Research

Microarray analysis of microRNA expression in the developing mammalian brain

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Abstract

Background: MicroRNAs are a large new class of tiny regulatory RNAs found in nematodes, plants, insects and mammals. MicroRNAs are thought to act as post-transcriptional modulators of gene expression. In invertebrates microRNAs have been implicated as regulators of developmental timing, neuronal differentiation, cell proliferation, programmed cell death and fat metabolism. Little is known about the roles of microRNAs in mammals.

Results: We isolated 18-26 nucleotide RNAs from developing rat and monkey brains. From the sequences of these RNAs and the sequences of the rat and human genomes we determined which of these small RNAs are likely to have derived from stem-loop precursors typical of microRNAs. Next, we developed a microarray technology suitable for detecting microRNAs and printed a microRNA microarray representing 138 mammalian microRNAs corresponding to the sequences of the microRNAs we cloned as well as to other known microRNAs. We used this microarray to determine the profile of microRNAs expressed in the developing mouse brain. We observed a temporal wave of expression of microRNAs, suggesting that microRNAs play important roles in the development of the mammalian brain.

Conclusion: We describe a microarray technology that can be used to analyze the expression of microRNAs and of other small RNAs. MicroRNA microarrays offer a new tool that should facilitate studies of the biological roles of microRNAs. We used this method to determine the microRNA expression profile during mouse brain development and observed a temporal wave of gene expression of sequential classes of microRNAs.

Background

MicroRNAs constitute a large class of small regulatory RNAs [1]. Their mechanism of action and the scope of their biological roles are beginning to be understood. The first two microRNAs were discovered as the products of heterochronic genes that control developmental timing in *Caenorhabditis elegans* [2]. In heterochronic mutants, the timing of specific developmental events in several tissues is altered relative to the timing of events in other tissues. These defects result from temporal transformations in the fates of specific cells; that is, certain cells acquire fates normally expressed by cells at other developmental stages. The molecular characterization of the heterochronic gene *lin-4* led to the surprising discovery that this gene encodes a 21-nucleotide non-coding RNA that regulates the translation of *lin-14* mRNA through base-pairing with the *lin-14* 3' UTR [3,4]. A second heterochronic gene, *let-7*, encodes another small non-coding RNA that is conserved in flies and mammals [5].

Biochemical and bioinformatic approaches have identified many genes that encode microRNAs in *C. elegans*, plants, *Drosophila melanogaster* and mammals [6-18]. Like the *lin-4* and *let-7* genes, other microRNAs encode 21-25-nucleotide RNAs derived from longer transcripts that are predicted to form stem-loop structures. More than 200 microRNAs are encoded by the human genome [8,14].

The biological roles of microRNAs are poorly understood. In *C. elegans*, *lin-4* and *let-7* act in developmental timing, and the microRNA *lsy-6* controls neuronal asymmetry [19]. In *Drosophila*, the microRNAs bantam and mir-14 act in the regulation of cell growth and cell death [20,21]. The mouse microRNA miR-181 has been implicated in the modulation of hematopoietic differentiation, and other mammalian microRNAs have been suggested to play roles in cancer [22,23].

Mature microRNAs are excised from a stem-loop precursor that itself can be transcribed as part of a longer primary RNA (pri-miRNA) [24]. The pri-miRNA appears to be processed by the RNAse Drosha in the nucleus, cleaving the RNA at the base of the stem-loop [25]. This cut defines one end of the microRNA. The precursor microRNA is then exported by Ran-GTP and Exportin-5 to the cytoplasm, where it is further processed by the RNAse Dicer [26,27]. Dicer recognizes the stem portion of the microRNA and cleaves both strands about 22 nucleotides from the base of the stem [25]. The two strands in the resulting double-stranded (ds) RNA are differentially stable, and the mature microRNA resides on the strand that is more stable [28,29]. Mature microRNAs can be found associated with the proteins eIF2C2 (an Argonaute-like protein), Gemin2 and Gemin3 and are thought to act in a protein-RNA complex with these and maybe other proteins [17,30].

The animal microRNAs studied so far act by reducing the levels of proteins from genes that encode mRNAs with sites partially complementary to microRNAs in their 3' UTRs [4,31]. The mechanism responsible is not understood in detail [32]. In contrast, although some plant microRNAs with partially complementary target sites also act by preventing translation, the majority studied so far cause the cleavage of target mRNAs at sites perfectly complementary to the microRNAs [33-38].

Determining spatial and temporal patterns of microRNA expression should yield insight into the biological functions of microRNAs. As the number of microRNAs identified has increased rapidly, the need for a method that allows for the parallel detection of microRNA expression has become apparent. Recent studies used a dot-blot technique to study 44 mouse microRNAs and northern blotting analysis to study 119 microRNAs from mouse and human organs [39,40].

In this study we cloned microRNAs from rat and monkey brains, developed a microRNA labeling method and used a microarray to monitor expression of microRNAs during mouse brain development. We determined the temporal expression pattern of 138 microRNAs in the mouse brain and found that the levels of 66 microRNAs changed significantly during development. We identified sets of genes with similar expression patterns, including genes that peaked in expression at different stages of development. More generally, the microRNA microarray we have developed can be used to determine the expression of all known microRNAs simultaneously under any set of experimental conditions or constraints.

Results and discussion

Identification of microRNAs from developing rat and monkey brains

To analyze microRNAs expressed in the developing mammalian brain, we cloned small 18-26-nucleotide RNAs from the neocortex and hippocampus of a 12-day postnatal rat (*Rattus norvegicus*) and from the cerebral wall of a 114-day-old fetal rhesus monkey (*Macaca mulatta*) (Table 1). In both species, by these stages most neurons have been generated and have begun synaptogenesis [41,42]. We identified a total of 1,451 sequences, 413 of which correspond to microRNA sequences on the basis of their potential to generate stem-loop precursors as predicted from corresponding sequences in the rat and/or human genomes. In all cases but one, the microRNAs we identified corresponded to known microRNAs from other species and defined 68 unique microRNAs (Table 1 and Additional data file 1). One of these microRNAs is new: it differs in sequence from any microRNA previously described and is conserved in the mouse and human genomes. We named this new microRNA rno-miR-421 (Figure 1 and Additional data file 2). As observed in similar studies, in addition to microRNAs a number of candidate small RNAs that do not fulfill all criteria of a microRNA were cloned (Additional data file 3) [9,43]. Of the 52 rat microRNA sequences we cloned, 27 had previously been cloned from rat primary cortical neurons

Table 1

Identity, frequency and size range of microRNAs cloned from the cortex and hippocampus of 12-day postnatal *R. norvegicus* and the cortex of a 114-day old *M. mulatta* fetus

Rattus norvegicus microRNAs			*Macaca mulatta* microRNAs		
Name	Number of times cloned	Size range	Name	Number of times cloned	Size range
rno-miR-421	2	21			
rno-let-7a	3	22	mml-let-7a	15	21
			mml-let-7a or c	1	18
rno-let-7b	1	23	mml-let-7b	20	22-23
rno-let-7c	10	22	mml-let-7c	9	21-22
rno-let-7d	1	22	mml-let-7d	1	22
			mml-let-7e	3	20-22
			mml-let-7f	3	22
			mml-let-7g	2	22
rno-let-7i	1	22	mml-let-7i	2	22
rno-miR-7	5	21			
			mml-miR-7-1	1	22
rno-miR-9	2	23	mml-miR-9	9	21-23
rno-miR-16	2	22	mml-miR-16	2	22
rno-miR-17-5p	3	23	mml-miR-17-5p	2	22-23
rno-miR-24	6	21-22			
			mml-miR-26a	3	21-22
rno-miR-26b	1	22			
rno-miR-28	1	22			
rno-miR-29a	4	22			
rno-miR-29b	7	22-23			
rno-miR-29c	2	20,22			
rno-miR-30b	1	22	mml-miR-30b	2	22
rno-miR-30c	3	23-24	mml-miR-30c	1	21
			mml-miR-33	2	20
rno-miR-92	2	22	mml-miR-92		
rno-miR-93	1	23			
rno-miR-99a	1	21	mml-miR-99a	4	20-22
rno-miR-99b	2	21,22	mml-miR-99b	2	22
			mml-mir-100	1	22
rno-miR-101b	1	22			
rno-miR-103	3	23	mml-miR-103	2	22-23
			mml-miR-103 or 107	1	21
rno-miR-124a	19	19-22	mml-miR-124a	97	18-23
rno-miR-125a	2	22,24	mml-miR-125a	4	22-23
rno-miR-125b	12	21-22	mml-miR-125b	17	20-22
			mml-miR-126	1	21
			mml-miR-126*	1	22
rno-miR-127	1	20			
rno-miR-128a	3	21-22	mml-miR-128a	9	22
rno-miR-128a or b	2	21	mml-miR-128a or b	17	18-21
rno-miR-128b	1	21	mml-miR-128b	8	22
rno-miR-129	2	21-22	mml-miR-129-2	1	22
rno-miR-130a	1	22			
rno-miR-132	6	22			

