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REVIEW

The functions and mechanisms of piRNAs in mediating mammalian spermatogenesis and their applications in reproductive medicine

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Abstract

As the most abundant small RNAs, piwi-interacting RNAs (piRNAs) have been identified as a new class of non-coding RNAs with 24–32 nucleotides in length, and they are expressed at high levels in male germ cells. PiRNAs have been implicated in the regulation of several biological processes, including cell differentiation, development, and male reproduction. In this review, we focused on the functions and molecular mechanisms of piRNAs in controlling spermatogenesis, including genome stability, regulation of gene expression, and male germ cell development. The piRNA pathways include two major pathways, namely the pre-pachytene piRNA pathway and the pachytene piRNA pathway. In the pre-pachytene stage, piRNAs are involved in chromosome remodeling and gene expression regulation to maintain genome stability by inhibiting transposon activity. In the pachytene stage, piRNAs mediate the development of male germ cells via regulating gene expression by binding to mRNA and RNA cleavage. We further discussed the correlations between the abnormalities of piRNAs and male infertility and the prospective of piRNAs' applications in reproductive medicine and future studies. This review provides novel insights into mechanisms underlying mammalian spermatogenesis and offers new targets for diagnosing and treating male infertility.

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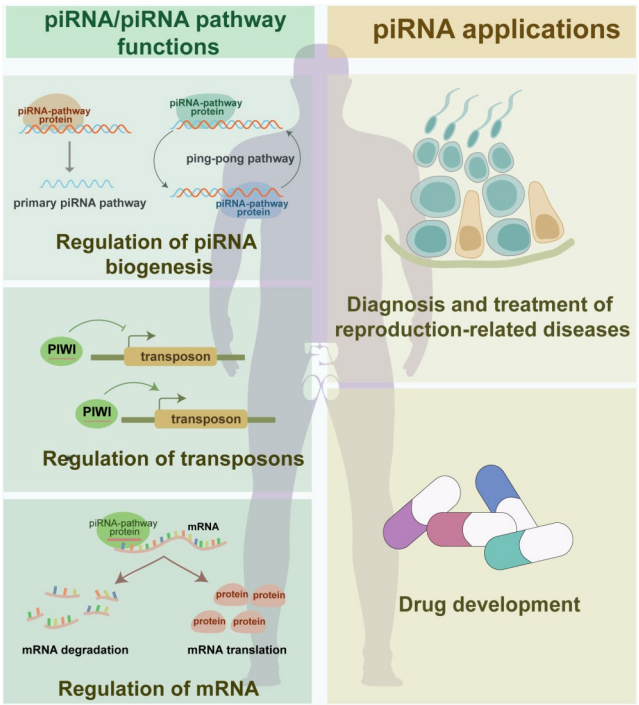
Graphical Abstract



The piRNA/piRNA pathway functions and applications. The piRNA/piRNA pathway is mainly involved in piRNA biogenesis, regulation of transposons, and binding to mRNAs to control spermatogenesis

Keywords piRNA pathways · piRNA biogenesis · Spermatogenesis · Transposons · PIWI · Male infertility

Introduction

PiRNAs and piRNA pathways

Non-coding RNAs are classified into short and long non-coding RNAs in terms of nucleotide length, as shown in Table 1. The short non-coding RNAs include microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), and piRNAs [1, 2]. MiRNAs inhibit mRNA translation or cause mRNA degradation by binding to fully or partially complementary sequences in the 3' untranslated regions (3' UTRs) of targeting message RNAs (mRNAs) [3, 4], while siRNAs silence gene expression by forming RNA-induced silencing complexes (RISC) to bind perfectly to complementary mRNAs [5]. In addition to binding to mRNAs, snoRNAs direct the cleavage of rRNA. The regulation of mRNA 3' end processing and selective cleavage by snoRNAs is crucial for regulating gene expression and appropriate protein synthesis [6]. Among all non-coding RNAs, piRNAs are the most abundant small RNAs, and they mediate gene expression by silencing transposable elements to maintain genome stability [7]. PiRNAs are enriched in the testis and ovary, and they play key roles in controlling spermatogenesis and oogenesis [8–11]. The orderly regulation of piRNA pathways ensures the correct pairing and segregation of chromosomes during meiosis to ensure the transmission and stability of genetic information [12].

The Piwi proteins were first identified in female *Drosophila* melanogaster, and deletion of *Piwi* leads to mitotic arrest of female germinal stem cells in *Drosophila* [26]. Knockdown of *Piwi* in *Caenorhabditis* elegans inhibits the self-renewal of germline stem cells [27]. PIWI proteins have been shown to be mainly expressed in male germ cells in mammals, and a diversity of PIWI proteins with temporal and spatial expression exists during germ cell development (Table 2) [28]. There are three types of PIWI proteins in mice, including PIWIL1 (also known as Miwi), PIWIL2 (Mili) and PIWIL4 (Miwi2) [29–31], while human PIWI proteins can be divided into four types, namely PIWIL1 (HIWI), PIWIL2 (HILI), PIWIL3 and PIWIL4 (HIWI2) [32, 33]. PiRNAs have been found to interact with PIWI proteins [34–39]. In mammals, piRNAs can guide PIWI proteins to silence transposons and regulate gene expression in cells [40]. However, the mechanisms of piRNA

Table 1 The characteristics and functions of non-coding RNAs in gene regulation and cellular processes

Non-coding RNAs	Lengths	Locations	Functions and mechanisms	References
MiRNAs	18 ~ 25 nt	Cytoplasm	MicroRNAs (miRNAs) form the RISC by binding to target mRNAs. This RISC complex degrades the mRNA or inhibits its translation, thus affecting the expression of the target genes.	[13, 14]
SiRNAs	21 ~ 23 nt	Cytoplasm	SiRNAs bind to the target mRNA through fully complementary pairing, which causes the degradation of mRNAs, thereby resulting in the silencing of the target genes.	[15, 16]
SnoRNAs	60 ~ 300 nt	Nucleolus	SnoRNAs regulate the rRNA processing, RNA cleavage, and translation as well as the oxidative stress response.	[17, 18]
PiRNAs	24 ~ 32 nt	Cytoplasm	PiRNAs form complexes with PIWI proteins and participate in various biological processes, e.g., maintenance of chromatin stability, post-transcriptional gene silencing, and transgenerational inheritance.	[19, 20]
LncRNAs	> 200 nt	Cytoplasm, nuclei	LncRNAs participate in gene expression regulation, cell fate determinations, cell cycle, and epigenetic regulation.	[21, 22]
CircRNAs	/	Cytoplasm	CircRNAs relieve the inhibitory effect of miRNA on target genes and upregulate the expression levels of the target genes.	[23–25]

Table 2 Expression, functions and interacting proteins of four PIWI proteins in mammals

