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Small non-coding RNAs in male reproduction

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ABSTRACT

Male reproductive functions are strictly regulated in order to maintain sperm production and fertility. All processes are controlled by precise regulation of gene expression, which creates specific gene expression programs for different developmental stages and cell types, and forms the functional basis for the reproductive system. Small non-coding RNAs (sncRNAs) are involved in gene regulation by targeting mRNAs for translational repression and degradation through complementary base pairing to recognize their targets. This review article summarizes the current knowledge on the function of different classes of sncRNAs, in particular microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), during male germ cell differentiation, with the focus on sncRNAs expressed in the germline. Although transcriptionally inactive, mature spermatozoa contain a complex population of sncRNAs, and we also discuss the recently identified role of sperm sncRNAs in the intergenerational transmission of epigenetic information on father's environmental and lifestyle exposures to offspring. Finally, we summarize the current information on the utility of sncRNAs as potential biomarkers of infertility that may aid in the diagnosis and prediction of outcomes of medically assisted reproduction (MAR).

Key words: small non-coding RNA, miRNA, piRNA, male germline, sperm, fertility

1. Introduction

The core function of the male reproductive system is to produce male gametes, spermatozoa, and to enable their storage, transport and delivery to the female reproductive system for fertilization. All male reproductive functions, including hormonal control by the hypothalamus-pituitary-gonadal axis, differentiation of germ cells (spermatogenesis), maturation of sperm in the epididymis and production of semen secretions, are dependent on the accurately regulated gene expression programs. Besides other regulatory mechanisms, small non-coding RNA (sncRNA)-mediated gene regulation has a critical contribution to male reproduction [1]. sncRNAs are short (20-35 nt) RNA molecules that are abundant in eukaryotic genomes, and mediate a broad range of gene regulatory processes that control diverse cellular functions and development [1]. sncRNAs mostly act at the posttranscriptional level by binding specific RNAs in the cytoplasm through complementary base pairing, and targeting them for degradation, thereby negatively affecting gene expression [2,3]. In addition, sncRNAs can target genomic regions to affect gene expression at the transcriptional level by inducing epigenetic changes [2,3].

In the male reproductive system, sncRNAs participate in the regulation of both somatic and germ cells activities. In this review article, however, we will focus on their regulatory roles in the male germline. A variety of different sncRNAs are expressed in male germ cells, where they are involved in posttranscriptional and epigenetic gene regulation [4]. The two best-characterized classes of sncRNAs in male germ cells are microRNAs (miRNAs, 22-25 nt) and PIWI-interacting RNAs (piRNAs, 24-34 nt). Somewhat less known class of sncRNAs are the transfer RNA-derived small RNAs (tsRNAs, 30-40 nt) that are particularly abundant in mature spermatozoa [5].

Disruption of sncRNA pathways at any stage of germline development can impair sperm production and lead to male subfertility or infertility, thus possibly contributing to the observed adverse trend in reduced number and quality spermatozoa in human population [6]. Considering the association of aberrant expression of sncRNAs with abnormal sperm production, sncRNAs have clinical relevance as potential indicators or biomarkers of male subfertility/infertility and other disorders of male reproductive health [7,8]. In addition, cumulative evidence shows that sperm sncRNAs can transmit epigenetic information on father's acquired conditions such as metabolic or psychiatric disorders to the offspring, therefore, they not only contribute to male reproductive health, but also play a role in the health of the offspring [9]. This further highlights the significance of understanding sncRNA functions in the germline to improve human health.

In this review, we summarize the current knowledge of the roles of different classes of sncRNAs in spermatogenesis and male fertility, particularly focusing on miRNAs and piRNAs. Furthermore, we discuss the recently identified roles of sperm sncRNAs in the intergenerational transmission of epigenetic information on father's environmental and lifestyle exposures to the offspring. Most of the functional studies have been done using model organisms, and therefore, we focus on mouse processes if not otherwise stated. The available human studies and the conservation of spermatogenic processes between mammalian species suggest that similar mechanisms take place in human as well. Finally, we will summarize current information on the benefits of sncRNAs as potential biomarkers for the diagnosis of human infertility and predicting outcomes of medically assisted reproduction (MAR).

2. Male germ cell differentiation

The male germline development is initiated during early embryogenesis around the time of embryo implantation when primordial germ cells (PGCs) are formed [10]. PGCs undergo proliferation and migrate to the gonadal ridge where they are directed to male gametogenesis and give rise to pro-spermatogonia. These cells cease proliferation and become arrested at the G0 phase of the cell cycle, and this quiescent state continues until they transform into spermatogonial stem cells (SSCs) that are capable of self-renewing and support spermatogenesis throughout adult life [11].

Postnatal spermatogenesis is a complex differentiation program that culminates in the production of fertile spermatozoa [12]. Spermatogenic cells are organized within the seminiferous tubule of the testis together with somatic Sertoli cells that provide physical, nutritional and signaling support to germ cells. When receiving specific signals, SSCs that reside close to the basement membrane initiate differentiation and become differentiating spermatogonia, which then enter the meiotic pathway as spermatocytes. The prophase of the first meiotic division (preleptotene, leptotene, zygotene, pachytene and diplotene phases) is a long process that takes several days, and includes homologous chromosome pairing, synaptonemal complex formation, genetic crossing-over and recombination, and finally reduction division to produce secondary spermatocytes, which rapidly undergo the second meiotic division to form haploid round spermatids [13].

The final haploid differentiation or spermiogenesis consist of dramatic morphological transformation during which spermatids acquire all specific structures required for the function of spermatozoa [14]. These include nuclear compaction and re-shaping, development of an acrosome and flagellum and shedding of cytoplasm. Mature spermatozoa are finally released into the lumen of seminiferous tubules via the process of spermiation and they leave the testis via efferent ducts to the epididymis. During transition of spermatozoa through the long epididymal tubule, they mature and gain potential for motility and fertilization, and are then stored in the cauda epididymis before ejaculation [15]. The duration of spermatogenesis is around 35 days in mice and 74 days in humans [16–18]. The epididymal maturation takes additional 7-14 days in mice and 2-4 days in human [19–21].

2.1. Dynamics of gene expression during spermatogenesis

The development of male germ cells, from fetal period until postnatal spermatogenesis, includes drastic epigenetic changes and dynamic gene expression patterns [22–27] (Fig. 1). Fetal germ cells undergo genome-wide erasure of DNA methylation, followed by re-establishment of DNA methylation marks in pro-spermatogonia [28]. The genome-wide DNA demethylation provides an opportunity to reprogram the genome for the next generation, but also challenges the cells with temporal removal of epigenetic silencing marks from repetitive transposable elements, which has to be dealt with to prevent their aberrant expression.