Table 1 *(Continued)*

Identity, frequency and size range of microRNAs cloned from the cortex and hippocampus of 12-day postnatal *R. norvegicus* and the cortex of a 114-day old *M. mulatta* fetus

rno-miR-136	2	23	mml-miR-136	1	23
			mml-miR-137	1	23
rno-miR-138	5	23-24			
rno-miR-139	1	23			
			mml-miR-140	1	22
rno-miR-140*	1	22			
rno-miR-142-3p	1	23			
rno-miR-145	1	23	mml-miR-145	2	22
rno-miR-146	2	23			
			mml-miR-149	2	23
rno-miR-150	4	22-23			
rno-miR-154	1	22			
			mml-miR-181a or 213	4	20-25
			mml-miR-181b	1	24
			mml-miR-181c	1	21
rno-miR-185	2	22-23	mml-miR-185	1	23
rno-miR-191	3	23-24			
			mml-miR-195	1	22
rno-miR-213	1	22			
			mml-miR-221	3	22-23
rno-miR-300	1	21			
rno-miR-323	1	22			
rno-miR-324	4	23			
rno-miR-325	1	22			
rno-miR-338	5	23			
rno-miR-342	1	25			
rno-miR-345	1	22			
Total	152		Total	261	

The rat (rno) and monkey (mml) microRNA names are indicated. Two microRNA names are assigned to the same clone when the cloned sequence is too short to distinguish between the microRNAs. mml-miR-7 and mml-miR-129 are encoded by three and two distinct genomic loci, respectively, although the sequences immediately adjacent to these microRNA sequences differ. The sequences we cloned for mml-miR-7-1 and mml-miR-129-2 were one base longer than that shared by the microRNAs, allowing us to determine the loci from where they originated, as indicated by -1 and -2. Notation follows the miRNA registry guidelines [53].

[11]. For 21 of the 52 microRNAs from rat and 14 of the 40 microRNAs from monkey we isolated only a single clone, indicating that our surveys are not saturated. By contrast, we isolated microRNA miR-124a 19 times from rat and 97 times from monkey. Mouse miR-124a as well as miR-128, miR-101 and miR-132 have been reported to be expressed specifically in brain [15]. We found that rat miR-138 also was expressed only in brain (Additional data file 4).

MicroRNA microarrays for the study of temporal and spatial patterns of microRNA expression

Previous analyses of microRNA expression have relied on dot blots, northern blots and cloning strategies [8,11-14,18,39,40]. A highly scalable approach using a microarray would facilitate the analysis of microRNA expression patterns for a large number of samples and is feasible now that many mammalian microRNAs have been identified.

We arrayed 138 oligonucleotides complementary to microRNAs (probes) corresponding to the 68 mammalian microRNAs we isolated from rat and monkey brains, to 70 mammalian microRNAs isolated by others from a variety of mouse tissues and mammalian cell lines, and to predicted microRNAs. In addition, we included a set of control probes as well as 19 probes corresponding to presumptive small RNAs that we and others identified but that do not satisfy all the criteria for a microRNA (see below and Additional data file 5). Probes had a free amine group at the 5' terminus and

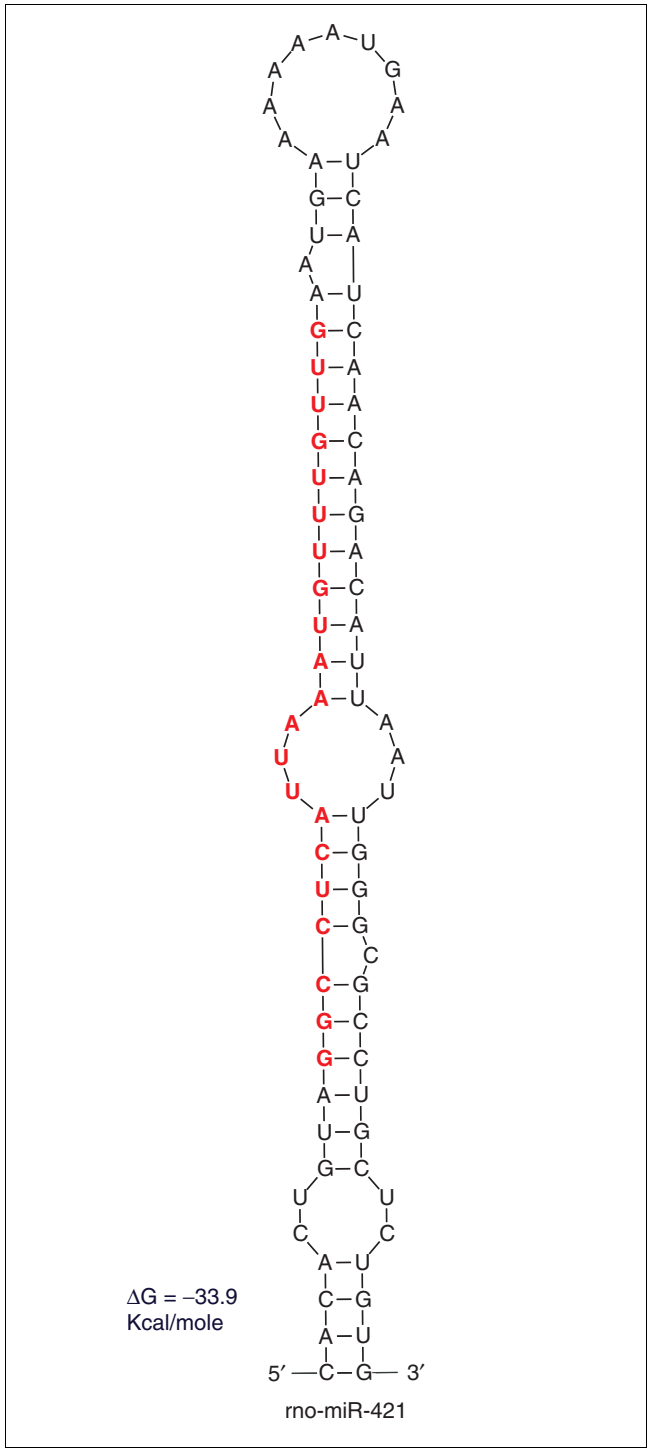

Figure 1
Predicted stem-loop structure of a novel mammalian microRNA, rno-miR-421. The stem-loop structure was predicted from sequences adjacent to rno-miR-421 in the rat genome. The cloned (mature) sequence is shown in red. The predicted secondary structure and the free energy calculation (ΔG, kcal/mole) were generated by the mfold software [52].

were printed onto amine-binding glass slides and covalently linked to the glass surface. All probes were printed in quadruplicate (Additional data file 5).