PIWI proteins	Mice	Humans	Functions	Expression	Abnormal phenotypes	PiRNA pathway-related proteins	References
PIWIL1	Miwi	HIWI	Protection of mRNA of male germ cells from premature degradation and translation	From pachytene spermatocytes to round spermatids	Arrest at the early spermatid stage	TDRKH, ADAD2, MAEL	[41]
PIWIL2	Mili	HILI	Promoting germline stem cell division and differentiation through translational regulation	From primordial germ cells (PGCs) to round spermatids	Arrest at the round spermatid stage	GPAT2, RHOX10, EXD2, MOV10L1, TDRD1, ADAD2, TEX15	[42–44]
PIWIL3	/	HIWI3	Transient maintenance of piRNA biogenesis and genome integrity	Oocytes and early embryos	Defective chromosome segregation and abnormal early embryonic development	TDRKH, PNLDC1	[45–47]
PIWIL4	Miwi2	HIWI2	Inhibition of aberrant retrotransposon activation	The PGCs of fetal testis	Arrest in the early stages of meiosis I	EDX1, TDRD12, GTSF1, MVH/DDX4, TDRD9	[31, 48]

generation vary in different species and tissues. Here, we focused on the piRNA generation mechanisms in mammals.

During mammalian spermatogenesis, piRNAs are formed via two stages. At the first wave beginning at the embryonic stage, pre-pachytene piRNAs can be detected in fetal and perinatal germ cells [49, 50]. Pachytene piRNAs are mainly detectable in pachytene spermatocytes [51]. These two waves of piRNA biogenesis play different roles. Pre-pachytene piRNAs are primarily involved in silencing transposons, while pachytene piRNAs mainly participate in gene regulation [52]. There are two main pathways for piRNA generation, including the primary processing pathway and the ping-pong cycle pathway. In the primary processing pathway, precursor RNA transcription of pachytene piRNAs is initiated by MYB proto-oncogene-like 1 (A-myb) and subsequently cleaved to generate piRNA intermediates by phospholipase D family member 6 (MitoPLD) [51]. MitoPLD is located at the mitochondrial outer membrane, and it can process piRNAs from their longer immature forms into shorter intermediate forms, namely pre-piRNAs [53]. PARN-like ribonuclease domain-containing exonuclease 1 (PNLDC1) interacts with Tudor structural domain protein TDRKH, which has been proposed to be a piRNA trimming cofactor [54]. Pre-piRNAs are cleaved to mature length by PNLDC1 and 2'-O-methylation into functional forms by HEN methyltransferase 1 (HENMT1) [55–57]. The absence of HENMT1 leads to unmethylation of piRNAs and decreases in their abundance and length [58]. In the ping-pong cycle pathway, primary piRNAs bind to PIWI proteins and cleave complementary mRNAs, which results

in the production of secondary piRNAs with a 5'end. These secondary piRNAs are associated with PIWI proteins and cleave complementary target mRNAs again, which generates primary piRNAs with a 3'end. After modification by HENMT1, primary piRNAs are matured into functional molecules. The primary piRNAs and secondary piRNAs are generated in a continuous ping-pong cycle pathway [59–61]. The generation process of piRNAs is illustrated in Fig. 1. Any error in the primary processing pathway or the ping-pong pathway can lead to the impaired piRNA biogenesis. In 2020, ribosomes have been identified as key players in pachytene piRNA formation, and they participate in piRNA generation through three distinct mechanisms [62]. First of all, the binding of ribosomes to cleavage products stabilizes the cleavage products for loading PIWI. Ribosomes maintain a stepwise division from the 5' UTR to the 3' UTR in piRNA biogenesis. Secondly, the extended ribosome itself acts as a powerful helicase to remove secondary structures and RNA-binding proteins. Finally, ribosomes provide a platform for biological and regulatory proteins to bind to pachytene piRNA precursors [62]. Ribosomes have been shown to direct the fragmentation of mRNAs to produce 3' UTR piRNAs [63].

PiRNAs and their regulation of spermatogenesis

Spermatogenesis is a complex process that comprises three main stages, including self-renewal and differentiation of spermatogonial stem cells (SSCs), two meiotic divisions of spermatocytes, and spermiogenesis with the morphological changes from round spermatids to elongated spermatids [64], and it is derived from primordial germ cells (PGCs) [65]. In the human testis, spermatogonia are classified into type A spermatogonia and type B spermatogonia, while type A spermatogonia are subdivided into the A_{dark} and A_{pale} spermatogonia [66]. A_{dark} spermatogonia are the quiescent SSCs, while A_{pale} spermatogonia are the self-renewing SSCs [67]. Type B spermatogonia further differentiate into spermatocytes that undergo two meiotic divisions to generate spermatids with morphic maturation to form spermatozoa. Unlike human testis, type A spermatogonia in mice are divided into A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia, which is followed by A_{1-4}, intermediate spermatogonia, and type B spermatogonia [68]. This process of spermatogenesis requires the cooperation of various kinds of male germ cells with testicular somatic cells, including Sertoli cells, myoid cells, and Leydig cells [69]. Spermatogenesis is precisely regulated by genetic and epigenetic factors. Notably, numerous studies have highlighted that epigenetic factors, including piRNAs and other ncRNAs, are involved in mediating spermatogenesis.

PiRNAs are essential for the development of male germ cells and somatic cells through multiple mechanisms. Firstly, piRNAs bind to PIWI proteins to form a RISC that effectively inhibits transposons by guiding their methylation and recombination [11, 70]. There are three major types

