BIOINFORMATICS

Transcriptome-wide analysis of small RNA expression in early zebrafish development

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ABSTRACT

During early vertebrate development, a large number of noncoding RNAs are maternally inherited or expressed upon activation of zygotic transcription. The exact identity, expression levels, and function for most of these noncoding RNAs remain largely unknown. miRNAs (microRNAs) and piRNAs (piwi-interacting RNAs) are two classes of small noncoding RNAs that play important roles in gene regulation during early embryonic development. Here, we utilized next-generation sequencing technology to determine temporal expression patterns for both miRNAs and piRNAs during four distinct stages of early vertebrate development using zebrafish as a model system. For miRNAs, the expression patterns for 198 known miRNAs within 122 different miRNA families and eight novel miRNAs were determined. Significant sequence variation was observed at the 5' and 3' ends of miRNAs, with most extra nucleotides added at the 3' end in a nontemplate directed manner. For the miR-430 family, the addition of adenosine and uracil residues is developmentally regulated and may play a role in miRNA stability during the maternal zygotic transition. Similar modification at the 3' ends of a large number of miRNAs suggests widespread regulation of stability during early development. Beside miRNAs, we also identified a large and unexpectedly diverse set of piRNAs expressed during early development.

Keywords: microRNA; zebrafish; piRNA; development

INTRODUCTION

The importance of small RNA-mediated gene regulation has been increasingly recognized in recent years, playing multiple roles during development (Pauli et al. 2011). miRNAs and endogenous siRNAs have been shown to regulate gene expression by silencing specific genes, whereas piRNAs have been implicated mainly in genome protection and/or maintenance in germ cells via silencing of transposable elements (Bartel and Chen 2004; Aravin et al. 2007; Brennecke et al. 2007; Houwing et al. 2007; Czech et al. 2008; Flynt and Lai 2008; Tam et al. 2008). miRNAs have been identified in organisms as diverse as viruses, unicellular algae, plants, worms, flies, fish, and mammals (Bartel 2004; Zhao et al. 2007; Skalsky and Cullen 2010). For those miRNAs thus far analyzed, expression patterns are highly regulated, both temporally and spatially (Wienholds et al. 2005; Landgraf et al. 2007; Ruby et al. 2007). miRNAs mainly exert their effects by blocking translation and/or destabilizing mRNAs (Lim et al. 2005; Giraldez et al. 2006; Baek et al. 2008; Guo et al. 2010). piRNA expression is thought to be mostly restricted to germ cells and some somatic cells in flies (Halic and Moazed 2009; Malone et al. 2009; Haase et al. 2010). Mature miRNAs are 22–23 nucleotides (nt) in length. They are derived from longer primary transcripts (pri-miRNAs) that contain multiple stem–loop structures that undergo two sequential cleavages by the enzymes Drosha and Dicer to produce mature small RNA duplexes (Hutvagner et al. 2001; Ketting et al. 2001; Lee et al. 2002, 2003; Cai et al. 2008). Generally, one of the two strands is then incorporated into an RNA Induced Silencing Complex (RISC) with one or more Argonaute proteins (Schwarz et al. 2003). In contrast to miRNAs, piRNAs are slightly larger (25–30 nt) and, at least in flies and fish, are derived from a reciprocal amplification loop in which primary piRNAs associate with Piwi proteins to direct cleavage of transposable element mRNAs (Brennecke et al. 2007; Houwing et al. 2008). The initial cleavage products then serve to promote production of sense-strand secondary piRNAs which, in turn, generate more primary piRNAs by pairing with and directing cleavage of antisense RNAs derived from discrete repetitive genomic loci, referred to as piRNA clusters (Brennecke et al. 2007; Malone et al. 2009).
This model of biogenesis is referred to as the Ping-Pong model, because the production of piRNAs from one strand drives the generation of piRNAs from the other strand (Brennecke et al. 2007).

Zebrafish is widely used as a model system to study early vertebrate development (Kimmel et al. 1995). Previous work has shown that miRNAs play important functional roles during cell specification and differentiation (Giraldez et al. 2005; Flynt et al. 2007; Mishima et al. 2009). In zebrafish, miRNA expression patterns have been extensively examined using direct cloning and microarray analyses (Giraldez et al. 2005; Wienholds et al. 2005; Thatcher et al. 2007). These studies have shown that most miRNAs are not highly expressed during the first 12 h of zebrafish development, but that the overall pattern becomes increasingly diverse and complex as development proceeds (Chen et al. 2005; Wienholds et al. 2005; Thatcher et al. 2007). Despite the seeming lack of diversity during the earliest stages of development, miRNA function is clearly essential, as maternal-zygotic Dicer mutant fish show severe defects and die by 7 dpf (Giraldez et al. 2005). One of the most abundant early expressed families of miRNAs is the miR-430 family, which functions to induce deadenylation, degradation, and clearance of maternal miRNAs, facilitating the maternal-zygotic transition (Giraldez et al. 2006). A similar phenomenon occurs in Xenopus laevis through the action of the miR-427 family (Lund et al. 2009).