We developed a method for preparing microRNA samples for microarray analysis. Several methods for mRNA sample labeling for microarray analysis have been described [44-47], but none is suitable for labeling RNAs as small as microRNAs. To fluorescently label small RNAs we adapted strategies for RNA ligation and reverse transcription PCR (RT-PCR) devised for microRNA cloning [12-14]. Briefly, 18-26-nucleotide RNAs were size-selected from total RNA using denaturing polyacrylamide gel electrophoresis (PAGE), oligonucleotide linkers were attached to the 5' and 3' ends of the small RNAs and the resulting ligation products were used as templates for an RT-PCR reaction with 10 cycles of amplification. The sense-strand PCR primer had a Cy3 fluorophore attached to its 5' end, thereby fluorescently labeling the sense strand of the PCR product. The PCR product was denatured and then hybridized to the microarray. As in microarray analysis, the labeled sample used for hybridization is referred to as the target. Significant biases in amplification, a problem when amplifying heterogeneously sized mRNAs, are less likely in the case of microRNAs because of their short uniform lengths. MicroRNA cloning frequencies obtained using a similar amplification strategy correlate well with expression levels as assayed by quantitative northern blots [7]. Because RNA is amplified before hybridization, relatively low amounts of starting material may be used with this method [8,11-14,18,39,40].

We optimized the conditions for hybridization to our microarray. The small sizes of microRNAs leave little opportunity for oligonucleotide (array probe) design to achieve homogeneous probe-target melting temperatures. Differences in melting temperatures are expected to result in greater non-specific binding if hybridizations are performed at low temperatures (to allow the detection of probe-target pairs with low melting temperatures) and in less specific binding if hybridizations are performed at high temperatures (to specifically detect probe-target pairs with high melting temperatures). To assess this issue we included control probes with two internal mismatches on the microarray for a subset of the microRNA probes (Additional data file 5). We tested a range of hybridization temperatures, and, on the basis of the signal of microRNA probes versus control probes, we determined that a hybridization temperature of 50°C was a reasonable compromise between sensitivity and specificity (data not shown).

Even at 50°C, specificity as assayed by comparing microarray spot signal intensities from matched and mismatched probes varied among the microRNAs assayed. As expected, specificity at 50°C was negatively correlated with calculated melting temperatures (Figure 2a). In all cases the cumulative signal from 10 hybridizations for the mismatched probe was equal

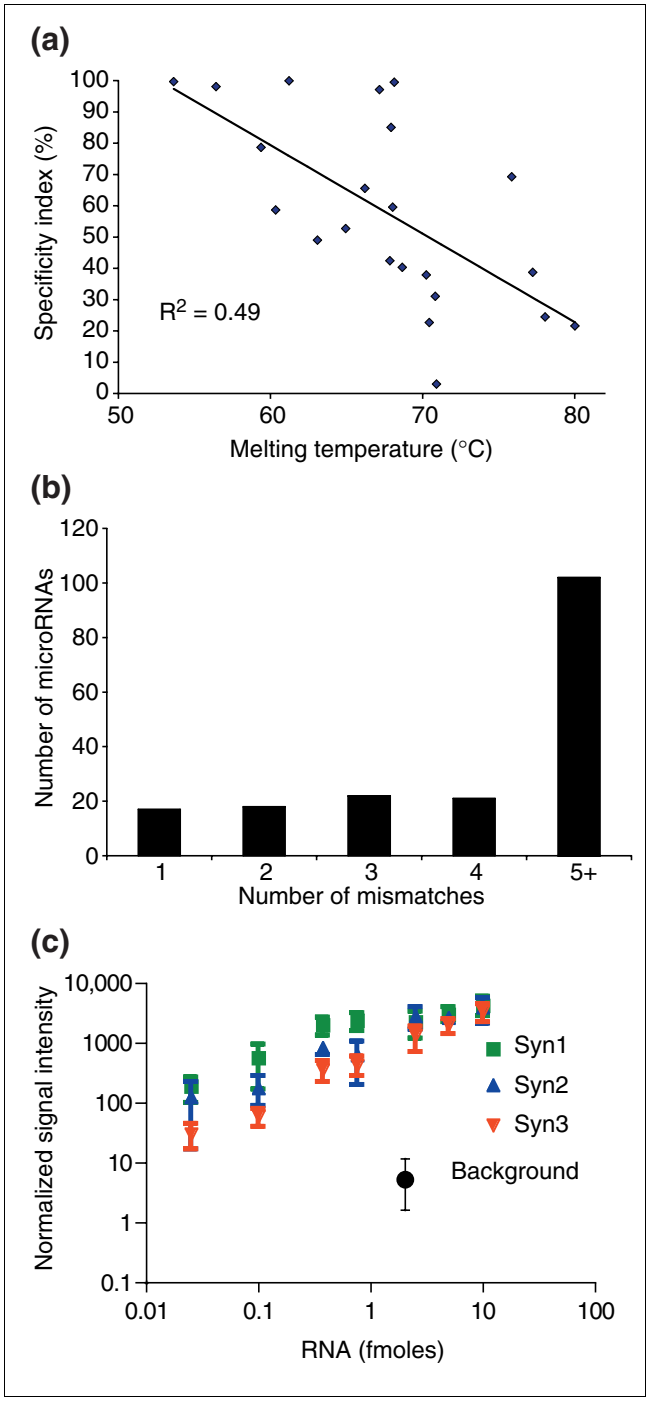

Figure 2

Figure 2
MicroRNA microarray specificity and quantification. **(a)** Specificity was assayed using a set of 23 microRNA and mismatched probe pairs (two mismatches). Average mean spot intensities from 10 independent hybridizations at 50°C were added to give a total signal for probes corresponding to a given microRNA as well as for probes with two mismatches to the microRNA. Mismatch probe design and sequences are described in Additional data file 6. A specificity index was calculated as 100 × (probe signal - mismatched probe signal)/probe signal. Melting temperatures for the microRNA probes were calculated using the nearest neighbors method [54]. The specificity index is plotted against the calculated melting temperature for each microRNA probe pair. Correlation of melting temperature and specificity index is significant (p = 0.004, Student's t-test). **(b)** Number of mismatches between microRNAs based on all known mouse microRNAs (the miRNA Registry 3.0 [53]). Each microRNA was aligned pairwise to every other microRNA and was assigned to the group (number of mismatches) corresponding to the least number of mismatches to another microRNA. **(c)** Quantification of microarray data using three synthetic RNAs: syn1, syn2 and syn3. Each data point is the average of two independent labelling/hybridization reactions. Probes for the three synthetic RNAs were printed in quintuplicate on the microarray. RNAs were used at 0.025, 0.1, 0.375, 0.75, 2.5, 5 and 10 fmoles. For comparison, the background signal of the array is shown. For more details, see Additional data file 5.

to or lower than that for the microRNA probe, but differences in the ratio of the matched to mismatched probe signal ranged widely (Figure 2a). Given these data, we do not expect the microRNA microarray to distinguish reliably between microRNAs that have only one or a few mismatches. This limitation is alleviated somewhat by the fact that for most microRNAs that have been identified the most closely related paralogs differ by five mismatches or more (Figure 2b). The signal from a mismatched control probe is likely to be caused by cross-hybridization with the microRNA for which it was designed, as other control probes corresponding to unrelated mRNA subsequences or synthetic probes that do not correspond to known microRNAs did not show signals above background (Additional data file 5). Microarray results for closely related microRNAs should be interpreted with caution, as differences in the apparent expression of a given microRNA could be dampened or exaggerated depending on the expression of the paralogs (Figure 2a).

