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# Cloning and expression profiling of testis-expressed microRNAs

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## Abstract

Using a new small RNA cloning method, we identified 141 miRNAs from the mouse testis, of which 29 were novel. The 141 miRNAs were mapped onto all chromosomes but the Y chromosome and 2/3 of these miRNA genes exist as clusters. ~70% of these miRNA genes were located in intronic or intergenic regions, whereas the remaining miRNAs were derived from exonic sequences. We further validated these cloned miRNAs by examining their expression in multiple mouse organs including developing testes and also in purified spermatogenic cells using semi-quantitative PCR analyses. Our expression profiling assays revealed that 60% of the testis-expressed miRNAs were ubiquitously expressed and the remaining are either preferentially (35%) or exclusively (5%) expressed in the testis. We also observed a lack of strand selection during testicular miRNA biogenesis, characterized by paired expression of both the 5' strands and 3' strands derived from the same precursor miRNAs. The present work identified numerous miRNAs preferentially or exclusively expressed in the testis, which would be interesting targets for further functional studies.

### Keywords

microRNAs, small RNAs; non-coding RNAs; germ cells; spermatogenesis, testis

## Introduction

MicroRNAs (miRNAs) have been identified in various organisms including primates, rodents, birds, fish, worms, flies and viruses (Cullen, 2006; Kim and Nam, 2006). The majority of these miRNAs are highly conserved across species, suggesting that they are important regulators of molecular and cellular processes. miRNAs are believed to function as posttranscriptional suppressors through binding to their target mRNAs by base-pairing and subsequently inducing either translational repression or mRNA destabilization (Kim, 2005). Several recent studies show that miRNAs are involved in the regulation of various cellular processes, including cell differentiation (Chen, 2004), cell proliferation (Brennecke et al., 2003), and apoptosis (Xu et

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**NOTE:** We attempted to submit novel miRNA sequences to the miRBase and GenBank, but were told that the official names and accession numbers will be assigned only after this manuscript is accepted for publication in a journal. Therefore, we used temporary names (mirt1−29) in the manuscript.

al., 2003). These findings suggest that miRNAs play an important role in the control of gene expression in higher eukaryotes.

Spermatogenesis is a complex process through which the male gametes, spermatozoa, are produced. A striking feature of spermatogenesis is that spermatogenic cells exhibit strictly regulated spatiotemporal gene expression and profoundly repressed translation in meiotic and haploid male germ cells (Eddy, 1998; Grimes, 2004; Kleene, 2001). This is in part achieved through sequestering mRNAs in translationally inactive ribonucleoprotein particles (Eddy and O'Brien, 1998; Hecht, 1988). Recent data show that chromatoid bodies in meiotic and early haploid male germ cells express components of the miRNA biogenetic pathway (Kotaja et al., 2006), suggesting that miRNAs may be involved in translational repression during spermatogenesis. If miRNAs play a role in the regulation of gene expression, one would expect that numerous miRNAs should be expressed by the spermatogenic cells and some of them should be testis-specific. If miRNAs play essential roles in spermatogenesis, aberrant small RNA expression may be involved in male infertility, and agents that can mimic small RNA function may be used as future male contraceptives. Cloning and preliminary characterization of testis-expressed miRNAs would be the first step toward these ultimate goals. Therefore, we attempted to clone miRNAs from the mouse testis using a new cloning method that we recently established (Ro et al., 2007a), which allows for the sensitive detection and cloning of small RNAs simply using PCR and agarose gel electrophoresis. Here, we report the cloning and expression profiling of 141 testis-expressed miRNAs.

## Materials and methods

### Isolation of mouse spermatogenic cells

Spermatogenic cells were isolated as described previously (Wang et al., 2005). Populations of cells highly enriched for specific spermatogenic cell types were prepared from CD-1 mice (Charles River Laboratories) using the Sta Put method based on sedimentation velocity at unit gravity (Bellve, 1993). Sertoli cells and spermatogonia were isolated from 7 day-old mice. Adult mice (60–70 day-old) were used for isolation of adult pachytene spermatocytes, spermatids, and spermatozoa. Purities of recovered germ cell populations were assessed on the basis of cellular morphology under phase optics and were >95% for pachytene spermatocytes and round spermatids, respectively, and >85% for all other cell types.

### Small RNA isolation

Small RNAs were isolated from 15 different mouse tissues [brain, heart, liver, spleen, lung, kidney, stomach, small intestine, colon, ovary, uterus and developing testes at post natal day 7 (P7), P14, P21 and adult (8−10 weeks of age)] using a mirVana™ miRNA isolation kit (Ambion) according to the manufacturer's instructions. Briefly, 50 to 250 mg of tissue was homogenized in 10 volumes of the Lysis/Binding buffer. A 1/10 volume of miRNA Homogenate Additive was added and incubated on ice for 10 min. Total RNA was extracted by adding an equal volume of Acid-Phenol:Chloroform. Small RNAs were extracted from the total RNA using a filter cartridge with 100 μl of preheated (95 °C) elution solution. The remaining total RNA was also isolated using another filter cartridge with 100 μl of preheated (95 °C) elution solution. The concentration of small RNA and total RNA was measured using a NanoDrop, ND-1000 spectrophotometer (NanoDrop Technologies).

### Cloning of miRNAs

Small RNA isolation and cloning were performed as described (Ro et al., 2007a). Briefly, small RNAs isolated from four developing testes (P7, P14, P21 and adult) and brain were polyadenylated followed by ligation to a 5′ RNA adapter (for oligo sequences see Supplementary Table 1). Reverse transcription was performed to generate small RNA cDNAs,

which were then amplified using PCR. The PCR products were directly subcloned into pcDNA3.1 TOPO vector (Invitrogen) for sequencing analyses.

### Bioinformatic analysis

DNA sequences were analyzed by Vector NTI Suite v.6.0 (InforMax) to locate small RNA sequences in the cloning vector. Each small RNA was then mapped to the mouse genome using the BLAST program in the UCSC Genome Browser (Karolchik et al., 2003). The strand origins, the starting and ending nucleotides in the genome, were determined based upon the BLAST search results. To identify miRNAs, all small RNAs were initially searched in the miRBase (http://microrna.sanger.ac.uk/) (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). If a small RNA completely or partially matched any registered miRNA from different organisms and its size ranged from 20−24 nt, we classified it as a miRNA. To further confirm the identity, we selected a fragment of ~200 bp genomic sequence flanking the small RNA at both the 5’ and 3’ ends and used this for predicting secondary structure of the miRNA precursor (stem-loop formation) using the MFOLD program (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) (Zuker, 2003). If a small RNA formed a stem-loop, its size ranged from 20−24, and it had not been registered in the miRBase, we classified it as a new miRNA. Genes encoding these miRNAs were then located on chromosomes, and miRNA gene clusters were determined according to the proximity of their genomic locations. The size, the 5’ first start site, and cluster distributions were calculated using Microsoft Excel. The data were plotted and graphed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

### Semi-quantitative RT-PCR analyses of miRNAs

Preparation of the small RNA complimentary DNA (srcDNA) library and semi-quantitative PCR analyses of miRNAs were performed as described (Ro et al., 2006). All oligos used are shown in Supplementary Table 1. Semi-quantitative PCR analyses using srcDNAs were performed such that the PCR cycle numbers were empirically determined to ensure that each of the amplification reactions was in the exponential range. Based upon expression levels of each of the miRNAs analyzed in 15 tissues and 2 purified spermatogenic cell populations, five values (0−4) were assigned: 4 for high, 3 for medium, 2 for low, 1 for little or none and 0 for none.

