MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary

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Abstract

MicroRNAs (miRNAs) are endogenous non-coding small RNAs that can regulate the expression of complementary mRNA targets. Identifying tissue-specific miRNAs is the first step toward understanding the biological functions of miRNAs, which include the regulation of tissue differentiation and the maintenance of tissue identity. In this study, we performed small RNA library sequencing in adult mouse testis and ovary to reveal their characteristic organ- and gender-specific profiles and to elucidate the characteristics of the miRNAs expressed in the reproductive system. We obtained 10 852 and 11 744 small RNA clones from mouse testis and ovary respectively (greater than 10 000 clones per organ), which included 6630 (159 genes) and 10 192 (154 genes) known miRNAs. A high level of efficiency of miRNA library sequencing was achieved: 61% (6630 miRNA clones/10 852 small RNA clones) and 87% (10 192/11 744) for adult mouse testis and ovary respectively. We obtained characteristic miRNA signatures in testis and ovary; 55 miRNAs were detected highly, exclusively, or predominantly in adult mouse testis and ovary, and discovered two novel miRNAs. Male-biased expression of miRNAs occurred on the X-chromosome. Our data provide important information on sex differences in miRNA expression that should facilitate studies of the reproductive organ-specific roles of miRNAs.

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Introduction

MicroRNAs (miRNAs) are endogenous non-coding small RNAs ~22 nucleotides (nt) in length that can regulate the expression of complementary mRNA targets (Bartel 2004, Meister & Tuschl 2004). Since the first miRNA, *Lin-4*, was identified (Lee *et al.* 1993, Wightman *et al.* 1993), more than 800 miRNAs have been discovered in animals by using various experimental approaches (e.g., forward genetic methods and sequencing of small RNA libraries), computational predictions, or combined strategies (Berezikov *et al.* 2006). Although the functions of miRNAs in animals are largely unknown, some are believed to regulate tissue differentiation and the maintenance of tissue identity (Ambros 2004, Wienholds & Plasterk 2005, Kloosterman & Plasterk 2006). Recent evidence also suggests that miRNAs exhibit tissue-specific effects during vertebrate development (Wienholds & Plasterk 2005). Ason *et al.* (2006) compared miRNA expression among various vertebrate species by *in situ* hybridization. Their results indicate that the timing

and location of miRNA expression are not strictly conserved; instead, miRNA expression may depend on the particular structure and function that is needed.

The patterns of gene expression in meiotic and haploid germ cells are repressed by post-transcriptional control (Eddy 1998, Kleene 2001, Grimes 2004). This is partly due to sequestration of mRNAs in translationally inactive free-messenger ribonucleoprotein particles (Eddy & O'Brien 1998). However, recent studies indicate that miRNA is also involved in post-transcriptional repression during spermatogenesis. Yu *et al.* (2005) reported that *Mirn122a* down-regulates the expression of transition protein 2 mRNA by mRNA cleavage in the mammalian testis. Moreover, Kotaja *et al.* (2006) have found that the chromatoid body, a perinuclear cytoplasmic cloud-like structure, in male germ cells serves like a somatic glycine-tryptophan body (GW-body), also known as a mammalian processing body (P-body), which is a cytoplasmic focus involved in the post-transcriptional regulation of gene expression. These findings stimulate us to further studies of post-transcriptional small RNA

pathways involved in the reproductive system. Analysis of the expression profiles of miRNAs in reproductive tissues of interest and subsequent identification of tissue-specific miRNAs is the first step toward understanding the biological functions of these molecules. Cloning of miRNAs has contributed greatly to an accelerated advance in miRNA profiling (Lau *et al.* 2001, Lee & Ambros 2001, Lagos-Quintana *et al.* 2002), but the total number of clones identified for mouse testis and ovary was relatively small in most previous studies (Yu *et al.* 2005, Takada *et al.* 2006, Watanabe *et al.* 2006, Ro *et al.* 2007a, 2007b). Here, we sequenced more than 20 000 small RNAs from adult mouse testis and ovary to produce a miRNA expression profile of each reproductive organ and revealed their differences in terms of gender.

Results

Small RNA sequencing

We obtained 10 852 small RNA clones from 768 sequences in adult mouse testis. The testis-derived small RNAs were classified as follows: mouse miRNAs, 6630 (159 genes); piwi-interacting RNAs (piRNAs), 1474; rRNAs, 314; tRNAs, 95; small nuclear RNAs (snRNAs), 1; small nucleolar RNAs (snoRNAs), 5; and mRNA, 199 (Fig. 1). We also obtained 11 744 small RNA clones from 768 sequences in adult mouse ovary. The ovary-derived small RNAs were as follows:

mouse miRNAs, 10 192 (154 genes); piRNAs, 58; rRNAs, 236; tRNAs, 49; snRNAs, 3; snoRNAs, 8; and mRNAs, 59 (Fig. 1).

miRNA profiling of adult mouse testis and ovary

All of the known miRNA cloning profile data, including cloned sequence information (ID, representative clone sequence, location in the 5'- and 3'-strand duplex of each miRNA stem-loop, clone count, cloning frequency), are presented in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental. All sequences of the known miRNAs cloned in this study are shown in Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental. Genes encoding miRNAs cloned from mouse testis and ovary were found on all chromosomes but the Y-chromosome. There was no significant chromosome bias in the distribution of the cloned miRNA genes between testis and ovary, except for miRNA genes on the X-chromosome (Table 1). The size distribution of the known miRNA clones derived from testis (22 nt, 44.1%; 23 nt, 26.6%; and 21 nt, 16.8%) was similar to that of clones derived from ovary (22 nt, 42.3%; 23 nt, 26.9%; and 21 nt, 17.4%; Fig. 1).