To determine the detection range of the microarray, we synthesized three artificial RNAs with the characteristics of microRNAs. These RNAs were phosphorylated RNA oligonucleotides of 20-23 bases; their sequences were chosen at random and were without any significant sequence similarity to known mammalian microRNAs (see Additional data file 5 for details). We titrated these RNAs into total mouse RNA samples, labeled them and hybridized them to a microarray that in addition to microRNA probes included probes corresponding to these three RNAs, called syn1, syn2 and syn3. Figure 2c shows the correlation between the amount of the RNAs and the microarray signal intensities. For comparison, the background signal for the array is also shown. All three RNAs were reliably detected at levels as low as 0.1 fmoles. The dynamic

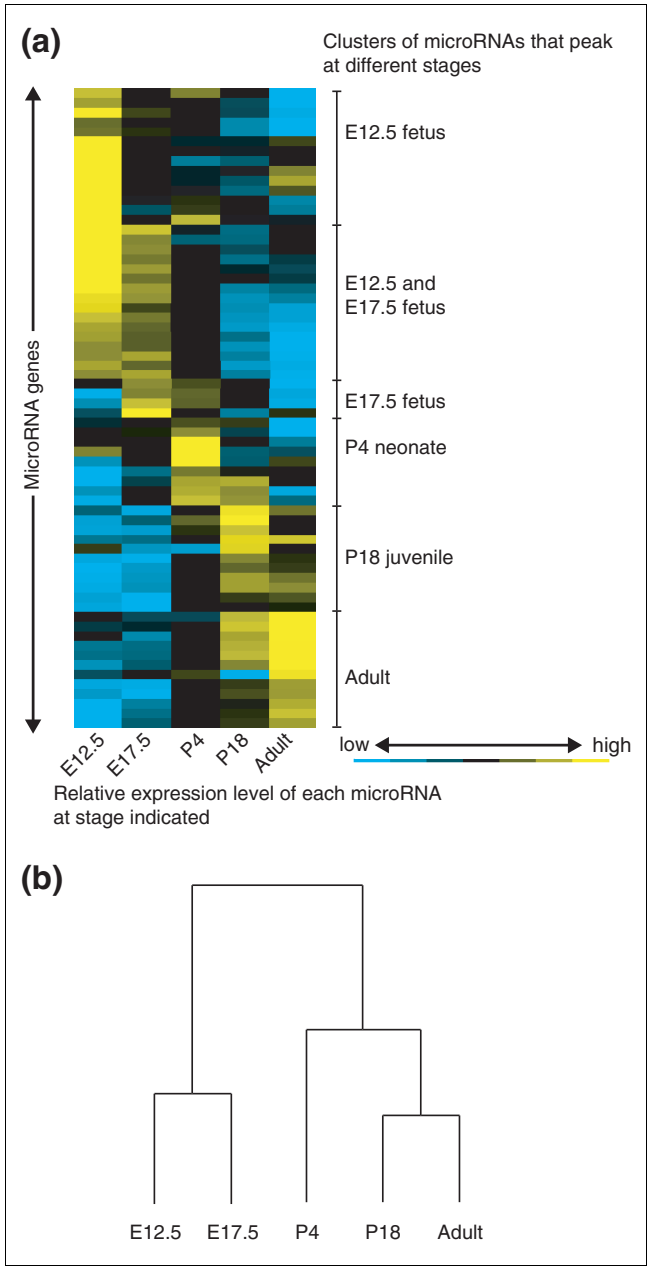

Figure 3
Profile of microRNA expression in the developing mouse brain. **(a)** Relative expression levels for the 66 microRNAs that changed significantly (ANOVA, $p < 0.001$) and more than twofold are shown in five columns corresponding to the five time points. Colors indicate relative signal intensities. The microRNA expression profile was sorted using a hierarchical clustering method, and major clusters are shown ordered according to the time that expression peaks. Gene names and a quantitative description of microRNA expression levels are presented in Additional data file 6. **(b)** Developmental time points were grouped using the same hierarchical clustering method and gene set as in (a).

range of the array was from 0.1 fmoles to at least 10 fmoles, or two orders of magnitude.

Analysis of microRNA expression during mouse brain development

We isolated small RNAs from mice at five developmental stages: embryonic days 12.5 and 17.5 (E12.5 and E17.5), postnatal days 4 and 18 (P4 and P18) and 4-month-old adults. E12.5-E17.5 spans a period of major neuronal proliferation and migration in the mouse brain, in particular the birth and subsequent migration of most neurons in the ventricular zone epithelium [48]. Between postnatal days P4 and P18, major sensory inputs are established. For example, eye opening occurs around P13 and is thought to result in activity-dependent neuronal remodeling [49].

We purified and size-selected RNA from whole mouse brains. For each sample, the products of four independent RNA amplifications based on two independent RNA ligations were hybridized to the array. A detailed description of our analysis of the microarray data is presented in Additional data file 5. Of the 138 microRNAs and 19 small RNAs represented by the probe set, 116 (74%) were expressed robustly (more than 75-fold over the level of background controls) at least at one time point. Of these, 83 (71%) changed significantly during the period surveyed (analysis of variance, ANOVA, $p < 0.001$) and 66 (57%) changed more than twofold. Of the microRNAs we cloned from rat and monkey and for which probes against the corresponding mouse homologs were present on the microarray, we detected 97% robustly.

We grouped microRNAs that changed more than twofold in expression during the period analyzed using a hierarchical clustering algorithm (Figures 3a,4) [50]. A group of microRNAs peaked at each of the developmental time points. The signal from 34 of the 66 probes that changed more than twofold peaked in the fetus (E12.5 and E17.5), suggesting roles in early development (Figure 4a). Nine and eleven microRNAs peaked during the neonate (P4) and juvenile (P18) stages, respectively. Twelve microRNAs had the highest signals at the adult stage (Figure 4b). These data indicate that murine brain development involves a wave of expression of sequential classes of microRNAs (Figure 3a).

We also grouped the developmental time points according to their microRNA expression pattern using hierarchical clustering. We found that samples from stages that are developmentally proximal had the most similar microRNA expression patterns (Figure 3b), indicating that a microRNA expression profile can be a marker of developmental stage. Examination of the temporal clusters revealed that probes with similar sequences showed correlated expression, as exemplified by miR-181a, miR-181b, miR-181c, smallRNA-12 (Figure 4a) and miR-29a, miR-29b and miR-29c (Figure 4b), respectively. Given our observation that the microRNA microarray can detect mismatched sequences, it is possible that this correlation among closely related family members is an artifact of hybridization.

(a)

MicroRNA	Oligo	Oligo sequence	
mmu-miR-23b	EAM261	GTGGTAATCCCTGGCAATGTGAT	
mmu-miR-139	EAM206	AGACACGTGCACTGTAGA	
mmu-miR-29b	EAM119	AACACTGATTTCAAATGGTGCTA	miR-29 family
mmu-miR-29c	EAM279	TAACCGATTTCAAATGGTGCTA	
mmu-miR-29a	EAM268	AACCGATTTCAGATGGTGCTAG	
mmu-miR-132	EAM137	CCGACCATGGCTGTAGACTGTTA	
mmu-miR-125b	EAM105	TCACAAGTTAGGGTCTCAGGGA	
mmu-miR-129	EAM289	AACAAGCCCAGACCGCAAAAAG	
mmu-miR-22	EAM255	ACAGTTCTTCAACTGGCAGCTT	
mmu-let-7h[14]	EAM182	AACTGTACACACTACTACCTCA	
mmu-miR-128b	EAM195	GAAAGAGACCGGTTCACTGTGA	miR-128 family
mmu-miR-128a	EAM194	AAAAGAGACCGGTTCACTGTGA	

(b)