### Generation of the heat map representing expression profiles of 122 miRNAs

Values assigned to represent expression levels of 122 miRNAs in 15 mouse organs and 2 purified spermatogenic cell populations were copied from the Microsoft Word file of Supplementary Table 6 and pasted into a spread sheet in Microsoft Excel. An Excel macro file (named heatmap.bas) was downloaded from a website (http://www.senorjosh.com/archives/2003/04/heatmap_tool_vba.shtml) and installed by importing into the Visual Basic Editor using the “File->Import File” command. The heat map was generated by running the macro on the data in the spread sheet. A more detailed step-by-step procedure is available in the website described above or available upon request.

## Results

### Cloning of miRNAs from the mouse testis

We recently developed a method for small RNA cloning, which eliminates the use of radioactive labeling and cumbersome denaturing polyacrylamide gel electrophoresis purification procedures (Ro et al., 2007a). In this study, we isolated small RNA fractions directly from the developing testes, and the small RNAs were polyadenylated followed by addition of a 5’-adaptor. The resulting products were then reverse-transcribed using a primer

containing oligo dT at the 3' end and an adaptor sequence at the 5' end. After PCR amplification of the cDNA products using both adaptor primers, the PCR products were visualized on agarose gel by electrophoresis (Fig. 1A). At this step, the major bands were excised out for purification and subsequent cloning and sequencing procedures. In P21 and adult testes, two major PCR products (~20−30 bp + 100 bp adaptor sequence) were detected (Fig. 1A). The lower bands corresponded to the expected sizes of miRNAs (~20 bp + 100bp adaptor sequence), while the other one showed much higher abundance and appeared to be larger (30 bp + 100 bp adaptor sequence) than the size of miRNAs (Fig. 1A). These were later identified as Piwi-interacting RNAs (piRNAs) (Ro et al., 2007a). A total of ~1,200 clones were sequenced and 141 miRNAs were identified (Supplementary Table 2). Among these miRNAs, 112 had been identified previously (Watanabe et al., 2006; Yu et al., 2005) and the remaining 29 were novel (Table 1). Among the 29 novel miRNAs, 20 miRNAs showed sequence homology with some known piRNAs and 9 miRNAs shared sequence homology with miRNAs found in other organisms (Table 1). Additionally, 7 miRNAs displayed sequence homology with not only mRNAs but also piRNAs or miRNAs. This type of sequence homology was also observed in the 141 miRNAs identified in this study (Supplementary Table 2), suggesting that potential interactions among miRNAs, piRNAs and mRNAs may exist. The size of cloned miRNAs ranged from 20 to 24 nt (Fig. 1B), with 22 nt being the most common size (48%). Analyses of the nucleotides at both the 5' and 3' ends of these miRNAs revealed that uridine (U) was the most common first nucleotide at the 5' end (60%), whereas the 3' end showed no base preference (Supplementary Table 3).

## Chromosomal distribution and miRNA gene clusters

Genes encoding these miRNAs were found on all chromosomes but the Y chromosome (Fig. 2A and Supplementary Table 4). Interestingly, 27 out of the 141 miRNAs (19%) were located on the X chromosome (Fig. 2A). A total of 19 miRNA gene clusters (miRcs) containing 94 miRNA genes (67%) were identified, and these clusters were randomly distributed across the mouse genome (Fig. 2B). The average size of a miRNA gene cluster is ~6.0 kb, containing an average of ~ 5 miRNA genes (Supplementary Table 5). The longest cluster, miRc-19 (62,361 bp) on chromosome X, contains 20 miRNAs. miRNA clusters are found on both the plus (58%) and minus (42%) strands. The majority of miRNA genes (~70%) were located in intergenic or intronic regions, whereas the remaining 30% were derived from exonic sequences (Supplementary Tables 2 and 5).

## Expression profiling for 122 testis-expressed miRNAs

Large-scale expression profiling efforts have been hindered due to the cumbersome nature and low sensitivity of conventional Northern blot analysis. We recently developed a PCR-based method for small RNA detection and quantification (Ro et al., 2006). Using this method, we validated all of the miRNAs cloned from the mouse testes by examining their expression in 15 mouse tissues and two purified spermatogenic cell types (pachytene spermatocytes and round spermatids). In the semi-quantitative PCR analyses, we empirically determined the cycle number for each of the semi-quantitative PCR analyses so that all PCR reactions were maintained within the exponential range. Based upon their expression profiles, we grouped these testis-expressed miRNAs into three categories: ubiquitous expression (evenly abundant expression in all the tissues and cells analyzed), testis-specific expression (expressed exclusively in developing, adult testes and/or 2 isolated spermatogenic cells), and preferential expression (preferentially expressed in more than one, but not all the tissues). Three representative expression patterns are shown in Fig. 3A. mir-16 was expressed in all of the tissues tested with evenly abundant levels (Fig. 3A, upper panel), suggesting that mir-16 is a "housekeeping" miRNA and thus can be used as a loading control. The new miRNAs cloned in this study, mir-t6 and mir-t25, displayed preferential (Fig. 3A, middle panel) and testis-specific (Fig. 3A, lower panel) expression patterns, respectively. We further assigned five

values (0−4) to represent differential expression levels of miRNAs in the different tissues and cells examined (4 for high, 3 for medium, 2 for low, 1 for little to none and 0 for none) (Supplementary Table 6).The heat map representing the expression profiles of all 122 miRNAs analyzed in this study showed that 73 (60%) miRNAs were ubiquitously expressed, 43 (35%) were preferentially expressed in the testis and several other tissues, and 6 (5%) were exclusively detected in the testis (Fig. 3B).

### miRNAs highly preferentially or exclusively expressed in the testis

Our expression profiling assays identified 6 miRNAs that were exclusively expressed in the mouse testis and 49 miRNAs that were preferentially expressed in a limited number of organs including the testis. Further analyses of the expression profiles of these 49 miRNAs revealed 22 miRNAs that were highly preferentially expressed in the testis (the highest levels in the testis or germ cells and much lower levels in <7 organs), which we refer to as testis-preferential miRNAs hereafter. The sequence information and expression profiles of these 28 testis-specific and testis-preferential miRNAs are summarized in Table 2. The majority of these testis-specific or testis-preferential miRNAs displayed similar developmental expression patterns with lower levels before P7 and higher levels at or after P14 (Table 2 and Fig. 3B). The increased levels of miRNA expression during testicular development may result from changes in miRNA levels in germ cells, but potential contributions of miRNAs from somatic cell types cannot be excluded (it is known that multiple types of somatic cells including Sertoli cells, Leydig cells, and peritubular myoid cells co-exist with germ cells and all cells are involved in development). To further assess their cellular origins, we analyzed expression levels of 28 testis-specific or testis-preferential miRNAs in 6 purified testicular cell populations including Sertoli cells, spermatogonia, pachytene spermatocytes, round spermatids, elongated spermatids and vas spermatozoa (Fig. 4). Three types of expression patterns were detected: ubiquitous expression in all 6 testicular cells examined, e.g. mir-t28 (Fig. 4A, upper panel); preferential expression in meiotic (pachytene spermatocytes) and haploid (round and elongated spermatids, as well as spermatozoa) germ cells, e.g. mir-t13 (Fig. 4A, middle panel); exclusively expressed meiotic and haploid germ cells, e.g. mir-t3 (Fig. 4A, lower panel). The heat map representing the expression of the 28 testis-specific or testis-preferential miRNAs in 6 testicular cell types revealed that 16 of the 28 miRNAs displayed evenly abundant expression across all testicular cell types examined, and 9 miRNAs were confined to germ cells. All of the 28 miRNAs showed the highest expression levels in pachytene spermatocytes, or round and elongated spermatocytes (Fig. 4B), which is consistent with the expression data from the developing testes (Fig. 3B and Table 2), suggesting that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis.