Postnatal spermatogenesis is characterized by orchestrated, tightly regulated gene expression programs giving rise to cell type-specific transcriptomes [29]. Particularly, meiotic and postmeiotic processes include unique mechanisms that require the expression of germline-specific proteins and non-coding RNAs, such as piRNAs [30–32]. Furthermore, dramatic chromatin changes during meiotic events are accompanied by genome-wide pervasive transcription that generates exceptionally diverse transcriptome that has to be accurately monitored and regulated [31]. After pervasive transcription, another challenge in gene expression is faced in late haploid cells that undergo transcriptional silencing due to chromatin compaction when histones are replaced by sperm-specific chromatin-packing proteins, protamines [33]. mRNAs needed in these cells have to be therefore transcribed in earlier cell types and stored for long periods to ensure material for translation when the chromatin is transcriptionally silenced. Germ cells need specific gene regulatory mechanisms to cope with these epigenetic and posttranscriptional challenges, and one important regulatory level is provided by the small non-coding RNA pathways.

3. miRNA pathway

microRNAs (miRNAs) are ~ 22 nt long sncRNAs that mainly function in posttranscriptional regulation of gene expression through complementary base paring to target mRNAs to silence them [34]. According to miRBase [35], approximately 2700 mature miRNAs have been identified in humans and around 2000 in mice. miRNAs are well conserved among metazoan species. Just like protein-coding mRNAs, miRNAs are expressed in a cell type-, tissue-, developmental stage, or disease-specific manner [34], and they can also have systemic effects due to their ability to transfer from one cellular compartment to another via body fluids [36]. miRNAs are widely involved in physiological processes such as proliferation, differentiation, growth, metabolism, cellular development, embryogenesis as well as disease conditions [37].

The canonical miRNAs biosynthesis pathway begins with the transcription of longer miRNA precursors (pri-miRNA) from endogenous genes by RNA polymerases II (Figure 2A). The precursor forms a hairpin loop that serves as a substrate for Microprocessor, which is a heterodimeric protein complex containing DROSHA endonuclease and its partner protein DGCR8. DROSHA cuts the stem of the pri-miRNA in the nucleus, and the resulting ~60 nt stem-loop pre-miRNA is then transported to the cytoplasm by Exportin 5 for further cleavage by the endonuclease DICER to generate miRNA duplex containing the miRNA guide strand paired to its passenger strand [34,38,39]. The miRNA duplex is then loaded into Argonaute (AGO) proteins that keep only the guide strand miRNA, releasing the passenger strand for degradation. In addition to the canonical pathway, miRNAs can be produced by non-canonical pathways that are not dependent on either DROSHA or DICER [34]. For example, some miRNAs (so called "mirtrons") are produced from introns that can undergo debranching and enter to the miRNA biogenesis pathway as pre-miRNAs, bypassing the processing by DROSHA [40]. miRNAs can also be produced by non-canonical pathways from endogenous short-hairpin RNAs (shRNAs), or from miRNA gene products that are produced as part of another type of small RNA genes, such as tRNA-like molecules or small nuclear RNAs (snRNAs) or small nucleolar RNAs (snoRNAs) [34].

Together with AGO proteins, mature miRNAs form an effector complex known as the RNA-induced silencing complex (RISC) where miRNA sequence serves as a guide to direct AGO proteins to target mRNAs [41]. RISC binding initiates mRNA degradation or translational repression, depending on the sequence complementarity between the guide and the target, the set of other effectors recruited by each specific AGO, and the catalytic activity of the AGO protein involved [34,41]. There are four AGO proteins in mammals (AGO1, AGO2, AGO3 and AGO4). Among these, AGO2 is well-known for its endonuclease activity and it is mostly involved in miRNA-mediated mRNA silencing functions, while other AGO proteins do not have robust slicing activities [42]. One miRNA can target several mRNA and a certain mRNA can be targeted by several miRNAs, which create complex miRNA-mRNA regulatory networks, the extent of which are not yet fully understood [34].

3.1. Expression and function of miRNAs during spermatogenesis

miRNAs are dynamically expressed during the development of male germ cells from embryonic germ cells to mature sperm, and each developmental stage and germ cell type is characterized by specific miRNA profiles [43]. Several studies using miRNA microarrays, RT-PCR or small RNA sequencing have identified miRNAs that are highly, exclusively or preferentially expressed in the testis and at the specific phases of male germ cell development and differentiation [4,44,45]. For example, miRNA expression profiling revealed differentially expressed miRNAs in PGCs isolated at different embryonic stages during their colonization of gonadal ridge and initiation of sex-specific pathways [46], suggesting the roles for miRNAs already in gonadal sexual fate and development. Similar studies have been conducted to identify miRNA expression profiles during postnatal spermatogenesis from SSCs to mature sperm [43].

The functional importance of miRNAs in different germ cell types has been revealed using cell culture and knockout mouse models [25]. One study identified miRNAs preferentially expressed in SSCs (miR-21, miR-34c, -182, -183, and -146a) by high-throughput sequencing, and showed that transient inhibition of miR-21 increased apoptosis and reduced the SSC potency, indicating that miR-21 is important for maintaining the SSC population [47]. Another study reported miR-202 to be required for SSC maintenance using a SSC cell culture model [48]. The importance of miR-202 for spermatogenesis was also demonstrated *in vivo* using a miR-202 knockout mice that had compromised fertility with reduced undifferentiated spermatogonial pool, lowered sperm counts and problems in meiotic progression [49,50]. Other examples of miRNAs shown to be involved in the regulation of undifferentiated vs. differentiating state of spermatogonia include miR-146 [51], as well as miR-221 and miR-222 [52]. The deletion of undifferentiated spermatogonia-expressed Mir-17-92 cluster in mice resulted in small testes and lower number of epididymal sperm and increase in the expression of its paralog Mir-106b-25 expression, suggesting functional co-operation of these two clusters (Tong et al., 2012).

