MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing

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ABSTRACT: Small non-coding RNAs, such as microRNAs (miRNAs), are involved in diverse biological processes including organ development and tissue differentiation. Global disruption of miRNA biogenesis in Dicer knockout mice disrupts early embryogenesis and primordial germ cell formation. However, the role of miRNAs in early folliculogenesis is poorly understood. In order to identify a full transcriptome set of small RNAs expressed in the newborn (NB) ovary, we extracted small RNA fraction from mouse NB ovary tissues and subjected it to massive parallel sequencing using the Genome Analyzer from Illumina. Massive sequencing produced 4,655,992 reads of 33 bp each representing a total of 154 Mbp of sequence data. The Pash alignment algorithm mapped 50.13% of the reads to the mouse genome. Sequence reads were clustered based on overlapping mapping coordinates and intersected with known miRNAs, small nucleolar RNAs (snoRNAs), piwi-interacting RNA (piRNA) clusters and repetitive genomic regions; 25.2% of the reads mapped to known miRNAs, 25.5% to genomic repeats, 3.5% to piRNAs and 0.18% to snoRNAs. Three hundred and ninety-eight known miRNA species were among the sequenced small RNAs, and 118 isomiR sequences that are not in the miRBase database. Let-7 family was the most abundantly expressed miRNA, and mmu-mir-672, mmu-mir-322, mmu-mir-503 and mmu-mir-465 families are the most abundant X-linked miRNA detected. X-linked mmu-mir-503, mmu-mir-672 and mmu-mir-465 family showed preferential expression in testes and ovaries. We also identified four novel miRNAs that are preferentially expressed in gonads. Gonadal selective miRNAs may play important roles in ovarian development, folliculogenesis and female fertility.

Key words: miRNA / ovary / oocyte / microRNA / ncRNA

Introduction

Gene expression can be modified at many levels, including transcriptional, posttranscriptional and translational. Many genes, however, do not result in a functional protein and instead encode for what are known as non-coding RNAs. Such non-coding RNAs include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), polyadenylated non-coding RNAs, microRNAs (miRNA), piwi-interacting RNAs (piRNAs) and RNA species yet to be discovered. miRNAs have received extraordinary attention due to their likely role in widespread regulation of mRNA metabolism, at both the transcriptional and the post-transcriptional levels (Doench and Sharp, 2004; Carthew and Sontheimer, 2009). There are currently 579 known mouse miRNAs (miRBase v14.0) and 721 human miRNAs (miRBase v14.0; Griffiths-Jones et al., 2006). miRNAs are derived from hairpin containing pre-miRNAs after endonucleolytic processing by Drosha (also known as Rnasen) and Dicer enzymes (Meister and Tuschl, 2004). miRNAs are thought to regulate gene expression by either repressing or blocking translational mechanism by base-pairing with the target mRNA, usually in the 3′-untranslated (UTR) region (Olsen and Ambros, 1999), although translational activation has also been reported (Filipowicz et al., 2008).

miRNA expression profiles differ between tissues and developmental stages. To better understand roles of miRNAs in the ovary, several studies have assessed expression profile of miRNAs in the ovary using.
different technologies. Ro et al. (2007) discovered 122 miRNAs, including 15 novel miRNAs, in the adult mouse ovary by sequencing 800 cDNAs corresponding to the small RNA fraction. Mishima et al. (2008) sequenced 11 744 cDNAs derived from small RNAs in the adult ovary and found that 154 known miRNAs are expressed in the adult ovary and discovered 1 novel miRNA. In a separate study, the miRNA microarray analysis of periovulatory granulosa cells identified a total of 212 mature miRNAs (Fiedler et al., 2008). mir-132 and 212 were highly up-regulated in the granulosa cells following LH/hCG induction. All three of the previous studies focused on the adult ovary, where most of the cell types are somatic in origin, with various stages of folliculogenesis throughout the ovary. The only study to assess miRNA expression in the developing ovary, used miRNA microarrays to assess the effect of germ-cell-specific transcription factor Nobox deficiency on miRNA expression in the newborn (NB) ovary (Choi et al., 2007). A total of 177 known miRNAs were shown to be expressed in the NB ovary, with little apparent effect of Nobox deficiency on their expression (Choi et al., 2007). Microarray analysis is limited to known miRNAs, with little information gained regarding other small RNAs or novel RNAs.