The highly cloned miRNAs (i.e., greater than 2% of the entire miRNA clone population in testis or ovary) included *Mirn15b*, *Mirn20a*, *Mirn30b*, *Mirn30c*, *Mirn34a*, *Mirn34b*, *Mirn93*, *Mirn99a*, *Mirn125b*,

Figure 1 Bioinformatic analysis of small RNA clones derived from adult mouse testis (left) and ovary (right). The sequences composed of 16–30 nt were extracted as valid small RNAs and were compared with various RNA databases. Novel miRNA candidates (i.e., those not matching known RNA sequences) were determined by secondary structural analysis followed by small RNA detection using Ago2-immunoprecipitation. Size distribution of the cloned miRNAs (lower panels). The nt length of each cloned miRNAs is shown on the X-axis; the percentage of different nt lengths in the sized miRNAs among the total miRNA population is shown on the Y-axis. piRNAs, piwi-interacting RNAs; rRNAs, ribosomal RNAs; tRNAs, transfer RNAs; snRNAs, small nuclear RNAs; snoRNAs, small nucleolar RNAs; mRNA, messenger RNA.

Table 1 Cloning profiles of microRNAs (miRNAs) from adult mouse testis and ovary by small RNA library sequencing.

Testis					Ovary				
Mature miRNA (<i>Mirn</i>)	%	Chr	Cluster ^a	Remark ^b	Mature miRNA (<i>Mirn</i>)	%	Chr	Cluster ^a	Remark ^b
Highly cloned miRNAs ^c									
<i>125b</i>	13.2	16		<i>125b-5p</i> (#2)	<i>125b</i>	11.7	16		<i>125b-5p</i> (#2)
<i>191</i>	5.8	9			<i>21</i>	11.3	11		
<i>34a</i>	5.0	4			<i>199a*</i>	7.7	9		<i>199a-3p</i> (#2)
<i>15b</i>	3.8	3			<i>99a</i>	4.1	16		
<i>30c</i>	3.7	4			<i>145</i>	4.0	18	C1	
<i>99a</i>	3.5	16			<i>351</i>	3.5	X		
<i>30b</i>	3.0	15			<i>214</i>	3.0	1		
<i>449</i>	3.0	13		<i>449a</i> (#2)	<i>143</i>	2.7	18	C1	
<i>20a</i>	3.0	14			<i>93</i>	2.4	5		
<i>34b</i>	2.4	9		<i>34b-5p</i> (#2)					
<i>199a*</i>	2.2	9		<i>199a-3p</i> (#2)					
<i>742</i>	2.2	X							
<i>202-5p</i>	2.1	7		(#1)					
<i>143</i>	2.1	18							
<i>93</i>	2.1	5							
Exclusive miRNAs ^d									
<i>742</i>	2.23	X	C4		<i>542-5p</i>	0.33	X		
<i>741</i>	1.01	X	C3		<i>Let7b-3p</i>	0.14	15		<i>Let7b*</i> (#1&2)
<i>883a-3p</i>	0.82	X	C4		<i>708-5p</i>	0.13	7		<i>708</i> (#1&2)
<i>124a</i>	0.65	14							
<i>204</i>	0.38	19							
<i>471</i>	0.24	X	C3						
<i>470</i>	0.20	X							
<i>547</i>	0.18	X	C2						
<i>463</i>	0.15	X	C3						
<i>743</i>	0.15	X	C4	<i>743a</i> (#2)					
<i>201</i>	0.14	X	C2						
<i>425-5p</i>	0.12	9		<i>425</i> (#1&2)					
<i>465-5p</i>	0.11	X		<i>465a-5p</i> (#2)					
<i>742-5p</i>	0.11	X	C4	<i>742*</i> (#1&2)					
Mature miRNA (<i>Mirn</i>)	T/O	Chr	Cluster ^a	Remark ^b	Mature miRNA (<i>Mirn</i>)	O/T	Chr	Cluster ^a	Remark ^b
Predominant miRNAs ^e									
<i>449</i>	103.0	13		<i>449a</i> (#2)	<i>126-5p</i>	29.9	2		
<i>34c-3p</i>	33.8	9	C5	<i>34c*</i> (#2)	<i>320</i>	22.7	14		
<i>34c</i>	29.2	9	C5		<i>Let7d*</i>	16.2	13		
<i>34b-3p</i>	27.9	9	C5	(#1)	<i>181a</i>	12.9	2		
<i>34b</i>	24.4	9	C5	<i>34b-5p</i> (#2)	<i>214</i>	8.8	1		
<i>203</i>	14.6	12			<i>99b-3p</i>	7.1	17		<i>99b*</i> (#1&2)
<i>18</i>	9.4	14			<i>351</i>	6.6	X	C6	
<i>350</i>	8.9	1			<i>450</i>	6.5	X	C6	<i>450a-5p</i> (#2)
<i>138</i>	8.7	9			<i>181b</i>	6.0	1		
<i>34a</i>	7.8	4			<i>145</i>	5.9	18		
<i>19b</i>	7.6	14			<i>503</i>	5.9	X	C6	
<i>151-5p</i>	7.6	15		(#1)	<i>322</i>	5.8	X	C6	
<i>19a</i>	5.3	14			<i>21</i>	5.7	11		
					<i>126-3p</i>	5.0	2		

%, Percentage of mRNA genes in the total miRNA clone population in testis or ovary; Chr, chromosome; T/O, testis/ovary ratio for miRNA in cloning frequency; O/T, ovary/testis ratio for miRNA in cloning frequency.