Many miRNAs involved in the control of meiotic and postmeiotic differentiation have also been reported, including miR-10a and members of the miR-34 family (miR-34a, 34b, 34c, 499a, 499b, and 499c) [53,54]. miR-34c is highly expressed in spermatocytes and round spermatids [53,55,56], and inhibition of miR-34c in cultured spermatocytes seems to prevent germ cell from testosterone deprivation-induced apoptosis [56]. Interestingly, overexpression of miR-34c induces a shift in the transcriptome towards the germ lineage transcriptome in HeLa cells, suggesting that it has a role in enhancing germ cell properties of the cells committed to germline lineage [53]. The expression of miRNAs from the miR-449 cluster is also drastically up-regulated by the transcription factors CREM and SOX5 upon meiotic initiation, but the deletion of miR-449 gene in mice did not affect spermatogenesis and fertility [57]. miR-34b/c expression was upregulated in miR-449-null testes, suggesting that these miRNA cluster have redundant activities [57]. Mice deficient for two miRNA loci of the miR-34 family, miR-34b/c and miR-449, were infertile with oligoasthenoteratozoospermia, further supporting the co-operation of the miRNAs belonging to this family [58,59]. miRNAs from miR-34b/c and miR-449 clusters are also present in mature sperm along with other type of sncRNAs [9,59], suggesting that they could be involved in the regulation of sperm function, for example in the transmission of epigenetic information to the offspring (discussed later).

In haploid spermatids, miRNAs have been shown to regulate the correct timing of the expression of transition proteins and protamines, therefore contributing to histone-protamine transition during sperm chromatin condensation. A testis-specific miRNA miR-469 was shown to target Transition protein 2 (*Tnp2*) and Protamine 2 (*Prm2*) mRNAs to prevent the premature expression of these proteins in spermatocytes and round spermatids [60]. The expression of miR-469 was in turn shown to be regulated by a gonadotropin-regulated testicular helicase (GRTH/DDX25) [60]. DDX25 was also shown to affect the expression levels of other miRNAs in addition to miR-469, therefore it appears to have a broader role in the control of miRNA-mRNA networks to support haploid differentiation of spermatids [61]. Another miRNA, miR-

122a, that is expressed in late stages of male germ cells differentiation has also been shown to directly control TNP2 expression by inducing mRNA cleavage [62].

3.2. Effects of the miRNA pathway disruption on male fertility

Due to critical role of miRNAs in the regulation of wide variety of physiological processes, dysregulated miRNA expression is widely implicated in different diseased conditions [63,64]. Knockout mouse models have proven that the functional miRNA pathway is also required for male reproduction, and the disruption of miRNA biogenesis components and miRNA effector proteins compromise the development of male germ cells, consequently causing infertility [43]. miRNA biogenesis and function is essential for embryonic development [65,66], and therefore, conditional knockout mouse models are needed to understand the role of miRNA pathway in the male germ line.

Using this approach, *Dicer1* was deleted in mouse embryonic testes in PGCs (*Tnap-Cre*), which resulted in impaired the proliferation of PGCs and spermatogonia, as well as arrested postnatal spermatogenesis at pachytene spermatocyte phase and the gradual loss of spermatogenic cells [67]. In the same study, *Ago2* was also deleted using the same Cre line, but *Ago2*-deficient testis did not show any spermatogenic defects [67]. Another mouse model revealed that deletion of *Dicer1* in the male germline just before birth (*Ddx4-Cre*) in pro-spermatogonia results in severe cumulative defects in meiotic and postmeiotic germ cells [55]. Interestingly, deletion of *Dicer1* after birth in postnatal spermatogonia (*Ngn3-Cre* and *Stra8-Cre*) resulted in somewhat less severe, but still infertile, phenotype with defective haploid differentiation, including problems in chromatin organization and shaping and condensation of sperm head [68–71]. The spermatogenesis of knockout mice with even later deletion of *Dicer1* in haploid cells (*Prm1-Cre*) escaped the most dramatic defects, but still postmeiotic differentiation was affected with compromised sperm head morphology and chromatin integrity [72]. Altogether, these mouse models clearly shows that DICER is required throughout male germ cell differentiation, with early deletion accumulating more spermatogenic problems.

The role of miRNA pathways on male germ cell differentiation has also been studied using knockout mouse models for the Microprocessor components DROSHA and DGCR8. The comparison of the testicular phenotypes of *Dicer1* and *Drosha* knockout mice (*Stra8-Cre*) showed that *Drosha* knockout display even more severe spermatogenic disruption than *Dicer1* knockout [70]. On the other hand, mutant mice with *Dgcr8* gene deleted in the germline just before birth (*Ddx4-Cre*) were also infertile with defective spermatogenesis, although the defects were less severe than with *Dicer1* mutant mice [55,73]. The differences in the testicular phenotypes of *Dgcr8* mutant and *Drosha* mutant [70,73] may originate from the different mouse models using different timing of Cre expression, but also from additional functions of DGCR8 and DROSHA in the male germline outside the Microprocessor complex

Disruption of miRNA pathway in male germline induce misregulation of a large number of protein-coding genes, indicating an important role of miRNA-mediated gene regulation for the progress of spermatogenesis [69,70,73]. In addition to protein-coding mRNAs, repeat elements were misregulated in the absence of DICER. Deletion of *Dicer1* before birth (*Ddx4-Cre*) induced the upregulation of transposable elements of the SINE (short interspersed nuclear element) family in spermatocytes [55]. Interestingly, in another *Dicer1* knockout mouse model with *Dicer1* deletion in postnatal spermatogonia (*Ngn3-Cre*), transposon expression was unaffected [68], perhaps due to escape of embryonic germ cells from *Dicer1* silencing in this mouse model. Instead, *Dicer1* knockout spermatocytes showed dramatic induction of major satellite repeat expression [68], and later study revealed that DICER directly targets major satellite repeat transcripts to downscale the expression of pericentric heterochromatin during meiotic progression [74].

Not only miRNA biogenesis factors but also the effector AGO proteins have been targeted to study their role in the regulation of spermatogenesis. AGO proteins are expressed in all tissues, including testis [75]. AGO4 expression is particularly strong in testis where it localized to the transcriptionally silenced sex body in spermatocytes [76]. Interestingly, *Ago4* deletion induced spermatogenic problems, with too early initiation of meiosis and incorrect sex body assembly, leading to disrupted MSCI [76]. Deletion of *Ago4* led also to decreased expression of X-chromosomal miRNAs that are known to escape MSCI [77,78].

4. piRNA pathway

PIWI-interacting RNAs (piRNAs) are small regulatory RNAs of 23–31 nucleotides in length that are predominantly expressed in the male germline [79–82]. The majority of animals including insects, mammals, nematodes and fish have piRNAs and PIWI proteins [83]. Like siRNAs and miRNAs, piRNAs bind to the Argonaute family of proteins and use complementary base-pairing rules to direct these proteins to target genes [84]. However, their biogenesis pathway differs from other small non-coding RNAs in both *Drosophila* and mammals, and unlike miRNAs and siRNAs, piRNAs are produced from single-stranded precursor transcripts independently of DICER (Figure 2B). piRNAs also possess 2′-O-methyl-modified 3′ termini and serve as guides for the PIWI subfamily Argonaute proteins that are exclusive to the germline, rather than ubiquitously expressed AGO subfamily of proteins involved in the miRNA and siRNA pathways [32,85]. In mouse, there are three PIWI proteins, PIWIL1/MIWI, PIWIL2/MILI, and PIWIL4/MIWI2 that have differential expression patterns during male germ cell differentiation, PIWIL4 being expressed in fetal germ cells, PIWIL2 in fetal germ cells and postnatal germ cells until early round spermatids, and PIWIL1 in postnatal late spermatocytes and round spermatids [32].

