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Cloning and Identification of Novel MicroRNAs from Rat Hippocampus

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Abstract MicroRNAs (miRNAs) are small regulatory molecules post-transcriptionally suppressing mRNA activity. Many miRNAs in various organisms have been cloned but many unknown miRNAs remain to be identified. Here we describe the cloning of six new miRNAs from rat hippocampus. Among them, four were not found in the rat miRBase, but were identical to their human and/or mouse homolog, therefore they were designated as rno-miR-92b, rno-miR-146b, rno-let-7g, and rno-miR-551b. The other two were derived from the other arms of the known miRNA precursors of rno-miR-330 and rno-miR-384, and were not found in miRBase of all organisms. They were designated as rno-miR-330* and rno-miR-384*. The expression of these miRNAs was confirmed by RNA-tailing and primer-extension real-time reverse transcription-polymerase chain reaction. These six miRNAs were expressed at significantly higher levels in the hippocampus than in other tissues, including cerebral cortex, heart, liver, lung and kidney. miR-384* was 10 times more abundant than miR-384 in rat hippocampus, but little difference was found between miR-330* and miR-330 expression in the same tissue.

Keywords microRNA; cloning; hippocampus

MicroRNAs (miRNAs) are approximately 22 nt non-coding RNAs that are expressed in a wide range of eukaryotic organisms. They are transcribed from the genome as pri-miRNA precursors and cleaved by the Drosha endonuclease in the nucleus to give approximately 70 nt pre-miRNAs with a characteristic hairpin structure. Following the export of pre-miRNAs to the cytoplasm by Exportin-5, the loop region of the hairpin is removed by the Dicer endonuclease. A strand of double-stranded RNA, which corresponds to the mature miRNA, is incorporated into a large protein complex termed the RNA-induced silencing complex (RISC), where it functions to guide RISC to complementary mRNA targets. The RISC either inhibits translation elongation or triggers mRNA degradation, depending upon the degree of complementarity of the miRNA with its target [1,2]. Thirty percent of protein-encoding genes in human are predicted to be regulated by miRNAs [3]. Increasing evidence has suggested that miRNAs have important regulatory roles in a broad range of biological processes including developmental timing, cellular differentiation, proliferation, apoptosis, and oncogenesis [4–6]. To date, many miRNA genes have been identified in different eukaryotic organisms and deposited in miRBase (http://microrna.sanger.ac.uk/sequences/) [7,8]. The number of miRNA genes in human, mice and rats from the miRBase are 474, 373 and 234, respectively. Bioinformatic prediction revealed that the total number of human miRNAs is estimated to be at least 800–1000 [9,10]. Clearly, there are still many unknown miRNAs to be identified. To obtain better insight into the biological function of this class of small RNA molecules in general, and of individual miRNAs in particular, it will be essential to identify all miRNAs that are expressed from a genome. Cloning miRNA from tissues and sequencing them provides direct evidence for their presence.

Hippocampus is a place of adult neurogenesis, where new neurons are continually generated [11,12]. Growing evidence supports a role for adult hippocampal neurogenesis in health and disease [13,14].

In order to clone new miRNAs that might be related to

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adult neurogenesis, we constructed a cDNA library using miRNAs extracted from rat hippocampus. Among the 75 miRNA clones identified, four miRNA sequences were not found in the rat miRBase but were identical to mouse miRNA sequences, and two sequences were not found in any other organism. These novel miRNAs were expressed at significantly higher levels in the hippocampus than in other tissues.

Materials and Methods

Small RNA isolation

Sprague-Dawley rats were anesthetized and killed by decapitation. Hippocampi were carefully dissected and frozen immediately to –80 ºC. Eight hippocampi from four rats were pooled for the extraction of mRNAs and small-sized RNAs (less than 200 nt) using the RNeasy lipid tissue mini kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. Four RNeasy mini spin columns (Qiagen) were used to bind mRNAs extracted from 400 mg of tissue. The flow-through from four columns was pooled and loaded onto another column after adding 1.4 volumes of 100% ethanol. The small RNA fraction was washed and eluted with 30 μl of RNase-free water. The concentration of RNAs was measured by the ultraviolet absorbance at 260 nm. The integrity of mRNAs eluted from the previous four columns was verified by 1% agarose electrophoresis. Small-sized RNAs were examined by electrophoresis using 3.5% NuSieve agarose gels (Cambrex, Rockland, USA). Small-sized RNAs were also extracted from cerebral cortex, heart, liver, lung and kidney of Sprague-Dawley rats in the same way.