In zebrafish, piRNAs are mainly expressed in ovaries and testes (Houwing et al. 2007, 2008). Ziwi-mutant fish, which lack the zebrafish PIWI protein, exhibit extensive apoptosis in adult germ cells and are sterile (Houwing et al. 2007). This suggests an essential role in genome maintenance, but the exact role and mechanism of action for these small RNAs remains mostly unknown.

Recently, the utilization of next-generation sequencing technologies has revolutionized our ability to dissect transcriptomes, even for RNAs expressed at low levels. Here, we used RNA-Seq to examine the expression of miRNAs and piRNAs across four stages of early zebrafish development. We discovered the presence of large numbers of miRNAs, both maternally deposited and zygotically transcribed. Many miRNAs show widespread variation at their 3' ends, mostly by the addition of nontemplate-directed nucleotides that are added in a developmental stage-specific manner. We also show that piRNAs are extensively expressed throughout early embryonic development, suggesting a broad role during the earliest cell divisions.

RESULTS

Small RNA sequencing

We isolated total RNA from zebrafish embryos at four different stages of early development (256-cell, sphere, shield, and 1 dpf) representing 2.5, 4, 6, and 24 h post fertilization (hpf), respectively. We chose these stages because they coincide with key gene expression changes during early vertebrate development. At the 256-cell stage, the majority of RNAs are maternally deposited. The sphere stage represents the time during which the embryo shifts from utilization of many maternally deposited RNAs and initiates zygotic transcription. During the shield stage, gastrulation continues to generate the three germ layers. By 1 dpf, the major organ systems have formed and hearts are beating. To examine gene expression patterns during these specific stages, small RNA libraries (15–30 nt) were prepared for high-throughput sequencing using the Illumina platform (Hafner et al. 2008). We constructed independent libraries for each stage, and generated a total of 29,963,921 sequence reads. In order to validate the reliability of the different high-throughput sequencing runs, experimental duplicates of small RNA libraries were prepared from sphere stage RNA, and independently sequenced. A significant correlation was observed between the results from the two independent libraries (R² = 0.89; Supplemental Fig. 1). In addition, the miRNA profiles revealed by our sequencing data across all four stages were largely consistent with prior miRNA expression analyses (Chen et al. 2005; Wienholds et al. 2005; Thatcher et al. 2007).

Analysis of the size distribution of all reads within each library revealed at least three classes between 18 and 30 nt. The major size class peaked at 22–23 nt (Fig. 1A). Based on the sequences and genomic positions of these reads, this class represents miRNAs. From all four stages, ~55% of the total reads were identified as miRNAs, representing 198 distinct miRNAs (based on sequence alignment with miRBase Release 16) (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008). At the sphere stage, 99% of the miRNA reads were derived from just one family, the miR-430 family. To better examine the diversity of noncoding reads, we grouped identical sequences, which showed that a wide diversity of small RNAs were recovered (Fig. 1B,C). Examination of the reads after such a grouping revealed a second size class peaking at 26–28 nt (Fig. 1A; Fig. 5A,B, below). Based on RNA sequences and genomic mapping, these RNAs are derived primarily from repetitive elements. The smallest size class peaked at 18 nt and consists almost entirely of tRNA-derived small RNAs (see below) and rRNA-related small RNAs (data not shown).

miRNA expression analysis

Analysis of the sequencing reads showed that 198 known miRNAs were detected that could be grouped into 122 families. Most of the miRNA reads were derived from just one arm of the hairpin structures that constitute each precursor miRNA. Short reads corresponding to the loop and the other precursor arm (star sequence arm) were also detected, but at much lower frequencies, consistent with proposed miRNA biogenesis models (Kim et al. 2009).
However, we also found exceptions in which the star reads were much more prevalent than the mature reads, as previously reported (Soares et al. 2009; Jagadeeswaran et al. 2010; Yang et al. 2011) (e.g., miR-735 and miR-135b; see Supplemental Table 1 for full list). An extreme example of diverse read lengths with different 5' and 3' ends was detected for reads derived from miR-2190, one of the most recently annotated zebrafish miRNAs (Soares et al. 2009). We found multiple small RNAs derived from this locus with different ends, suggesting random cleavage at multiple sites across the proposed precursor hairpin (see Supplemental Table 1). The miR-2190 putative hairpin overlaps with two rRNA genes in the zebrafish genome, and that fact, together with the observed sequence heterogeneity, suggests that miR-2190 is more likely to be a product of rRNA degradation rather than an authentic miRNA.