The genomic locations of the 6 testis-specific and 22 testis-preferential miRNAs were further analyzed (Supplementary Table 7 and Fig. 2B). 21 of the 28 miRNA genes were located in intergenic regions (75%), 3 were mapped to introns, and 4 were from exons or both exons and introns. Interestingly, 11 of the 28 miRNA genes were on the X chromosome, suggesting that the X chromosome encodes more miRNAs preferentially or exclusively expressed in spermatogenic cells. In addition, three testis-specific miRNAs (mir-t19, mir-t25 and mir-t27) were located on the miRNA clusters miRc-16 and miRc-19 (Fig 2B). The miRc-16 located on chromosome 17 encoded two miRNAs (mir-t19 and mir-t20), while all of the 11 X-linked testis-specific or testis-preferential miRNA genes were located within the miRc-19 (Fig. 2B). More interestingly, each of these 11 X-linked miRNA genes within the miRc-19 was flanked by repetitive sequences at both the 5’ and the 3’ sides and all of them are clustered within a region of ~62 kb, where no mRNA-coding genes are found. The significance of this unique genomic structure of X-linked miRNAs deserves further investigation. Since most, if not all, of the miRNAs within the same clusters show testis-specific or testis-preferential expression, they may be transcribed from the same pre-miRNA and play roles related to spermatogenesis.

### Paired expression of miRNAs in the testis

Precursor miRNAs (pre-miRNAs) form a stem-loop structure in which the 5' strand base-pairs with the 3' strand in a partially complementary fashion. The current "thermodynamic stability" theory of miRNA biogenesis asserts that, although both the 5'- and the 3' strands in the stem-loop structure of a pre-miRNA are diced out by DICER, only the strand with less thermodynamic stability (called miRNA or guide strand) will be incorporated into the RISC and thus be accumulated (Schwarz et al., 2003) and functional. The other strand (also called miRNA* or passenger strand) is subject to degradation through an unknown mechanism. Only in rare cases where both strands display identical or similar thermodynamic stability, can they be co-accumulated. However, we recently found that the phenomenon of strand selection occurs in a tissue-dependent manner (Ro et al., 2007b). In certain tissues including the testis, most, if not all, of the miRNAs are actually expressed and accumulated as sister pairs (miRNAs and miRNA*), whereas in some other tissues either the miRNA or miRNA* strands are detectable (Ro et al., 2007b). During sequence analyses of the 141 miRNAs identified in this study, we found that 60 were the sister miRNAs of the known "unpaired" miRNAs (Supplementary Table 2). For example, mir-t27 and the known mir-741 are derived from the 5'-strand and the 3'-strand of the same pre-miRNA, and thus are a sister miRNA pair (Fig. 4A). Similarly, mir-t23/mir-742 (Fig. 4B) and mir-t25/mir-t24 (Fig. 4C) are two other sister miRNA pairs. The stem-loop structures and expression profiles of the three paired miRNAs (mir-t27 and mir-741; mir-t23 and mir-742; mir-t25 and mir-t24) are shown as representatives in Fig. 4. Interestingly, the expression profile of each of the paired miRNAs is unique although they are derived from the same pre-miRNAs, suggesting that the steady-state levels of each strand of a miRNA pair are independently regulated. We predicted the sister miRNAs for all of the "unpaired" miRNAs identified in this study, and these predicted sister miRNAs were further validated by examining their expression in multiple mouse tissues. As expected, they were all detected in our expression profiling analyses (see miRNAs marked with asterisks in the Supplementary Table 6 for their detailed expression profiles).

## Discussion

Spermatogenesis is a complex process consisting of three phases: mitotic phase (self-renewal of male germ line stem cells, multiplication and differentiation of spermatogonia), meiotic phase (chromosomal replication, recombination and two consecutive meiotic cell divisions of spermatocytes) and haploid phase (also called spermiogenesis, differentiation of spermatids into spermatozoa). Meiosis and spermiogenesis are unique cellular processes to germ cells in the male. Unique processes often require unique genes/gene products to execute specific functions, which may explain why the majority of the testis-specific mRNAs are expressed during the meiotic and haploid phases (Schultz et al., 2003; Shima et al., 2004). Given that numerous testis-specific or testis-preferential mRNAs are expressed during the meiotic and haploid phases of spermatogenesis, there must be numerous miRNAs exclusively or preferentially expressed in the testis during the same period of development if these miRNAs function through targeting mRNAs. The identification of 6 testis-specific miRNAs and numerous miRNAs that are highly preferentially expressed in the testis (Table 2) in the present study strongly suggests that testicular miRNAs are involved in the regulation of gene expression.

In addition to the expression of unique genes/gene products, the meiotic and haploid phases of spermatogenesis are also characterized by high transcriptional activity but suppressed translational activity (Braun, 1998; Eddy and O'Brien, 1998; Kimmins and Sassone-Corsi, 2005; Kleene, 1993; Sassone-Corsi, 2002). Highly active transcription in the meiotic phase may result from the requirement for numerous gene products during this lengthy (~10 and 24 days in mice and humans, respectively), complex (involving chromosomal replication,

recombination, two consecutive cell divisions, etc), and unique (only occurs in oocytes and spermatocytes) cellular process (Eddy and O'Brien, 1998). In mice, the active transcription persists and may even be enhanced during the early haploid phase (up to step 9) because mRNAs for making proteins required for late spermiogenesis (steps 9−16) must be transcribed before chromatin condensation and nuclear elongation, which start at step 9. Since miRNAs are initially derived from primary miRNAs (pri-miRNAs), which are transcribed by RNA polymerase II and modified (5' capping and 3' tailing ) in the nucleus using similar machinery as used in mRNA production (Bartel, 2004; Kim, 2005), pri-miRNAs must be produced before transcription ceases at step 9.

miRNAs appear to regulate gene expression mostly at the post-transcriptional level (Ambros and Chen, 2007; Bartel, 2004). Interestingly, the time window during which the number and levels of testicular miRNA expression reach a peak coincides with active transcription and suppressed translation in late meiotic and early haploid germ cells. This finding suggests that these testicular miRNAs may play a role in the translational suppression of mRNAs. mRNAs that are subject to translational suppression are those that function in late spermiogenesis (after step 9), where transcriptional machinery is no longer available. Therefore, it is likely that miRNAs are transcribed together with their target mRNAs and the transcripts, to be translated at later stages, are then selected for suppression by their targeting miRNAs. Meanwhile, interactions between miRNAs and their target mRNAs may also affect the stability of mRNAs and thus control expression levels of those genes that are transcribed and immediately translated. Recent reports have shown that chromatoid bodies (CB) appear to be the site of interaction between miRNAs and their target mRNAs because in addition to mRNAs and miRNAs, many of the components of the miRNA biogenetic pathway including AGO2/AGO3 and DICER are present in the CBs (Kotaja et al., 2006). It has been suggested that CBs serve as RNA processing centers, where mRNAs and miRNAs synthesized in the nucleus and exported through the nuclear pores are collected and sorted such that some mRNAs are subject to miRNA-mediated translational suppression and others are connected to the translational machinery for protein production (Kotaja and Sassone-Corsi, 2007). It is equally possible that CBs may only collect and/or store mRNAs subject to translational suppression and their targeting miRNAs, and thus serve as repression centers. Nevertheless, the fact that miRNA expression peaks at late meiotic and haploid phases suggests that these miRNAs are likely to be involved in the regulation of gene expression through translational suppression and/or destabilization of mRNAs.