Cloning of miRNAs

The miRNA cloning strategy described by Fu *et al.* [15] was used with some modifications. Small RNAs (2 μg) were polyadenylated at 37 ºC for 30 min in a 50 μl reaction volume with 5 U poly(A) polymerase using a poly(A) tailing kit (Ambion, Austin, USA). The poly(A)-tailed small RNAs were recovered by phenol/chloroform extraction and ethanol precipitation with 5 μg of glycogen as a carrier. A 5′ adapter, 5′-CGACUGGAGCACGAGGACACUGACA-UGGACUGAAGGAGUAGAAA-3′ (GeneRacer kit; Invitrogen, Carlsbad, USA), was ligated to poly(A)-tailed RNA using T4 RNA ligase (Ambion) in a 20 μl reaction volume at 37 ºC for 1 h. The ligation products were extracted by phenol/chloroform and precipitated with ethanol with 5 μg glycogen as carrier. The recovered RNA was reverse transcribed using a 5′-extended and one-base anchored oligo-dT reverse transcription (RT) primer [GCTGTCAACGATACGCTACGTAACGGCATGACA-GTG$(T)_{24}$(A/G/C)] and 200 U SuperScript III reverse transcriptase (Invitrogen) at 50 ºC for 1 h. The cDNA was amplified by polymerase chain reaction (PCR) using primers5′-CGAGGATCCGACTGGAGCACGAGGACAC-TGA-3′ and 5′-CGAGGATCCGCTGTCAACGAT-ACGCTACGTAAC-3′. Pfu DNA polymerase (Tiangen, Beijing, China) was used to ensure high-fidelity amplification. After 25 cycles of amplification at an annealing temperature of 52 ºC, the PCR products were extracted by phenol/chloroform and precipitated with ethanol. The DNAs were then digested with *Bam*HI and separated on a 3% NuSieve GTG low-melting agarose gel. DNA fragments of approximately 140 bp were excised from the gel and recovered by phenol/chloroform extraction and ethanol precipitation. DNA fragments were then inserted into a *Bam*HI cut pUC19 vector (New England Biolabs, Ipswich, USA). Clones with inserts of approximately 140 bp in length were submitted for sequencing.

Bioinformatic analysis

The cloned RNA sequences were compared to sequences in miRBase. Sequences not found in the miRNA database were subjected to BLAST analyses against the rat genome (http://www.ncbi.nlm.nih.gov/blast), and their flanking sequences were analyzed using the mFold Web server (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) [16] to evaluate the ability of putative precursor sequences to form thermodynamically stable hairpin structures.

Detection of miRNA expression by real-time RT-PCR

An RNA-tailing and primer-extension real-time RT-PCR assay [17] was developed based on the method of Shi and Chiang [18]. Poly(A)-tailed small RNA (2 μg) was reverse transcribed using 1.5 μg of one-base anchored oligo-dT RT primers with a 40 nt extension at their 5′-ends (as described above) and 200 U of SuperScript III (Invitrogen) according to the manufacturer's instructions. Primers were designed using Primer 3 software available online at http://frodo.wi.mit.edu/. Wherever possible, primers were designed to have similar properties so that different genes could be amplified in the same run. For miRNAs with a high or low GC content, primer lengths were changed to make the T_m approximately 60 ºC. For miRNAs with very low GC content, lower T_m was set for primer selection and PCR was carried out at a lower