Quantitation of miRNA expression patterns was determined based on the read frequency for each mature miRNA. Previous work demonstrated a significant correlation between read numbers and miRNA levels, with the caveat that bias cannot be completely eliminated due to secondary structures or other variables (Landgraf et al. 2007). Nevertheless, normalization between developmental stages has usually been performed using miRNA read numbers divided by the total number of genome matching reads in each library (Chen et al. 2005; Ameres et al. 2010). We initially analyzed expression patterns in this manner (Supplemental Fig. 3A). We found that this method was not appropriate, as overall transcriptional activity significantly increases as development proceeds, resulting in a large increase in the size of libraries derived from small RNA. Also, there is a large increase in the total number of piRNAs and miRNA reads mostly derived from the miR-430 family, which increased from 25% of the total miRNA reads at the 256-cell stage to 99% at the sphere stage. If the libraries are normalized using individual miRNA read numbers, divided by the total number of genome matching reads in each library, changes in the expression of individual miRNAs can become obscured by increased overall transcription as development proceeds, as well as by the extreme abundance of reads derived from just the miR-430 family. Thus, we normalized the values from each library based on the levels of two miRNAs that are present at moderate levels in all libraries (let-7 and miR-9). For this, we performed RT–qPCR to determine the levels of these miRNAs relative to U6 snRNA (Supplemental Fig. 2). We then used the relative values of let-7 and miR-9 to normalize the read frequencies for each library (Fig. 2; Supplemental Fig. 3B; Friedlander et al. 2009). Reassuringly, the expression patterns were quite similar, whether normalized to miR-9 levels or let-7 levels, even though the RT–qPCR levels for these miRNAs differ by an order of magnitude, and despite the fact that let-7a levels might be biased using deep-sequencing approaches (Linsen et al. 2009). Normalization based on miR-9 or let-7 resulted in very different heat maps compared with that obtained when total genome matching reads of each library were used for normalization (Supplemental Fig. 3).

Quantitation and normalization of sequencing data to analyze gene expression patterns is not trivial and is subject to ongoing research and debate (Meyer et al. 2010). Thus, as a further test of whether normalization based on miR-9 or let-7 is valid, we used the weighted trimmed mean of the log expression ratios (the trimmed mean of M values [TMM]) to normalize the data (Robinson and Oshlack 2010). TMM uses raw data to estimate appropriate scaling factors.
that facilitates analysis of differential expression patterns. When we used TMM normalization, we reassuringly found that the derived heat maps were very similar to those obtained after normalization with either miR-9 or let-7 (Supplemental Fig. 3C). Finally, we used Northern blots on six miRNAs with differing raw read numbers to confirm our normalization method, and the results completely supported our heat maps (Supplemental Fig. 4).

Developmentally, we found that 180 miRNAs belonging to 109 different families are present at the 256-cell stage. Because this is before the onset of zygotic transcription, these miRNAs are maternally deposited and, interestingly, are present at relatively high levels, suggesting an important role during the earliest stages of development (Newport and Kirschner 1982; Schier 2007). A total of 88 out of the 109 miRNA families that are expressed at the 256-cell stage are down-regulated at least twofold as development and zygotic transcription proceeds. The 10 most abundant miRNAs expressed at this stage and their subsequent changes in expression are listed in Table 1. For the sphere state, a total of 182 different miRNAs were detected. Many of the maternally inherited miRNAs that were detected at the 256-cell stage were not observed by the sphere stage. From the sphere stage onward, miRNAs can be roughly divided into two distinct groups based on their expression patterns during early development. The first group is composed of 60 miRNA families that are expressed at low levels across all four stages, while the second group is composed of 48 miRNAs that show decreased expression at the sphere stage, followed by at least a twofold increase in expression as development proceeds (Fig. 2). The miRNAs in the second group appear to be more broadly expressed.

Identification of novel miRNAs

Analysis of all sequencing reads during early zebrafish development resulted in the identification of eight miRNAs that had not been previously reported in the zebrafish genome (Table 2). For this, we analyzed all small RNA reads from the four developmental stages using the miRDeep algorithm (Friedlander et al. 2008). Following this pipeline, predicted miRNAs were filtered for novel miRNA identification by comparison to sequences deposited in the miRNA Registry. To increase accuracy, miRDeep-predicted novel miRNAs that genomically overlap with tRNA, rRNA, or transposable elements, were eliminated, and our analyses were restricted to only those that were identified in at least two different developmental stages and/or two independent libraries. With this restriction, five novel candidate miRNAs were identified, two of which are conserved across vertebrates, whereas the other three appear to be zebrafish specific (Supplemental Fig. 5). The remaining three were discovered independent of miRDeep and match miRNA sequences reported for other species. For these three, we aligned small RNAs to known miRNA hairpin sequences of other species in conjunction with secondary structure analyses of the corresponding...
zebrafish genomic loci. Of the three, two show conservation of the mature miRNA strand, while one shows conservation of the passenger strand. For all eight new zebrafish miRNAs, we validated their presence by examining and ensuring that their flanking sequences and corresponding genomic loci fit existing models of miRNA biogenesis (see Supplemental Material, Novel miRNA Sequences). To independently confirm the expression of these miRNAs, we performed Northern blots on total RNA from the same four stages of development as used for deep sequencing. As expected, these miRNAs were expressed at low levels, but we were still able to detect bands of the appropriate size for the mature sequences for six out the eight novel miRNAs and bands corresponding to pre-miRNAs for five of the eight (Fig. 3).