^aCluster (C1-6) indicates miRNA gene clusters. ^bRemark shows the current miRNA IDs in the miRBase version 11.0 that are different from those in the miRBase version 9.1 employed in this study. In Remark, the hash 1 symbol (#1) indicates miRNAs that were the unregistered opposite-strand miRNAs of the known 'unpaired' miRNAs in the miRBase version 9.1 and are now registered in version 11.0; the hash 2 symbol (#2) indicates miRNA IDs that has been renamed in the miRBase version 11.0. ^cGreater than 2% of the total miRNA clone population in testis or ovary. ^dGreater than 0.1% in one gonad and none in the other gonad. ^eMore than fivefold difference between testis and ovary in cloning frequency, and greater than 0.1% in either gonad.

Mirn143, *Mirn191*, *Mirn199a*, *Mirn202-5p*, *Mirn449*, and *Mirn742* in testis and *Mirn21*, *Mirn93*, *Mirn99a*, *Mirn125b*, *Mirn143*, *Mirn145*, *Mirn199a**, *Mirn214*, and *Mirn351* in ovary. These results are summarized in Table 1. Five miRNAs (*Mirn93*, *Mirn99a*, *Mirn125b*, *Mirn143*, and *Mirn199a**; 23 and 29% of the entire

miRNA clone population in testis and ovary respectively) were common between adult testis and ovary. We next directed our attention to the miRNAs that were cloned exclusively either from testis or from ovary (Table 1 and Supplementary Table 3, which can be viewed online at www.reproduction-online.org/supplemental).

The following miRNAs were cloned only from testis: *Mirn124a*, *Mirn201*, *Mirn204*, *Mirn425-5p*, *Mirn463*, *Mirn465-5p*, *Mirn470*, *Mirn471*, *Mirn547*, *Mirn741*, *Mirn742*, *Mirn742-5p*, *Mirn743*, and *Mirn883a-3p* (greater than 0.1% of the total miRNA clone population in testis), and 35 others. Similarly, those cloned only from ovary were: *Let7b-3p*, *Mirn542-5p* and *Mirn708-5p* (greater than 0.1% of the total miRNA clone population in ovary), and 45 others. Although some of the miRNAs, for example, *Mirn742* and *Mirn741*, were highly cloned, others represented relatively small proportions in cloning frequency (Supplementary Table 3). Based upon the cloning profiles, male-biased expression occurred on the X-chromosome. Approximately, 79% of miRNA genes detected exclusively from testis were derived from the X-chromosome (at cutoff of 0.1% cloning frequency), whereas 33% of miRNAs cloned exclusively from ovary were from the X-chromosome (Table 1). Similarly, with no cutoff, X-linked miRNA genes in the miRNAs cloned exclusively from testis and ovary were 31 and 13% respectively. All exclusive-miRNAs (without cutoff) examined in this study are shown in Supplementary Table 3. Furthermore, the miRNAs that were differentially detected between testis and ovary by at least fivefold and greater than 0.1% of the entire miRNA clone population in either gonad were: *Mirn18*, *Mirn19a*, *Mirn19b*, *Mirn34a*, *Mirn34b-3p*, *Mirn34b*, *Mirn34c*, *Mirn34c-3p*, *Mirn138*, *Mirn151-5p*, *Mirn203*, *Mirn350*, and *Mirn449*, totaling 13 genes in favor of the testis, and *Let7d**, *Mirn21*, *Mirn99b-3p*, *Mirn126-3p*, *Mirn126-5p*, *Mirn145*, *Mirn181a*, *Mirn181b*, *Mirn214*, *Mirn320*, *Mirn322*, *Mirn351*, *Mirn450*, and *Mirn503*, totaling 14 genes in favor of the ovary (Table 1). Table 1 summarizes characteristic miRNA signatures in testis and ovary.

We also examined paired miRNAs that are the 5'- and 3'-strand miRNAs derived from the same pre-miRNAs. In this small RNA library sequencing analysis, 55 paired miRNA genes (stem-loop sequences) were detected in mouse gonads (Table 2). Paired miRNA genes with the ratio of 5'/3'-strand clone count ranging from 1:5 to 5:1 (i.e., 0.2–5.0) were 42 and 34% of all paired miRNA genes in testis and ovary respectively. In these paired miRNA genes, most, if not all, 5'- and 3'-strand miRNAs were almost evenly cloned at least either in testis or in ovary. The other paired miRNA genes were cloned preferentially either 5'-strand or 3'-strand, without sex dependence. Interestingly, four paired miRNA genes (*Let7d*, *Mirn22*, *Mirn126*, and *Mirn425*) showed sex-dependent expression (Table 2 and Supplementary Table 1). For example, cloning frequency for *Let7d* (5'-strand) were three times more abundant than that of *Let7d** (3'-strand) in testis. On the other hand, *Let7d** were four times more abundant than *Let7d* in ovary. We also found that 12 miRNAs (*Let7e-3p*, *Mirn16-2-3p*, *Mirn92a-1-5p*, *Mirn103-2-5p*, *Mirn107-5p*, *Mirn195-3p*, *Mirn328-5p*, *Mirn350-5p*, *Mirn351-3p*, *Mirn361-3p*, *Mirn449c-3p*,

and *Mirn670-3p*) cloned in this study were the sister miRNAs of the known 'unpaired' miRNAs (see miRNAs marked with #3 in Table 2 and Supplementary Table 3).