As the development of male germ cells proceeds, different classes of piRNAs are produced: "Fetal piRNAs" in fetal germ cells, as well as three classes of postnatal piRNAs, including "pre-pachytene piRNAs", "pachytene piRNAs" and "hybrid piRNAs” [86]. Fetal piRNAs bind to the PIWI proteins PIWIL2 and PIWIL4 in order to silence transposable elements (TEs) [87]. The majority of postnatal piRNAs are transcribed from specific genomic loci known as piRNA clusters as long transcripts prior to their processing into piRNAs. Pre-pachytene piRNAs that mainly associated with PIWIL2 are expressed prior to the meiotic pachytene stage from the pre-pachytene piRNA clusters. Pre-pachytene piRNAs are mostly derived from the 3′ untranslated region (UTR) regions of protein-coding genes. Although pre-pachytene piRNAs includes some TE-targeting piRNAs, the functions of these piRNAs produced from genic regions are still largely unknown [86,88]. Pachytene piRNAs are derived from non-TE intergenic clusters and their expression is highly induced by a transcription factor A-MYB at the pachytene stage of the meiotic prophase I, and they associate with PIWIL1 and PIWIL2 [32,86]. Pachytene piRNAs are highly abundant, and they cover more than 90% of the piRNAs expressed in the testis in mice [86]. Hybrid piRNAs combine the characteristics of pre-pachytene piRNAs and pachytene piRNAs [86]. Pre-pachytene, pachytene and hybrid piRNA clusters have been identified also in human, which indicates that the function of piRNA pathway is conserved across species [89].

4.1. piRNA biogenesis and function

piRNAs are produced by primary processing pathway from long precursors, as well as secondary ping-pong amplification pathway [85]. Long single-stranded piRNA precursors are transcribed from the piRNA clusters by RNA polymerase II, and they undergo standard mRNA processing, such as 5′-capping and polyA tailing. piRNA precursors are then processed by endonucleolytic cleavage to produce the monophosphorylated 5′-end of the piRNA precursor, which allows PIWI protein binding and initiates the production of phased trailing pre-piRNAs with extended 3′ end, which are then shortened by a 3′–5′ exonuclease [88,90]. The length of the mature piRNA is defined by the area protected by the bound PIWI protein from exonucleolytic cleavage, therefore, each member of PIWI protein family bind piRNAs of distinct sizes that is approximately 26 nt, 28 nt, and 30 nt for PIWIL2, PIWIL4, and PIWIL1, respectively [91]. An endonuclease located on outer mitochondrial membrane, PLD6, has been shown to be involved in the production of trailing pre-piRNAs, linking the production of piRNAs to the mitochondrial surfaces [32]. The ping-pong amplification of piRNAs is initiated by a cleavage of the complementary RNA target that is bound by the ”initiator piRNA”-PIWI complex to commit the RNA to produce a ”responder piRNA” from its 5’end. The responder piRNA can in turn act as an initiator piRNA, and in this way, amplify the signal [32,85].

The best-characterized function of piRNA is safeguarding the germline genome by suppressing transposon expression during the reprogramming of the epigenome in fetal germ cells [28]. The cytoplasmic piRNA-PIWI complex can bind and cleave the TE transcripts to silence them posttranscriptionally. On the other hand, nuclear piRNA-PIWI complexes can bind premature TE transcripts on the chromatin and recruit epigenetic modifiers to block transcription via the methylation of lysine 9 of Histone 3 (H3K9) and DNA methylation [32,92]. This is a highly critical function considering the potential detrimental consequences of transposon expression and integration to new locations in a genome, which would compromise genomic integrity.

Apart from transposon silencing, growing evidence suggests that the PIWI-piRNA machinery has a broader role in the posttranscriptional regulation of gene expression, including the regulation of non-coding and protein-coding genes. Particularly pachytene piRNAs that are devoid of TE-targeting sequences, has been shown to have diverse functions in meiotic and postmeiotic male germ cells [93]. Analysis of PIWIL1-associated RNAs using crosslinking and immunoprecipitation (CLIP) assays followed by RNA-sequencing has demonstrated its interaction with a diverse range of cellular mRNAs, suggesting its involvement in the posttranscriptional regulation of gene expression [94,95]. Interestingly, pachytene piRNAs have been shown to be involved in the elimination of many mRNAs in spermatids in mice, either through PIWIL1-mediated mRNA slicing, or mRNA decay by deadenylation by recruiting the CAF1 deadenylase [94–96]. In addition, a set of pachytene piRNAs bound to PIWIL1 can actually have a positive effect on gene expression by promoting the translation of a large number of spermiogenic mRNAs with the help of translation initiation factor eIF3f and the ARE-binding protein HuR, and specific base-pairing of piRNAs with the 3'UTRs of their target mRNAs [97].

4.2. piRNA pathway and male fertility

piRNA pathway is essential for germline development in the majority of species. While disruption of piRNA pathway causes infertility in both sexes in flies and zebrafish, in mice PIWI proteins and many piRNA pathway proteins are necessary only for male fertility [98–100]. Knockout of *Piwil4, Piwil2* and *Piwil1* genes in mice all resulted in a complete spermatogenic arrest at specific steps of differentiation, reflecting the timing of their expression. *Piwil4* knockout mice had reduced germ cell number and a profound spermatocyte arrest occurring before the pachytene phase of meiosis, which was associated with defective transposon silencing during epigenetic resetting in fetal male germ cells [92,98]. Mice deficient in *Piwil2* were sterile due to complete cessation of spermatogenesis in early prophase of the first meiosis, from the zygotene to the early pachytene stage, resembling the phenotype of *Piwil4* knockout [92,100]. PIWIL1 is expressed later during male germ cell differentiation, and *Piwil1* deletion resulted in a later phenotype with spermatogenic arrest at the early stages of round spermatid development, demonstrating the essential function of PIWIL1 in regulating haploid differentiation and the morphological transformation of round spermatids into spermatozoa [99,101].