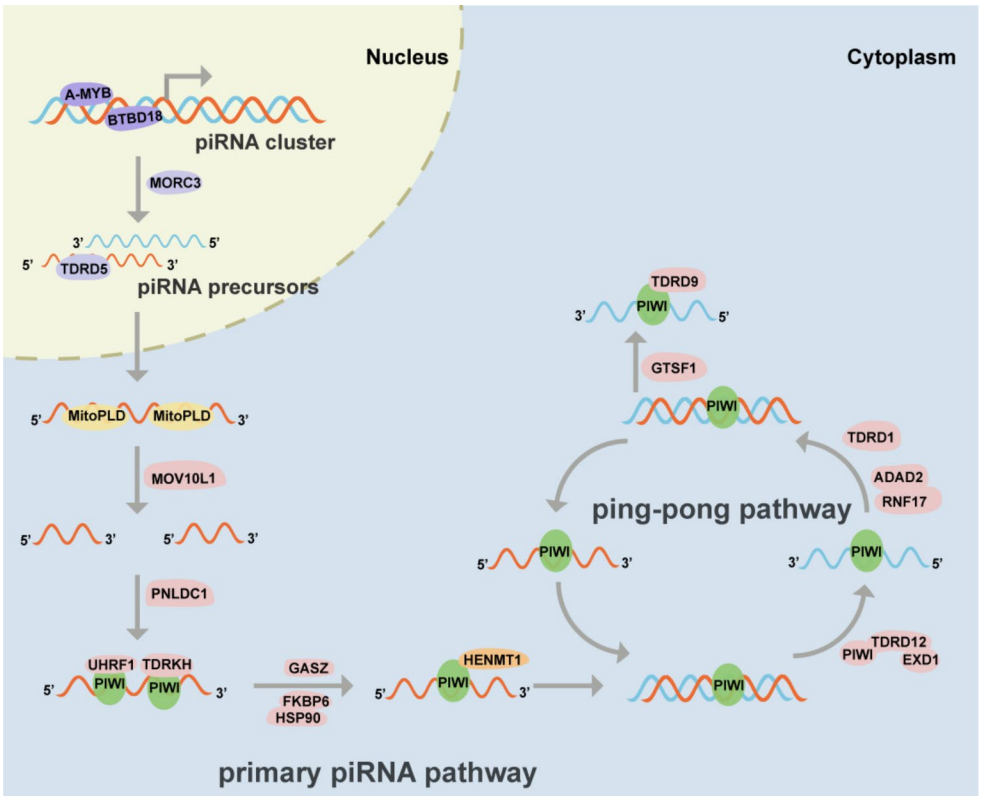


Fig. 1 The piRNA pathway proteins are involved in piRNA biosynthesis. PiRNA biosynthesis can be divided into two main processes, namely primary piRNA pathway and ping-pong pathway. Primary piRNAs generate the initial piRNAs with the beginning of the ping-pong pathway. These two processes are involved in piRNA generation and functions. Different proteins are required for the primary piRNA pathway and the ping-pong pathway

of transposons in mammals, including LINES, SINES, and LTRS, while piRNAs are mainly involved in the regulation of LINES [49, 71, 72]. Secondly, piRNAs can bind to complementary sequences of mRNAs or lncRNAs to promote their degradation, transport, or post-transcriptional regulation. Pachytene piRNAs negatively regulate mRNAs, lncRNAs, and LINE-1 retrotransposons in late spermatocytes, which is largely dependent on retrotransposon sequences and pseudogenes [73]. Thirdly, piRNAs are highly enriched in late spermatocytes and round spermatids [74]. Abnormality of piRNAs/PIWI leads to developmental disorders of male germ cells in *Drosophila*, zebrafish, nematode, mice, golden hamsters, and humans. Notably, piRNAs are involved in the development and maturation of male germ cells by affecting chromosome remodeling, epigenetic modifications, and apoptosis, and conversely, piRNA dysfunction leads to male infertility [10, 75–80]. Finally, piRNA clusters contain partial or complete transposon sequences, and piRNA systems can distinguish between friends and enemies and initiate responses [81]. As such, piRNAs protect the genome of male germline from invasion by new transposable elements [82, 83]. In this review, we elucidated the roles and mechanisms of piRNAs in controlling spermatogenesis, including genome stability, gene expression regulation, and male germ cell development, to illustrate the indispensable functions of piRNAs in maintaining male fertility and reproductive health.

The functions of piRNAs in controlling mammalian spermatogenesis

Abnormality in the expression of one or more genes involved in the piRNA pathway leads to aberrant spermatogenesis, and the phenotypes of mice with piRNA pathway defects include abnormal activation of germ cell transposons, meiotic arrest, and spermiogenesis disorder, which eventually results in male infertility [31, 84–87]. Several proteins, including Mili, Mov10-like RISC complex RNA helicase 1 (Mov10l1), and Ring finger protein 17 (Rnf17), have been identified as key components involved in the piRNA pathway [88–91]. PiRNAs play different roles in the pre-pachytene and pachytene stages. During the pre-pachytene stage, piRNAs play a critical role in maintaining genome stability by inhibiting transposon activity [49, 92]. Pachytene piRNAs are mainly involved in regulating mRNA degradation and translation during the post-meiotic stages of spermatogenesis [41, 93, 94]. Notably, piRNAs interact with mRNAs, as evidenced by the findings that piRNAs mediate the stability and translation of mRNAs and that mRNAs in turn participate in the biogenesis and functional regulation of piRNAs [74, 88]. The targets of piRNAs are expressed in the testis, with 74.93% of them being retrotransposons [95]. Therefore, piRNAs are committed to protecting the genome from transposons and participate in the regulation of protein-coding genes. These two processes act synergistically at different stages of testis development [96]. To elucidate the diverse mechanisms by which piRNA function, we summarized the proteins mainly involved in the pre-pachytene piRNA biogenesis and pachytene piRNA biogenesis as well as the deficiency of proteins in the piRNA pathways with spermatogenesis arrest, as we illustrated in Fig. 2.

The pre-pachytene piRNA pathway and spermatogenesis

A series of pre-pachytene piRNA-related proteins have been identified in mammalian spermatogenesis, including Mili [30], Miwi2 [31], Pnldc1 [57], Mov10l1 [97], the homeobox transcription factor Rhox10 [98], Exonuclease domain-containing 1 (Exd1) [99], Glycerol-3-phosphate acyltransferase 2 (Gpat2) [100], Gametocyte-specific factor 1 (Gtsf1) [101], Tdrd12 [102], ATPase activity of mouse Vasa homolog (Mvh) [103], and MORC family CW-type zinc finger 3 (Morc3) [104]. The main function of piRNAs is to inhibit transposon activity during de novo DNA methylation [105, 106]. Mili is expressed in the cytoplasm of prospermatogonia, while Miwi2 is present in the cellular nuclei of these cells. Both Mili and Miwi2 are required for DNA methylation of TE sequences. *Mili* acts as an upstream factor of Miwi2, and it is involved in the regulation of Miwi2 nuclear localization [107]. As a functional partner of Miwi2, *Tdrd9* mutation in fetal testes results in abnormal piRNA profiles in prospermatogonia [108]. In spermatogonia, LINE-1 repression is accomplished primarily through the following three pathways: the Piwi-piRNA pathway, CpG promoter DNA methylation, and G9a-mediated H3K9me2 [109]. Here, we discussed the repression of LINE-1 by the Piwi-piRNA pathway in spermatogonia. PiRNAs complement the LINE-1 sequences and maintain genome stability by directing the Piwi proteins Mili to cleave and disrupt LINE-1 transcripts via the RNA interference (RNAi)-like mechanism [110]. Deletion of *Mili* severely impacts the self-renewal and differentiation of SSCs by regulating translation [30, 103, 111]. In *Mili* null mice, spermatogonia are absent and exhibit a Sertoli cell-only (SCO) phenotype [44]. During the transition of PGCs to prospermatogonia, Rhox10 transcriptionally activates *Mili*, and Mili protein drives the piRNA pathway to mediate the repression of the LINE-1 promoter, thereby inhibiting transposons [112]. Loss of *Rhox10* leads to an aberrant number of SSCs and a progressive spermatogenesis disorder [98, 113].