More than 50% of all the known miRNA genes exist as clusters on different chromosomes (except the Y chromosome) (Bartel, 2004). However, the physiological significance of multiple miRNAs encoded by genes located within the same cluster remains unknown. One possibility is that they are derived from the same pri-miRNAs, which are transcribed under the control of the same promoters. The co-production of these miRNAs may also suggest that they are functionally related. For example, they may target the same mRNAs or they may target genes which belong to the same molecular pathways. This notion is supported by the finding that 11 miRNAs located within the same miRNA gene cluster (miRc-19) are either testis-specific or are highly preferentially expressed in the testis, suggesting that these miRNAs may have a role limited to spermatogenesis.

There are less than 80 mRNA-encoding genes on the Y chromosome and the majority of these genes are involved in sex determination and sperm production (Hughes et al., 2005; Tilford et al., 2001). Interestingly, among the 429 mouse miRNAs in the miRBase and the 29 novel miRNAs that we cloned, none are encoded by genes on the Y chromosome. Lack of Y-linked miRNAs may simply be because we have not yet identified them due to fewer miRNA genes present on the Y chromosome, similar to their mRNA-encoding counterparts. Alternatively, the Y chromosome may not encode miRNAs at all. In contrast, the X-chromosome encodes

~15−20% of all the known miRNAs, all of which are expressed in the testis (S. Ro, J. Michaels and W. Yan, unpublished data). Previous studies have shown that the X chromosome is enriched of spermatogonial genes (Wang et al., 2001) and that X-linked protein-encoding genes become inactivated during the meiotic phase (Monesi, 1965; Turner et al., 2005; Wang et al., 2005). It would be interesting to see if these X-linked miRNAs are also subject to meiotic sex-chromosome inactivation.

Our finding that all testicular miRNAs are expressed and accumulated as sister pairs suggests that the “thermodynamic stability” theory (Schwarz et al., 2003) on selective accumulation of miRNA and miRNA* strands is not universally followed during natural miRNA biogenesis. We recently found that both strands of an miRNA pair could target equal numbers of genes and that both are able to suppress the expression of their target genes (Ro et al., 2007b). More interestingly, co-expression and co-accumulation of sister miRNAs may be functionally significant for a number of reasons. First, production of two miRNAs from the same precursor increases the efficiency of miRNA biosynthesis. Since paired expression appears to be limited to certain organs including the testis, the expression of miRNAs* which are normally degraded in some tissues may suggest that these organs may require expression of more miRNAs to regulate their specialized functions. Second, paired expression means the total number of miRNAs expressed should double in these tissues. A pair of sister miRNAs may target the same genes or different genes that belong to the same molecular pathway. If this is true, the paired expression supports a “killing two birds with one stone” model in miRNA biosynthesis and target selection.

In summary, we cloned 141 miRNAs from the mouse testes and experimentally validated their expression in multiple mouse tissues and spermatogenic cells. Expression profiling assays identified 28 miRNAs highly preferentially or exclusively expressed in the testis. Co-expression/co-accumulation of miRNAs and their corresponding miRNAs* is one of the characteristics of the testis-expressed miRNAs. Further analyses of the targets of these testis-expressed miRNAs and their physiologic functions will help us gain more insight into the regulation of spermatogenesis.

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## References

Ambros V, et al. A uniform system for microRNA annotation. Rna 2003;9:277–9. [PubMed: 12592000]

Ambros V, Chen X. The regulation of genes and genomes by small RNAs. Development 2007;134:1635–41. [PubMed: 17409118]

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97. [PubMed: 14744438]

Bellve AR. Purification, culture, and fractionation of spermatogenic cells. Methods Enzymol 1993;225:84–113. [PubMed: 8231890]

Braun RE. Post-transcriptional control of gene expression during spermatogenesis. Semin Cell Dev Biol 1998;9:483–9. [PubMed: 9813196]

Brennecke J, et al. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 2003;113:25–36. [PubMed: 12679032]

Chen X. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 2004;303:2022–5. [PubMed: 12893888]

Cullen BR. Viruses and microRNAs. Nat Genet 2006;38(Suppl):S25–30. [PubMed: 16736021]

Eddy EM. Regulation of gene expression during spermatogenesis. Semin Cell Dev Biol 1998;9:451–7. [PubMed: 9813192]

Eddy EM, O'Brien DA. Gene expression during mammalian meiosis. Curr Top Dev Biol 1998;37:141–200. [PubMed: 9352186]

Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res 2004;32:D109–11. [PubMed: 14681370]

Griffiths-Jones S, et al. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006;34:D140–4. [PubMed: 16381832]

Grimes SR. Testis-specific transcriptional control. Gene 2004;343:11–22. [PubMed: 15563828]

Hecht NB. Post-meiotic gene expression during spermatogenesis. Prog Clin Biol Res 1988;267:291–313. [PubMed: 3070565]

Hughes JF, et al. Conservation of Y-linked genes during human evolution revealed by comparative sequencing in chimpanzee. Nature 2005;437:100–3. [PubMed: 16136134]

Karolchik D, et al. The UCSC Genome Browser Database. Nucleic Acids Res 2003;31:51–4. [PubMed: 12519945]

Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 2005;6:376–85. [PubMed: 15852042]

Kim VN, Nam JW. Genomics of microRNA. Trends Genet 2006;22:165–73. [PubMed: 16446010]

Kimmins S, Sassone-Corsi P. Chromatin remodelling and epigenetic features of germ cells. Nature 2005;434:583–9. [PubMed: 15800613]

Kleene KC. Multiple controls over the efficiency of translation of the mRNAs encoding transition proteins, protamines, and the mitochondrial capsule selenoprotein in late spermatids in mice. Dev Biol 1993;159:720–31. [PubMed: 8405691]

Kleene KC. A possible meiotic function of the peculiar patterns of gene expression in mammalian spermatogenic cells. Mech Dev 2001;106:3–23. [PubMed: 11472831]

Kotaja N, et al. The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc Natl Acad Sci U S A 2006;103:2647–52. [PubMed: 16477042]

Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. Nat Rev Mol Cell Biol 2007;8:85–90. [PubMed: 17183363]

Monesi V. Differential rate of ribonucleic acid synthesis in the autosomes and sex chromosomes during male meiosis in the mouse. Chromosoma 1965;17:11–21. [PubMed: 5833946]

Ro S, et al. A PCR-based method for detection and quantification of small RNAs. Biochem Biophys Res Commun 2006;351:756–63. [PubMed: 17084816]

Ro S, et al. Cloning and Expression Profiling of Testis-Expressed piRNA-like RNAs. RNA. 2007aIn Press

Ro S, et al. Tissue-dependent paired expression of miRNAs. Nucleic Acids Research. 2007bIn Press

Sassone-Corsi P. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 2002;296:2176–8. [PubMed: 12077401]

Schultz N, et al. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci U S A 2003;100:12201–6. [PubMed: 14526100]

Schwarz DS, et al. Asymmetry in the assembly of the RNAi enzyme complex. Cell 2003;115:199–208. [PubMed: 14567917]

Shima JE, et al. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 2004;71:319–30. [PubMed: 15028632]

Tilford CA, et al. A physical map of the human Y chromosome. Nature 2001;409:943–5. [PubMed: 11237016]

Turner JM, et al. Silencing of unsynapsed meiotic chromosomes in the mouse. Nat Genet 2005;37:41–7. [PubMed: 15580272]

Wang PJ, et al. An abundance of X-linked genes expressed in spermatogonia. Nat Genet 2001;27:422–6. [PubMed: 11279525]