annealing temperature in a separate run. All of the selected sequences were further analyzed with Primer Express Version 3.0 (Applied Biosystems, Foster City, USA) to determine the probability of dimerization and self-priming. miRNA-derived cDNAs were amplified by real-time PCR for 40 cycles using SYBR green real-time PCR master mix kit (Toyobo, Osaka, Japan) on a MiniOpticon (BioRad, Hercules, USA). miRNA-specific primers (**Table 1**) and a common 3′ primer were used for the amplification. Most miRNAs were amplified at an annealing temperature of 60 ºC. For miRNAs with a very low GC content, PCR was carried out at different annealing temperatures (according to the T_m of their primers) using the gradient function of the MiniOpticon. The relative expression level of each miRNA was analyzed by Opticon Monitor 3 software (BioRad) using 5S RNA as a reference gene for the normalization. Amplification specificity was checked by melting curve analysis and electrophoresis of the PCR products on a 3.5% NuSieve GTG agarose gel.

Table 1 **Sequences of primers used in this study**

Primer	Sequence
miR-384*	5′-TGTAAACAATTCCTAGGCAATGT-3′
miR-384	5′-ATTCCTAGAAATTGTTCACAAAA-3′
miR-330*	5′-CTCTGGGCCTGTGTCTTAGG-3′
miR-330	5′-CACAGGGCCTGCAGAGAA-3′
miR-92b	5′-ACTCGTCCCGGCCTCCGA-3′
miR-146b	5′-TGAGAACTGAATTCCATAGGCTGT-3′
let-7g	5′-TGAGGTAGTAGTTTGTACAGT-3′
miR-551b	5′-CGACCCATACTTGGTTTCAG-3′
5sR30	5′-TCTCGTCTGATCTCGGAAGC-3′
3′ primer	5′-GCTGTCAACGATACGCTACG-3′

Results

Identification of miRNAs from a rat hippocampus cDNA library

Sixty clones were initially analyzed by DNA sequencing. Bioinformatic analysis showed that 28% of the cloned RNAs were breakdown products of tRNA or rRNA, 31% were concatamers of 5′ tags and 3′ RT primer sequences without miRNA-derived cDNAs in between, 36% were miRNA-containing clones, and 5% might be breakdown products of mRNA. Next, all the clones were compared on agarose gels with sequencing-verified miRNA-containing clones after *Bam*HI digestion. After precise size screening, approximately 73% of clones proved to be miRNA-containing clones.

Seventy-five clones were identified as miRNAs. Their names and clone numbers are listed in **Table 2**. Of these, 67 clones correspond to known miRNAs. Four sequences (six clones) were not found in the rat miRBase but were identical to hsa-miR-92b, mmu-miR-146b, mmu-let-7g, and mmu-miR-551b. Their flanking genomic sequences could form miRNA precursor-specific hairpin structures (**Fig. 1**), and were therefore designated as rno-miR-92b, rno-miR-146b, rno-let-7g, and rno-miR-551b. Two novel sequences were not found in any organisms. They were derived from the other arm of the known miRNA

Table 2 **Seventy-five microRNAs (miRNAs) identified as clones from a rat hippocampus cDNA library**

miRNA	Clones	miRNA	Clones	miRNA	Clones
rno-let-7c	7	rno-let-7f	1	rno-let-7i	1
rno-miR-9	3	rno-mir-21	1	rno-miR-22	1
rno-miR-23b	1	rno-miR-24	9	rno-miR-26a	6
rno-miR-27a	1	rno-miR-27b	1	rno-miR-29b	1
rno-miR-29c	1	rno-miR-30a-3p	3	rno-miR-30a-5p	1
rno-miR-30c	1	rno-miR-99a	1	rno-miR125b	8
rno-miR-128b	2	rno-miR-129*	1	rno-miR-132	2
rno-miR-138	3	rno-miR-181a	5	rno-miR-181b	2
rno-miR-186	1	rno-mir-221	2	rno-miR-376b*	1
rno-miR-92b	**2**	**rno-miR-146b**	**1**	**rno-let-7g**	**2**
rno-miR-551b	**1**	**rno-miR-330***	**1**	**rno-miR-384***	**1**

The cloned novel miRNA sequences are in bold.