A critical event in the mammalian female gonadal development is the development of the finite pool of primordial follicles that will be used for reproduction and hormonal homeostasis. Mouse female germ cells, following mitotic proliferation in the gonadal ridge, exist as germ cell clusters and enter meiosis I approximately at the 13.5, and by the time of birth, arrest in the diplotene stage of the first meiotic division (Wilhelm et al., 2007). Germ cell clusters formed in the embryonic gonad break down shortly after birth with resulting large loss of oocytes and formation of primordial follicles (Pepling and Spradling, 2001; Choi and Rajkovic, 2006). The primordial follicle endowment, as well as the subsequent rate of primordial ovarian follicle loss, likely determines reproductive life span (Pangas and Rajkovic, 2006). We and others have previously shown that multiple oocyte-specific genes such as Nobox, Figlo, Sohlh1, Sohlh2, Lhx8 and Mater are expressed in the NB ovaries and that these genes are essential at various stages of ovarian development and early embryogenesis (Soyal et al., 2000; Ballow et al., 2006; Choi et al., 2008). NB ovary is an important developmental time point in the mouse, when germ cell clusters breakdown and primordial follicles form. We therefore hypothesized that novel miRNAs preferentially expressed in the NB ovary exist and may have important roles in ovarian development and early folliculogenesis. We utilized massive parallel sequencing on the Genome Analyzer Platform from Illumina (San Diego, CA, USA) to determine small RNA NB ovary transcriptome (Morozova et al., 2009).

**Materials and Methods**

**Small RNA library construction and sequencing**

Mouse C57BL/6/J2SScEv strain was used to harvest tissues. All experimental and surgical procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Ovaries from 54 wild-type female NB (within 24 h of delivery) mice were isolated and stored in RNAlater solution (Ambion, Austin, TX, USA) for RNA extraction. Total RNA was extracted using mirVana™ miRNA isolation kit (Ambion) following the manufacturer’s total RNA isolation procedure. Ten micrograms of total RNA was size fractionated on 15% tris-borate-EDTA (TBE) urea polyacrylamide gel and small RNA fraction in the range 18–30 nucleotides was extracted. 5’ and 3’ RNA adapters were ligated to the gel extracted small RNA fraction, and cDNA was generated according to the manufacturer (Illumina). The resulting cDNA was amplified and sequenced using the Illumina’s Genome Analyzer.

**Small RNA genome mapping**

Small RNA genome mapping and quantification was performed as described previously (Morin et al., 2008) as well as by the Pash alignment algorithm (Kalafus et al., 2004; Coarfa and Milosavljevic, 2008). The Pash algorithm was used to map the reads onto the mouse genome assembly (NCBI Build 37, mm9) and mapping parameters were guided by both the number of reads mapped as a measure of sensitivity and the number of mappings to known RNAs as a measure of specificity. Read mappings were clustered based on overlapping mapping coordinates and intersected with known miRNAs (http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=miRNA), snoRNAs (http://gene.fudan.sh.cn/snoRNAbase.nsf), piRNA Bank (Sai Lakshmi and Agrawal, 2008) and repeats (http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=msk).

**Novel miRNA prediction**

Read clusters not intersecting with known RNAs and consisting of at least 100 reads were identified as putative novel miRNAs. The cutoff of 100 reads was chosen arbitrary to enrich for more abundant small RNAs. The mouse putative novel RNAs were mapped to human and rat using Blast (Altschul et al., 1997), UCSC Alignment Nets and UCSC Alignment Chains (Kent et al., 2003) for isolating conserved putative small RNAs across three species. miR-abela (Sewer et al., 2005) was used to identify putative small RNAs with miRNA hairpin structures.

Novel miRNA prediction was approached as described previously (Morin et al., 2008). In essence, this method uses RNAfold (Hofacker, 2003) to determine whether genomic regions flanking sites yielding small RNAs are able to fold into miRNA-like hairpins. MiPred, which relies on an RF algorithm, then determines whether each hairpin has structural properties in the genomic sequence (Pedersen et al., 2005) was used to identify putative small RNAs with miRNA hairpin structures. Novel miRNA prediction was approached as described previously (Morin et al., 2008). In essence, this method uses RNAfold (Hofacker, 2003) to determine whether genomic regions flanking sites yielding small RNAs are able to fold into miRNA-like hairpins. MiPred, which relies on an RF algorithm, then determines whether each hairpin has structural properties similar to known miRNAs (Jiang et al., 2007). A second approach, termed miRDeep, was specifically designed to use aligned small RNA reads to identify signatures of Drosha and Dicer cleavage in addition to a pre-miRNA-like structure (Friedlander et al., 2008). Predictions from both methods were refined using EvoFold, which identifies evolutionarily conserved hairpins based on the conservation of structural properties in the genome sequence (Pedersen et al., 2006).