Knockout mouse models for other proteins involved in piRNA biogenesis and function, including Tudor domain-containing proteins (TDRD5, TDRD6, TDRD7, TDRD12, TDRKH, and RNF17), as well as DDX4, PLD6, PNLDC1, ASZ1, MAEL, GTSF1 and HENMT1, have also corroborated the significance of piRNA pathway in male fertility [102]. In addition, recent studies have shown that specific piRNA clusters in mice are needed for normal sperm function. Deletion of the piRNA cluster on the chromosome 18 resulted in acrosome dysgenesis, severe sperm head dysmorphology and failure in fertilization due to impaired motility [103]. On the other hand, deletion of the piRNA cluster on the chromosome 6 caused defects in acrosome reaction and sperm motility, and early embryos generated by intracytoplasmic sperm injection (ICSI) of the cluster-deleted sperm had a delayed first cleavage and eventual embryonic lethality at the two-cell stage [104]. These results suggest that the piRNAs expressed from piRNA clusters do not only have role in sperm production, but also in early embryonic development. Importantly, dysregulation of the piRNAs system has also been associated with male infertility in humans. According to a comprehensive survey of rare genetic variation causing non-obstructive azoospermia (NOA) in humans, eleven NOA patients were identified to have recessive variation in six piRNA biogenesis genes (PLD6, PNLDC1, RNF17, TDRD9, TDRD12, TDRKH) [105].

4.3. Germ granules and piRNA pathway

The biosynthesis and function of piRNAs is strongly associated with the germ cell-specific cytoplasmic ribonucleoprotein (RNP) granules that are commonly called germ granules [106,107]. Germ granules are membraneless RNP condensates that are formed by phase separation, sequestering RNAs and RNA-binding proteins from their environment when they reach a certain concentration [107]. In mice, two types of germ granules are formed during postnatal spermatogenesis that serves as platforms for piRNA biogenesis and function [107–109]. The first one of them, the intramitochondrial cement (IMC), forms between the clusters of mitochondria, being particularly prominent in pachytene spermatocytes during the time of pachytene piRNA production. As discussed before, many proteins involved in the piRNA biogenesis are indeed localized on the mitochondrial membranes [110–112]. Isolation of the IMC from spermatocytes using anti-PIWIL2 antibody confirmed the accumulation of the piRNA biogenesis proteins in the IMC, indicating the IMC as a central hub for piRNA production [109].

The chromatoid body (CB) is another type of germ granule that appears a bit later than the IMC. CB precursor granules form in late spermatocytes and then condense into a single big granule in the cytoplasm of haploid round spermatids [107]. The protocol developed for the isolation of CBs enabled the identification of its full protein and RNA composition [113,114]. piRNAs and PIWI proteins PIWIL2 and PIWIL1 are enriched in the CB, and additionally, it contains a broad range of different RNA-binding proteins, including proteins implicated in the piRNA pathway, as well as a wide variety of non-coding RNAs and protein-coding mRNAs [114]. The presence of piRNAs, PIWI proteins, potential target RNAs and proteins known to support the function of the piRNA pathway suggest a role for the CB as a platform for piRNA-targeted RNA regulation [107,114,115]. The importance of germ granules and their functions as coordinators of the piRNA pathway is substantiated by their conservation in lower organisms like *Drosophila* and *C. elegans* [106,116,117]. Although the germ granules have remained poorly characterized in humans, they have been shown to be present in the human male germline, for example using specific markers to visualize CBs in human round spermatids [115].

5. Sperm small non-coding RNAs

Due to the highly compacted genome, spermatozoa are largely transcriptionally inactive [118], and the amount of RNA in sperm is very low. Sperm also lacks abundant ribosomal RNA, which indicates low number of ribosomes and therefore, low protein synthesis activity. Low amount of RNA has challenged the identification and analysis of sperm RNAs. However, the RNA sequencing technologies have enabled detailed studies on sperm RNA content and indeed revealed the complex population of RNAs, including both coding and non-coding transcripts and several species of sncRNAs [5,119–123]. The limited transcriptional and translational activity argues against the function of sncRNAs in the regulation of gene expression in mature sperm. Indeed, sperm sncRNAs may have intergenerational functions, serving as carriers of intergenerational epigenetic information from a father to offspring [9].

5.1. Different classes of sperm small non-coding RNAs

The sperm RNA pool is composed of several classes of sncRNAs (< 40 nt), including miRNAs, piRNAs, transfer RNA (tRNAs)-derived small RNAs (tsRNAs) and ribosomal RNA (rRNA)-derived small RNAs (rsRNAs) [121,122,124,125]. The most abundant types of sncRNAs in sperm are tsRNAs and rsRNAs [5,124,125]. While sperm rsRNAs have remained poorly characterized, tsRNAs have been under more active investigation. tsRNAs are quite heterogeneous population of sncRNAs that can be derived from both the 5' and 3' termini of precursor tRNA molecules or mature cytoplasmic tRNAs, and their sizes varies from 10 to 45 nucleotides [126,127]. They are also heavily modified, providing additional regulatory level to their stability and function [126]. The biogenesis of tsRNAs is an evolutionarily conserved process, which is highly responsive to dynamic cellular conditions [126,127]. The functional diversity of tsRNAs is currently being investigated, and their range of functions is yet to be fully understood. Nevertheless, they possess the capability to regulate gene expression in a manner similar to miRNAs and piRNAs [126,127].

tsRNAs are relatively scarce in the testis where piRNAs dominate as the most abundant species of sncRNAs, but in the mature sperm, tsRNAs dominate together with rsRNAs. Interestingly, the abundance of tsRNAs in sperm was shown to increase when they pass through the epididymal duct in mice [122,123]. There is evidence suggesting that, during epididymal transit, spermatozoa gain its tsRNA payload from the epididymosomes secreted by the epithelial cells of the epididymis [122,123]. Another study, however, challenged this finding by showing that the sperm cytoplasmic droplets also contribute to the sperm tsRNA payload [124]. Due to their germline-specific biosynthesis machinery [32], piRNAs, on the other hand, are believed to be produced during spermatogenesis and then retained in the mature sperm. Further investigation is needed to fully understand and validate the cellular origin of sperm RNA profile and the molecular mechanisms involved.