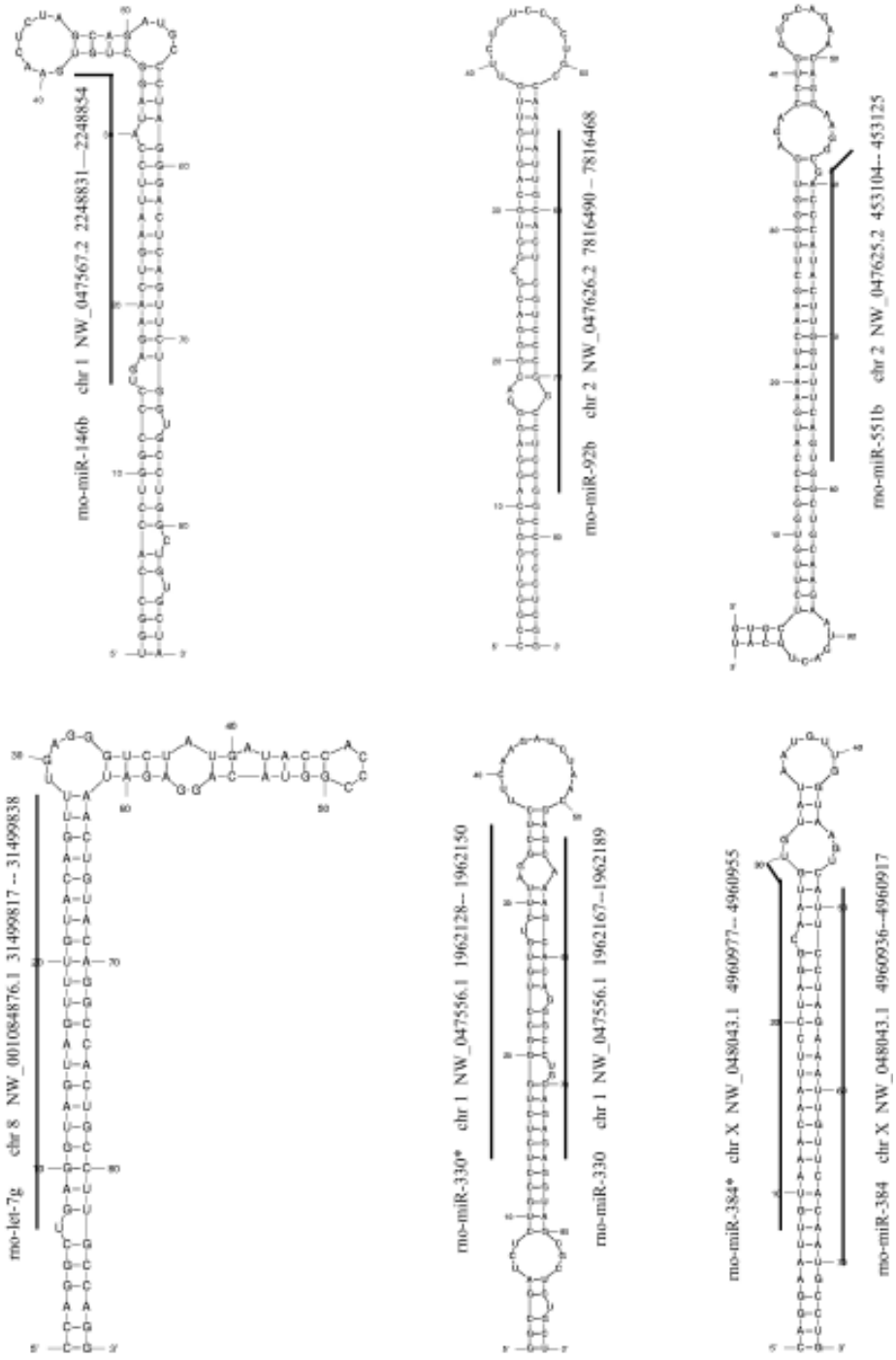

Fig. 1 Predicted secondary structures of microRNA (miRNA) precursors from rat hippocampus

The positions of the novel miRNA sequences in the rat genome are indicated.

precursors rno-miR-330 and rno-miR-384, and were designated as rno-miR-330* and rno-miR-384*. The new miRNAs were submitted to the miRNA Registry website at http://microrna.sanger.ac.uk/registry for official annotation. Their precursor structures are shown in **Fig. 1**.