Wang PJ, et al. Differential expression of sex-linked and autosomal germ-cell-specific genes during spermatogenesis in the mouse. Hum Mol Genet 2005;14:2911–8. [PubMed: 16118233]

Watanabe T, et al. Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. Genes Dev 2006;20:1732–43. [PubMed: 16766679]

Xu P, et al. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 2003;13:790–5. [PubMed: 12725740]

Yu Z, et al. MicroRNA Mirn122a reduces expression of the posttranscriptionally regulated germ cell transition protein 2 (Tnp2) messenger RNA (mRNA) by mRNA cleavage. Biol Reprod 2005;73:427–33. [PubMed: 15901636]

Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406–15. [PubMed: 12824337]

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

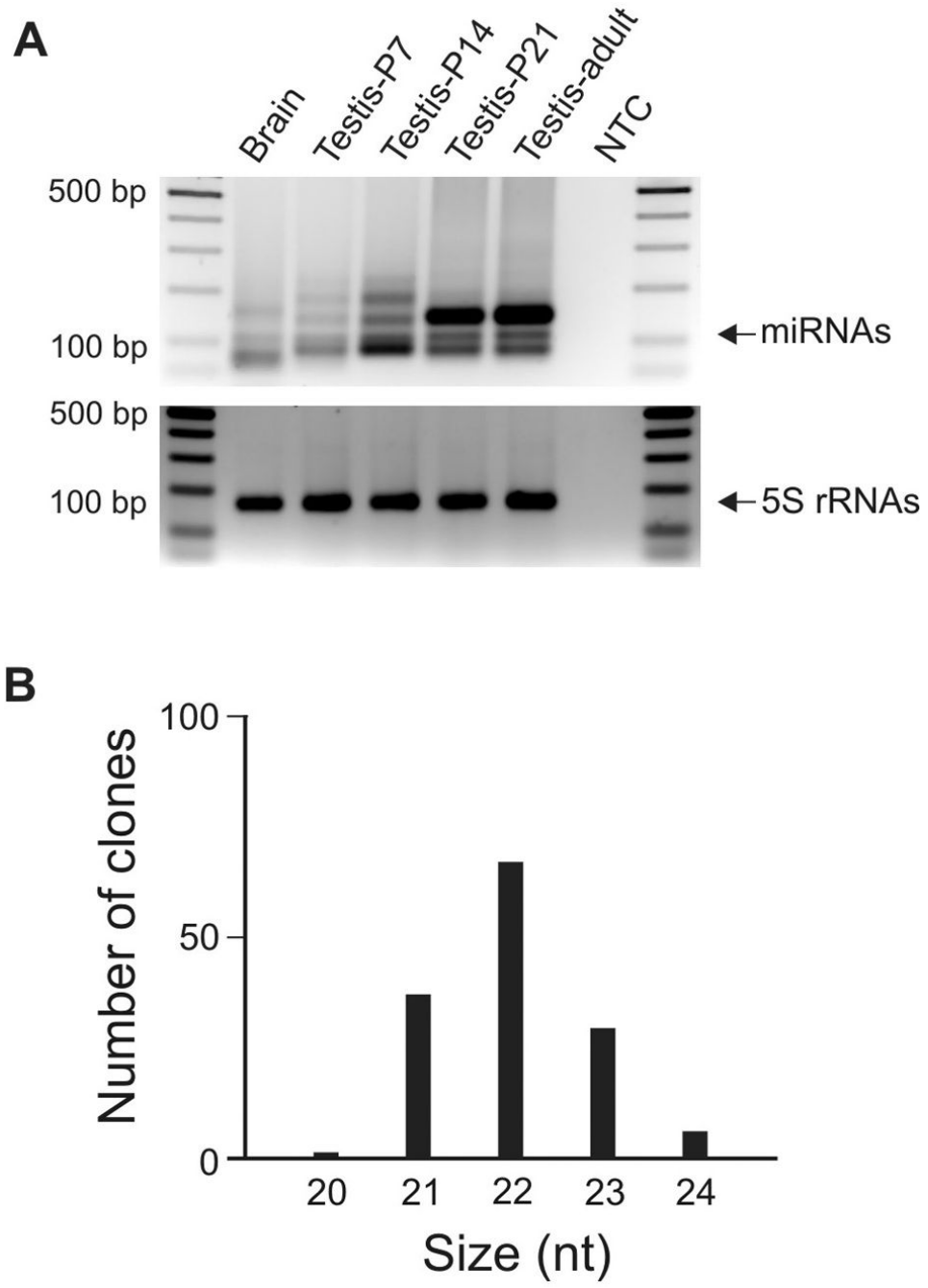


**Fig. 1.**

Cloning of miRNAs from the mouse testis. (A) PCR products amplified using small RNA cDNAs of brain, the developing [postnatal day 7 (P7), P14, and P21] and adult testes. The lower bands with the expected size (~120 nt) of miRNAs (arrow) was gel-extracted and subcloned for sequencing. A DNA ladder on each side indicates the size of the fragments. NTC stands for non-template control. (B) Size distribution of 141 miRNAs cloned from mouse testes aged P21 and adult. The miRNAs range from 20 to 24 nt in length, with 22 nt being the predominant size.