Real-time PCR

Real-time PCR analysis was performed to confirm selected data obtained using cloning. Thirteen miRNAs (*Let7d**, *Mirn15b*, *Mirn21*, *Mirn34a*, *Mirn99a*, *Mirn124a*, *Mirn125b*, *Mirn145*, *Mirn191*, *Mirn199b*, *Mirn204*, *Mirn351*, and *Mirn542-5p*) that were cloned predominantly or exclusively either from testis or from ovary were examined by real-time PCR using commercially available kits. The level of expression was compared among seven organs (lung, heart, liver, kidney, spleen, testis, and ovary). Our analysis revealed that 11 out of the 13 miRNAs (i.e., all except *Mirn15b* and *Mirn124a*) were expressed higher in either ovary or testis compared with the others. *Mirn34a*, *Mirn191*, *Mirn204*, and *Let7d**, *Mirn21*, *Mirn99a*, *Mirn125b*, *Mirn145*, *Mirn199b*, *Mirn351*, *Mirn542-5p* were expressed preferentially in testis and ovary respectively (Fig. 2). No significant differences in the expression levels of *Mirn15b* and *Mirn124a* were detected between testis and ovary (data not shown), and *Mirn124a* is considered to be highly specific for the central nervous system (CNS; Lim *et al.* 2005, Mishima *et al.* 2007).

For each of the 11 miRNAs (*Mirn21*, *Mirn34a*, *Mirn99a*, *Mirn125b*, *Mirn145*, *Mirn191*, *Mirn199b*, *Mirn204*, *Mirn351*, *Mirn542-5p*, and *Let7d**), we compared the ratio of testis/ovary expression identified by cloning with the ratio quantified by real-time PCR to assess whether the number of cloned cDNAs for a given miRNA corresponded to its level of tissue or organ expression. For example, the testis/ovary ratio for *Mirn351* was 0.15 ((35 *Mirn351* clones/6630 total known miRNA clones in testis)/(358/10 192 in ovary)), and the *Mirn351* expression level was 0.11 (0.11:1.00 = testis:ovary) by real-time PCR. Our results are summarized in Fig. 3. The ratios calculated from the cloning data were in substantial agreement with those determined from real-time PCR data, except for data on *Mirn125b*. These results suggest that the number of times a particular miRNA is cloned in small RNA sequencing correlates well with its actual level of expression.

Novel miRNAs

We next evaluated whether any of our cloned cDNAs might represent novel miRNA genes. After comparison with existing gene databases to exclude sequences matching known RNAs (Fig. 1), the remaining small RNAs were evaluated *in silico* for the ability of their putative precursor sequences to form thermodynamically stable stem-loop structures. After secondary structural analysis, a total of 69 (14 genes) and 7 clones

Table 2 Paired-microRNAs (miRNAs) cloned in adult mouse testis and ovary.

Pre-miRNA (<i>Mirn</i>)	5'-str	3'-str	Ratio of 5'/3'-str	Chr	Remark ^a
Testis					
99a	235	1	235.000	16	
30b	202	1	202.000	15	
125b-2	881	5	176.200	16	
93	139	1	139.000	5	
16-1	127	1	127.000	14	
202	144	2	72.000	7	
99b	67	1	67.000	17	
145	45	1	45.000	18	
195	23	2	11.500	11	
322	31	3	10.333	X	
Let7g	10	1	10.000	9	
15a	54	6	9.000	14	
425	8	1	8.000	9	†
34c	114	22	5.182	9	
181a-2	6	2	3.000	2	
Let7d	6	2	3.000	13	†
470	13	5	2.600	X	
30a	11	5	2.200	1	
31	4	2	2.000	4	
362	2	1	2.000	X	
106b	7	4	1.750	5	
34b	159	91	1.747	9	
30e	4	4	1.000	4	
324	1	1	1.000	11	
361	1	1	1.000	X	#3
126	2	4	0.500	2	†
532	2	6	0.333	X	
199a-1	45	151	0.298	9	
181c	1	4	0.250	8	
29c	2	15	0.133	1	
350	2	29	0.069	1	#3
142	2	30	0.067	11	
92a-2	1	18	0.056	X	
742	7	148	0.047	X	
214	1	23	0.043	1	
140	1	71	0.014	8	
Ovary					
125b-2	1196	2	598.000	16	
99a	419	1	419.000	16	
351	358	3	119.333	X	
125a	106	1	106.000	17	
20a	83	1	83.000	14	
15b	110	2	55.000	3	
93	254	5	50.800	5	
181a-2	119	3	39.667	2	
202	147	4	36.750	7	
744	27	1	27.000	11	
542	34	2	17.000	X	
503	82	5	16.400	X	
Let7a-1	39	3	13.000	13	
99b	137	11	12.455	17	
154	8	1	8.000	12	
Let7e	7	1	7.000	17	
322	190	28	6.786	X	
34c	6	1	6.000	9	
Let7b	77	14	5.500	15	
15a	26	5	5.200	14	
22	6	2	3.000	11	†
126	92	31	2.968	2	†
106b	10	4	2.500	5	
34b	10	5	2.000	9	
151	4	3	1.333	15	
132	5	4	1.250	11	
30a	7	6	1.167	1	
434	1	1	1.000	12	
361	2	3	0.667	X	#3
30e	3	5	0.600	4	

Table 2 Continued.