MicroRNA	Oligo	Oligo sequence	
hsa-miR-199b	EAM235	GAACAGATAGTCTAAACACTGGG	miR-199 family
mmu-miR-199b	EAM282	GAACAGGTAGTCTAAACACTGGG	
mmu-miR-130a	EAM159	ATGCCCTTTTAACATTGCACTG	
mmu-miR-18	EAM225	TATCTGCACTAGATGCACCTTA	
mmu-miR-19b	EAM237	TCAGTTTTGCATGGATTTGCACA	
mmu-miR-181a	EAM226	ACTCACCGACAGCGTTGAATGTT	miR-181 family
mmu-miR-181b	EAM227	AACCCACCGACAGCAATGAATGTT	
mmu-miR-181c	EAM228	ACTCACCGACAGGTTGAATGTT	
smallRNA-12	EAM156	ACTCACCGAGAGCGTTGAATGTT	
mmu-miR-9	EAM276	TCATACAGCTAGATAACCAAAGA	
mmu-miR-324-5p	EAM133	ACACCAATGCCCTAGGGGATGCG	
mmu-miR-320	EAM175	TCGCCCTCTCAACCCAGCTTTT	
mmu-miR-149	EAM216	GGAGTGAAGACACGGAGCCAGA	
mmu-miR-134	EAM202	TCCCTCTGGTCAACCAGTCACA	

(c)

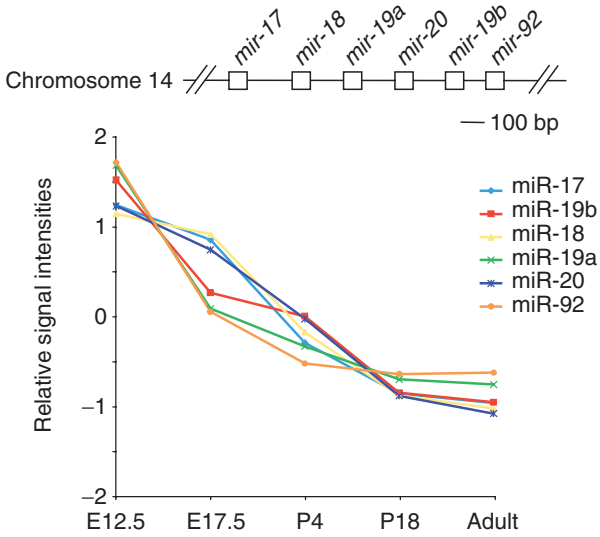

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Examples of co-regulated microRNAs.**(a)** MicroRNAs with a sharp peak at the E12.5 stage. Methods were as described for Figure 3. Brackets indicate closely related sequences. **(b)** MicroRNAs with a single sharp peak at the adult stage. **(c)** Co-regulation of microRNAs derived from the *mir-17* cluster from chromosome 14. To compare signal intensities, data were transformed to give a mean of 0 and a standard deviation of 1.

We found that four of the 66 RNAs that changed more than twofold were small RNAs rather than microRNAs. The temporal regulation of these small RNAs indicates that they may play a role during development.

Several mouse microRNAs are clustered closely in the genome, suggesting that they might be expressed from a single precursor transcript or at least share promoter/enhancer elements. We searched all known microRNA clusters in the mouse genome to attempt to identify coordinately controlled clustered microRNAs. We sought clusters with the following features: first, the clustered microRNAs are not all members of the same family; second, the microRNAs have no or few paralogs; and third, the microRNAs are detected robustly on our microarray and their expression changes significantly during the timecourse studied. The *mir-17* cluster on chromosome 14 fulfills all these criteria. Figure 4c shows that the expression of all six microRNAs in this cluster is indeed highly co-regulated.

Validation of microarray results using northern blots

To validate our microarry results, we performed northern blots of eight microRNAs that were robustly expressed at least at one point during development according to our microarray data. The relative changes of microRNA expression assayed using microarray analysis and northern blots were consistent (Figure 5). For example, on a northern blot miR-29b was almost undetectable at the embryonic and P4 stages but appeared at P18 and was strongly expressed in the adult. The microarray data showed a similar pattern. In only a few cases did there seem to be discrepancies; for example, relative levels of expression of miR-138 at P4 compared to adult differed between the northern blots and the microarrays. As is the case for mRNAs, small differences may be seen between the methods and northern blot analysis is superior to microarrays for quantitative analysis [51]. Nonetheless, microarrays offer a high-throughput method that generally captures changes in microRNA expression.

Conclusions

Here we describe the development of a microarray technology for profiling the expression of microRNAs and other small RNAs and apply this technology to the developing mammalian brain. Recently, Krichevsky *et al.* described the temporal expression of 44 microRNAs during mouse brain development [39]. Their study used a dot-blot array approach and direct labeling of microRNAs using radioactivity instead of a glass microarray and RT-PCR/fluorescent labeling, as we used in our study. Despite differences in sample selection as well as in the number of microRNAs analyzed, there is good agreement between the overlapping aspects of the two datasets. Our strategy has the potential to be highly scalable, allowing high-throughput analysis of samples with limiting starting material.

MicroRNA microarrays offer a new tool that should facilitate studies of the biological roles of microRNAs. We speculate that some of the developmentally regulated microRNAs we describe in this report play roles in the control of mammalian brain development, possibly by controlling developmental timing, by analogy to the roles of the *lin-4* and *let-7* microRNAs in *C. elegans*.

Materials and methods

MicroRNA cloning

We isolated RNAs and cloned microRNAs from *R. norvegicus* and *M. mulatta* using methods described previously [13], except that the samples were not dephosphorylated during the cloning procedure.

Microarray printing and hybridization

Microarray probes were oligonucleotides (named EAM followed by a number) with sequences complementary to microRNAs. Each probe was modified with a free amino group linked to its 5' terminus through a 6-carbon spacer (IDT) and was printed onto amine-binding slides (CodeLink, Amersham Biosciences). Control probes contained two internal mismatches resulting in either C-to-G or T-to-A changes (Additional data file 6). Printing and hybridization were done using the protocols from the slide manufacturer with the following modifications: the oligonucleotide concentration for printing was 20 μM in 150 mM sodium phosphate pH 8.5, and hybridization was at 50°C for 6 h. Printing was done using a MicroGrid TAS II arrayer (BioRobotics) at 50% humidity.

Sample and probe preparation

Whole brains from three to eight C57BL/6 mice were pooled. Starting with 250 μg of total RNA for each time point, 18-26-nucleotide RNA was purified on denaturing PAGE gels. The samples were divided, and the following cloning steps were done independently twice for each time point. 3' and 5' adaptor oligonucleotides were ligated to 18-26-nucleotide RNA followed by reverse transcription, essentially as described for microRNA cloning [13]. Briefly, a RNA-DNA hybrid 5'-pUUUaaccgcgaattccagt-idT-3' (Dharmacon: X, RNA; x, DNA; p, phosphate; idT, inverted [3'-3' bond] deoxythymidine) was ligated to the 3' end and 5'-acggaattcctcactAAA-3'