We also cloned six new short sequences. After an analysis of their 5′ and 3′ flanking genomic sequences using the mFold program, we could not find canonical miRNA precursor hairpin structures. These sequences might be breakdown products of mRNA.

Several of the miRNAs we cloned differ with those reported by others in having one or two additional or missing bases at either the 3′ or 5′ end.

Detecting the expression of the novel miRNAs by real-time PCR

When novel miRNAs are cloned, Northern blots are generally used to confirm the presence of miRNA in size-fractionated RNA. However, Northern blots require large amounts of RNA and many rare miRNAs are present at undetectable levels by standard Northern blot protocols. Therefore, we developed an RNA-tailing and primer-extension real-time RT-PCR assay and confirmed the expression of the newly cloned miRNAs. Using this much more sensitive method, we analyzed the relative expression levels of six new miRNAs and miR-384 and miR-330. The results showed that these eight miRNAs were expressed at significantly higher levels in the hippocampus than in the other tissues (**Table 3** and **Fig. 2**). A single dissociation peak on the thermal melting curve and a single band on the agarose gel signified the amplification specificity for these miRNAs (**Fig. 3**). Comparing the expression of miR-384* and miR-330* with their opposite strand miRNA, we found that miR-384* was 10 times more abundant than miR-384 in rat hippocampus, whereas little difference was found between miR-330* and miR-330 expression in the same tissue.

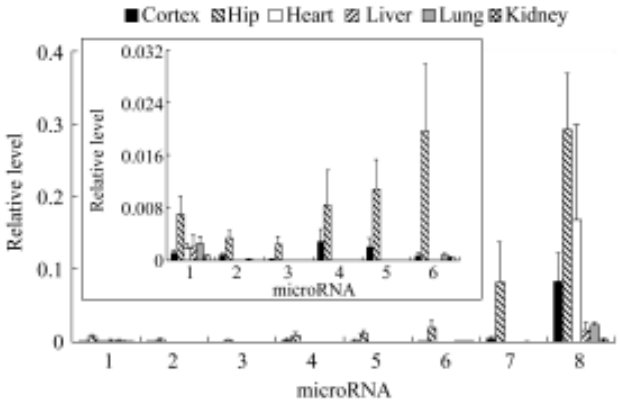

Fig. 2 Relative expression level of eight microRNAs in the cortex, hippocampus (Hip), heart, liver, lung and kidney of Sprague-Dawley rats

Data are represented as mean±SEM from three rats. 1–8, miR-146b, miR-330*, miR-330, miR-384, miR-551b, miR-92b, miR-384*, and let-7g.

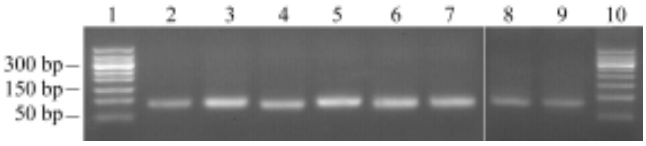

Fig. 3 Specificity of amplifying microRNAs (miRNAs) by real-time reverse transcription-polymerase chain reaction

Single bands were detected on agarose gel for eight miRNAs amplified from rat hippocampus. 1 and 10, 50 bp DNA ladder; 2, miR-384*; 3, miR-330*; 4, miR-92b; 5, miR-146b; 6, miR-330; 7, miR-384; 8, miR-551b; 9, let-7g.