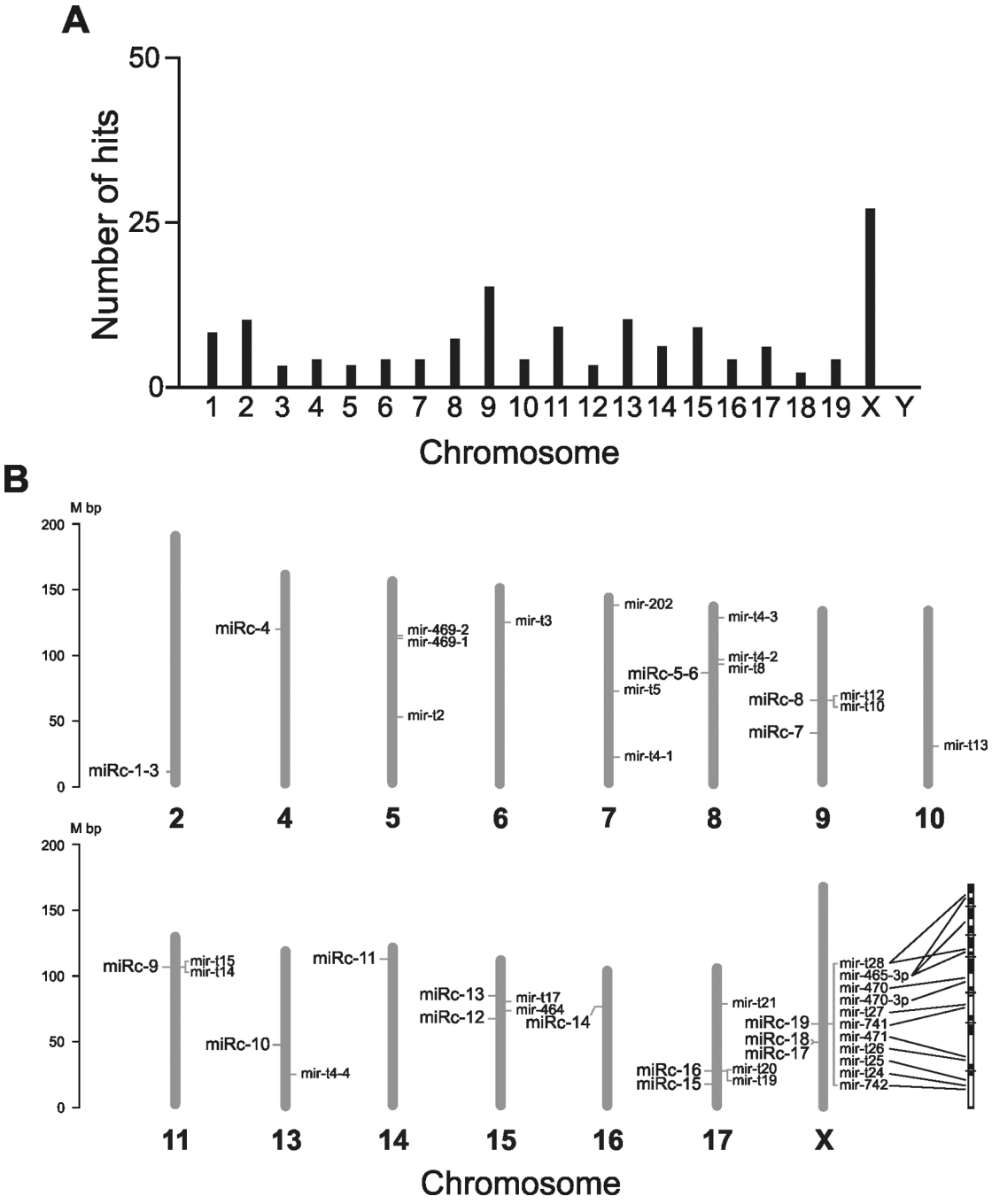


**Fig. 2.**

Chromosomal distribution of miRNA genes and miRNA gene clusters identified in this study. (A) Chromosomal distribution of the 141 miRNA genes in the mouse genome. (B) Chromosomal distribution of 19 miRNA gene clusters and 28 miRNA genes exclusively or preferentially expressed in the testis. The mouse chromosomes were drawn to scale and aligned by their centromere position. Nineteen miRNA clusters (miRcs1−19) are indicated on the left and the 28 testis-specific or testis-preferential miRNAs are marked on the right. The miRc-19 genomic fragment containing the 11 X-linked testis-specific or testis-preferential miRNAs is enlarged and shown to the right of the X chromosome. The solid bars represent repetitive sequences and the open bars stand for the unique miRNA-coding sequences.

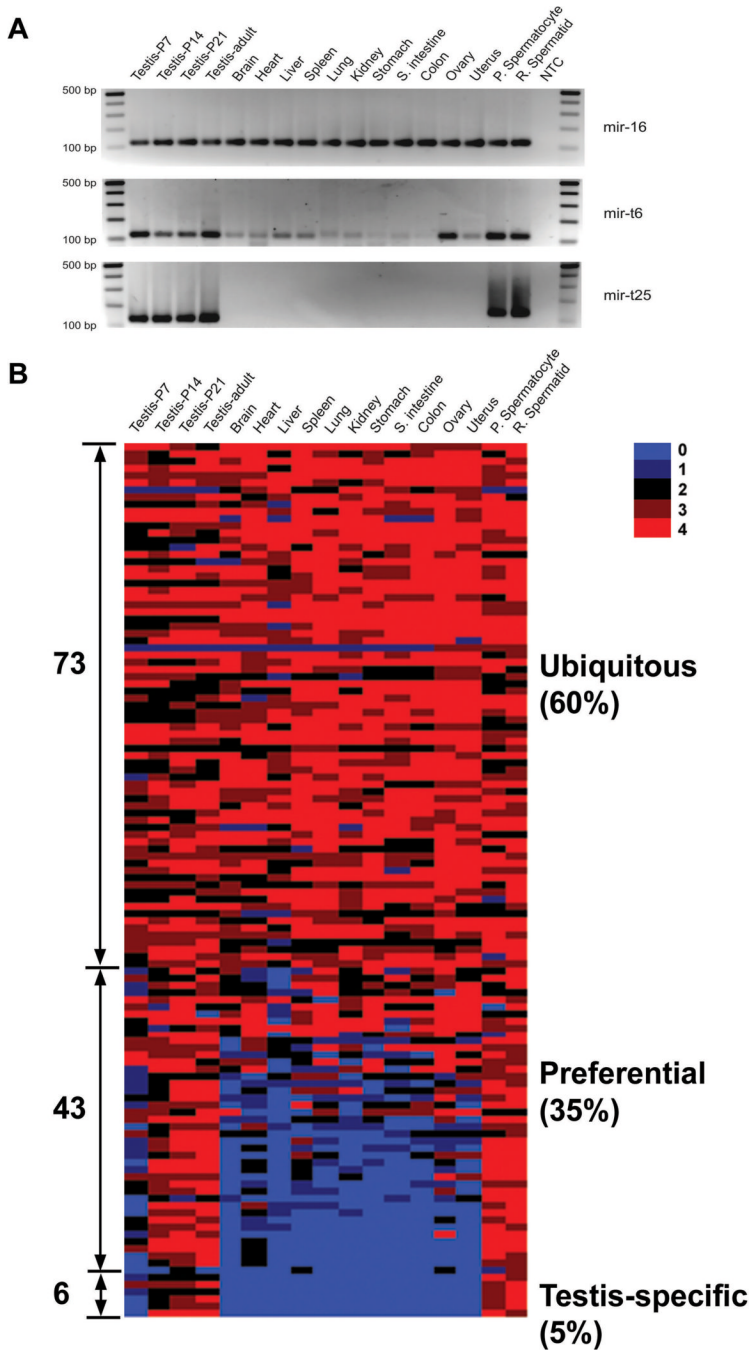


**Fig. 3.**

Expression profiling for 122 miRNAs cloned from the mouse testis. Levels of miRNAs in 15 mouse tissues and two purified spermatogenic cells [pachytene (P.) spermatocytes and round (R.) spermatids] were determined using a semi-quantitative PCR analysis. (A) Expression profiles of mir-16, mir-t6 and mir-t25, representing the ubiquitous, preferential, and testis-specific expression patterns, respectively. A DNA ladder on each side indicates the size of the fragments. NTC stands for non-template control. (B) The heat map representing expression levels of the 122 miRNA in 15 mouse tissues and two purified spermatogenic cells [pachytene (P.) spermatocytes and round (R.) spermatids].