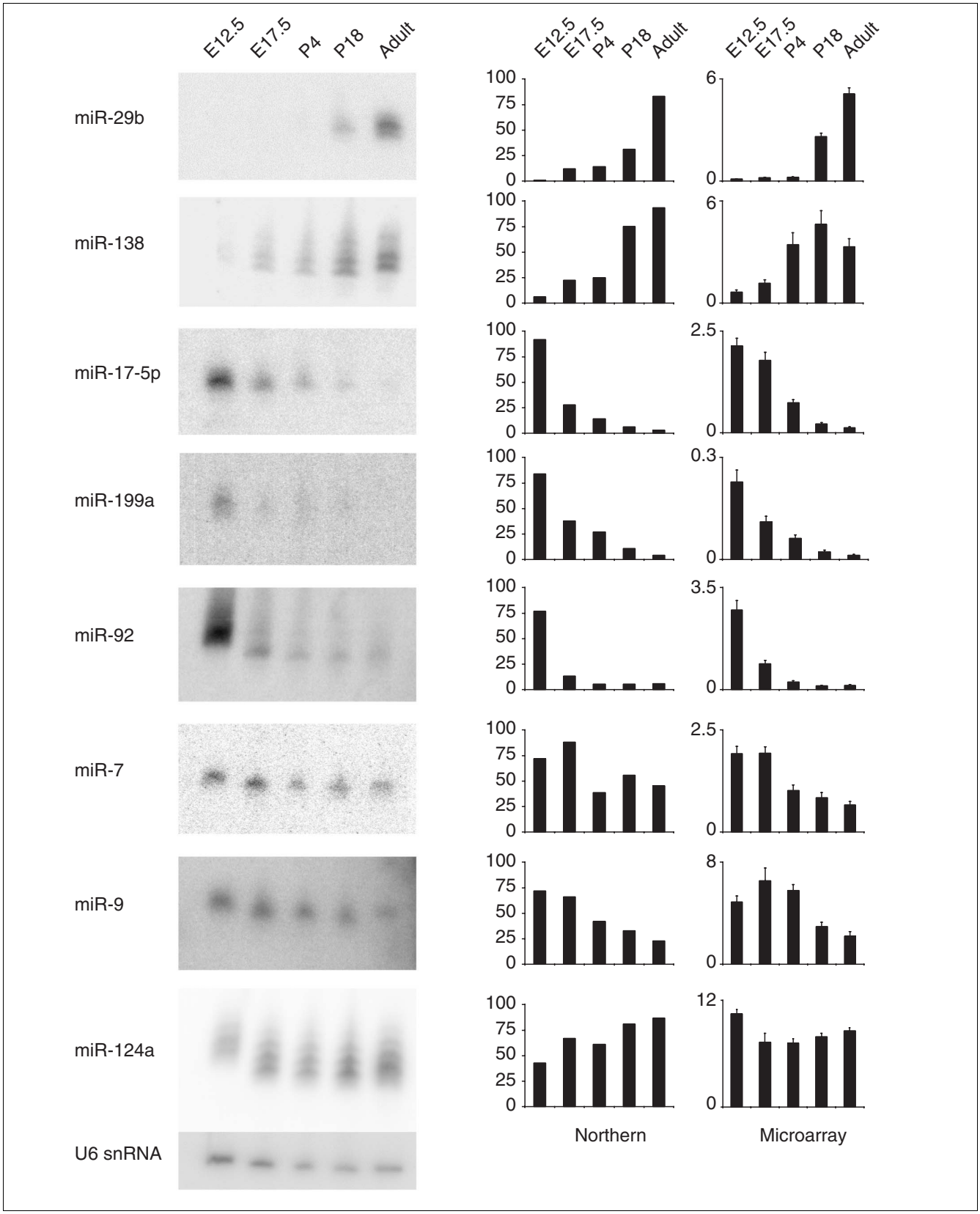

Figure 5 *(see legend on next page)*

Figure 5 *(see previous page)*
Comparison of microarray data with representative developmental northern blots of microRNAs. Northern blots were prepared and microarray analysis was done using the same starting material. For each microRNA, northern blots (left panel) and the microarray hybridization signals (right panel) are shown. Quantification of northern blots is also shown (middle panel). *Y*-axis for the microarray data refers to the averaged mean signal intensities (× 10^{-3}), and error bars are standard errors of the mean. Northern blots were done using 20 μg of total RNA in each lane. Because northern blots were exposed for different lengths of time, the intensities of the signals on northern blots cannot be directly compared to those from the microarrays. A probe against U6 snRNA was hybridized to the same blots for comparison.

(Dharmacon) was ligated to the 5' end. The ligation products were divided into two aliquots, and the following steps were done independently twice for each time point. Ligation products were reverse transcribed and amplified by 10 rounds of PCR (40 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C). For PCR, the oligonucleotides used were: oligo1 5'-Cy3-ACGGAATTC-CTCACTAAA-3' and oligo2 5'-TACTGGAATTCGCGGTTAA-3'. The PCR product was precipitated, washed and resuspended in hybridization buffer (5× SSC, 0.1% SDS, 0.1 mg/ml sheared denatured salmon sperm DNA).

Data acquisition and analysis

Microarray slides were scanned using an arrayWoRx biochip reader (Applied Precision), and primary data were analyzed using the Digital Genome System suite (MolecularWare) and Spotfire DecisionSite (Spotfire). Cluster analysis was performed using the CLUSTER/TreeView software [50]. For details concerning microarray data analysis see Additional data file 5. The predicted stem-loop RNA structures were generated using the mfold (version 3.1) software [52].

Northern blots

Northern blots were performed as described [14]. Twenty micrograms of total RNA were loaded per lane. A probe for the mouse U6 snRNA (5'-TGTGCTGCCGAAGCGAGCAC-3') was used as loading control. The probes for the northern blots had the same sequences as the corresponding EAM oligonucleotides printed on the microarray (see Additional data file 6). The blots were stripped by boiling for 5 min in distilled water and reprobed up to four times.

The probes used were: EAM119 (miR-29b), EAM125 (miR-138), EAM224 (miR-17-5p), EAM234 (miR-199a), EAM131 (miR-92), EAM109 (miR-7), EAM150 (miR-9) and EAM103 (miR-124a).

Additional data files

The following additional data files are available with the online version of this article: A file (Additional data file 1) with details of rat microRNA precursors: using the assembly of the rat genome [55] we identified candidate genomic locations for all of our rat microRNAs that have orthologs in the mouse but that have not been described previously for the rat. An alignment of the top BLAST hit of each mouse microRNA precursor sequence (The miRNA Registry, Release 3.2) against the rat genome sequence (public release draft genome assembly, version 3.1) is shown in this file. In addition, predicted precursor secondary structures are shown for each rat microRNA gene for which the precursor sequence differs from that of the corresponding mouse precursor. We used the mfold algorithm to make secondary structure predictions [52].

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A file (Additional data file 4) showing rno-miR-138 brain specific expression. A northern blot shows that rno-miR-138 expression was restricted to brain. The probe was identical to EAM125. Total RNA isolated from various adult rat tissues (Ambion) was size-separated on denaturing PAGE (12 μg per lane), transferred to a nylon membrane and used for hybridization. Equal loading was verified using a probe for U6 snRNA (data not shown).

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An Excel file (Additional data file 7) with the primary microarray data corresponding to the E12.5 time point; an Excel file (Additional data file 8) with the primary microarray data corresponding to the E17.5 time point; an Excel file (Additional data file 9) with the primary microarray data corresponding to the P4 time point; an Excel file (Additional data file 10) with the primary microarray data corresponding to the P18 time point; an Excel file (Additional data file 11) with the primary microarray data corresponding to the Adult time point. All data in Additional files 7-11 were exported from the Digital Genome System suite (MolecularWare). The primary microarray data will also be submitted to the Gene Expression Omnibus (GEO) database [56].

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Additional data files

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A file with details of rat microRNA precursors

Additional data file 2
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Additional data file 4
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An Excel file with the primary microarray data corresponding to the P4 time point

Additional data file 10
An Excel file with the primary microarray data corresponding to the P18 time point