5.2. Small non-coding RNAs as intergenerational mediators of epigenetic information

Human epidemiological studies have revealed intriguing associations between parental preconception exposures to toxicants, lifestyle factors, nutrition status or traumatic stress-induced conditions and the specific phenotypic parameters in offspring [128]. These associations suggest that the information of parental acquired conditions can be transmitted to offspring, which is reflected in the offspring health. This type of non-genetic inheritance that can occur in the absence of continued direct environmental influence is mediated via epigenetic changes in the germline, and is defined as epigenetic inheritance. During recent years, the use of experimental animals have continuously improved our understanding of the mechanisms of epigenetic inheritance [129]. It has become evident that exposures to various environmental factors can induce epigenetic changes, including changed levels of DNA methylation and sncRNAs in gametes [9,129–134]. These epigenetic changes in sperm allows passing the information on father's acquired traits and conditions to the offspring.

Although epigenetic inheritance is likely to be executed by several different epigenetic mechanisms, sperm sncRNAs have been convincingly demonstrated to be able to act as carriers of epigenetic information to future generations [9]. Sperm sncRNAs are responsive to environmental exposures. The most thoroughly examined paternal exposures involve traumatic stress and diet-induced metabolic stress, as well as exposures to environmental toxicants, such as endocrine disrupting chemicals (EDCs) [9,135]. For example both high-fat diet and low-protein diet can modify the levels of sperm sncRNAs in mice, and tsRNAs appear to be particularly sensitive for dietary changes [121,122,133]. Environmentally-induced differences in sperm epigenome have also been reported in humans. For examples weight loss after bariatric surgery, or endurance exercise of normal weight individuals induce changes in sperm epigenome [136,137]. Interestingly, the effects of environmental factors on the epigenome of human sperm can occur relatively quickly. For instance, a high-sugar diet has been shown to alter the levels of transfer RNA-derived small RNA (tsRNA) in sperm within just two weeks [138].

Mouse studies provide evidence that sperm sncRNAs are not only responsive to environmental exposures, but they are able to carry the epigenetic information on father's diet-induced metabolic condition to the offspring [9]. The functional role of sperm tsRNAs in epigenetic inheritance has been demonstrated by sperm RNA microinjection experiments – microinjection of sperm RNAs after diet exposures into normal zygotes was able to transmit the information on acquired phenotype in the offspring [121,122]. Another microinjection study showed that the injection of sperm RNAs from males exposed to early life stress into normal zygotes also affected the behaviour and metabolism in offspring, and this was suggested to be mediated by altered miRNA expressions [139]. The role of miRNAs in epigenetic inheritance was also supported by the study showing that injection of nine miRNAs elevated in sperm of mice exposed to chronic variable stress [140] into the zygote affected stress responses in the offspring [141]. While sperm piRNA levels are also responsive to environmental exposures [9], their role in paternal epigenetic inheritance in mammals is still unexplored. Interestingly, deletion of a specific piRNA cluster in mice (pi6 on chromosome 6) affected the survival of embryos derived from homozygous sperm, suggesting possible postfertilization function [104]. While information is still scarce in mammals, piRNAs in non-vertebrae have well-established roles in epigenetic inheritance [142].

The exact mechanisms of how sperm sncRNAs transmit information to offspring is still unclear, but the current hypothesis is that they could bring about changes in the epigenome and gene expression of the zygote when released to the oocyte in fertilization, and in this way transmit fathers acquired conditions to the developing embryo. This scenario is supported by the study showing that sperm tsRNAs could regulate genes associated with endogenous MERVL retroelement in mouse embryonic stem cells [122].

6. Small non-coding RNAs as biomarkers for human fertility

Infertility affects a large number of couples globally, and in around 50% of the cases, male factor is involved [143]. Male infertility is diagnosed when semen parameters fall below the World Health Organization (WHO) reference values [144], however, aetiology remain undefined for most men with infertility. Furthermore, semen parameters are not always predictive of natural fecundity or MAR outcomes [145–147]. Especially in the case of idiopathic or unexplained infertility when a male shows normal semen parameters, the diagnostic and predictive tools are very limited [143]. Therefore, there is a high demand for the improvement of the traditional diagnostic tools in the hand of the andrologists.

The cell type-specific expression patterns of sncRNA during spermatogenesis, as well as their critical role in the control of male reproductive functions makes them potential biomarkers for the diagnosis of sub/infertility and design of infertility treatments. sncRNAs are also relatively stable and they can be secreted outside the cells where they have been produced. In addition, in case of tissue damage due to disease or exposure to toxicants, sncRNAs produced in the tissue can leak into surrounding body fluids [148]. Therefore, they are often found in the body fluids, either inside extracellular vesicles or in association with RNA-binding proteins [149], providing possibility to detect them in serum of in the seminal fluid. For these reasons, sncRNAs are widely used as biomarkers for many diseases and conditions such as cancer, viral infections, nervous system disorders, cardiovascular disorders and diabetes [149].

6.1. Small non-coding RNA levels in sperm of sub/infertile men

Sperm sncRNA profile reflects the earlier processes taking place during sperm production and maturation, and therefore, sncRNAs in sperm are considered as potential biomarkers for spermatogenic defects and fertility. Several studies have been conducted to identify differences in the levels of specific types of sperm sncRNAs in sub/infertile vs. fertile men [8]. For example, miR-34b/c and miR-449a that are specifically expressed during late phases of spermatogenesis and required for normal production of sperm [58,150], have been reported to have reduced levels in subfertile individuals and infertile patients associated with spermatogenic failure [151]. Other sncRNA classes in addition to miRNAs have also been analysed, for example, some differentially expressed individual piRNAs have been identified in the sperm samples of asthenozoospermic or oligospermic patients compared to the normozoospermic controls [151]. However, knowing that oligo-, astheno-, and teratozoospermia can be diagnosed using inexpensive macroscopic and microscopic sperm analysis, the usefulness/feasibility of sncRNA analysis in this type of sub/infertility remains unexplored.

The evaluation of idiopathic infertility with normal sperm morphology and motility could benefit from sncRNAs as additional biomarkers. In search for novel diagnostic tools for idiopathic male infertility, one study identified 57 miRNAs that were differentially expressed in the sperm of fertile and infertile normozoospermic men [152]. Sperm miRNA levels have also been associated with fertilization rate, blastocyst rate or high-quality embryo rate after *in vit*ro fertilization [153,154], suggesting that the sperm miRNAs could be predictive for MAR outcomes.

Similarly, levels of some piRNAs were correlated with sperm concentration and fertilization rate after ICSI in males with idiopathic male factor infertility [155]. The responsiveness of the sperm sncRNA profile to environmental exposures somewhat challenges the use of sperm sncRNAs as biomarkers for male idiopathic infertility, because it is still undetermined which changes in sperm sncRNA levels caused by the environment affect sperm function during fertilization, and which ones do not affect fertility but instead transmit epigenetic information to the offspring.