Table 3 Relative expression level of eight microRNAs (miRNAs) in the cortex, hippocampus, heart, liver, lung and kidney of Sprague-Dawley rats (mean±SEM)

miRNA	Cortex	Hippocampus	Heart	Liver	Lung	Kidney
miR-146b	0.0011 ±0.000488	0.007112 ±0.002738	0.001796 ±0.000842	0.002051 ±0.001776	0.002586 ±0.001027	0.000917 ±0.000134
miR-330*	0.000785 ±0.000299	0.00342 ±0.001081	Neg.	Neg.	0.000202 ±0.000092	0.000049 ±0.000007
miR-330	0.000187 ±0.000092	0.002383 ±0.001328	Neg.	Neg.	0.000028 ±0.000014	0.000007 ±0.000001
miR-384	0.002928 ±0.001784	0.008475 ±0.005264	Neg.	Neg.	Neg.	Neg.
miR-551b	0.002089 ±0.001402	0.010974 ±0.004294	Neg.	Neg.	0.000021 ±0.000015	Neg.
miR-92b	0.000745 ±0.000451	0.019671 ±0.010179	Neg.	Neg.	0.00076 ±0.000334	0.000592 ±0.000157
miR-384*	0.005261 ±0.002256	0.082367 ±0.05538	0.000271 ±0.000222	0.00015 ±0.000108	0.000297 ±0.000198	0.000185 ±0.000090
let-7g	0.083773 ±0.038969	0.291367 ±0.078699	0.167873 ±0.13186	0.016014 ±0.011252	0.024367 ±0.002997	0.003505 ±0.000801

Neg., negtive.

We also detected the expression of these eight miRNAs in other tissues of rats, including cerebral cortex, heart, liver, lung and kidney. miR-330*, miR-330, and miR-92b were negative in the heart and liver. miR-384 was negative in the heart, liver, lung and kidney. miR-551b was negative in the heart, liver and kidney. The others were expressed at much lower levels, as shown in **Fig. 2**. Negative samples and those containing very low levels of template usually showed non-specific amplification, as shown by melting curve analysis and *C*t values above 32.

One of the six new short sequences that did not meet the "biogenesis" criteria was also detected for its expression by real-time RT-PCR and got a negative result. All no-template controls and non-reverse transcription controls showed to be negative or their *C*t values were above 32.

Discussion

The central nervous system already has the highest number of specifically expressed miRNAs [19–21]. Cloning many new miRNAs will be more difficult, as shown by the fact that we found only six novel miRNAs from 75 clones. These six miRNAs meet both the "expression" and "biogenesis" criteria for bona fide miRNAs put forward by Ambros *et al*. [22]. Their higher expression in the hippocampus suggests that they might have important roles in hippocampal function.

The exact roles of most miRNAs are not known. Establishing the spatial and temporal expression pattern of miRNAs will help in narrowing down the potential target genes. Three real-time PCR methods have been reported for determining the concentration of individual mature miRNAs [18,23,24]. We developed an RNA-tailing and primer-extension real-time RT-PCR method based on the method of Shi and Chiang [18] with some modifications [17]. This method has a high dynamic range, can detect low abundance miRNAs, and requires much less RNA than Northern blots and microarrays. Different miRNAs from various samples can be assessed in a single run. Compared with the other two methods, we could use a common primer for RT, which greatly reduced the cost.

Some of the cloned miRNAs are from the 5′ arm and others are from the 3′ arm of their precursors. However, more and more miRNAs are found originating from both arms of a hairpin precursor. It is believed that the miRNAs with biological function are usually abundant, as the RISC might have protected them from degradation. The opposite strands of miRNAs are thought to be non-functional byproducts of the reaction catalyzed by the Dicer endonuclease and should be less abundant. Our results showed that two miRNAs originating from the same hairpin precursor might express at similar (like miR-330 and miR-330*) or significantly different levels (like miR-384 and miR-384*). This phenomenon was also observed by Cummins *et al*. [25] and thus brought about several questions. When both of them are present in the same tissues at high levels, do they function together or separately? Is it possible that certain miRNA functions in some tissues, while its partner from the same hairpin precursor functions in other tissues? These questions deserve further investigation.

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