Pre-miRNA (Mirn)	5'-str	3'-str	Ratio of 5'/3'-str	Chr	Remark ^a
324	3	6	0.500	11	
181c	1	3	0.333	8	
Let7d	12	50	0.240	13	†
199a-1	176	793	0.222	9	
107	2	21	0.095	19	#3
532	1	18	0.056	X	
328	1	19	0.053	8	#3
103-2	1	48	0.021	2	#3
214	6	313	0.019	1	
29a	1	65	0.015	6	
140	1	183	0.005	8	

5'-str, Clone count of 5'-strand miRNAs; 3'-str, clone count of 3'-strand miRNAs; Ratio of 5'/3'-str, ratio of 5'/3'-strand miRNA clones; Chr, chromosome.

^aIn Remark, the hash 3 symbol (#3) indicates that the unregistered opposite-strand miRNAs of the known 'unpaired' miRNA genes in the miRBase version 11.0 were detected in the pre-miRNA genes; the dagger symbol (†) shows that pre-miRNA genes showed reciprocal expression of 5'- and 3'-strand miRNAs between testis and ovary.

(5 genes) were identified as novel miRNA candidates in testis and ovary respectively. We further examined the 19 candidates using Argonaute2 (Ago2)-immunoprecipitation to determine whether they are indeed novel

miRNAs. Out of the 19 candidates, 4 were specifically detected in the immunoprecipitates with anti-mouse Ago2 antibody from mouse testis lysate (Fig. 4A). Specific bands for the other 15 genes were undetectable

Figure 2 Real-time PCR analysis of adult mouse organs. (A) The expression of eight miRNAs (*Mirn21*, *Mirn34a*, *Mirn99a*, *Mirn125b*, *Mirn145*, *Mirn191*, *Mirn199b*, and *Mirn351*) that were each highly cloned from testis or ovary. (B) The expression of three miRNAs (*Let7d**, *Mirn204* and *Mirn542-5p*) that were cloned exclusively either from testis or from ovary. Real-time RT-PCR analysis was performed using 5SrRNA or U6 snRNA as an internal control. The expression of each miRNA is given relative to its expression level in testis or ovary with the higher level of expression assigned a value of 1. The Y-axis includes integer numbers. Values are means $\pm$ s.d.

Figure 3 Correlation of the number of miRNA clones identified by cloning with the expression level of the miRNAs determined by real-time PCR. (A) The ratio (testis/ovary) of the percentage of each of the 11 miRNAs (*Let7d**, *Mir21*, *Mir34a*, *Mir99a*, *Mir125b*, *Mir145*, *Mir191*, *Mir199b*, *Mir204*, *Mir351*, and *Mir542-5p*) among the total miRNA population identified by cloning. If the percentage of a miRNA gene in the total miRNA clone population was higher in testis than in ovary, the miRNA was assigned a value of 1. (B) The expression ratio (testis/ovary) for each of the 11 miRNAs quantified by real-time PCR. If the expression level of a particular miRNA was higher in testis than in ovary, the miRNA was assigned a value of 1.

(data not shown). We assigned tentative miRNA names (*MirG1*–*4*) to the four novel miRNA genes in this study. Although *MirG1* was detected exclusively in testis (Fig. 4B), *MirG2*, *MirG3*, and *MirG4* were widely expressed in most, if not all, organs examined (Table 3). Sanger Data Base (miRBase) has been just recently updated to version 11.0 (as of April, 2008). After additional analysis for homology for the four novel RNAs with miRBase version 11.0, we found that two of the four miRNAs are newly registered miRNAs (i.e., *Mir883a-3p* and *Mir883b-5p*, see Table 3). Data on

the novel miRNAs (representative sequence, clone count in testis and ovary, expression level of miRNA in seven organs analyzed by semi-quantitative PCR, locus in the mouse genome, and minimum free energy (ΔG) of the miRNA/miRNA binding-site duplex) are summarized in Table 3.

Discussion

Small RNA sequencing

We have produced detailed miRNA profiles of adult mouse gonads. We achieved a high level of efficiency using this method: 61% (6630 miRNA clones/10 852 small RNA clones) and 87% (10 192/11 744) for adult mouse testis and ovary respectively. Some studies on miRNA expression profiling by cloning methods were previously reported (Cummins *et al.* 2006, Takada *et al.* 2006, Landgraf *et al.* 2007). Cummins *et al.* (2006) discovered a novel miRNA in human colorectal cancer cells through a combination of cloning and serial gene expression analysis, referred to as miRNA serial analysis of gene expression (miRAGE), although their cloning efficiency was only 25% (68 376 miRNA clones/273 986 small RNA clones). Takada *et al.* (2006) developed a new cloning method, termed miRNA amplification profiling (mRAP), which they applied to various mouse embryos and adult organs, with a cloning efficiency of 16% (11 988 miRNA clones/77 436 small RNA clones).

Figure 4 Analysis of novel miRNAs cloned from mouse testis and ovary using Ago2-immunoprecipitation followed by semi-quantitative PCR analysis. (A) *MirG1*–*4* were detected in the immunoprecipitate with anti-mouse Ago2 (anti-mAgo2); on the other hand, they were undetectable in that with non-immune IgG. *Let7c* was used for a positive control. (B) A representative gel picture showing the expression level of *MirG1* in adult mouse organs. A DNA ladder in each gel indicates the size of the fragments (bp).

Table 3 Novel microRNAs (miRNAs) cloned from adult mouse testis and ovary.