6.2 Seminal fluid small non-coding RNAs as biomarkers

Seminal plasma can be easily separated from sperm, allowing its analysis for diagnostics purpose. Seminal plasma is a composite fluid produced by testis and accessory sex glands, and it regulates many processes required for sperm transport and function [156]. Human semen contains a high number of membrane-enclosed extracellular vesicles that are packed with many different types of sncRNAs, the most abundant being miRNAs, Y RNAs, rsRNAs and tsRNAs [157]. sncRNA composition of the seminal fluid is likely to reflect the spermatogenic activity in the testis, and therefore, seminal plasma sncRNA analysis is considered as a promising approach for diagnosis of male infertility [7]. For the diagnosis of azoospermia (the absence of sperm in the ejaculate), the seminal plasma sncRNA analysis provides a potential tool to differentiate between non-obstructive azoospermia due to spermatogenesis failure and obstructive azoospermia, the latter being characterized by full testicular spermatogenesis and high likelihood to obtain sperm for MAR treatment using testicular biopsy. Testicular sperm retrieval is also used as a treatment of non-obstructive azoospermia, and in these cases, seminal plasma sncRNA analysis could predict the success of sperm retrieval, which is greatly dependent on the type of spermatogenic defect. Some studies have indeed revealed that semen extracellular sncRNA content (including miRNAs, piRNAs, tsRNAs) can be used to predict the presence of sperm in testicular tissue and the success of testicular sperm retrieval in non-obstructive azoospermia patients [7,158–161].

7. Conclusions

Active research on the expression and functions of sncRNAs in both experimental animals and humans has demonstrated their essential role in the maintenance of spermatogenesis and male fertility. Clinical studies on subfertile/infertile men have also underlined the potential of sncRNAs as robust biomarkers for the assessment of male fertility. Furthermore, recent findings on the function of sncRNAs in sperm in epigenetic inheritance and their ability to modulate gene expression in early embryos after fertilization make them relevant candidate biomarkers for the prediction of MAR outcomes. miRNAs have attracted much attention in the search for new biomarkers. However, given the critical functions of piRNAs during spermatogenesis and the abundance of tsRNAs in mature spermatozoa, these classes of sncRNAs deserve much more future research to understand their full potential as biomarkers. Altogether, sncRNAs have important contribution to male reproductive health, and the characterization of their functions and expression signatures does not only provide important information about the mechanisms of spermatogenesis, but also advance the development of better tools for the diagnostics of infertility and design of infertility treatments.

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Conflict of interest

None declared.

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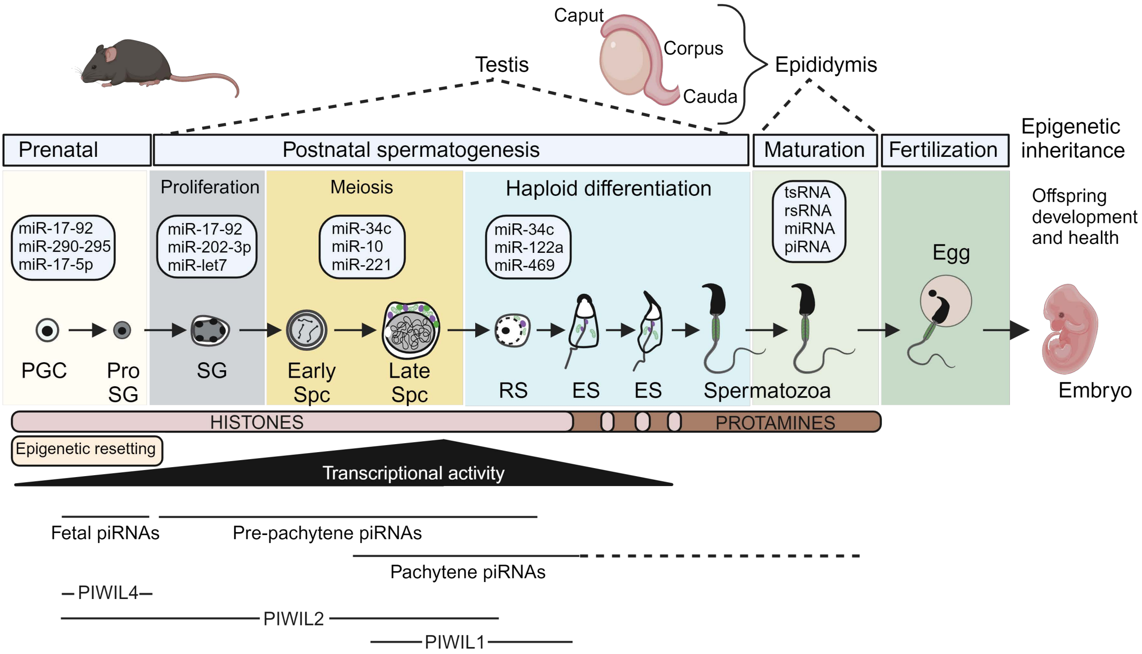
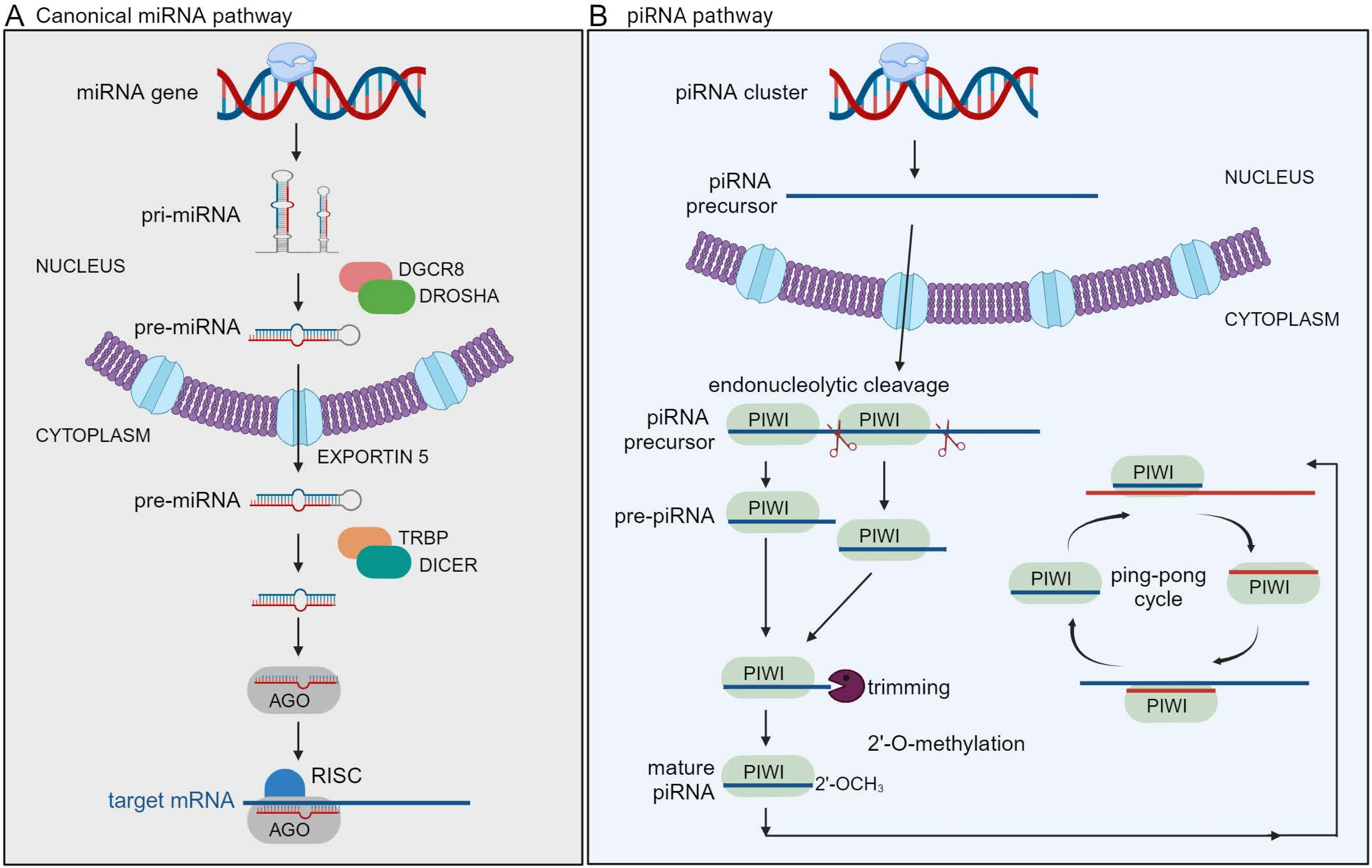
Figure Captions