Additional data file 11
An Excel file with the primary microarray data corresponding to the Adult time point

mmu-let-7a-1 *Mus musculus* let-7a-1 precursor RNA

```
>gi|34874105|ref|NW_047490.1|Rn17_2012 Rattus norvegicus chromosome 17 WGS
               supercontig

Mouse: 1       ttcactgtgggatgaggtagtaggttgtatagttttagggtcacacccaccactgggaga 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   3523419 ttcactgtgggatgaggtagtaggttgtatagttttagggtcacacccaccactgggaga 3523478

Mouse: 61      taactatacaatctactgtctttcctaaggtgat 94
               |||||||||||||||||||||||||||||||||
Rat:   3523479 taactatacaatctactgtctttcctaaggtgat 3523512
```

mmu-let-7a-2 *Mus musculus* let-7a-2 precursor RNA

```
>gi|34865068|ref|NW_047799.1|Rn8_2323 Rattus norvegicus chromosome 8 WGS
               supercontig

Mouse: 3        gcatgttcccaggttgaggtagtaggttgtatagtttagagttacatcaagggagataac 62
                ||||| ||||||| |||||||||||||||||||||||||||||| ||||||||||||||
Rat:   14144995 gcatgctcccaggctgaggtagtaggttgtatagtttagagttacaacaagggagataac 14145054

Mouse: 63       tgtacagcctcctagctttccttgggacttgcac 96
                |||||||||||||||||||||||||||||||||
Rat:   14145055 tgtacagcctcctagctttccttgggacttgcac 14145088
```

mmu-let-7b *Mus musculus* let-7b precursor RNA

```
>gi|34934010|ref|NW_047783.1|Rn7_2307 Rattus norvegicus chromosome 7 WGS
               supercontig

Mouse: 4       gggtgaggtagtaggttgtgtggtttcagggcagtgatgttgcccctccgaagataacta 63
               ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Rat:   1610759 gggtgaggtagtaggttgtgtggtttcagggcagtgatgtcgcccctccgaagataacta 1610818

Mouse: 64      tacaacctactgccttccctga 85
               |||||||||||||||||||||
Rat:   1610819 tacaacctactgccttccctga 1610840
```

mmu-let-7c-1 *Mus musculus* let-7c-1 precursor RNA

```
>gi|34933964|ref|NW_047354.1|Rn11_1874 Rattus norvegicus chromosome 11 WGS
               supercontig

Mouse: 1        tgtgtgcatccgggttgaggtagtaggttgtatggtttagagttacaccctgggagttaa 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   16398264 tgtgtgcatccgggttgaggtagtaggttgtatggtttagagttacaccctgggagttaa 16398323

Mouse: 61       ctgtacaaccttctagctttccttggagcacact 94
                |||||||||||||||||||||||||||||||||
Rat:   16398324 ctgtacaaccttctagctttccttggagcacact 16398357
```

mmu-let-7c-2 *Mus musculus* let-7c-2 precursor RNA

```
>gi|34934010|ref|NW_047783.1|Rn7_2307 Rattus norvegicus chromosome 7 WGS
               supercontig

Mouse: 1       acggcctttggggtgaggtagtaggttgtatggttttgggctctgccccgctctgcggta 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1610341 acggcctttggggtgaggtagtaggttgtatggttttgggctctgccccgctctgcggta 1610400

Mouse: 61      actatacaatctactgtctttcctgaagtggccgc 95
               |||||||||||||||||||||||||||||||||||
Rat:   1610401 actatacaatctactgtctttcctgaagtggccgc 1610435
```

mmu-let-7e *Mus musculus* let-7e precursor RNA

```
>gi|34854887|ref|NW_047555.1|Rn1_2077 Rattus norvegicus chromosome 1 WGS
               supercontig

Mouse: 1       cgcgccccccgggctgaggtaggaggttgtatagttgaggaagacacccgaggagatcac 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1805450 cgcgccccccgggctgaggtaggaggttgtatagttgaggaagacacccgaggagatcac 1805509

Mouse: 61      tatacggcctcctagctttccccaggctgcgcc 93
               |||||||||||||||||||||||||||||||||
```

```
Rat:   1805510 tatacggcctcctagctttccccaggctgcgcc 1805542
```

hsa-let-7i Homo sapiens let-7i precursor RNA

```
>gi|34865737|ref|NW_047776.1|Rn7_2300 Rattus norvegicus chromosome 7 WGS
              supercontig

Mouse: 1       ctggctgaggtagtagtttgtgctgttggtcgggttgtgacattgcccgctgtggagata 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   3609065 ctggctgaggtagtagtttgtgctgttggtcgggttgtgacattgcccgctgtggagata 3609006

Mouse: 61      actgcgcaagctactgccttgcta 84
               ||||||||||||||||||||||||
Rat:   3609005 actgcgcaagctactgccttgcta 3608982
```

mmu-mir-7-1 *Mus musculus* miR-7-1 precursor RNA

```
>gi|34873863|ref|NW_047487.1|Rn17_2009 Rattus norvegicus chromosome 17 WGS
              supercontig

Mouse: 1       ttggatgttggcctagttctgtgtggaagactagtgattttgttgtttttagataactaa 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   6715460 ttggatgttggcctagttctgtgtggaagactagtgattttgttgtttttagataactaa 6715519

Mouse: 61      aacgacaacaaatcacagtctgccatatggcacaggccacctctacag 108
               |||||||||||||||||||||||||||||||||||||||||||||||
Rat:   6715520 gacgacaacaaatcacagtctgccatatggcacaggccacctctacag 6715567
```

mmu-mir-7-2 *Mus musculus* miR-7-2 precursor RNA

```
>gi|34857743|ref|NW_047560.1|Rn1_2082 Rattus norvegicus chromosome 1 WGS
               supercontig

Mouse: 8        ccagccccgtttggaagactagtgattttgttgttgtgtctctgtatccaacaacaagtc 67
                ||||||| || |||||||||||||||||||||||||||||| |||  ||||||||||||||
Rat:   14956730 ccagccctgtctggaagactagtgattttgttgttgtgtctgtgt--ccaacaacaagtc 14956787

Mouse: 68       ccagtctgccacatggtgctggtca 92
                |||||||||||||||||| ||||||
Rat:   14956788 ccagtctgccacatggtgttggtca 1495681Q
```

mmu-mir-9-1 *Mus musculus* miR-9-1 precursor RNA

```
>gi|34858271|ref|NW_047626.1|Rn2_2148 Rattus norvegicus chromosome 2 WGS
              supercontig

Mouse: 1       cggggttggttgttatctttggttatctagctgtatgagtggtgtggagtcttcataaag 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   6714042 cggggttggttgttatctttggttatctagctgtatgagtggtgtggagtcttcataaag 6714101

Mouse: 61      ctagataaccgaaagtaaaaataacccca 89
               |||||||||||||||||||||||||||||
Rat:   6714102 ctagataaccgaaagtaaaaataacccca 6714130
```

mmu-mir-9-3 *Mus musculus* miR-9-3 precursor RNA

```
>gi|34857743|ref|NW_047560.1|Rn1_2082 Rattus norvegicus chromosome 1 WGS
               supercontig

Mouse: 1        ggaggcccgtttctctctttggttatctagctgtatgagtgccacagagccgtcataaag 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   15603655 ggaggcccgtttctctctttggttatctagctgtatgagtgccacagagccgtcataaag 15603714

Mouse: 61       ctagataaccgaaagtagaaatgactct 88
                |||||||||||||||||||||||||||||
Rat:   15603715 ctagataaccgaaagtagaaatgactct 15603742
```

mmu-mir-9-2 *Mus musculus* miR-9-2 precursor RNA

```
>gi|34853324|ref|NW_047616.1|Rn2_2138 Rattus norvegicus chromosome 2 WGS
              supercontig

Mouse: 1       gttgttatctttggttatctagctgtatgagtgtattggtcttcataaagctagataacc 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   6903262 gttgttatctttggttatctagctgtatgagtgtattggtcttcataaagctagataacc 6903203
```

```
Mouse: 61      gaaagtaaaaac 72
               ||||||||||||
Rat:   6903202 gaaagtaaaaac 6903191
```

mmu-mir-16-2 *Mus musculus* miR-16-2 precursor RNA

```
>gi|34857808|ref|NW_047625.1|Rn2_2147 Rattus norvegicus chromosome 2 WGS
               supercontig

Mouse: 5       cttgttccactctagcagcacgtaaatattggcgtagtgaaataaatattaaacaccaat 64
               |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   45354558 cttgttccgctctagcagcacgtaaatattggcgtagtgaaataaatattaaacaccaat 45354617

Mouse: 65      attattgtgctgctttagtgtgacagggata 95
               |||||||||||||||||||||||||||||||
Rat:   45354618 attattgtgctgctttagtgtgacagggata 45354648
```