Defects in the processing and maturation of piRNAs impede normal development of male germ cells. Pnldc1 is

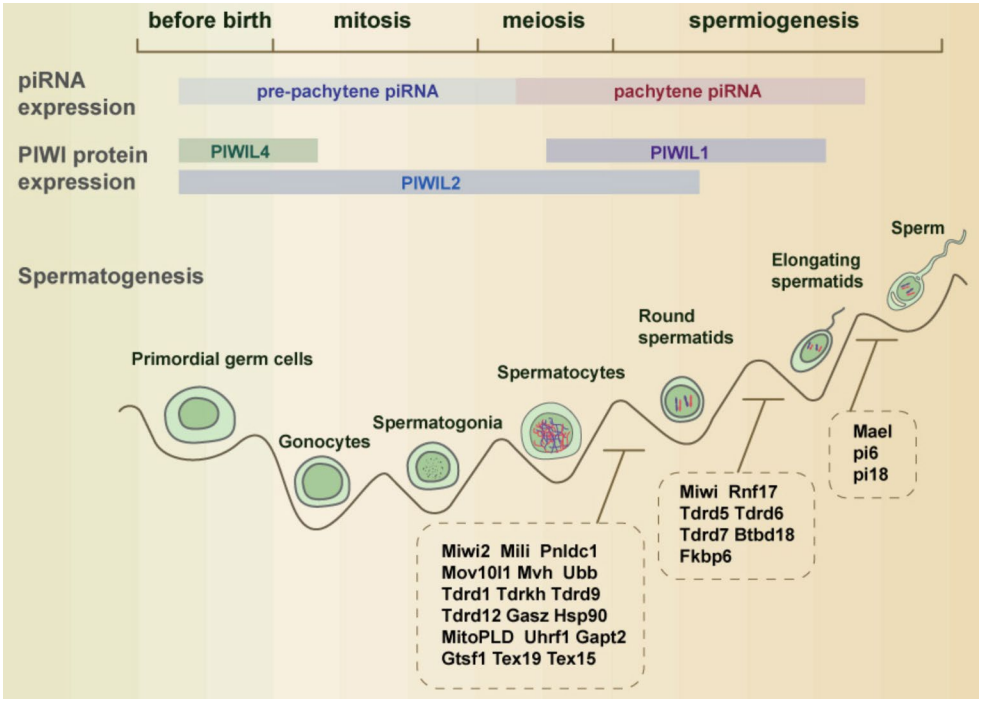


Fig. 2 The expression of piRNAs and PIWI proteins and the relationship between the loss of key proteins in the piRNA/piRNA pathway and spermatogenesis failure. The expression of piRNAs and PIWI proteins is spatio-temporal specific. According to the expression periods, piRNAs are classified into pre-pachytene piRNAs and pachytene piRNAs. Loss of key proteins of the piRNA/piRNA pathway results in spermatogenesis disorder

a trimmer of pre-piRNAs, and it is involved in the cleavage of pre-piRNAs [54, 85]. Abnormal alterations in the length of piRNAs, the number of pachytene piRNAs, and piRNA-processing proteins PIWIL1, PIWIL4, A-MYB and TDRKH, have been observed in non-obstructive azoospermia (NOA) with *PNLDC1* mutations [79]. Also, piRNAs 3'end processing is disrupted in *Pnldc1* mutant mice, which leads to an accumulation of 3'untrimmed piRNA intermediates, a reduction of pre-pachytene piRNAs and pachytene piRNAs, and defective spermatogenesis [57, 77, 114]. Lack of piRNA trimming and methylation in *Pnldc1* and *Henmt1* double knockout male mice results in the collapse of the piRNA pathway [115]. A 3' tail modification of mature piRNAs has been identified in mouse testes, and uridylation initiated by TUT4/7 is the predominant tail form of MIWI-bound piRNAs [116]. In addition, *Mov10l1* is an RNA helicase located upstream of *PNLDC1* and it is involved in piRNA 5' end processing. It is noteworthy that two *MOV10L1* mutations and one homozygous *MOV10L1* mutation are identified in NOA patients [117]. MOV10L1 has been found to be upstream of Piwi proteins during the primary processing of pachytene piRNAs [92]. In mice, Mov10l1 has a similar expression pattern to Mili, and it can bind to pre-piRNAs to initiate the piRNA pathway [97, 118]. Disruption of *Mov10l1* helicase activity results in the loss of pre-pachytene piRNAs, the activation of retrotransposons, early meiotic arrest, and male infertility [119, 120]. In testes of cryptorchid boys, expression levels of *MOV10L1*, *PIWIL2*, *PIWIL4*, and *TDRD9* are significantly reduced, suggesting that the impaired expression of these genes associated with transposon silencing may lead to genome instability and azoospermia [121]. Defective processing of piRNAs may be an important etiology of NOA patients, as evidenced by the integration of clinical data and in vivo studies.

MVH is crucial for processing intermediates into piRNAs, which ensures the silencing of transposable elements and the maintenance of male fertility [122]. *Mvh* mutation impairs piRNA binding to Miwi2 and impacts de novo methylation of transposons, resulting in piRNA cycle arrest and spermatocyte arrest at the meiotic stage and male infertility in mice [103]. *Gasz* (Germ cell protein with Ankyrin repeats, sterile alpha motif, and leucine Zipper) knockout mice exhibit phenotypes similar to *Mili* knockout mice, and Gasz may participate in the regulation of spermatogenesis by localizing or stabilizing multiple proteins to affect piRNA processing and synthesis [123].

EXD1 acts as an RNA adaptor in the PIWI-EXD1-TDRD12 (PET) complex [99]. The absence of Exd1 causes a reduction in the production of Mili-sliced piRNAs and the biogenesis of Miwi2 piRNAs [99]. In contrast to the phenotype observed in *Exd1*-deficient mice, Exd2 interacts with Mili and its mutation leads to abnormal regulation of specific piRNA clusters without affecting piRNA biogenesis [124]. Therefore, *Exd2* mutations have no obvious effect on male fertility, and the precise function of Exd2 in the piRNA pathway remains to be further elucidated. To elucidate the function of Exd1 and Exd2 in the piRNA pathway is important for understanding the mechanism that maintains genome stability during spermatogenesis [124].