**miRNA amplification**

Semi-quantitative PCR was performed as described previously (Ro et al., 2007) with modifications. Small RNAs from 11 different mouse tissues (brain, lung, heart, stomach, liver, muscle, kidney, uterus, adult ovary, testis and NB ovary) were isolated using the mirVana™ miRNA isolation kit (Ambion). The small RNA fraction was poly-adenylated at 37°C for 1 h with poly(A) polymerase (Ambion). The poly(A)-tailed small RNA fraction was treated with DNase I (Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min. RTQ primer (CGAAATTTCCGGCTCGAGGCA GCGCGACATGGCTGCTAGT TAAAGTTGGTACCGAGCTCGGATCC ACTAGTCC(T)25-) was annealed to the cDNA and reverse transcription was carried out with 200 U of SuperScript II reverse-transcriptase (Invitrogen). Following treatment with RNase H (Invitrogen), the cDNA was used to perform RT–PCR with a miRNA-specific primer, and a universal reverse primer, RTQ-UNIV (CGAATTTCCGGCTCGAGGCA), was used for
PCR amplification of each miRNA. miRNA-specific primer sequences and annealing temperatures used to verify NB ovary preferential miRNAs are listed in Supplementary Table S1. HotStart Taq (Qiagen, Valencia, CA, USA) was used to perform PCR. The annealing temperature was adjusted according to the Tm of each miRNA. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Results

Sequencing and annotation of small RNAs from the NB mouse ovary

The Genome Analyzer sequencing of NB ovary isolated small RNA fraction produced 4 655 992 Genome Analyzer reads of 33 bp each representing 153 673 763 bp of sequence data. After uniqueness filtering, the Pash method was used to map Solexa reads from small, non-coding RNAs onto the mouse reference genome. Several Pash runs were performed using different parameters in order to optimize the mapping of Solexa reads derived from small RNAs. Optimization of Pash mapping parameters was guided by both the number of reads mapped as a measure of sensitivity and the number of mappings to known RNAs as a measure of specificity. Sequences were mapped based on their overlaps with publicly available genome annotations including miRNAs, rRNAs, tRNAs, other small RNAs and genomic repeats. Read mappings were clustered based on overlapping mapping coordinates and intersected with known miRNAs, snoRNAs, piRNA clusters and genomic repeats (Table I). The most optimal Pash run mapped 50.13% (2 333 996 reads) of the reads. A total of 398 distinct miRNA sequences were identified in the NB mouse ovary transcriptome, out of 492 known miRNAs (Supplementary Table SII). One hundred and thirty-six out of 211 known snoRNAs (Supplementary Table SIII) and 564 390 out of 1 396 863 known piRNAs (Supplementary Table SIV) were found in the NB ovary small ovary transcriptome. Among reads that were mapped to the genome, 25.24% mapped to miRNAs, 25.5% to genomic repeats, 3.5% to piRNAs, 1.88% to tRNAs and 1% to rRNAs, snRNAs, scRNAs (small cytoplasmic RNAs), srpRNAs (signal recognition particle RNAs) and snoRNAs (Figs. 1A and Table II). Among 398 miRNAs expressed from mouse NB ovaries, 53.02% derived from intergenic regions, 40.95% from intronic regions, 3.27% from larger non-coding RNAs, 1.76% from exons and 0.75% from 3′UTR (Fig. 1B, Table III). These results indicate that mouse NB ovaries, composed of both the somatic and the germ cell components, express a large repertoire of small RNAs.

<table>
<thead>
<tr>
<th>Small RNA type</th>
<th>Known RNAs</th>
<th>RNAs overlapped by mappings</th>
<th>% RNAs overlapped by mappings</th>
<th>Mappings to RNA</th>
<th>( \frac{\text{Average mappings/RNA}}{} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>miRNA</em></td>
<td>492</td>
<td>398</td>
<td>80.89%</td>
<td>589 033</td>
<td>1479.98</td>
</tr>
<tr>
<td><em>snoRNA</em></td>
<td>211</td>
<td>136</td>
<td>64.45%</td>
<td>4156</td>
<td>30.5582353</td>
</tr>
<tr>
<td><em>piRNA clusters</em></td>
<td>1 396 863</td>
<td>564 390</td>
<td>40.40%</td>
<td>81 594</td>
<td>0.144570244</td>
</tr>
</tbody>
</table>

\( ^{*}\text{Known RNAs were obtained from UCSC Genome Browser miRNA track on mm9}, ^{\text{snoRNAbase} (http://gene.fudan.sh.cn/snoRNAbase.nd)}, \text{and} ^{\text{piRNA Bank (Sai Lalsheni and Agrawal, 2008)}}. \text{The average mappings/RNA for piRNA is lower than 1 because most reads that map to piRNA map to multiple locations of the piRNA in the genome.} \)
Novel small RNAs