We could not detect signals for the two remaining novel miRNAs, presumably due to expression at levels below the limits of detection. Nevertheless, based on the criteria used to identify these miRNAs, we believe they constitute newly detected miRNAs, presumably due to expression at levels below the limits of detection. Nevertheless, based on the criteria used to identify these miRNAs, we believe they constitute newly detected miRNAs.

**miRNA sequence variation**

Sequence variation at the 5' and 3' ends of mature miRNA sequences has been reported following deep-sequencing analyses (Landgraf et al. 2007; Morin et al. 2008; Lehrbach et al. 2009; Burroughs et al. 2010; Fernandez-Valverde et al. 2010; Kamminga et al. 2010). Such variation is due to mismatches between the reads and their corresponding genomic loci, with most mismatches detected at the 3' ends. We also observed significant sequence variation, mostly nucleotide additions at the 3' ends (Fig. 4A; Supplemental Table 2). Within the mature miRNA sequences, <1% of the reads differed from their genomic loci. In contrast, ~40% of the total reads were 1–2 nt longer at the 3' end than the mature sequence reported in miRBase, whereas much less 5' end variation was detected. In some cases, the additional nucleotides matched the sequence of the pre-miRNA, suggesting that cleavage events during miRNA processing are not always precise. In the case of miR-2190, sequence heterogeneity was observed at both the 5' and 3' ends, with no clear preference for specific precursor cleavage sites, again arguing against this being a bona fide miRNA.

We also detected significant 3' sequence variation (~10% of reads) due to the addition of nongenomically encoded nucleotides, mostly adenine, uracil, or both (Supplemental Fig. 6A). Rarely did we detect addition of cytosine or guanosine, although miR-181 had reads with guanosine addition and miR-738 had reads with cytosine addition. Among miRNA families, the composition and extent of sequence variation was nearly identical between family members (Fig. 4B). Additions at the 3' end in this manner (tailing) have been reported previously, with proposals that the extra adenine nucleotides might stabilize miRNA half-life (Katoh et al. 2009; Fernandez-Valverde et al. 2010; Burns et al. 2011). Our data are consistent with this idea, but also support the hypothesis that tailing with U residues may be a mechanism to target miRNAs for degradation. Support for this hypothesis is based on variation at the 3' end of miR-430 family members. As above, this family is the most

<table>
<thead>
<tr>
<th>miRNA family</th>
<th>Seed sequence</th>
<th>Raw read number at 256-cell</th>
<th>Fold change from 256-cell to sphere</th>
<th>Other members</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-430</td>
<td>AACUGCU</td>
<td>62,555</td>
<td>12.0</td>
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<tr>
<td>miR-21</td>
<td>AGCUUAU</td>
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<td>−55.6</td>
<td>-</td>
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<tr>
<td>miR-203</td>
<td>UGAAAUUG</td>
<td>22,723</td>
<td>−128.1</td>
<td>-</td>
</tr>
<tr>
<td>let-7</td>
<td>GAGGUAGG</td>
<td>15,332</td>
<td>−46.3</td>
<td>-</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>miR-100</td>
<td>ACCCGGUA</td>
<td>4,899</td>
<td>−46.5</td>
<td>miR-99</td>
</tr>
</tbody>
</table>

*aAlso includes miRNA reads with 3' end heterogeneity that does not match the genomic loci (see Supplemental Table 2).
abundant miRNA at the sphere stage, where it constitutes 97% of the reads and functions to target maternal mRNAs for degradation (Giraldez et al. 2005, 2006). We found that 3’ nontemplate-directed uracil addition among miR-430 family members is relatively low until the sphere stage, after which time increasing addition of U residues was observed. In contrast, the extent of modification by the addition of A residues was not significantly different across these stages. Increasing modification by the addition of U residues coincided with declining levels of detectable miR-430 from the sphere stage onward (Fig. 4C).