TDRD12, also known as ECAT8, is a unique piRNA biogenesis factor. The complex formed by Tdrd12 with Exd1 and Mili is mainly involved in the biogenesis of secondary piRNAs, while Tdrd12 does not affect the biogenesis of primary piRNAs [125]. Gpat2 is one of the Mili binding proteins, and Gpat2 may act as a scaffold to recruit various factors required for piRNA production, which is essential for primary piRNA biogenesis [126]. Knockdown of *Gpat2* results in the impaired piRNA biogenesis in SSCs and more apoptosis in neonatal spermatogonia [100]. Gtsf1 is a component of the Mili/Miwi2 complex, and deficiency of *Gtsf1* in mouse prospermatogonia results in abnormal localization of Miwi2. Loss of Miwi2-bound piRNAs leads to defective secondary piRNA biogenesis, reflecting that Gtsf1 is a key factor for piwi-piRNA cleavage of target mRNAs [101]. Morc3 recognizes and binds to H3K4me3 marks on the promoter regions of retrotransposon genes and piRNA clusters, thereby affecting the biogenesis of primary piRNAs. In the embryonic testes, Morc3 is involved in chromosome remodeling and regulates the transcription of piRNA precursors. In addition, Morc3 may be involved in the biogenesis of secondary piRNAs [104].

Collectively, these studies mentioned above implicate that piRNAs assume stage-specific regulation during spermatogenesis. In spermatogonia, a number of transposon-targeting piRNAs are generated, while piRNAs in primary spermatocytes play pivotal roles in the suppression of repetitive sequences and transposons. This stage-specific regulation is essential for maintaining genome stability and ensuring proper spermatogenesis [127].

The pachytene piRNA pathway and spermatogenesis

We summarized the pachytene piRNA pathway proteins affecting mammalian spermatogenesis, including Miwi [128], Tdrd1 [90], Tdrd5 [129], Tdrd9 [108], Ubiquitin-like with PHD and ring finger domains 1 (Uhrf1) [130], Tdrkh [131], MitoPLD [55], Rnf17 [88], Adenosine deaminase domain containing 2 (Adad2) [132], Testis expressed 19 (Tex19) [133], Testis expressed 15 (Tex15) [134], Ubiquitin B (Ubb) [135], BTB domain containing 18 (Btbd18) [136], Maelstrom (Mael) [137], and FKBP prolyl isomerase family member 6 (Fkbp6) [55, 138]. Next, we addressed the specific functions and molecular mechanisms of these proteins involved in piRNA biogenesis.

Miwi, a member of the PIWI protein family, is mainly expressed in male germ cells from pachytene spermatocytes to early spermatids [29]. *Miwi* deficiency affects centromere assembly in meiosis, resulting in an enhancement in chromosome missegregation at meiosis I, an increase in aneuploidy at meiosis II, and the death of spermatids [139]. Miwi/HIWI functions as piRNAs-guided mRNA degradation [96]. The Miwi/piRNAs mechanism plays a dual role in the regulation of spermatogenesis in mice, and translation activation occurs through the formation of Miwi/piRNAs/eIF3f/HuR supercomplex in round spermatids [128]. At elongating spermatids, the Miwi/piRNAs/CAF1 supercomplex forms and initiates extensive mRNA elimination in spermatids to regulate acrosome formation [128].

Members of the TDRD family (TDRD1-9, 12) are involved in the biogenesis of piRNAs or PIWI protein interaction, with the exception of TDRD3 [140]. However, the specific functions of each protein seem to be distinct [141–143], as shown in Table 3. Tdrd1 interacts with Mili, and it is involved in regulating retrotransposons via the Piwi pathway. *Tdrd1* deficiency leads to the derepression of LINE-1, and loss of DNA methylation of its regulatory elements causes mislocalization of Miwi2 from nuclei to cytoplasm [42]. Nevertheless, Tdrd1 does not affect piRNA biogenesis in spermatogenesis [43, 144]. Unlike Tdrd1, Tdrd9 is a functional partner of Miwi2 in the Piwi pathway. *Tdrd9* mutation

Table 3 Different functions of the TDRD family proteins

TDRDs	Alias	Interacting PIWI	Functions	Phenotypes of knockout mice	References
Tdrd1	Mtr-1	Mili	Ensuring correct nuclear localization of Miwi2; ensures the entry of correct transcripts into the normal piRNA pool	Arrest at the pachytene stage	[42, 158]
Tdrd2	Tdrkh	Miwi	Promotes the final step of piRNA biogenesis	Arrest at the zygotene stage	[131, 142, 159]
Tdrd4	Rnf17	Miwi	Suppressing the production of secondary piRNAs	Arrest at round spermatids	[88, 160]
Tdrd5	/	Miwi	Essential for pachytene piRNA biogenesis	Arrest at either zygotene spermatocytes or round spermatids	[129, 161, 162]
Tdrd6	/	Mili Miwi	Involved in spliceosome maturation and mRNA cleavage	Arrest at round and elongated spermatids	[144, 163]
Tdrd7	/		Suppresses LINE-1 retrotransposons	Arrested at round spermatid stage	[149]
Tdrd8	Stk31	Miwi	A critical player in fertilization	Affect fertility	[143, 151]
Tdrd9	/	Miwi2	Silencing Line-1 retrotransposon	Meiotic failure	[108]
Tdrd12	Ecat8	Mili Miwi2	Essential for the production of secondary piRNAs	Spermatocytes fail to proceed beyond the prepachytene stage	[102, 125]