Mim ^a	Representative sequence (5'–3')	Clones		Expression profiles by PCR ^b										Chr	Start	End	Str	ΔG	Remark
		T	O	H	Lu	Li	K	S	T	O									
G1	UAACUGCAACAGCUCACAGUUAU	55	0	0	0	0	0	0	4	0				X	63041432	63041454	-	-44.80	883a-3p ^c
G2	UACUGAGAAUUGGUAGCAGUCAC	1	0	2	2	1	1	3	2	0				X	63050600	63050622	-	-35.90	883b-5p ^c
G3	CAUCUAACCCUGGCCUUGACA	0	1	0	2	3	2	2	2	2				1	186523196	186523217	-	-41.70	
G4	GUAAAGGCUGGGCUUAGACGUGG	1	0	2	3	3	3	3	3	2				1	186523254	186523276	-	-41.70	

T, testis; O, ovary; H, heart; Lu, lung; Li, liver; K, kidney; S, spleen; Chr, chromosome; Str, strand.
^aWe assigned tentative miRNA names (MimG1–4) to the four genes in this study. ^bThe expression levels of the miRNAs were classified as follows: 4 for high, 3 for medium, 2 for low, 1 for little to none and 0 for none. ^cMimG1 and MimG2 are newly registered as *Mim883a-3p* and *Mim883b-5p* respectively, in the miRBase version 11.0.

Landgraf *et al.* (2007) cloned over 250 small RNA libraries from 26 different organ systems and cell types of human and rodents including mouse testis and ovary. Their cloning efficiencies were 23% (3075 miRNA clones/13 398 small RNA clones) and 90% (1217/1343) for mouse testis and ovary respectively. Although it could in part have resulted from differences in RNA complexity in these tissues, miRNA-cloning efficiencies in this study are higher than those in previous studies mentioned above, except for the cloning efficiency of mouse ovary by Landgraf *et al.* (2007). We concatenated more than 20 cDNAs into a single fragment prior to TA cloning. However, in our study, the average number of counts detected in a single sequencing reaction was 14.2 in testis and 13.2 in ovary, since it depends on the sequence read length of the instrument used. In addition, it is likely that our approach does not affect the proportions of the miRNAs in a given sample, as the expression of many of the miRNAs we identified by cloning was in substantial agreement with that determined by real-time PCR analysis (see Fig. 3). Moreover, Landgraf *et al.* (2007) indicated that the relative cloning frequencies of miRNAs represent a measure of miRNA expression. Several hundred miRNA clones may not be enough to accurately profile miRNA expression in tissues or organs. By real-time PCR analysis, we practically detected the expression of cloned miRNAs that are less than 0.1% of the total miRNA clone population in one organ (e.g., two clones of *Let7d** in testis; Fig. 2).

miRNA profiling of adult mouse testis and ovary

The most highly cloned miRNA in both testis (13.2%) and ovary (11.7%) was *Mim125b*. The expression of *Mim125* in developing mouse embryos and in adult mouse organs has been extensively studied (Lagos-Quintana *et al.* 2002, Miska *et al.* 2004, Kloosterman *et al.* 2006, Takada *et al.* 2006). These reports indicate that *Mim125* is expressed mainly in the CNS throughout mouse development. We confirmed the ovary-enriched expression of *Mim125b* in adult mouse by real-time PCR (Fig. 2). Several researchers cloned miRNAs from adult mouse testis and ovary (Lagos-Quintana *et al.* 2002, Yu *et al.* 2005, Takada *et al.* 2006, Watanabe *et al.* 2006, Ro *et al.* 2007a, 2007b). However, the total number of clones identified for each organ was relatively small in those studies. The focus was likely on the discovery of novel miRNAs rather than the organ-specific profiling of miRNA expression. Landgraf *et al.* (2007) showed an excellent atlas of mammalian miRNA expression including that of mouse testis and ovary by cloning. By comparison between their data and ours, *Mim15b*, *Mim16*, *Mim21*, *Mim29b*, *Mim34c*, *Mim143*, *Mim191*, *Mim449*, and *Mim741* in testis and *Mim26a*, *Mim143*, and *Mim322-5p* in ovary are common highly cloned miRNAs that are greater than 1% of the entire miRNA

clone population. However, there are also differences between the expression profiles obtained by their methods and ours (compare Supplementary Table 1 in this study with Table S6 in Landgraf *et al.* 2007). These differences may be explained by considering the total number of miRNA clones included in each analysis. They obtained 3075 and 1217 miRNA clones from mouse testis and ovary respectively. Another possible explanation for differences in miRNA profiles between their data and ours is a systematic bias in cloning methods, as indicated by Landgraf *et al.* (2007). Recently, Ro *et al.* (2007a) reported PCR-based expression profiles of 122 miRNAs cloned from 15 mouse tissues and two purified spermatogenic cell types using PCR. They found that 24 known miRNAs were preferentially expressed in testis, and that one known miRNA, *Mirn469*, was exclusively detected in testis. By comparison between our data and those by Ro *et al.*, 6 of the 14 testis-exclusive miRNAs shown in Table 1 in this study (i.e., *Mirn465*, *Mirn468*, *Mirn470*, *Mirn470**, *Mirn471*, and *Mirn741*) are the miRNAs that were expressed preferentially in testis in their PCR-based study. Yan *et al.* (2007) observed differential miRNA expression between neonatal (1-week-old) and adult (7-week-old) mouse testes by miRNA microarray analysis. Five miRNAs (*Mirn29*, *Mirn34a*, *Mirn34b*, *Mirn34c*, and *Mirn449*) were downregulated in the neonatal mice. Their results are in good agreement with our findings that these miRNAs were highly cloned from adult mouse testis (see Supplementary Table 1). It should be noted that the miRNAs that were cloned only from testis (i.e., testis-exclusive miRNAs) were preferentially derived from the X-chromosome (see Table 1 and Supplementary Table 3). These results are consistent with earlier findings (Landgraf *et al.* 2007, Ro *et al.* 2007a). These X-linked, testis-exclusive miRNAs may play specific roles related to spermatogenesis and meiotic sex-chromosome inactivation, as suggested previously (Ro *et al.* 2007a). In addition, despite great endeavors to sequence several thousands of miRNA clones from testis, no miRNA genes were detected on the Y-chromosome. The Y-chromosome may encode few, if any, miRNA genes.

