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Expression of microRNAs during embryonic development of *Xenopus tropicalis*

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Abstract

microRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression and have prominent roles during early embryo development and organogenesis. We set out to determine the expression pattern of miRNAs in the developmental model system, *Xenopus tropicalis*. We made probes to predicted primary miRNA transcripts and performed in situ hybridization. Our data show conserved and novel tissue-specific expression patterns during embryogenesis that suggest functional roles during development.

Keywords

Xenopus tropicalis; microRNA; miRNA; expression; in situ hybridization

1. Results and Discussion

microRNAs are a recently discovered class of small, non-coding RNAs encoded by the genome that regulate genes post-transcriptionally. They are transcribed by RNA Polymerase II as primary microRNAs (pri-miRNAs), which are then processed by the enzymes Drosha and Dicer to generate the mature single-stranded miRNA of ~22 nucleotides, which is incorporated into the RNA-induced silencing complex (RISC), characterized by the presence of the Argonaute family of proteins (Pasquinelli et al., 2005). This complex is responsible for the regulatory function of the miRNAs. Though microRNAs are present in all metazoan taxa (Hertel et al., 2006), few studies have addressed the *in vivo* function of these molecules during development. In order to understand the function of miRNAs during embryogenesis of the developmental model organism *Xenopus*, it is important to know the spatiotemporal expression profiles of these genes throughout development. Though other groups have cloned miRNAs from *X. laevis* embryos (Watanabe et al., 2005), there is as yet no data on the spatiotemporal expression of miRNAs during embryogenesis. Therefore, we generated approximately 1-kb digoxigenin-labeled antisense probes to 60 predicted *Xenopus tropicalis* miRNAs and hybridized them to neurula, tailbud, and tadpole stage embryos. Probes to 18 of the miRNAs gave spatially distinct patterns above background, and these are shown in Figure 1.

Many of the *X. tropicalis* miRNA expression patterns are conserved across animal species. miR-1a-1 is expressed in mesoderm in *Drosophila* (Aboobaker et al., 2005; Chen et al.,

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2006), zebrafish (Chen et al., 2006), and mouse (Zhao et al., 2007) and can clearly be seen in the trunk mesoderm of the neurula stage embryo and in somites at the tailbud stage (Fig. 1B'). Similarly, miR-133 is expressed in muscle tissue (Chen et al., 2006) (Fig. 1O'). Among other conserved expression patterns (Arora et al., 2007; Deo et al., 2006; Mansfield et al., 2004), miR-124 is highly expressed in the entire central nervous system (Fig. 2, N–R), miR-9 is expressed in the brain (Fig. 1, D–F' and Fig. 2 A–G), miR-7 is expressed in the eye (Fig. 1C'), and miR-10 is expressed in the posterior region of the tailbud embryo (Fig. 1G). These expression patterns are conserved across large evolutionary distances.

For the 42 probes that failed to detect distinct patterns of expression above background, we cannot say that this is due to a lack of expression. A negative result could be the result of very rapid processing of the primary transcript, low levels of expression, expression at a different stage of development, or a non-optimized hybridization probe or procedure. Some probes gave ubiquitous expression patterns, but we have not eliminated the possibility that these probes caused non-specific background staining. Probes that gave tissue-specific expression patterns were more enriched for miRNAs that are intergenic (17/18, 94%) than the total collection of probes (48/61, 79%), though the significance of this is not clear. miR-133b is the only intronic miRNA whose expression is investigated here. It is found in the second intron of a homolog of *Pkhd1*, whose expression in *Xenopus* is unreported, and where no ESTs have been found among the 1.2 million in genbank. In mice, however, this gene is expressed in the developing kidney (Nagasawa et al., 2002), a tissue in which miR-133b is not detected.

Using probes for the entire primary-miRNA transcript allowed us to differentiate between the expression patterns of genes that have identical mature miRNA sequences. The three paralogous miR-9 genes show unique but overlapping expression patterns (Fig. 1, D–F'). The most highly expressed at all stages is miR-9a-1. miR-9a-2 has very low levels of expression in the anterior-most portion of the neural plate at the neurula stage, but expression increases at tailbud stages. miR-9-3 displays intermediate expression levels at both these stages. Interestingly, during the tailbud stage, miR-9a-2 has a broader expression pattern than the other paralogs, encompassing not just the eye and forebrain, but also the hindbrain. However, by the tadpole stage, all show similar expression patterns to miR-9a-1 (Fig. 2A and data not shown).

A cluster of miRNAs containing miR-23b and miR-24a is found in the *X. tropicalis* genome, along with miR-27b (Griffiths-Jones et al., 2007). Non-overlapping probes to miR-23b and miR-24a revealed identical novel expression patterns, first appearing at stage 19 in the eye anlagen and posterior cells around the blastopore (Fig. 1, I–J'). The neural retina continues to express both miRNAs, as does the posterior-most mesenchyme, and therefore it is likely these miRNAs derive from the same primary transcript.