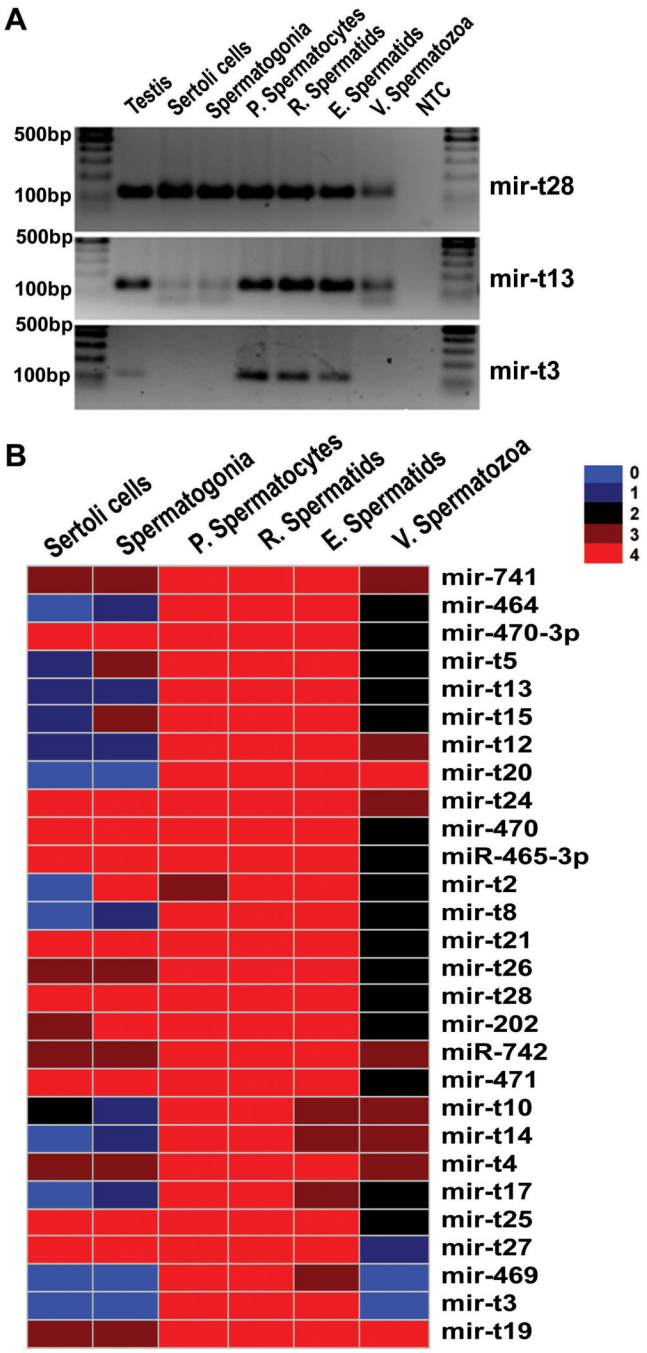


**Fig. 4.**

Expression levels of 28 testis-specific or testis-preferential miRNAs in purified Sertoli cells and 5 spermatogenic cell populations including spermatogonia (Sg), pachytene spermatocytes (P), round spermatids (rS), elongated spermatids (eS) and vas spermatozoa (vS). (A) Three representative gel pictures showing expression levels of mir-t28, mir-t13 and mir-t3. A DNA ladder on each side indicates the size of the fragments. NTC stands for non-template control. (B) The heat map representing expression levels of the 28 testis-specific or testis-preferential miRNAs in purified Sertoli cells and 5 spermatogenic cell populations.

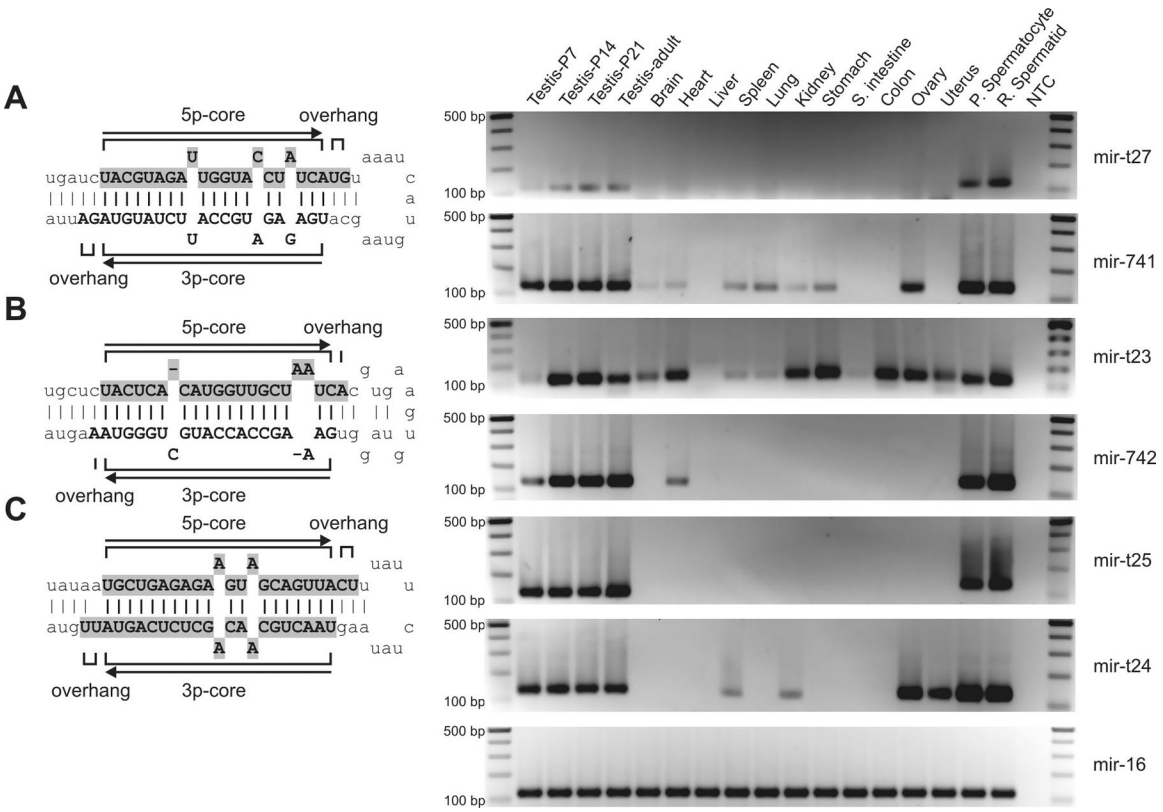


**Fig. 5.**

Paired expression of miRNAs in the testis. The stem-loop structure of pre-miRNAs for three pairs of miRNAs [mir-t27/mir-741 (A), mir-742/mir-t23 (B), and mir-t25/mir-t24 (C)] are shown on the left and PCR amplicons of the 6 miRNAs (3 pairs) amplified from 15 mouse tissues and two purified spermatogenic cell populations (pachytene spermatocytes and round spermatids) are on the right. All of the 6 miRNAs were cloned from the testis and the four novel miRNAs are highlighted in gray in the stem-loop structures. The core RNA sequences used for designing PCR primers are indicated by arrows. The house-keeping miRNA mir-16 was used as a loading control. P7, P14 and P21: postnatal days 7, 14, and 21; P. Spermatocyte: pachytene spermatocyte; R. spermatid: round spermatid; A DNA ladder on each side indicates the size of the fragments. NTC stands for non-template control.

## Table 1

Novel miRNAs cloned from mouse testis.

