogenesis. For instance, miR-20, miR-21, miR-100, miR-106b, miR-146a, miR-182, miR-183, miR-222 and miR-383 have been shown to be involved in the 'differentiation vs proliferation' balance in spermatogonial stem cells: miR-20, miR-100 and miR-106a are critical for spermatogonial stem cell proliferation through their interactions with Stat3 and Ccnd1 (He *et al.* 2013; Huang *et al.* 2017); regulation of retinoic acid by miR-146a (Huszar and Payne 2013) and cKIT by miR-222 (Yang *et al.* 2013) maintain the undifferentiated status of spermatogonial cells; inhibition of miR-21, which accounts for 11% of total miRNA expression in spermatogonia, increases apoptosis leading to a loss of cells (Niu *et al.* 2011). Meiosis is also regulated by miRNAs, as exemplified by miR-34c, miR-29 and miR-214 which target Nanos2, favouring the meiosis process (Yu *et al.* 2014; Hilz *et al.* 2017). Double knock out mice for miR-34b/c and miR-449 are infertile, due to a drastic reduction in sperm number, a motility loss and an increased proportion of decapitated sperm (oligoasthenoteratozoospermia) (Comazzetto *et al.* 2014; Yuan *et al.* 2015).

In bovine, enriched GO terms and Kegg pathways associated with predicted targets of miRNA preferentially expressed in testicular parenchyma sperm highlighted biological processes such as cell proliferation as well as response to stress and protein ubiquitination (Sellem *et al.* 2021). Interestingly, ER stress and UPR signalling cascades are involved in spermatogenesis, (Karna *et al.* 2020). Likewise, the ubiquitin–proteasome pathway plays a key role at several stages of spermatogenesis (meiosis, histone–protamine transition, acrosome biogenesis, and spermatozoa maturation) to remove many proteins and organelles and help the formation of condensed sperm. Knock-out mice lacking ubiquitylation enzymes such as UBE2J1 suffer from male sterility associated with flagella and acrosomal defects (Koenig *et al.* 2014). Interestingly, piRNAs are also involved in this pathway and have been shown to induce ubiquitination of MIWI, a process essential for maturation from late spermatid to sperm (Zhao *et al.* 2013). Of note, while silencing of retrotransposons in the germline during genome demethylation is the unifying function of piRNAs in mammals, they have also been shown to be crucial for spermatogenesis by regulating meiotically expressed protein-coding genes. Indeed, as spermatocytes reach the pachytene stage of meiosis, a second wave of piRNA transcription starts, producing a particular class of piRNAs termed pachytene piRNAs (Girard *et al.* 2006). These pachytene piRNAs have been shown to form a silencing complex (pi-RISC) containing MIWI and deadenylase CAF1 to induce deadenylation and subsequent decay of spermatogenic mRNAs in elongating spermatids, via a mechanism similar to, but distinct from, the classical miRNA/siRNA machinery (Gou *et al.* 2014; Goh *et al.* 2015). Recently, CRISPR/Cas9 was used to knock-out the expression of a pachytene piRNA precursor locus on mouse chromosome 18, leading to male sterility due to sperm head

dysmorphology, acrosome dysgenesis, poor motility and defects in interactions with zona pellucida impairing penetration (Choi *et al.* 2021).

Post testicular acquired sncRNAs: a role in embryo development?

Since sperm RNAs can be delivered to the oocyte during fertilisation and remain stable until the onset of embryonic genome activation (Ostermeier *et al.* 2004), it has long been hypothesised that they may impact early embryo development. Evidence of such a role has recently emerged, when Guo *et al* treated mouse mature spermatozoa with lysolecithin, pronase and RNases (RNase A and RNase H) to deplete sperm from RNAs and used treated sperm to perform intracytoplasmic sperm injection (ICSI). Reduced blastocyst formation rate and live birth rate of the embryos, as well as lower body weight of F1 mice were observed (Guo *et al.* 2017), suggesting that sperm RNAs, including sncRNAs, play critical roles in embryonic development and may act as an additional source of paternal hereditary information.