To further examine the effects of nucleotide addition on the levels of miR-430, we also analyzed the 3’ ends of miR-430 reads that lacked the last guanosine from the mature miRNA. These reads are likely to be derived from mature miR-430 RNAs that have undergone trimming and subsequent tailing at the time of library preparation. For these RNAs, we found a significant increase in adenosine addition at the sphere stage (Fig. 4D). In contrast, uracil addition for these reads was lowest at the sphere stage. Together with the modifications described above for mature miR-430 family members, the sequencing data are consistent with the hypothesis that U and A additions are associated with miRNA destabilization and stabilization, respectively.

To more broadly assess the dynamics of nucleotide addition between stages, we next examined the subset of modified miRNAs whose expression overlaps both the 256-cell stage and the sphere stage. For these miRNAs, we calculated the percentage of reads that showed U addition compared with all tailed reads. For each miRNA shown in Figure 4E, we show the extent of U modification at the 256 cell stage (blue) compared with U modification at the sphere stage (red). Increased levels of U modification were observed for the majority of miRNAs as the embryo proceeds from the 256-cell stage through the sphere stage. Interestingly, if all modifications on all miRNAs are examined across stages of development, we see a similar phenomenon with the majority of modifications detected at the 256-cell stage, followed by the sphere stage, and then decreasing levels of 3’ modification thereafter. This is shown by the extent and amounts of black lines above the x axis in Supplemental Figure 6B. The data are consistent with the idea that there is active stabilization/destabilization prior to and through the beginning of the maternal zygotic transition, after which multiple mechanisms are in play to control the expression and stability of a given miRNA.

**Zebrafish piRNA expression**

When we examined the total reads from all four developmental time points, a distinct subset of small RNAs was detected that derive from genomic regions that do not encode miRNA genes (Fig. 1). These RNAs represent a diverse array of sequences with a peak size distribution of ~26–28 nt, and they map to either repetitive or unique genomic sequences (Fig. 1). Their overall detection was clearly not saturated, as >80% were detected only once. Based on size, they most likely represent piRNAs (Fig. 5A,B). Three additional criteria suggest that these RNAs are bona fide piRNAs. First, 10,892 reads matched sequences previously reported to be piRNAs in zebrafish (Houwing et al. 2007). Second, in genome wide, a much, much larger number of reads (674,777 sequences from 1,068,353 reads from shield stage) mapped to transposable elements with a strong bias toward the antisense strand (Fig. 5C; Supplemental Fig. 7). Finally, for those reads that mapped to the sense strands of transposable elements, there was a strong preference for an A at position 10, while those derived from antisense strands showed a preference for a U at position 1. This is completely consistent with the Ping-Pong model of piRNA biogenesis (Supplemental Fig. 8).

Over 65% of the piRNA reads that originated from repetitive elements were derived from the LTRs of transposable elements (Supplemental Fig. 9). A different subset mapped back to unique genomic loci, primarily intergenic regions of the zebrafish genome. For both, the reads showed a periodicity and strand preference. Based on the genomic loci of both types of piRNAs, it appears that the majority of the piRNA reads are derived from long transcripts that are then subsequently processed into smaller RNAs. The genomic regions that give rise to these long transcripts are referred to as piRNA clusters (Brennecke et al. 2007; Houwing et al. 2007). We defined piRNA clusters as genomic fragments encoding at least 10 unique piRNAs with gaps no greater than 1 kb (Supplemental Table 3). By grouping reads into defined piRNA clusters, we were able to quantify expression patterns based on the abundance of clusters instead of absolute piRNA reads, allowing examination of the expression of piRNAs during early zebrafish development.
and testes (Houwing et al. 2007), but we detected widespread embryonic piRNA expression across all four developmental stages (Fig. 5D,E). Beginning at the sphere stage and becoming much more prevalent at the shield stage, we detected increased piRNA expression (Fig. 5D–G). The increase was not due to a decrease in other small RNAs, because the sequencing runs were not saturated and, for example, we detected a robust increase in piRNA reads between 256-cell and sphere stage embryos, coincident with the large increase in miR-430 reads. This suggests enhanced transcriptional activity of piRNA clusters and transposable elements, with a resultant increase in piRNA production that likely func-
FIGURE 5. (Legend on next page)
tions to maintain genome integrity during early cell divisions and as germ cells are set aside. Compared with piRNA expression data from adult ovaries and testes (GEO: GSE7131; http://www.ncbi.nlm.nih.gov/geo/ under series number GSE7131) (Houwing et al. 2007), there is a significant reduction in piRNA expression in adult tissues compared with the early developmental stages we examined. At least for ovaries and testes, the expression patterns in adult tissues most closely resemble those observed at the 256-cell stage. This is consistent with maternally contributed piRNAs being most similar in pattern to those detected in ovaries and testes.