in fetal testis affects the silencing of LINE-1 in pre-spermatogonia and piRNAs profiles [108]. Tdrd2, also known as Tdrkh, specifically recruits Miwi to drive the piRNA biogenesis. Miwi is lost in the chromatoid body when Tdrkh is defective in the testis, which causes spermatogenesis arrest at the round spermatid stage [145]. Unlike Tdrkh, MitoPLD deficiency in mice leads to meiotic arrest of spermatocytes due to affecting piRNA generation and distribution [84, 146]. Deletion of Uhrf1 in the testis results in a significant reduction of PIWI proteins and piRNA-associated proteins TDRKH and MVH [130]. Tdrd4, also known as Rnf17, has been shown to participate in the balance of the ping-pong cycle of piRNAs during meiosis, affect piRNA content by inhibiting the generation of secondary piRNAs, and enhance the expression of protein-coding genes crucial for the regulation of spermatogenesis by inhibiting the ping-pong cycle [88]. Interestingly, a testis-specific protein, namely Adad2, interacts with multiple RNA-binding proteins, including Mili, Miwi, Rnf17, and Ythdc2, has been found to be involved in piRNA biogenesis [132]. Adad2 guides Rnf17 to repress ping-pong activity during the biogenesis of pachytene piRNAs. During meiosis, *Adad2* knockout can cause mislocalization of Rnf17 followed by the loss of ping-pong suppression, which results in overproduction of secondary piRNAs and ultimately spermatogenesis arrest at the round spermatid stage [147, 148]. *Tdrd5* binds directly to the precursors of piRNAs and functions via selectively controlling the processing of the pachytene piRNAs precursors [129]. Tdrd6 interacts with Mili and Miwi, and *Tdrd6* knockout mice are arrested at the round spermatid stage and fail to form the elongated spermatids. Abnormal miRNA expression has been observed in *Tdrd6* knockout mice, and it remains unclear about whether Tdrd6 affects piRNA biogenesis [144]. In contrast to Tdrd6, LINE-1 expression is abnormal in *Tdrd7*$^{-/-}$ testis, and *Tdrd7* mutant mice have spermatogenesis arrest at the round spermatid stage [149]. Tdrd7 is associated with RNP remodeling at early spermatid stage, and Tdrd6 is related to structural maintenance at a later stage of spermatid development. Chromatoid bodies are important subcellular sites for piRNA biogenesis, containing a large number of proteins required for piRNA biogenesis [150]. Therefore, Tdrd7 may influence not only the expression of LINE-1 in spermatogenesis but also the expression and localization of other piRNA biogenesis proteins, e.g., Mili, Mvh, Mael, and Gasz [149]. Tdrd8, also known as Stk31, has been shown to interact with Miwi in mouse testis by LC/MS assay [151]. However, subsequent experiments found that disruption of *Stk31* does not affect male fertility [143], and the function of Stk31 in the testis needs to be further explored. TEX19 is directly associated with piRNAs through its VPTEL domain [133]. Unlike TEX19, TEX15 functions independently of piRNA biogenesis, and it may act as a nuclear effector protein downstream of the piRNA pathway to silence TEs in male germ cells [152]. Meanwhile, TEX15 has been shown to be essential for de novo DNA methylation of TEs regulated by MIWI2-piRNA pathway [134]. Ubb is essential for maintaining piRNA-metabolic proteins, and *Ubb* knockout in mice results in the reduction of piRNA metabolic process-related proteins and meiotic cell cycle arrest in male germ cells, which leads to azoospermia phenotype [135]. Deletion of *Btbd18* reduces the expression level of primary piRNA precursors, which severely impairs piRNA biogenesis. Mice lacking *Btbd18* experience a massive loss of spermatocytes due to apoptosis, leading to spermatogenesis arrest, azoospermia, and male infertility. Since no aberrant retrotransposon activation is observed, the phenotype of *Btbd18* null mice differs from *Miwi* knockout mice [136]. Btbd18 enhances the expression level of primary piRNA precursors by promoting transcriptional elongation [136]. During spermatogenesis, both A-MYB and TCFL5 regulate piRNAs. *A-Myb* is involved in piRNA generation as a transcription factor, and *A-Myb* null mice generate fewer pachytene piRNAs [51, 153]. Interestingly, half of the promoters of human pachytene piRNAs do not interact with A-Myb [153]. During early meiosis I, *A-Myb* initiates transcription of *Tcfl5* which binds to its own promoter and *A-Myb* promoter to form a mutually reinforcing positive feedback loop. Tcfl5 regulates the expression of genes required for piRNA maturation and stimulates transcription of evolutionarily young pachytene piRNA genes, while A-Myb is responsible for activating transcription in older pachytene piRNA genes [154]. Spermatogenesis in *Tcfl5*$^{-/-}$ and *Tcfl5*$^{+/-}$ mice is arrested at the pachytene/diplotene spermatocyte transition and round/elongating spermatid transition [155, 156], while spermatocytes in *A-Myb* mutant mice assume abnormal cell cycle progression [157].

The histone H3 family includes two major histone variants *CENP-A* and *H3.3* [164]. H3.3 is encoded by two different genes, namely *H3.3a* and *H3.3b*, with the same amino acid sequences [165, 166]. Loss of *H3.3b* in spermatocytes is associated with the increased expression of RLTR10B and RLTR10B2 retrotransposons as well as downregulation of piRNA clusters. This finding reflects the dual role of H3.3b in controlling spermatogenesis. H3.3 can positively regulate the expression of piRNAs required for meiotic chromatin inactivation and repression of repeat element transcription [167]. Mael is a conserved HMG box structural domain protein that is essential for mouse spermatogenesis [137, 168], and its MAEL domain may have potential nuclease activity or RNA binding capacity to affect piRNA biogenesis [169]. MAEL-related protein complex, including MIWI/PIWIL, TDRD6, TDRD4/RNF17, TDRD, STK31/TDRD8, and TDRD9, has been identified [170]. *Mael129* knockout mice exhibit spermatogenesis arrest

with acrosome and flagellar malformations, which may be caused by an imbalance between pachytene piRNAs and MIWI [170], and loss of *Mael* leads to a decrease in the levels of pachytene piRNAs in mouse testes [171, 172]. MAEL is mainly expressed in spermatid mitochondria in the human testis, and loss of *Mael* can lead to mouse mitochondrial dysfunction and asthenozoospermia [173]. It remains to be further uncovered whether MAEL affects piRNA biogenesis in human spermatogenesis. FKBP6 is required for spermatogenesis, and it is involved in piRNA biogenesis and synaptic complex formation [174]. Recently, *Fkbp6*-null testicular cells have been found to be arrested at the round spermatids, and *Fkbp6* deletion severely affects piRNA levels [175]. Moreover, FKBP6, a molecular chaperone of HSP90, reduces MIWI2-binding piRNAs in *Fkbp6*-null testes [138, 176]. Hsp90 regulates spermatogenesis by participating in the formation and/or stabilization of MILI-piRNA and MIWI2-piRNA complexes [177]. In addition, piRNAs can directly bind to mRNAs and affect their translation and stability, thereby regulating gene expression. High level of piR-003399 inhibits CDK6 expression and causes cell cycle arrest in mouse spermatogonia at G1 phase and abnormal sperm count, motility, and morphology [178]. The loss of pi6 and pi18 loci on mouse chromosomes 6 and 18 affects sperm motility and acrosome reaction, respectively, thereby preventing spermatids from penetrating the zona pellucida and resulting in male infertility. Pi6 and pi18 piRNAs primarily target mRNA rather than retrotransposons. Pi6 represses gene expression by cleaving mRNA encoding proteins required for spermatogenesis, and it is involved in the piRNA-piRNA precursor interaction network [179]. In summary, the piRNA pathway plays critical roles in spermatogenesis through three primary pathways, including participation in piRNA biogenesis, altering the status of the transposons, and regulating mRNA stability or translation.

Abnormal piRNAs and spermatogenesis failure and male infertility

Mutations or low piRNA levels in blood or semen have recently been linked to male infertility. High-throughput Illumina Hiseq technology has been used to compare the piRNA profiles of testes from unsuccessful sperm retrieval (USR) groups and successful sperm retrieval (SSR) controls. Interestingly, 553 piRNAs have been shown to be specifically expressed in NOA patients with successful sperm retrieval [180], suggesting that these piRNAs may be potential biomarkers for predicting successful sperm retrieval. RNA sequencing of seminal plasma reveals significantly fewer piRNA numbers in infertile patients compared to normal men. In addition, piR-31068, piR-31925, piR-43771, piR-43773, and piR-30198 have been identified as molecular hallmarks of male infertility [181]. In another study, low expression of piR-31704 and piR-39888 has been found in sperm of infertile men, and piRNA levels have been indicated to be correlated with sperm concentration and fertilization rate with intracytoplasmic sperm injection (ICSI) [182]. The levels of piR-1207 and piR-2107 in sperm and seminal plasma of asthenospermia patients are significantly lower than those of normal fertile individuals [183]. RNA sequencing of serum reveals that piR-26399 level exhibits a significant difference between males with reduced fertility and normal controls [184].