Recently, sperm sncRNAs gained at epididymis cauda were shown to be crucial for normal embryonic development after the blastocyst stage in mice (Conine *et al.* 2018). Indeed, blastocysts produced by ICSI with spermatozoa obtained from epididymis caput showed a significant overexpression of multiple genes encoding RNA-binding proteins and chromatin-associated factors during the preimplantation period, including overexpression of SMARCA5, a key protein involved in chromatin remodelling. These caput-derived embryos then failed to efficiently implant and develop. These preimplantation molecular defects as well as the post-implantation lethality could be rescued by microinjection of purified cauda-specific sncRNAs into caput-derived embryos, providing evidence that cauda sperm are epigenetically immature. Interestingly, microinjection of separate gel-purified sncRNA population was performed, using either 18–26 nt sncRNA fraction enriched with microRNAs or 26–40 nt sncRNA faction primarily comprising tRFs. Gene expression was globally restored only for the 18–26 nt fraction, suggesting that miRNA gained by sperm as they further transit the epididymis to cauda are required to support normal preimplantation gene regulation. In this respect, the dramatic increase in miR-100 expression observed from caput to cauda, at least in bovine sperm, may explain the observed SMARCA5 overexpression in caput-derived embryos compared to cauda-derived embryos, SMARCA5 being a target gene of miR-100. Of note, miR-100, is also highly expressed in bovine ejaculated sperm and has been proposed as one of the main factors associated with the initiation of pluripotency (Morikawa and Cserjesi 2004; Fei *et al.* 2010). Likewise, miRNA whose expression peak at epididymis cauda or ejaculated sperm in cattle were found to be associated with GO terms related to embryo development such as developmental process, embryonic morphogenesis as well as cell proliferation, differentiation, and migration (Sellem *et al.* 2021). In addition, miR-34c and miR-191, two highly expressed miRNA in bovine ejaculated sperm (Sellem *et al.* 2020) have been associated with murine embryo early development (Liu *et al.* 2012; Donkin and Barrès 2018; Le Blevec *et al.* 2020) and fertilisation rate and embryo quality in humans (Xu *et al.* 2020), respectively. Of note, sncRNA in the range 18–26 nt also contain short piRNAs, rsRNAs and tsRNA, which may also be involved in the observed phenotype. In this respect, some tsRNA peaking at epididymis cauda or ejaculated sperm in cattle have also been shown to contribute to early cleavage of porcine preimplantation embryos (Chen *et al.* 2020) and injection of tRNA-Gly-GCC fragments into zygotes results in slowdown of embryo development (Sharma *et al.* 2016).

Beyond their role in embryonic development, sncRNAs, especially tsRNAs, may have a role in trans-generational inheritance. A growing body of evidence published recently suggest that sperm sncRNA-encoded information is decoded in early embryos to control offspring phenotypes (Zhang *et al.* 2019). For instance, changes in sncRNA content was observed in sperm of F0 males subjected to a high-fat diet both in mouse (Grandjean *et al.* 2016) and rat (de Castro Barbosa *et al.* 2016), including differentially expressed miRNAs, tsRNAs and piRNAs. These changes were associated in F1 and F2 newborn offspring with reduced body weight and pancreatic beta-cell mass, as well as glucose intolerance, insulin resistance and type II diabetes. Sperm from F1 rat offspring also showed a modified sncRNAs expression profile. In particular, altered expression of sperm miRNA let-7c was observed in F0 rats and was transmitted to offspring, leading to a transcriptomic shift of let-7c targets, including AKT2, IGF2R and UCP2 which are involved in glucose metabolism and insulin-related biological processes.

Molecular mechanisms underlying this trans-generational inheritance have yet to be ascertained and may probably result from the combination or cooperation of several sncRNA species, including at least miRNAs et tsRNAs. Indeed miR-19b was identified as overexpressed in male mice fed a high-fat diet and its microinjection into naive zygotes leads to metabolic alterations in the resulting progeny (Grandjean *et al.* 2016). Similarly, a high-fat diet was shown to disturb the expression of about 11% of mouse sperm tsRNAs. Injection of mouse sperm tsRNAs from males subjected to a high-fat diet was shown to alter metabolic pathways in early embryos and to induce metabolic disorders in the F1 offspring (Chen *et al.* 2016).

Taken together, these results demonstrate that the dynamic remodelling of sperm sncRNA payload occurring during post testicular maturation has dramatic functional consequences on embryos preimplantation development, may downregulate a set of genes encoding RNA-binding proteins and

chromatin-associated factors, thus modifying the epigenetic program and have a global impact on offspring development.