Previous analysis of piRNAs in zebrafish showed that in adult ovaries and testes, the majority of piRNAs are derived from the plus strands of genome (Houwing et al. 2007). In contrast, during early development, we observed a preference for piRNAs from minus strands (Fig. 5F,G). Given the fact that zebrafish piRNAs have a strong bias toward the antisense strand of transposons, this suggests that the expressed transposable or repetitive elements that are associated with piRNAs at very early developmental stages are, at least partially, different from those expressed in adult ovaries and testes. It is not clear why such strand preferences exist at the different times of development. Regardless of strand of origin, the data suggest that piRNAs have important functions not only in adult ovaries and testes, but also at multiple stages of early development.

**tRNA-derived small RNAs in zebrafish**

In addition to miRNAs and piRNAs, we also detected a large number of other small noncoding RNAs. One particularly interesting subset (~225 different sequences; 56,311 total reads) was derived from tRNAs (Fig. 6). These RNAs matched the 5' and 3' ends of tRNAs with a size range between 18 and 28 nt (Fig. 6A,B; Supplemental Table 4). The 3' end reads contained the universal 3' CCA sequence that is added post-transcriptionally, indicating that these RNAs are derived from mature tRNAs, consistent with previous reports (Cole et al. 2009; Lee et al. 2009; Haussecker et al. 2010). Interestingly, the sizes of the 3' tRNA reads are mainly 18 nt, while the 5' tRNA reads are distributed more widely, suggesting that they might be under different selection mechanisms or have different functions (Fig. 6B). Given the nonrandom accumulation of these small RNAs from just the 5' and 3' ends, it is hard to argue that these RNAs are degradation products. Indeed, other reads across the entire mature tRNA sequence were observed at much, much lower frequencies (17-fold less at 256-cell and 1 dpf) (Fig. 6C). We note that the tRNA-derived fragments we detect are likely different from angiogenin-mediated tRNA fragments found in stress-induced cells, because the cleavage sites we detect are different from those reported (Thompson et al. 2008; Yamasaki et al. 2009; Emara et al. 2010).

**DISCUSSION**

**miRNA expression**

miRNAs are postulated to play important roles during the maternal-zygotic transition, when embryos reshape and reprogram the transcriptional landscape (Giraldez 2010). Using deep sequencing, we determined the expression patterns for 198 distinct miRNAs, and we identified five novel miRNAs and three new conserved zebrafish miRNAs expressed during early development. Maternally inherited miRNAs and a subset of miRNAs that are expressed very early in development are important for zebrafish embryogenesis (Giraldez et al. 2005, 2006). Compared with prior approaches involving direct cloning or microarrays, the increased sensitivity of miRNA detection with deep sequencing affords the opportunity to more completely define the expression patterns and, ultimately, the function of small noncoding RNAs during early vertebrate development, including those expressed in single cells or specific tissues (Wienholds et al. 2005; Thatcher et al. 2007). The 198 miRNAs that we identified and profiled can be classified into 122 miRNA families. A total of 70% of these families are expressed at or below the limits of detection using microarrays. Fortunately, for those miRNAs expressed at detectable levels using microarrays, there was a strong correlation between the data sets, lending confidence that data generated from our sequencing libraries are accurate (Wienholds et al. 2005; Thatcher et al. 2007).

Previous studies showed that zebrafish miRNA expression patterns become increasingly complex as development proceeds (Wienholds et al. 2005; Thatcher et al. 2007). Our results revealed more widespread miRNA expression at
earlier stages of development than previously observed. Beginning at the 256-cell stage, a large number (178 out of 216 currently known zebrafish miRNAs) of miRNAs are detectable, with several expressed at relatively high levels. These miRNAs are likely maternally deposited and may either regulate initial development or function similar to the miR-430 family to target maternally inherited mRNAs for degradation during the maternal-zygotic transition (Newport and Kirschner 1982; Giraldez et al. 2005; Schier 2007). We found that fully 99% of the miRNA reads detected during the sphere stage are derived from miR-430 family members. Interestingly, maternally inherited miRNAs appear to be rapidly down-regulated during the maternal-zygotic transition, raising the possibility that they are subject to similar forms of regulation as maternally inherited mRNAs. Beyond maternally inherited miRNAs, our analyses are also consistent with important early roles for a number of miRNAs, including miR-203, miR-1, and miR-133, which function to balance proliferation and differentiation during early development (Yi et al. 2008; Mishima et al. 2009). In contrast to previous results, we detected significant levels of let-7, raising the question as to its exact role in early development (Kloosterman et al. 2004; Wienholds and Plasterk 2005).

**Normalization of miRNA sequencing data**

Typically, total genome matching reads are used to account for differences in the sequencing depth of individual libraries when normalizing small RNA sequencing data. This certainly applies when biological replicates are being sequenced; for example, when comparing two independent libraries from the same developmental stage. However, if the overall transcriptome changes dramatically between samples, normalization based solely on total genome matching reads can obscure important biology (Robinson and Oshlack 2010). During early development, cell numbers are increasing dramatically, concomitant with extraordinary changes.