Many of the miRNAs that gave specific expression patterns are expressed in neuronal tissues. This correlates with previous studies in other vertebrates (Kapsimali et al., 2007), which show that neurons and their progenitors are enriched in the number of expressed miRNAs. In *X. tropicalis*, this includes let-7a, miR-7, -9, -18, -23, -24, -96, -98, -124, -130, -181, and -219 (Fig. 1). miR-429 is expressed in cells of the olfactory placode (Fig. 1, R and R'). miR-9a-1 is expressed broadly in the most anterior portions of the forebrain, where cells are proliferating and differentiation is limited (Papalopulu and Kintner, 1996), and in the mid- and hindbrain it is expressed in the proliferating ventricular zone (Fig. 2B–E). miR-124 is expressed in a complementary pattern, in the medial and lateral areas of the brain and neural tube, where differentiation will take place (Fig. 2N–R). This holds true in the eye as well, where miR-9a-1 is expressed more highly in the marginal zone and miR-124 is highly expressed in the differentiated neural retina (Figs. 2C vs 2N, respectively). These results agree with previous observations in other vertebrates (Krichevsky et al., 2006; Smirnova et al., 2005).

2. Experimental Procedures

Based on the nomenclature and position information obtained from miRbase (Griffiths-Jones et al., 2007) we used the primers in Table 1 to synthesize approximately 1-kb templates by PCR from genomic DNA. In almost all the templates, the pre-miRNA hairpin resides in the center of this probe. PCR products were incorporated into the pCR-II-TOPO vector (Invitrogen) and sequenced with T7 and SP6 to determine orientation. Either T7 or SP6 polymerase was used on these templates to synthesize antisense digoxigenin-labeled probes. *Xenopus tropicalis* embryo acquisition, fixation, and whole-mount in situ hybridization were carried out as described at <http://tropicalis.berkeley.edu/home> and in (Harland, 1991). Embryos stained in whole-mount were sectioned at 100µm using a vibratome (Technical Products International, Inc.).

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Figure 1.
Expression patterns of *X. tropicalis* miRNAs at neurula (A–R, dorsal view) and early tadpole stages (A'–R', lateral view, anterior to the left).

Figure 2.

Xenopus miRNAs exhibit tissue-specific expression patterns. A, G, M, and S are lateral views, anterior to the left. All others are 100uM coronal sections, dorsal at the top. (A–F) Stage 35 tadpole showing miR-9a-1 expression in sub-ventricular cells of the brain. (G–L) Stage 24 tailbud embryo showing miR-23b expression in the eye primordia and the posterior mesenchyme. (M–R) Stage 35 tadpole showing miR-124 expression in non-proliferative neural tissue. (S–X) Stage 35 tadpole showing miR-133b staining in head mesoderm, migrating hypaxial muscles, and somites.