It has been considered that the thermodynamic stability of the 5'-strand and the 3'-strand in the stem-loop structure of a pre-miRNA is important for preferential selection of the less stable one (designated as the miRNA or guide strand) and obliteration of the other one (designated as the miRNA* or passenger strand; Schwarz *et al.* 2003). However, Ro *et al.* (2007a, 2007c) recently reported that the strand selection in pre-miRNAs occurs in a tissue-dependent manner. In certain mouse tissues including the testis, most, if not all, of miRNAs were evenly co-expressed as sister pairs (5'- and 3'-strand miRNAs), whereas in some other tissues either the 5'-strand miRNA or the 3'-strand miRNA were detectable. In the present study, some paired miRNA

genes showed the guide strand-biased cloning profiles without sex dependence, others displayed co-cloning profiles or sex-dependent profiles (see Table 2). Although some, but not many, paired miRNAs may not be equally co-expressed in testis and ovary, it is likely that tissue-dependent strand selection occurs *in vivo*.

Novel miRNAs

In this study, we discovered four different novel miRNA genes using the minimum, operational criteria for miRNA identification (Ambros *et al.* 2003, Berezikov *et al.* 2006) followed by analysis of small RNAs associated with Ago2. Ago2 is one of the main components of RNA-induced silencing complex (Hutvagner & Simard 2008). Although it might be hereafter hard to find novel miRNAs that are expressed in low quantities, Ago2-immunoprecipitation would be valuable for discovery of novel miRNAs as well as for profiling of the tissue-specific miRNA expression.

Concluding remarks

We produced detailed miRNA expression profiles for adult mouse testis and ovary by small RNA library sequencing. Acquisition of cDNA clones as many as possible should be critical for the tissue-specific profiling of miRNA expression based on this type of analysis, as mentioned above. Although target mRNAs for the miRNAs revealed in this study are computationally predicted, identification of functional target mRNAs in reproductive organs remains to be elucidated. The miRNA expression profiles presented in this study would provide important information resources for facilitating studies of the reproductive organ-specific functions of miRNAs.

Materials and Methods

Animals and total RNA extraction

The Nippon Medical School Ethics Review Committee for Animal Experimentation approved our experimental protocols. Twelve BALB/c mice (8-weeks-old) were purchased from Japan SLC (Hamamatsu, Japan). The ovaries from female mice and testes, lungs, hearts, livers, kidneys, and spleens from male mice were excised. Total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions.

miRNA cloning

We cloned small RNA by the miRNA cloning protocol of Lagos-Quintana *et al.* (2002). Briefly, 50 µg of total RNA from mouse testis and ovary were used. Linker ligation, cDNA synthesis, and PCR-amplification of cDNAs were carried out. Then, we concatenated more than 20 cDNAs into a single

fragment using a *BanI* restriction enzyme (New England Biolabs, Ipswich, MA, USA), a DNA ligation kit ver. 2.1 (Takara Bio, Shiga, Japan), and a GeneClean III kit (Qbiogene, Irvine, CA, USA) prior to TA cloning. The concatenated products were then inserted into plasmids and sequenced. Sequencing and the following bioinformatics analysis were supported by Takara Bio DragonGenomics Center (Yokkaichi, Japan).

It was important to avoid contamination from other samples and molecular-weight makers during electrophoresis. Such contaminants considerably diminished the accuracy and efficiency of miRNA cloning. We avoided contamination by performing the cloning procedure separately for each sample, by using a special gel with a small plastic rod that divided the sample and marker lanes, and by using separate vats for each gel for ethidium bromide staining. We made small RNA libraries by excising a portion of a polyacrylamide gel containing species 18–24 nt in length to avoid contaminating our purified RNAs with piRNAs (Kim 2006).

Bioinformatic analysis of the sequence data

We performed a homology search for all cloned small RNAs and a secondary structural analysis for all novel miRNA candidates.

Step 1: extraction of the target sequences

Using Parcel Filtering Package software (Pasadena, CA, USA), the vector sequence, the 5' and 3' linkers, and their coupled sequences (CTGTAGGCACCTGAAA) were removed. Those extracted sequences composed of 16–30 nt were defined as valid small RNAs and were subjected to step 2.