Sperm-borne sncRNAs as fertility biomarkers?

Diagnosis of idiopathic diseases, as well as prediction of the disease course or monitoring the response to therapeutic approaches is a common challenge in the clinical field. In this respect, diagnostic, prognostic and predictive biomarkers have gained major interest. Proteins have long been suggested as biomarkers, however low sensitivity and specificity of detection, as well as the struggle in developing efficient detection methods, often limit their usefulness.

Next to proteins, circulating miRNAs were previously shown to be relevant biomarkers in several pathologies such as cancer or cardiovascular disease. In the last decade, the search for miRNAs biomarkers related to various conditions was an impressive research field, including in sperm fertility assessment. Of note, various fertility definitions were used according to studies and species, based on either spermogram parameters (sperm quantity, motility and absence of morphological abnormalities) or fertilisation/pregnancy rates after artificial insemination (especially for cattle) or other assisted reproductive technologies.

In humans, sperm miRNAs expression profiles were used to detect infertile patients, with or without morpho-functional sperm alterations (Alves *et al.* 2020; Momeni *et al.* 2020; Vashisht and Gahlay 2020). For instance, 50 and 42 miRNAs including miR-15, miR-16a, miR-19a, miR-34b, miR-34c-5p, miR-122, miR-449 and miR-1973 were shown to be upregulated while 27 and 44 miRNA were found to be downregulated in asthenozoospermia and oligoasthenozoospermia, respectively (Abu-Halima *et al.* 2013). Reduced expression of miR-10b and miR-135b was observed in semen of asthenospermic patients (Tian *et al.* 2017). Likewise, decreased expression of miR-25, miR-34b, miR-34c-5p, miR-122, miR-152, miR-192, miR-335, miR-449a was observed in oligozoospermic patients, while 12 miRNAs were significantly more abundant: Let-7b, let-7c, let-7g, miR-21, miR22, miR-30a, miR-148a, miR-221, miR-320a, miR-375, miR-423-3p and miR-423-5p (Muñoz *et al.* 2015). Six miRNAs (miR-125a-3p, miR-132-5p miR-151-5p, miR-195-5p, miR-320 and miR-935) were shown to be downregulated in case of teratozoospermia (Salas-Huetos *et al.* 2015). However, usefulness of these biomarkers is unclear, as fast and inexpensive technologies such as spermograms are already used to efficiently detect these abnormalities in infertile patients. As a biomarker, miRNA would provide an added value in order to diagnose idiopathic infertility or, for instance, refine current diagnosis and differentiate between obstructive and nonobstructive azoospermia without requirement of testicular biopsy. In this respect, miR-34b, miR-34c-5p, miR-429 and miR-122 were suggested to improve diagnosis of patients with nonobstructive azoospermia along with traditional techniques (Abu-Halima *et al.* 2014). In addition, 57 miRNAs were identified as differentially expressed between two groups of normozoospermic men having contrasting fertility levels, highlighting biological processes such as embryonic morphogenesis and chromatin modification, and suggesting that subfertility of these patients may be related to the dysregulation in early embryo of key genes targeted by these sperm miRNAs (Salas-Huetos *et al.* 2016). Likewise, expression of sperm miRNA miR-101-3p, miR-132-3p and miR-191-5p was shown to be associated with either fertilisation rate, blastocyst rate or high-quality embryo rate after *in vitro* fertilisation (Hua *et al.* 2019; Xu *et al.* 2020). Recently, 48 pairs of miRNAs, whose expression is strongly correlated in fertile men but disrupted in infertile patients, were identified (Corral-Vazquez *et al.* 2019). The most suitable diagnostic miRNA pairs were determined for asthenozoospermia (miR-942-5p/miR-1208), teratozoospermia (miR-296-5p/miR-328-3p), normozoospermic infertility (miR-34b-3p/miR-93-3p) and oligozoospermia (miR-139-5p/-miR-1260a).