The piRNA pathway genes, including *HENMT1*, *PIWIL1*, and *PIWIL2*, are highly expressed in male germ cells during normal spermatogenesis, and their expression levels are decreased in spermatogenesis failure accompanied by germ cell depletion [185]. Significantly, male infertility has been demonstrated to be associated with hypermethylation of the *PIWIL2* and *TDRD1* promoters, and epigenetic inactivation of PIWI pathway genes can lead to piRNA deficiency and LINE-1 hypomethylation [186]. Aberrant expression of piRNAs is associated with the impaired spermatogenesis, loss of sperm motility, and abnormal sperm morphology in azoospermia (Table 4). Mutations in genes responsible for piRNA generation, e.g., *PNLDC1* and *TDRD1*, contribute to spermatogenesis failure. Notably, mutations in *PNLDC1* have been found to cause azoospermia by whole exome sequencing (WES) in three studies [79, 187, 188]. Four *PNLDC1* mutations have been identified in 924 NOA patients, reflecting that mutations of *PNLDC1* affect meiosis of spermatocytes [79]. A novel *PNLDC1* mutation causing oligoasthenozoospermia has been identified by generating male mice with the *PNLDC1 R58G* mutation [188]. A compound heterozygous missense variant of *PNLDC1* has been further found as it causes spermatogenesis arrest [187]. Therefore, PNLDC1 is essential for maintaining normal spermatogenesis by affecting piRNA biogenesis. Mutations in *MOV10L1*, which is required for piRNA processing, have been identified in two of 414 patients with NOA or severe oligospermia [117]. HIWI and TDRD proteins have been shown to be critical for piRNA biogenesis [189, 190], and notably, *HIWI* and *TDRD* polymorphisms are highly related to male infertility [191]. Nine single nucleotide polymorphisms (SNPs) in four human *Piwi* genes have been identified by the SNP stream ® 12-plex platform and TaqMan methods. Interestingly, the *HIWI2 rs508485* has been found to be positively associated with the risk of azoospermia [192], which provides the first epidemiological evidence supporting the involvement of genetic polymorphisms of *Piwi* in spermatogenesis failure. Variants in piRNA pathway genes have been identified as risk factors for male infertility [191]. In addition, the SNP *rs77559927* in piRNA pathway *TDRD1* gene has been shown to be associated with

Table 4 Correlation of piRNAs and piRNA pathways with male infertility and spermatogenesis disorder

References	Methods of detection	Tissues or cells	Patients and numbers	PiRNA/piRNA pathway	Phenotypes
[79]	Whole exome sequencing (WES)	Blood	924 NOA patients	*PNLDC1*: rs200629089 rs141903829 rs754159168 c.607–2A→T	Error-prone meiosis arrest
[188]	WES	Blood	456 patients with male infertility	PNLDC1 p.R47G	Oligoasthenoteratozoospermia
[187]	WES	Blood	280 NOA patients	PNLDC1: p.E381K p.R476W	Affect the function of PNLDC1 protein
[117]	WES	Blood	414 NOA patients	MOV10L1: p.Ser816Ile p.Pro1032Argfs*53 p.Gly848Arg	Meiosis is arrested and piRNAs levels are relatively low
[192]	SNPstream® 12-plex platform and the Taqman method	Blood	490 patients with idiopathic azoospermia or oligozoospermia and 468 fertile controls	*HIWI2* rs508485	Increased risk of oligospermia
[191]	Multi-temperature single strand conformation polymorphism technique	Blood	226 NOA patients and 200 fertile controls	*HIWI2* rs508485 *HIWI3* rs11703684	Increased risk of azoospermia
[193]	Improved multiplex ligation detection reaction technique	Blood	342 cases of NOA and 493 fertile controls	*TDRD1* rs77559927	Spermatogenic impairment
[195]	Single-strand conformation polymorphism	Blood	30 infertile men with complete maturation arrest at spermatocytes and 70 fertile controls	*MOV10L1* gene polymorphisms	Increased risk of spermatogenesis defects
[180]	Small RNA-Seq	Testicular tissues	10 NOA patients (both SSR and USR groups)	553 testicular piRNAs	Biomarker for micro-TESE application
[181]	High-throughput sequencing	Seminal plasma	211 infertile patients (asthenozoospermia and azoospermia) and 91 fertile controls	piR-31068, piR-31925, piR-43771, piR-43773, piR-30198	Male infertility
[182]	Real-time PCR	Spermatozoa	186 patients with idiopathic male infertility	piR-31704, piR-39888	Correlate with sperm concentration and fertilization rate after ICSI
[184]	Small RNA-Seq	Serum	12 subfertile men and 4 fertile controls	piR-26399	Subfertility

NOA: non-obstructive azoospermia; SSR: successful sperm retrieval; USR: unsuccessful sperm retrieval; Micro-TESE: micro-dissection testicular sperm extraction

a risk of spermatogenesis disorder [193]. Notably, the expression levels of the *TDRD* gene family are significantly lower in NOA testicular tissues compared to the OA patients with normal spermatogenesis. Abnormal expression of *TDRD* family genes associated with the piRNA pathway may lead to male infertility [194]. *MOV10L1* polymorphisms have been found to be associated with male infertility in 30 infertile men diagnosed with spermatocyte arrest [195]. It is of particular significance to explore the mechanisms by which piRNA-related genes' polymorphisms control abnormal spermatogenesis. This would help us better understand the pathogenesis of male infertility and develop new treatments for this disease.

In conclusion, aberrant expression of piRNAs and piRNA pathway genes are associated with abnormalities in spermatogenesis and male infertility. Therefore, with the continuous development of single-cell RNA sequencing or RNA deep sequencing, more clues can be uncovered to better understand molecular mechanisms underlying the regulation of piRNAs in male germ cell development and reproductive system diseases, and new approaches would be developed for the diagnosis and treatment of male infertility.