**FIGURE 6.** Zebrafish tRNA-derived Fragments (tRF). (A) Graphic representation of tRF alignments to zebrafish tRNAs. Most reads mapped to the 5' and 3' ends or mature tRNAs. (B) Size distribution of all identified tRF reads from four developmental stages in terms of either unique read sequence abundance or total read abundance. (C) The raw abundance of the 5' tRF and 3' tRF reads at four developmental stages, as indicated.
in gene expression as differentiation proceeds. This creates a unique set of problems when normalizing sequencing data. We chose to normalize based on the relative expression levels of either miR-9 or let-7. These miRNAs were chosen because they are expressed at moderate levels across the four stages of early development that we examined. The resulting heat maps were very similar, whether using miR-9 or let-7, and Northern blots of six miRNAs were completely consistent with the derived heat maps. Interestingly, when we normalized using TMM, the resulting heat maps were also very similar to those obtained with either miR-9 or let-7. Thus, we believe that the strategy we adopted to normalize our small RNA sequencing data during early development is appropriate. Nevertheless, we realize that under conditions where differential gene expression patterns are changing dramatically, every available method has certain limitations, and other methods can certainly be used to quantitatively analyze small RNA sequencing data, e.g., TMM (Robinson and Oshlack 2010) and DESeq package from http://www-huber.embl.de/users/anders/DESeq/ (Anders and Huber 2010).

**Sequence variation**

miRNA 3' tailing and trimming were previously reported in worms, flies, mouse, and human cells (Landgraf et al. 2007; Katoh et al. 2009; Lehrbach et al. 2009; Ameres et al. 2010; Burroughs et al. 2010; Fernandez-Valverde et al. 2010). We detected many tailed miRNAs containing additional nontemplate-directed nucleotides, mostly A and U, primarily at 3' ends (Fig. 4A). The extent of 3' variation may actually be higher than that reported here, since in some cases (e.g., let-7) the extra A and U residues match the pre-miRNA flanking sequence, so their origin is not clear. The temporal pattern of A versus U tailing suggests that the addition of A residues might stabilize miRNA half-life, whereas U addition may promote degradation. This seems to hold true for the miR-430 family, but may apply more broadly, since we detected a large percentage of tailed miRNAs during very early development, with decreasing modification at the later stages. It is possible that different mechanisms regulate miRNA half-life during the maternal zygotic transition compared with later stages, but our data suggest a common mechanism.

**piRNA expression**

piRNAs have been reported to be specifically expressed in the germline to maintain genome integrity (Halic and Moazed 2009). We identified many piRNAs in 256-cell stage embryos, consistent with maternal inheritance. We also found a peak of piRNA expression at the shield stage, with readily detectable levels out to 1 dpf. Except for the shield stage, the pattern is similar to that reported in mouse embryos (Ohnishi et al. 2010). However, the robust expression levels at the shield stage, when the germ layers are forming, may indicate an important function for piRNAs during early development. Previous work has reported that piRNAs can induce deadenylation and decay of nanos, a maternal mRNA in *Drosophila* embryos (Rouget et al. 2010). In zebrafish, primordial germ cells (PGCs) are first specified during early embryogenesis and give rise to germ cells after proper migration and further differentiation. Zebrafish PGCs show maximal migration activity amongst a far larger number of somatic cells at the beginning of gastrulation (5.5–6 hpf) (Raz 2003; Blaser et al. 2005). The shield stage coincides with the onset of gastrulation, suggesting that the widespread expression of piRNAs that we detect at this time may play an important role in the maintenance and proper migration of PGCs. Only a very limited number of PGCs have formed or are forming during this time, raising the possibility of piRNAs originating from other cells beside PGCs in embryos. Consistent with a requirement for piRNAs during these early stages, morpholino-mediated knockdown of Ziwí protein inhibited and/or blocked proper germ-cell migration (H Dai, C Wei, and JG Patton, unpubl.). Future studies are needed to understand the temporal and spatial manner of piRNA expression and their specific functions during early embryonic development.

**TRNA-derived small RNAs**

Small tRNA-derived fragments were previously reported in different cell lines (Cole et al. 2009; Lee et al. 2009; Haussecker et al. 2010). Our sequencing analyses expand the number of species that express this class of small RNA, indicating that the biogenesis and function of tRNA-derived small RNAs is conserved, suggesting an important role. Several lines of evidence raise the possibility that these small RNAs function in gene silencing in a Dicer-independent manner (Cole et al. 2009; Haussecker et al. 2010). It will be important to determine how or whether these RNAs regulate gene expression, especially in light of the fact that these RNAs are themselves temporally regulated during development.