Step 2: comparing the sequences of the clones with those of known RNAs

The small RNA sequences from step 1 were analyzed for homology with known RNAs and mouse genomic DNA sequences, including miRNA (mouse and non-mouse), piRNAs, rRNAs, tRNAs, snRNAs, snoRNAs, mRNA, and genomic DNA. The databases used were: miRNA (mature and pre), Sanger Data Base version 9.1 (<http://microrna.sanger.ac.uk/sequences/index.shtml>); piRNA, NCBI Entrez Nucleotide database (<http://www.ncbi.nlm.nih.gov/entrez/>); rRNA, the European rRNA database (<http://bioinformatics.psb.ugent.be/webtools/rRNA/>); tRNA, the Genomic tRNA database (<http://lowelab.ucsc.edu/GtRNAdb/>); sn/snoRNA, RNAdb (<http://research.imb.uq.edu.au/rnad/>); and NONCODE (<http://www.noncode.org/>); mRNA, NCBI Reference Sequence Release18 (<ftp://ftp.ncbi.nih.gov/refseq/>); Mouse genome, UCSC Genome Bioinformatics Site (mm8, Build 36, February 2006 Assembly; <http://genome.ucsc.edu>). In our search, we defined the top-hit results with greater than 90% *Mus musculus* homology as valid if they met our criteria for sequence error, erroneous PCR amplification, and 3', 5' variation.

Those clones with 100% homology to mouse genomic DNA but that did not match with known RNAs were subjected to step 3. The cloned small RNAs were compared with the above databases on 31 March 2007. Subsequent additions and changes to these databases are not reflected in our analysis.

Step 3: secondary structural analysis

The two-dimensional pre-miRNA configurations of our novel miRNA candidates were predicted as per Mineno *et al.* (2006). Briefly, 198 nt of genomic sequence were added to the candidate sequences (88 nt at each end). Each candidate sequence was divided into 110-nt windows and subjected to two-dimensional analysis along its entire length, using RNAfold software from the Vienna RNA Secondary Structure Package (Hofacker 2003). Those configurations with the least free energy and that met the following criteria were termed novel miRNA candidates: i) contains a stem-loop configuration, ii) cloned mature miRNA sequence portion consists of more than 16 nt in its double-stranded region, iii) the loop is less than 20 nt long, iv) the internal loop is less than 10 nt long, and v) the bulge is less than 5 nt long. Furthermore, novel sequences with overlapping positions in the genome were grouped together. The remaining candidates were then subjected to PCR analysis described below.

Real-time PCR analysis of known miRNAs

Real-time PCR was performed on an ABI7300 (Applied Biosystems, Foster City, CA, USA) using various mirVana qRT-PCR primer sets (Ambion, Austin, TX, USA) and a SYBR ExScript RT-PCR kit (Takara Bio), or with TaqMan miRNAs assays (Applied Biosystems), a High capacity cDNA archive kit (Applied Biosystems), and Absolute QPCR ROX mix (Abgene, Rochester, NY, USA), according to the manufacturers' instructions. As an endogenous control, 5S rRNA or U6 snRNA was used. The primers used for *Let7d** (catalog number: 30208), *Mir15b* (30061), *Mir21* (30102), *Mir34a* (30168), *Mir99a* (30205), *Mir125b* (30022), *Mir145* (30047), *Mir191* (30079), *Mir199b* (30090), *Mir351* (30266), and 5S rRNA (30302) were purchased from Ambion. The primers used for *Mir124a* (part number: 4373295), *Mir204a* (4373313), *Mir542-5p* (4378110), and U6 snRNA (4373381) were from Applied Biosystems.

Ago2-immunoprecipitation and PCR analysis of novel miRNAs

After bioinformatic analysis of the sequence data, we further validated novel miRNAs by using a combination of Ago2-immunoprecipitation (Azuma-Mukai *et al.* 2008) followed by PCR-based miRNA detection (Ro *et al.* 2006). Briefly, 50 µl Dynabeads protein G slurry (Invitrogen) was immobilized with 20 µg mouse anti-mouse Ago2 monoclonal antibody (clone 2D4, Wako Pure Chemical Industries, Osaka, Japan). One hundred fifty micrograms of adult mouse testis were homogenized in 1.5 ml of a cell lysis solution (provided in miRNAs isolation kit, Wako) using a Polytron PT1200C homogenizer (Kinematica AG, Lucerne, Switzerland) for 10 s at 4 °C, and then 1.5 ml of the cell lysis solution was added into the homogenized solution. Following incubation for 15 min on ice, testis lysate was centrifuged at 20 000 g for 20 min at 4 °C and filtered through a 0.8 µm Supor Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI, USA). One milliliter of the filtered lysate was incubated with 25 µl of the anti-Ago2-Dynabead protein G for

incubation for 60 min at 4 °C. After immunoprecipitation, Ago2-associated RNAs were isolated from the immunoprecipitate according to the manufacturer's protocol (Wako). We confirmed that the immunoprecipitate contained mouse Ago2 protein of ~100 kDa in size by western blot (data not shown). Non-immune mouse IgG (Sigma) was used as a control for Ago2-immunoprecipitation. Preparation of the cDNA library using the Ago2-associated RNAs and semi-quantitative PCR analysis of the above-mentioned novel miRNA candidates were performed, as reported previously (Ro *et al.* 2006). The expected cDNA sizes for mature miRNAs are ~120 bp. PCR information (primer sequence, annealing temperature, and PCR cycle) is shown in Supplementary Table 4, which can be viewed online at www.reproduction-online.org/supplemental. Complimentary DNA libraries were also generated from small RNAs isolated from seven mouse organs including heart, lung, liver, kidney, spleen, testis, and ovary. The expression levels of novel miRNAs in the seven organs were examined by semi-quantitative PCR and scored as 4 for high, 3 for medium, 2 for low, 1 for little to none and 0 for none.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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