Applications of piRNAs in reproductive medicine

The applications of piRNAs in the male reproductive system

PiRNAs are essential for regulating male germ cell development, normal spermatogenesis and male fertility. In the field of male reproduction, piRNAs might have significant implications as shown in Fig. 3. First of all, piRNAs may be

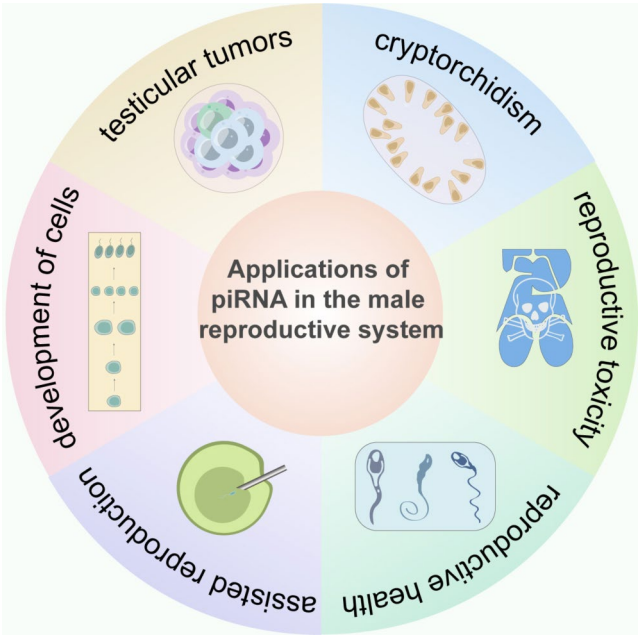


Fig. 3 Prospective of piRNAs in the male reproductive system. The applications of piRNAs in the male reproductive system include the following six aspects: as a standard for assessing male reproductive health, selecting high-quality sperm in ART, the diagnosis and treatment of male infertility, the diagnosis and therapy of male reproductive system tumors, biomarkers of reproductive toxicity, and exploring novel mechanisms underlying male germ cell development

used as biomarkers to assess male reproductive health based upon the quality and quantity of piRNAs in serum or seminal plasma, and piRNAs can be employed for the diagnosis and treatment of reproduction-related diseases. PiRNAs can also be utilized as biomarkers to predict residual spermatogenic conditions in NOA patients. In testicular tissues from NOA patients, a total of 959 piRNAs were significantly differentially expressed between successful and failed sperm extraction groups by RNA-seq, while 553 piRNAs were completely absent in the failed sperm extraction group [180]. Therefore, these piRNAs can be utilized as markers for the development of assisted reproductive technology (ART). Cryptorchidism has been found to be deficient in normal piRNA formation, which affects the development of normal testicular tissues [196, 197]. The expression pattern of piRNAs during spermatogenesis and maturation is closely related to the quality and quantity of sperm, and thus piRNAs can be used as biomarkers of sperm for the diagnosis and treatment of male infertility.

PiRNAs also have potential applications in the treatment of reproductive tumors. The majority of piRNAs in testicular germ cell tumors (TGCT) have been found to be lost by RNA sequencing [198]. It has been found that loss of piRNA defense in carcinoma in situ and TGCT cells results in a reduced ability to prevent chromatin instability [198], since neither in germ cell neoplasia in situ (GCNIS) cells nor TGCT cells express PIWI/piRNA pathway genes with no piRNA biogenesis [199, 200]. In particular, piRNAs may have applications in treating tumors because they can inhibit the proliferation and metastasis of cancer cells [201].

PiRNAs can be further used as biomarkers for reproductive toxicity. Fluoride has been shown to change the expression of piRNAs and their lysosomal signaling pathway in the testis, thereby causing testicular damage [202]. The toxicity of nickel nanoparticles (Ni NPs) has been demonstrated to affect spermatogenesis, sperm motility, and fertilization ability. Furthermore, piR-32362,259 enhances Ni NPs-induced GC-1 cell damage by regulating the PI3K/AKT signaling pathway [203]. Together, abnormalities of piRNAs are closely related to the occurrence of male infertility, testicular cancer, and other diseases, and piRNAs can be applied as biomarkers for the diagnosis and treatment of reproductive system diseases.

The applications of piRNAs in drug development

Katalin Kariko and Drew Weissman won the 2023 Nobel Prize in physiology or medicine, for their discoveries on nucleoside base modifications that enable the development of effective mRNA vaccines against COVID-19. At present, the development and application of RNA drugs have aroused great interest. In 2018, two drugs received FDA approval, including Onpattro (patisiran) for the treatment of hereditary amyloidosis and Givlaari (givosiran) for the treatment of acute intermittent porphyria [204, 205]. During COVID-19, Moderna and Pfizer/BioNTech have successfully developed an mRNA vaccine against the Corona Virus [206]. Moreover, non-coding RNAs can be used as targets of drug resistance in cancer cells. By constructing the patient-derived xenograft tumor mouse model, treatment with siRNA targeting carcass has been found to restore sorafenib resistance [207]. The length of piRNAs has been found to be correlated with translation, and short piRNAs result in the impaired MIWI/piRNAs translational activation and inhibit mRNA translation [208]. Future studies may be focused on designing drugs to add exogenous piRNAs to restore normal spermatogenesis. In human spermatogenesis, *PNLDC1*, *MOV10L1*, and *HIWI* mutations have recently been shown to cause azoospermia by affecting piRNA processing [79, 117, 190]. In the near future, small molecule drugs targeting proteins related to these piRNA pathways can be designed to repair the abnormal biosynthesis of piRNAs, rescue spermatogenesis and treat male infertility. It is feasible to achieve this goal by designing small molecule drugs that target piRNAs and their pathway proteins by binding to piRNAs or interfering with piRNA interactions with PIWI or MIWI proteins, thereby affecting piRNA functions. These small molecule compounds can

be discovered and optimized by high-throughput screening, bioinformatics analysis, gene expression profiling, and functional experiments. This would offer perspective for the treatment and prevention of male infertility, other reproductive system diseases, and reproductive system tumors.

Conclusions and perspective

In summary, we addressed the functions and mechanisms of piRNAs in regulating spermatogenesis and their abnormality or mutations in male infertility. In general, the piRNA pathway participates in the regulation of spermatogenesis through three main pathways, including the inhibition of transposons, participating in piRNA biogenesis, and binding to mRNAs. We also discussed the correlations between the abnormalities of piRNAs and male infertility. Finally, we pointed out potential applications of piRNAs in reproductive medicine and drug design. Furthermore, piRNAs play essential roles in male reproduction and tumor formation. Future studies on piRNAs might be focused on the following aspects: (i) the roles and regulatory mechanisms of piRNAs in controlling self-renewal and differentiation of SSCs, meiosis of spermatocytes, and spermatogenesis of spermatids; (ii) the functions of piRNAs in mediating the testis environment or niche, particularly somatic cells, including Sertoli cells, myoid cells, and Leydig cells; (iii) the regulatory networks formed by piRNAs, other non-coding RNAs, and genes or proteins to regulate normal spermatogenesis; and (iv) the important applications of piRNAs as biomarkers for the diagnosis of male infertility and cancers as well as novel drug development for the treatment of these diseases.

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Data availability The data is available upon the request from the corresponding author.

Declarations

Competing interests The authors declare no competing financial interest.

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