**MATERIALS AND METHODS**

**Small RNA purification, cloning, and sequencing**

Zebrafish embryos were collected at the 256-cell stage, sphere stage, shield stage, and 1 d post fertilization (dpf). Total RNA was isolated from embryos using Trizol. RNAs were fractionated on 15% denaturing polyacrylamide gels, and small RNAs between 15 and 30 nt were excised and purified. cDNA libraries were generated using specific linkers and RT/PCR, as previously described (Hafner et al. 2008). Libraries were sequenced in the Genome Technology Core of Vanderbilt University using the Illumina sequencing platform. The numbers of sequencing reads from the four developmental stages are listed in Supplemental Table 5.
3' ligation adaptor: AMP-5'-pCTGTAGGCACCATTCAATdideo xyC-3'.
5' ligation adaptor: 5'-ACACUUUUCCCUACGACGCUUU CCGAUC-3'.
RT primer: ATGGATGTGGCTACAG.
PCR forward primer: 5'-AATGATACGGCCACCACCAGAACACT CTTCTCTACAGCGACG-3'.
PCR reverse primer: 5'-CAACGAGAGAGCGGATACTTGATG GTGCCTACAG-3'.

Small RNA identification

Initial reads were processed to remove the linker sequences using an in-house dynamic alignment algorithm, which allows one mismatch and a minimal of five starting nucleotides in the linker sequences. Small RNAs matching the zebrafish genome (Zv8) from Ensembl (http://www.ensembl.org) were retrieved with Bowtie, a short read alignment algorithm widely used for aligning short DNA sequences, using the default parameters which allow, at most, two mismatches in the “seed of 28 nt” with maximum quality values of 70 (Langmead et al. 2009). After elimination of reads derived from known mRNAs, tRNAs, rRNAs, and snRNAs, miRNAs were next annotated based on a perfect match to miRNA hairpin sequences as reported in miRbase (http://www.mirbase.org/ftp.shtml). Small reads either aligned to known hairpin sequences from other species listed in miRBase, transposable elements, rRNAs, tRNAs, snRNAs, and other known small RNAs (UCSC genome browsers). To ensure the authenticity of these predicted miRNAs, only small RNAs that appeared in at least two developmental stages were retained. From among the 327 novel miRNAs predicted by mirDeep, five were finally recognized as authentic novel miRNAs.

qRT–PCR

qRT–PCR assays (Taqman RT kit and Taqman miRNA custom assays, ABI) were performed to measure the expression levels of let-7a and mir-9. Relative levels were normalized to U6 snRNA. cDNAs were synthesized from 10 ng of total RNA extracted from zebrafish embryos. Products of RT reactions without reverse transcriptase served as a negative control, and each measurement was derived from three biological replicates. Expression levels were determined based on the threshold cycle values (Ct) of each miRNA relative to that of U6 and assigned as 2^(-ΔΔCt).

Northern blots

A total of 18–23 μg of total RNA (10 μg for mir-R-430) was extracted from zebrafish embryos at different developmental stages, separated on 15%–20% denaturing polyacrylamide gels, and transferred to membranes for Northern blot analysis of known miRNAs following procedures as previously described (Thatcher et al. 2007). For novel miRNA detection, 13–15 μg of small RNAs (<200 nt) were enriched from 220–250 μg of total RNA extracted from 1 dpf zebrafish embryos using mirVana miRNA isolation kits (Applied Biosystems). The sequences of miRNA Northern probes (miRNA StarFire probes, IDT) were complementary to the mature sequences for let-7a, mir-455, mir-21, mir-22a, mir-200b, and mir-430b. For the novel miRNAs, the probes were as follows:

novel miRNA 1: GGTAACCATTTGGCTTCCATTGT;
novel miRNA 2: AACTGGTAAACATTAGCCCTTCA;
novel miRNA 3: ACTGGCTACCAAGGATTGGTACA;
novel miRNA 4: CAGTATCGAGGATTTCATT;
novel miRNA 5: CCTTCCAGTGAGGCACAC;
novel miRNA 8: GAAAGCCTTCTCCACITC.

miRNA expression profiles

Absolute read numbers were collected and sorted into miRNA families based on sequence records in miRBase. We defined miRNA families as those miRNAs sharing the same seed sequence along
with high-sequence similarity toward the 3’ end. We also normalized expression levels across four developmental stages relative to \(let-7a\) and \(miR-9\) levels, as determined using qRT–PCR. Read numbers were transformed into log2 values and displayed with a MultiExperiment Viewer (Saeed et al. 2003, 2006) (http://www.tm4.org/mev/). The raw log2 value of all single miRNAs and miRNA families throughout development are listed in Supplemental Table 6.

**DATA DEPOSITION**

Sequencing data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the series number GSE72722. Public data with series number GSE7131 are also available at http://www.ncbi.nlm.nih.gov/geo/.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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