Regarding livestock species, several studies have been performed on bull and boar and specific sperm-borne miRNA expression profiles have been associated with semen functional parameters, *in vitro* embryo development or field fertility. For instance miR-93, miR-106b, miR-100, miR-122, miR-184, miR-486-5p and miR-2285n have been associated with sperm motility in cattle (Capra *et al.* 2017) and let-7d/e in porcine (Curry *et al.* 2011). Many miRNAs differentially expressed between fertile and sub-fertile bulls have also been identified, such as bta-miR-9-5p, bta-miR-10a-5p, bta-miR-19b-3p, bta-miR-27a-5p, bta-miR-34c, bta-miR-98, bta-miR126-5p, bta-miR-142-5p, bta-miR-148b-3p, bta-miR-182, bta-miR-320a, bta-miR-329a, bta-miR-449a, bta-miR-502-5p, bta-miR-1249-3p, bta-miR-1839, bta-miR-2284y (Fagerlind *et al.* 2015; Perkins *et al.* 2020). The expression level of nine miRNAs (miR-9-5p, miR-34c, miR-423-5p, miR-449a, miR-5193-5p, miR-1246, miR-2483-5p, miR-92a, miR-21-5p) were significantly correlated to non-return rate after insemination with sexed semen (Keles *et al.* 2021). Differential expression of 11 miRNAs was observed between high and low fertility bulls, including miR-33b, miR-126-5p, miR-205, miR-505, miR-532, miR-500 and miR-542-5p which were overexpressed in high fertility bulls and miR-15a, miR-29, miR-216b and miR-339a which were downregulated in high fertility bulls (Alves *et al.* 2019; Menezes *et al.* 2020). In a recent study, two miRNAs (miR-221 and mir-621) were found to be down- and up-regulated in high-fertility relative to low-fertility boars, respectively (Alvarez-Rodriguez *et al.* 2020).

More recently, evidence has been provided regarding the value of other sncRNA classes as fertility biomarkers. For instance, two groups of sperm samples, producing either high or low rates of good quality embryos, were compared

by sncRNA-Seq. Ten differentially expressed tsRNAs were identified, including three 5′-tRNA halves, two 3′-tRNA halves, four were 5′-tRFs and one i-tRF. Among them, five were downregulated in the low-quality semen group (two GlyGCC derived tsRNAs, two ThrTGT and one GluTTC) and five were upregulated (two ProAGG, one ProTGG, one AsnATT and one ArgCCG). In addition, six differentially expressed 28S rsRNAs and one 18S rsRNAs were also identified between the two sperm quality groups (Hua *et al.* 2019). Likewise, sperm tsRNAGln-TTG has been suggested as a potential diagnostic biomarker based on its role in the first cleavage of a porcine embryo as well as development of human embryos (Chen *et al.* 2021). A more exhaustive list of sncRNAs related to semen quality, *in vitro* embryo development, fertility and human infertility (spermogram abnormalities) is provided in Supplementary Table S1.

As already noticed, many biomarker studies were based on a small number of extreme samples due to the cost of NGS technology, leading likely to numerous false positives. Further studies, based on a larger set of samples, accounting for the diversity of the general population, will undoubtedly be required to identify robust and relevant fertility biomarkers.

Similar to previous work done to improve the prediction using sperm functional assessment (Sellem *et al.* 2015), a combination of several sncRNAs biomarkers will undoubtedly be required to reach a good predictive value. Novel statistical approaches, based on deep learning, have also to be implemented to deal with hundreds of sncRNA biomarkers and to combine biomarkers with other functional parameters in order to improve diagnosis as well as fertility prediction.

Conclusions

Ejaculated sperm carry thousands of sncRNAs, including miRNAs, piRNAs, rsRNAs and tsRNAs, whose functions remain to be elucidated. Some are a legacy of spermatogenesis and may be indicative of normal semen production. Some are gained along sperm maturation and may promote acquisition of sperm motility and fertilising capacity or may have a role in embryo development or paternal epigenetic inheritance. In line with these hypotheses, numerous studies in humans and livestock species have established a link between sperm-borne sncRNAs and spermatogenesis defects, embryo development in ART strategy and fertility. However, biological knowledge is still lacking, and further work is still needed to identify robust lists of biomarkers and combine the most relevant ones to improve diagnosis and treatment.

Supplementary material

Supplementary material is available online.

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Data availability. All the fastq files focusing on bovine sperm and published by our team have been stored at ENA, European Nucleotide Archive, under the primary accession numbers PRJEB33940 and PRJEB41989.

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