| Chr.[a] | ID[b] | Sequence (5′-3′) | Length | Homology |
|---|---|---|---|---|
| 3 | mir-t1 | TGATGCTAGCAATTTCCTTTTCT | 23 | piR-106547 mRNA |
| 5 | mir-t2 | GCCTGATTTGAACGTCAACAG | 21 | |
| 6 | mir-t3 | GTATGATTTTGAGCTCATGTAA | 22 | piR-106547 mRNA |
| 7+ | mir-t4−1 | CAGTAGCTCACACCTGTAATCC | 22 | |
| 8+ | mir-t4−2 | CAGTAGCTCACACCTGTAATCC | 22 | |
| 13+ | mir-t4−3 | CAGTAGCTCACACCTGTAATCC | 22 | |
| 7 | mir-t5 | TGGAGAAGAAAGGACACAGCCCGG | 24 | like piR-109074 |
| 7 | mir-t6 | TTCCTATGCATATACTTCTTT | 21 | hsa-202−5p mRNA |
| 8 | mir-t7 | AACATTCATTGTTGTCGGTGGG | 22 | human mir-181d mRNA |
| 8 | mir-t8 | TGAGCTTTGGGATTTGATGCTAG | 23 | like piR-112655 |
| 9 | mir-t9 | GACAGCCTCGGTGTCCCAGCTGC | 23 | piR-123118 |
| 9 | mir-t10 | TGGAACAATTTCTGTAAATTTCCT | 24 | osa-mir-417 like piR-125507 |
| 9 | mir-t11 | TCCTGCTATGATGATGCTCAGCTA | 24 | like piR-106020 |
| 9 | mir-t12 | TTAACTTTTACCACCTTGCTT | 21 | like piR-105942 mRNA |
| 10 | mir-t13 | GTTGTTCTTTGACCGTCACATGT | 23 | like piR-138637 mRNA |
| 11 | mir-t14 | TTTCCTATATTTTTGTTTGTGA | 22 | like piR-118437 |
| 11 | mir-t15 | TGCTACTGTACCATGCTGCTTCC | 23 | |
| 15 | mir-t16 | TTAACTACGGGAAGGTGAGCCTG | 23 | like piR-105411 |
| 15 | mir-t17 | TATGTGATTAGTTCAGAATCTGGT | 24 | mRNA |
| 16 | mir-t18 | AACCCGTAGATCCGATCTTGTG | 22 | rno-mir-99a |
| 17 | mir-t19 | TAGTTTGGTGGGTTTTGGGAA | 21 | |
| 17 | mir-t20 | TAGAGTTCCTGGTCTTTAATTTGC | 24 | like piR-133441 |
| 17 | mir-t21 | TAGGAGCCTTAGAGACTGCTGGT | 23 | mRNA |
| 19 | *mir-t22 | AGCTTCTTTACAGTGTTGCCTT | 23 | mir-107−5p mRNA |
| X | mir-t23 | TACTCACATGGTTGCTAATCA | 21 | rat piR-174026 |
| X | mir-t24 | TAACTGCAACAGCTCTCAGTATT | 23 | piR-126141 |
| X | mir-t25 | TGCTGAGAGAAGTAGCAGTTACT | 23 | piR-139287 |
| X | mir-t26 | TGAAAGGTGCCATACTATGTAT | 22 | like rat piR-177608 |
| X | mir-t27 | TACGTAGATTGGTACCTATCATG | 23 | piR-137720 |
| X | mir-t28 | TATTTAGAATGGCGCTGATCT | 21 | like mir-465 piR-133736 |
| X | mir-t29 | TGAACAGTGCCTTTCTGTGTAG | 22 | mir-201−3p like rat piR-174532 |

[a] Multi-copy miRNA genes are indicated by a "+" next to the chromosome (Chr.) numbers.

[b] The official miRNA IDs will be issued once the paper is accepted for publication per information from the miRNA Registry.

**Table 2**

Twenty eight miRNAs preferentially or exclusively expressed in the mouse testis[1].

| Chr. | miRNA (5′-3′) | ID | Exp. Cat. | No. Ti. | Testis | | | | Br | He | Li | Sp | Lu | Ki | St | Si | Co | Ov | Ut | Spe | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | P7 | P14 | P21 | ad | | | | | | | | | | | | P | R |
| X | TGAGAGATGCCATTCTATGTAG | mir-741 | P | 13 | 3 | 4 | 4 | 4 | 1 | 1 | 0 | 2 | 2 | 1 | 2 | 0 | 0 | 3 | 0 | 4 | 4 |
| 15 | TACCAAGTTTATTCTGTGAGATA | mir-464 | P | 12 | 0 | 2 | 3 | 3 | 0 | 3 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 4 | 4 |
| X | AACCAGTACCTTTCTGAGAAGA | mir-470−3p | P | 12 | 3 | 2 | 4 | 4 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 2 | 3 |
| 7 | TGGAGAAGAAAGGACACAGCCCGG | mir-t5 | P | 11 | 1 | 3 | 4 | 4 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 4 | 4 |
| 10 | GTTGTTCTTTGACCGTCACATGT | mir-t13 | P | 11 | 1 | 3 | 4 | 4 | 0 | 2 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 4 | 4 |
| 11 | TGCTACTGTACCATGCTGCTTCC | mir-t15 | P | 11 | 1 | 2 | 3 | 4 | 0 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 2 | 3 | 3 | 4 | 4 |
| 9 | TTAACTTTTACCACCTTGCTT | mir-t12 | P | 10 | 0 | 4 | 4 | 4 | 0 | 2 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 4 | 4 |
| 17 | TAGAGTTCCTGGTCTTTAATTTGC | mir-t20 | P | 10 | 1 | 4 | 4 | 4 | 0 | 2 | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 4 | 4 |
| X | TAACTGCAACAGCTCTCAGTATT | mir-t24 | P | 10 | 3 | 3 | 3 | 3 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 4 | 3 | 4 | 4 |
| X | TTCTTGGACTGGCACTGGTGA | mir-470 | P | 10 | 2 | 3 | 4 | 4 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 3 | 3 |
| X | GATCAGGGCCTTTCTAAGTAGA | miR-465−3p | P | 10 | 2 | 3 | 3 | 4 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 4 | 4 |
| 5 | GCCTGATTTGAACGTCAACAG | mir-t2 | P | 9 | 0 | 2 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 2 | 4 | 4 |
| 8 | TGAGCTTTGGGATTTGATGCTAG | mir-t8 | P | 9 | 0 | 4 | 4 | 4 | 0 | 3 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| 17 | TAGGAGCCTTAGAGACTGCTGGT | mir-t21 | P | 8 | 0 | 3 | 3 | 4 | 0 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 4 | 4 |
| X | TGAAAGGTGCCATACTATGTAT | mir-t26 | P | 8 | 2 | 4 | 4 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 4 |
| X | TATTTAGAATGGCGCTGATCT | mir-t28 | P | 8 | 2 | 3 | 3 | 4 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3 |
| 7 | AGAGGTATAGCGCATGGGAAGA | mir-202 | P | 7 | 1 | 3 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 | 4 |
| X | GAAAGCCACCATGCTGGGTAA | miR-742 | P | 7 | 2 | 4 | 4 | 4 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 |
| X | TACGTAGTATAGTGCTTTTCACA | mir-471 | P | 7 | 3 | 4 | 4 | 4 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 |
| 9 | TGGAACAATTTCTGTAAATTTCCT | mir-t10 | P | 6 | 0 | 3 | 3 | 4 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3 |
| 11 | TTTCCTATATTTTTGTTTGTGA | mir-t14 | P | 6 | 0 | 2 | 3 | 4 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| 7 | CAGTAGCTCACACCTGTAATCC | mir-t4 | P | 5 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 3 |
| 15 | TATGTGATTAGTTCAGAATCTGGT | mir-t17 | TS | 6 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3 |
| X | TGCTGAGAGAAGTAGCAGTTACT | mir-t25 | TS | 6 | 3 | 3 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| X | TACGTAGATTGGTACCTATCATG | mir-t27 | TS | 6 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| 5 | TGCCTCTTTCATTGATCTTGGTGT | mir-469 | TS | 5 | 0 | 2 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| 6 | GTATGATTTTGAGCTCATGTAA | mir-t3 | TS | 5 | 0 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| 17 | TAGTTTGGTGGGTTTTGGGAA | mir-t19 | TS | 5 | 0 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3 |

Based upon expression levels of each of the miRNAs analyzed in 15 tissues and 2 purified spermatogenic cell populations, five values (0−4) were assigned: 4 for high, 3 for medium, 2 for low, 1 for little or none and 0 for none.

[1] Abbreviations: Chr., Chromosome; Exp. Cat., Expression category; No. Ti., Number of tissues and cells expressing the miRNA; Ad, Adult; Br, brain; He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney; St stomach; Si, small intestine; Co, colon; Ov, ovary; Ut, uterus; Spe, spermatogenic cells; P, pachytene spermatocytes; R, round spermatids.