Figure 1. Expression of small non-coding RNAs during differentiation of male germ cells. During prenatal development, primordial germ cells (PGC) and pro-spermatogonia (Pro-SG) undergo epigenetic resetting to erase all DNA methylation mark and establish new, germline-specific marks, including paternal imprinting. Postnatal spermatogenesis includes three phases, proliferation of spermatogonia (SG), meiosis of spermatocytes (Spc), and haploid differentiation, during which round spermatids (RS) undergo morphological transformation to elongating spermatids (ES) and finally to mature spermatozoa. When released from the seminiferous epithelium of the testis, spermatozoa are transported to the epididymis where they undergo final maturation during the epididymal transit through caput, corpus and cauda parts of the epididymis. Spermatocytes and round spermatids actively transcribe their genome, but chromatin compaction due to replacement of histones with protamines gradually silences transcription, and mature spermatozoa are transcriptionally inactive. Germ granules participate in the transcriptome regulation, and the two most prominent ones, the intermitochondrial cement (IMC; green dots) in spermatocytes and the chromatoid body (CB; purple dots) in late spermatocytes and round spermatid, are indicated. Fetal piRNAs are expressed in pro-spermatogonia where they are associated with PIWIL4 and PIWIL2 for transposon silencing during the epigenetic resetting. Pre-pachytene piRNAs are expressed during early postnatal spermatogenesis and they associated with PIWIL2. Massive production of pachytene piRNAs is induced in pachytene spermatocytes where they bind to PIWIL1 and PIWIL2. Some selected miRNAs are also indicated to highlight their function in specific cell types. Spermatozoa contain a complex population of tsRNAs, rsRNAs, miRNAs and piRNAs that are able to mediate intergenerational transmission of epigenetic information to the offspring via the process of epigenetic inheritance, therefore affecting offspring development and health.

Figure 2. Biogenesis of miRNAs and piRNAs. (A) Canonical miRNA pathway. miRNA biogenesis begins with the transcription of a long primary miRNA precursors (pri-miRNA) by RNA polymerase II. The pri-miRNA folds to form a hairpin substrate that is processed by enzymes DROSHA and DGCR8 into pre-miRNAs (~60 nt), then exported to the cytoplasm by EXPORTIN 5. The pre-miRNA is finally processed by DICER into ~22 nt miRNAs duplex. The other strand of the duplex is selected to remain associated with AGO proteins to form RNA-induced silencing complex (RISC) that binds to the target RNA using complementary base-pairing between the miRNA and the target. (B) piRNA pathway in male germ cells. piRNA biogenesis in postnatal male germ cells begins with the transcription of a long piRNA precursor from a genomic piRNA cluster. PIWI proteins associate with the piRNA precursor and guide the endonucleolytic cleavage to induce the production of a string of tail-to-head phased pre-piRNAs from the precursor. Subsequently, the 3’ end of pre-piRNAs is trimmed by an exonuclease and finally 2′-O-methylated. PIWI-associated mature piRNA can recognize complementary antisense transcripts to induce so-called ping-pong cycle that functions as a piRNA amplification loop.


Caput
Corpus
Cauda
Testis
Epididymis
Prenatal
Postnatal spermatogenesis
Maturation
Fertilization
Epigenetic inheritance
Proliferation
Meiosis
Haploid differentiation
miR-17-92
miR-290-295
miR-17-5p
miR-17-92
miR-202-3p
miR-let7
miR-34c
miR-10
miR-221
miR-34c
miR-122a
miR-469
tsRNA
rsRNA
miRNA
piRNA
Offspring development and health
Egg
PGC
Pro SG
SG
Early Spc
Late Spc
RS
ES
ES
Spermatozoa
Embryo
HISTONES
PROTAMINES
Epigenetic resetting
Transcriptional activity
Fetal piRNAs
Pre-pachytene piRNAs
Pachytene piRNAs
PIWIL4
PIWIL2
PIWIL1


A Canonical miRNA pathway
miRNA gene
pri-miRNA
NUCLEUS
DGCR8
DROSHA
pre-miRNA
CYTOPLASM
EXPORTIN 5
pre-miRNA
TRBP
DICER
AGO
RISC
target mRNA
AGO
B piRNA pathway
piRNA cluster
piRNA precursor
NUCLEUS
CYTOPLASM
endonucleolytic cleavage
piRNA precursor
PIWI
PIWI
pre-piRNA
PIWI
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PIWI
trimming
2'-O-methylation
mature piRNA
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ping-pong cycle
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