Read clusters not intersecting with known RNA species and consisting of at least 100 reads were further considered as putative novel miRNAs. We identified a total of 30 ‘novel’ miRNAs that met our previously established criteria for miRNA (Morin et al., 2008). We examined a subset of the predicted miRNAs for multi-tissue expression pattern. Most of the examined putative miRNAs were ubiquitously expressed. However, four novel miRNAs, novel 11, novel 12, novel 18 and novel 20, derived from four distinct regions in the genome (Table V) showed preferential expression in the reproductive tract (Fig. 4A). However, the expression was not exclusive to the ovaries. One miRNA, mmu-mir-1981, was initially classified as a novel miRNA but was recently identified in mouse ES cells (Babiarz et al., 2008). mmu-mir-1981 expression was confined to the gonads (Fig. 3A).

A total of 522 distinct sequences not overlapping known RNA species were identified in addition to the novel putative miRNAs and did not appear to derive from the pre-miRNA transcripts (Supplementary Table SV). Twenty-five out of the 522 such transcripts shared 100% conservation with rat and human sequences, whereas 40 transcripts shared 90% or higher conservation with rat and human. We examined the expression of one of the conserved small RNAs that was not predicted to derive from pre-miRNA, NovelRNA 361, and found that this transcript was preferentially expressed in the gonads (Fig. 4A). Interestingly this transcript was derived from an intron within Astn2 gene, which is preferentially expressed in the brain, turbinates, eye and dorsal root ganglion (Wheeler et al., 2008). These findings suggest that some of these unidentified transcripts may play an important function during gonadal development.

Identification of isomiR sequences in the NB ovary miRNAs

It has been previously shown that miRNA variants are detectable on massive parallel sequencing (Morin et al., 2008). These multiple mature variants have been referred to as isomiRs (Morin et al., 2008). The origin of isomiRs is most likely due to the Dicer or Drosha variant cleavage within the pre-miRNA hairpin loop, although the reason for this variant cleavage is unknown. We identified eight isomiRs with reads more than 100 (Supplementary Table SVI).
mmu-mir-135 and mmu-mir-871 isomiRs were most abundant, with 1249 and 472 reads detected on sequencing. mmu-mir-135 is expressed in multiple tissues; however, the isomiR detected in the NB ovary, mmu-mir-135a-2-3p, was expressed preferentially in the gonads (Fig. 4B). mmu-mir-871 is expressed from the X chromosome and shows preferential expression in the gonads, whereas its isomiR, mmu-mir-871-3p, is also expressed preferentially in the gonads (Fig. 4B). Interestingly, mmu-mir-871 originates from the same cluster as mmu-mir-465, which we previously showed to be expressed preferentially in the gonads (Fig. 3B). Both mmu-mir-135a-2-3p and mmu-mir-871-3p isomiR sequences were absent from the mouse miRBase database, corroborating RT–PCR results that these sequences are preferentially expressed in the gonads.

### Discussion

NB ovary is characterized by heterogenous group of structures including germ cell clusters and primordial follicles. It is during this stage of
ovarian development that many oocytes within the cluster are eliminated to yield a final pool of primordial follicles (Pangas and Rajkovic, 2006). A number of germ cell specific RNA binding proteins have been identified such as MSY2, DAZLA, and NANOS3, and their respective knockouts show the importance of such proteins and RNA metabolism in the female gonadal development (Ruggiu et al., 1997; McNeilly et al., 2000; Tsuda et al., 2003; Yang et al., 2006). Conditional knockouts of Dicer1 and Argonaute2 in growing mouse oocytes (Murchison et al., 2007; Kaneda et al., 2009), two important enzymes in miRNA biogenesis, show normal ovarian development, but post-ovulation oocyte maturation is defective likely due to perturbed completion of meiosis I. The role of miRNAs during NB ovary development is unclear. We have previously shown that a number of germ cell specific transcriptional regulators are highly expressed in the NB ovary (Choi and Rajkovic, 2006). We therefore examined the population of small RNAs in the NB ovary. We used massive parallel sequencing to determine the small RNA transcriptome in the 18–30 nucleotide range in the NB mouse ovary, and identified 398 known miRNAs and 30 novel small RNAs predicted to be miRNAs.