Table 1

Primers used to make pri-miRNA probes

Name	Primer 1	Primer 2
let-7a	TGGCGTTCCACACTTGTGAAGAC	CTCCCCATCAAAAGCTCAATAAG
let-7c	GCTGCATTGCATTTGCCAAC	TTCACCTCAATTAACTTCCCTCTACTG
let-7e-2	GTCACCTTTGTGGCACTGCTGTG	AGGAAAATTCCCTCCCCAATG
let-7f	CAACATCCCTCCGTGCCTGTATC	CCCCTCCCTTTCGAGAGAAATC
let-7g	GGGAAAACATCTTCTGAAACG	TTGCTAGGGATTTAGTTGACCTTAC
let-7i	GTGTGCAAAAATTAGAGCATGACAG	CAGTCTGTGCCCTTTACTTTG
mir-1a-1	GGAAACATCTTACCTTACAGC	CGGCATTTCACGAGGAGGCAGG
mir-7-1	GCGCAGCCTCATTATGTAAGG	GTAACACTGAGAAAACTAGGC
mir-7-2	GGAGAAAAGAAGGTACAGTG	GGCTACAAATGATATTGAAGTG
mir-9a-1	CACACGGGATTCTGGGAATC	GATCCAATCAGATGACTATG
mir-9a-2	GGAATTGTAGTCTGGGTTTTAG	GTCACCTTGGACTGGAATGGG
mir-9-3	CACACGGGATTCTGGGAATC	GATCCAATCAGATGACTATG
mir-9b	GTGTGCCTGTTTGTCTCTGC	GATATTTGAGGAAGGGAAGATG
mir-10b	GGGTTCGTCTTTTACATGG	CTCCTATTGTCTATCTCTGG
mir-10c	TGCCCTTAGCCTTCTGTCTTATC	TAGCCTAAATGTGCCAGCGG
mir-16c	GGGGAGGATACAAAGATGGCTTC	GCTAAGGCATGTGAGAGAACTGGC
mir-18a	GATAATCCCAAGCATCTAAAC	GAGCAACAGTGAGTGTCC
mir-20a	CTTTAGCTGAATTTTCAAGTAGAG	GGTGATAATAGTCTAAGCTG
mir-22	TGCTTGATGTGTTGCTTCTCTCC	CCACCCTGGGACAATATGGTAGTC
mir-23a	GTGAACAGGTGCGAGAGTGAC	GTTCTCTGATAAATCTGTGGACTG
mir-23b	GCCTTCCATCCTTTCTGTCTG	CACATGATAACTGCTGGGATG
mir-24a	CTGTGGCTGAGCTATGATAATCTG	GGTAACGGAGGGAGAACTGG
mir-26a	GTGTTTTGTGTGACTGTGAC	CAGTGGAGATAGAGGGAAC
mir-29d	GCCCTTAGTCAATGGATATGC	CACGGTGTGTACCTCTAAATCTGTAG
mir-30b	GAGGCACAATGCAGTGAATGG	TGACCACAGAAGCACAATGTTAGAG
mir-30c-1	CTGTGGCTGAGCTATGATAATCTG	GGAAACTTTGGCTAGGAATCAAG
mir-34a	TGCCTGCCGTTTTAATTCAGTC	ACAGTTAGCACCACCACTTGGG
mir-34b-3	TTCTGGTAAACGGAATCACTACACC	GCCATTTCCTGCAATTTTAAAGTGC
mir-34b-4	TTCAGAATACCTGGGACCTGGGTC	ACAACACTTTGGATCTAAATGCCAG
mir-92a-1	GTGTTACTGTCTCTTAAAGAG	CACCTGTAGCATTATATTGAC
mir-96	CAGCACCACAGAAGAAGAATACTGC	TGGAGGATGGATTAAGGGGC
mir-98	AAGCATCTGAATCCTCTGCTCG	GAATACCCCATTTGAACTGAGCC
mir-99	GACTCTCAAGCATTTCCTCAACG	CATTGCCCTTTTCCCTG
mir-100	TAAACACCGTAGCCAGAGCACC	TTCCACCAAGAGCAGAACAAGTC
mir-103-1	GCCATTTGACCAGTAATCCAGTG	CCATGTTGGGTGTGGTTTAGTGC
mir-103-2	GGGCTCCTTACTGTCTGTGTATG	AATGGCTGGGTTGCTGATGG
mir-124	GGGGATTGGGCAGGTGCGATTG	CACCTGATGCAGATTGTGGATAG
mir-125a	GGTGAATCTCAGTCCATACACAGTAG	GGGGATGCAAAGCACAAGATATAAG
mir-125b-1	GGTGCTAAATGCAGCCGATG	GCGTTCAATAACCGATATGCAGTC
mir-125b-2	GGATCTTAAACGCAAGGCAGC	TTCACCTCTCGCAGACAACAGACAG
mir-129-2	TGAGTAAATGTGCCCCCTAGTGC	TCAGAAGAAAAATCCTCCCTCGC
mir-130a	CCCATGTGCCAAAAAGCATAGC	CATGCTCTGTGTATTCTGGGCAG
mir-133b	GGAGCTGCTGCACCTTTGTG	CCCATTACCTTTCCCTGCC
mir-135-1	CAGTGATATAACACAGGAAATGGCG	TGGCTTCCATCCCTACATGAGAC
mir-137-2	GCAAATTCCTCAACCATAGAAGG	TGCAAGCAGGAAAGAAGACATGAG
mir-143	TGGAAGCAGAGGAAATTGCG	CCAGCCATTATGGATTGCTCTC
mir-153-1	TGTTATTATCTAGGGGGTGCCTGTC	TAGGGACACTGGGAATGTTCCGG
mir-153-2	GAAGGAGAACATCAAATTGGTGGC	GCACAGACAATAAATCAGCAGTGC
mir-181a-2	GCACACAATATGCTAAGACACTTTC	GTAAGGTAACCCAATCCTACAAAAC
mir-194-2	GCTGTGAGGATAGCCTGATGTAGTG	TCATATAGACGTTTCTGCTGTGC
mir-196	ACCATGCCTTGCAGTCAGACAG	GAAAACCTAATGTCATTGCCCCAC
mir-199a	GTAGGGTGCGATATGAAAGCTGTAG	CTGGCTGAAAGATGAAATGACTGC
mir-200a	CTAGTGTTTGAATCCTCTGGGGAG	AGGCAAGGTGTTACAATGCTGTG
mir-210	CCCACCTGATTTATGTGTG	CAGCATCTGTCTTTTGTG
mir-214	CATAATCTCTTTGGCTTTGG	GCACTTAAACATAGGTTTAC
mir-217	TGAAGCCATTCCCACTAACGG	ATTGGTAGACCTTGCGGAGAGG
mir-218	AGCCAACGGAAAAGTTGCTTC	GACATCCCTGGTTTGTGTAACGAC
mir-219	GGAAGAGAACTGATGGCAATGACTG	GGAAGAGAACTGATGGCAATGACTG
mir-222	CATGCGATTGTCTTACGGGG	CCAGCAGACAATGTAGCTGTTGC
mir-338-1	CATCTCTGGAACCATGAATAAG	CTGGGACTGTGTGCGTATTG