We also identified a significant expression of piRNAs, likely originating from the oocytes within the NB ovaries, and snoRNAs. piRNAs interact with PIWI proteins and knockouts of individual PIWI family members result in male sterility; however, female fertility is not affected by such knockouts (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). The significance of piRNAs in the NB ovaries is unclear, although recent evidence suggest that piRNAs are expressed in the Drosophila and mouse female germlines (Chambeyron et al., 2008; Tam et al., 2008) and important in germline silencing of transposable elements. Whether redundant, piRNA-independent pathways exist in the female germline and suppress transposable elements remains to be seen. It is perhaps of interest that almost 522 transcripts matched un-annotated sites in the genome, one of which showed preferential expression in the gonads. The role of such transcripts in the general nucleic acid metabolism of the germ cells remains to be established.

We showed that the total number of miRNA sequences mapped to the X chromosome more than any other chromosome, followed by chr2, chr1 and chr10. The number of miRNA genes is highest on chromosomes 2, chrX and chr12, and thus perhaps it is not...
unexpected that chrX and chr2 express more miRNA transcripts than other chromosomes. However, correlation between gene density on a particular chromosome, and expression is not uniform. For example, more reads mapped to chr10 than chr12, despite six times as many miRNA genes on chr12 rather than chr10. The significance of the preferential expression of miRNAs from certain chromosomes in the NB ovary is unclear.

We discovered a total of 30 potential novel miRNAs using methodologies described previously (Morin et al., 2008). The number of novel small RNA species mapped in our study is much higher than previously described in the adult ovaries (Ro et al., 2007; Mishima et al., 2008) and is largely due to the use of massive parallel sequencing technology. For example, Mishima et al. (2008) only sequenced 10,000 clones from the adult ovary and mapped them to only 154 known miRNA genes, whereas we obtained more than 4,000,000 reads that mapped to 398 known miRNA genes. There are also significant differences between NB and adult ovaries. RNA contribution in adult ovaries is mostly derived from the somatic cell component, whereas NB ovaries are highly enriched in the germ cell component. The novel miRNAs we discovered were not very abundant based on the sequence count, with total number of reads varying between 12 and 1,100. Most of these miRNAs were expressed ubiquitously, although few did show preferential expression in the gonads. The isomiRs expressed in the NB ovary were not abundant with read counts numbering less than 100 for most of them. It is interesting to note that the top two expressed isomiRs, mmu-mir-135a-2-3p and mmu-mir-871-3p, were both preferentially expressed in gonads, suggesting that gonadal milieu plays a role in processing of these transcripts.
The role of any of these miRNAs in ovarian follicular development, either novel or isomiRs, is unknown. Moreover, it is unclear whether these miRNAs are redundant in their function, as has been suggested for other miRNAs (Miska et al., 2007). Future studies ablating specific miRNAs using transgenic technologies will help us better understand the role of these clusters as well as individual miRNAs in gonadal development and ovarian folliculogenesis.

### Table V

<table>
<thead>
<tr>
<th>Name</th>
<th>Genomic location</th>
<th>Most abundant mature sequence</th>
<th>Count</th>
<th>*Expression profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>novel 42</td>
<td>chrX:141473887-141473957</td>
<td>TAATAGCCAGAAGCTGGAAGAACC</td>
<td>12</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 165</td>
<td>chr:8:98487444 -98487507</td>
<td>TGGGTATAAAGGACTGACACCC</td>
<td>17</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 166</td>
<td>chrX:159312853-159312922</td>
<td>TGGAGAGATGCTGACCC</td>
<td>21</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 215</td>
<td>chr1:60168183-60168229</td>
<td>CGTTTCCCAGGCGGGG</td>
<td>22</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 220</td>
<td>chr:5:34529822-34529868</td>
<td>CGCCGCAGGGGGGCGGG</td>
<td>18</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 221</td>
<td>chr1:52289212-52289295</td>
<td>CGGCGCGGGGGGGCCGGG</td>
<td>18</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 567</td>
<td>chr:11:9729088-97298125</td>
<td>TGAAGTAAAGGATGTTGA</td>
<td>223</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 572</td>
<td>chr12:110986103-110986159</td>
<td>TGCCCCCTCCAGGAGACCTTCT</td>
<td>29</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>novel 635</td>
<td>chr:2:158646319-158646376</td>
<td>CCCTGGGAGGAGACGTGATTC</td>
<td>20</td>
<td>N/A</td>
</tr>
<tr>
<td>novel 766</td>
<td>chrX:136845487-136845531</td>
<td>TCTGGAGGACATGTTTGA</td>
<td>49</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*aUbiquitous, expression in all examined tissue types; Specific, preferential expression in gonads; N/A, PCR not amplified or data not available.*

### Supplementary data


### Acknowledgements

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Identification of small RNAs in the newborn ovaries


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