mmu-mir-24-1 *Mus musculus* miR-24-1 precursor RNA

```
>gi|34873863|ref|NW_047487.1|Rn17_2009 Rattus norvegicus chromosome 17 WGS
               supercontig

Mouse: 1       ctccggtgcctactgagctgatatcagttctcatttcacacactggctcagttcagcagg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1862421 ctccggtgcctactgagctgatatcagttctcatttcacacactggctcagttcagcagg 1862362

Mouse: 61      aacaggag 68
               ||||||||
Rat:   1862361 aacaggag 1862354
```

mmu-mir-24-2 *Mus musculus* miR-24-2 precursor RNA

```
>gi|34851559|ref|NW_047534.1|Rn19_2056 Rattus norvegicus chromosome 19 WGS
               supercontig

Mouse: 1       gcctctctccgggctccgcctcccgtgcctactgagctgaaacagttgattccagtgcac 60
               ||||||| | |||||||||||||| ||||||||||||||||||||||||||||||||||
Rat:   10919959 gcctctccctgggctccgcctcctgtgcctactgagctgaaacagttgattccagtgcac 10919900

Mouse: 61      tggctcagttcagcaggaacaggagtccagccccc-taggagctggca 107
               |||||||||||||||||||||||||||||||||| ||||||||||||
Rat:   10919899 tggctcagttcagcaggaacaggagtccagcccccataggagctggca 10919852
```

mmu-mir-26b *Mus musculus* miR-26b precursor RNA

```
>gi|34877332|ref|NW_047816.1|Rn9_2340 Rattus norvegicus chromosome 9 WGS
               supercontig

Mouse: 1       tgcccgggacccagttcaagtaattcaggataggttgtggtgctgaccagcctgttctcc 60
               |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Rat:   17163277 tgcccgggacccagttcaagtaattcaggataggttgtggtgctggccagcctgttctcc 17163336

Mouse: 61      attacttggctcgggggccggtgcc 85
               |||||||||||||||||||||||||
Rat:   17163337 attacttggctcgggggccggtgcc 17163361
```

mmu-mir-28 *Mus musculus* miR-28 precursor RNA

```
>gi|34869631|ref|NW_047356.1|Rn11_1876 Rattus norvegicus chromosome 11 WGS
               supercontig

Mouse: 1       ggtccctaccttcaaggagctcacagtctattgagttgcctttctgattctcccactaga 60
               ||||||||||  |||||||||||||||||||||||| | ||||||||||||||||||||
Rat:   31535044 ggtccctacccgcaaggagctcacagtctattgagttccttttctgattctcccactaga 31534985

Mouse: 61      ttgtgagctgctggagggcaggcact 86
               ||||||||| |||||||||||||||
Rat:   31534984 ttgtgagctcctggagggcaggcact 31534959
```

mmu-mir-29a *Mus musculus* miR-29a precursor RNA

```
>gi|34855362|ref|NW_047689.1|Rn4_2212 Rattus norvegicus chromosome 4 WGS
               supercontig

Mouse: 1       accccttagaggatgactgatttcttttggtgttcagagtcaatagaattttctagcacc 60
```

```
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   28825083 acccctagaggatgactgatttcttttggtgttcagagtcaatagaattttctagcacc 28825024

Mouse: 61       atctgaaatcggttataatgattgggga 88
                ||||||||||||||||||||||||||||
Rat:   28825023 atctgaaatcggttataatgattgggga 28824996
```

mmu-mir-29c *Mus musculus* miR-29c precursor RNA

```
>gi|34881453|ref|NW_047404.1|Rn13_1926 Rattus norvegicus chromosome 13 WGS
              supercontig

Mouse: 1        atctcttacacaggctgaccgatttctcctggtgttcagagtctgtttttgtctagcacc 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   2146548 atctcttacacaggctgaccgatttctcctggtgttcagagtctgtttttgtctagcacc 2146607

Mouse: 61       atttgaaatcggttatgatgtaggggga 88
                ||||||||||||||||||||||||||||
Rat:   2146608 atttgaaatcggttatgatgtaggggga 2146635
```

mmu-mir-29b-1 *Mus musculus* miR-29b-1 precursor RNA

```
>gi|34855362|ref|NW_047689.1|Rn4_2212 Rattus norvegicus chromosome 4 WGS
               supercontig

Mouse: 1        aggaagctggtttcatatggtggtttagatttaaatagtgattgtctagcaccatttgaa 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   28825451 aggaagctggtttcatatggtggtttagatttaaatagtgattgtctagcaccatttgaa 28825392

Mouse: 61       atcagtgttct 71
                ||||||||||||
Rat:   28825391 atcagtgttct 28825381
```

mmu-mir-29b-2 *Mus musculus* miR-29b-2 precursor RNA

```
>gi|34881453|ref|NW_047404.1|Rn13_1926 Rattus norvegicus chromosome 13 WGS
              supercontig

Mouse: 1        cttctggaagctggtttcacatggtggcttagatttttccatctttgtatctagcaccat 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   2146020 cttctggaagctggtttcacatggtggcttagatttttccatctttgtatctagcaccat 2146079

Mouse: 61       ttgaaatcagtgttttaggag 81
                |||||||||||||||||||||
Rat:   2146080 ttgaaatcagtgttttaggag 2146100
```

mmu-mir-30b *Mus musculus* miR-30b precursor RNA

```
>gi|34867094|ref|NW_047780.1|Rn7_2304 Rattus norvegicus chromosome 7 WGS
             supercontig

Mouse: 1       atgtaaacatcctacactcagctgtcatacatgcgttggctgggatgtggatgtttacgt 60
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Rat:   418464 atgtaaacatcctacactcagctgtcatacatgagttggctgggatgtggatgtttacgt 418405
```

mmu-mir-30c-1 *Mus musculus* miR-30c-1 precursor RNA

```
>gi|34871316|ref|NW_047719.1|Rn5_2243 Rattus norvegicus chromosome 5 WGS
              supercontig

Mouse: 1        accatgttgtagtgtgtgtaaacatcctacactctcagctgtgagctcaaggtggctggg 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   2519905 accatgttgtagtgtgtgtaaacatcctacactctcagctgtgagctcaaggtggctggg 2519846

Mouse: 61       agagggttgtttactccttctgccatgga 89
                |||||||||||||||||||||||||||||
Rat:   2519845 agagggttgtttactccttctgccatgga 2519817
```

mmu-mir-30c-2 *Mus musculus* miR-30c-2 precursor RNA

```
>gi|34875263|ref|NW_047813.1|Rn9_2337 Rattus norvegicus chromosome 9 WGS
               supercontig

Mouse: 1        gagtgacagatattgtaaacatcctacactctcagctgtgaaaagtaagaaagctgggag 60
                |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
```

```
Rat:   22164044 gagtgacagatactgtaaacatcctacactctcagctgtgaaaagtaagaaagctgggag 22164103

Mouse: 61       aaggctgtttactctctctgcctt 84
                |||||||||||||||||||||||||
Rat:   22164104 aaggctgtttactctctctgcctt 22164127
```

mmu-mir-92-2 *Mus musculus* miR-92-2 precursor RNA

```
>gi|34881658|ref|NW_048049.1|RnX_2574 Rattus norvegicus chromosome X WGS
               supercontig

Mouse: 1       tgcccattcatccacaggtggggattggtggcattacttgtgttagatataaagtattgc 60
               ||||||||||||||||||||||||||| ||| |||||||||||||||||| ||||||||||
Rat:   2017453 tgcccattcatccacaggtggggattagtgccattacttgtgttagataaaaagtattgc 2017394

Mouse: 61      acttgtcccggcctgaggaagaaa 84
               ||||||||||||||||||||||||
Rat:   2017393 acttgtcccggcctgaggaagaaa 2017370
```

mmu-mir-93 *Mus musculus* miR-93 precursor RNA

```
>gi|34871632|ref|NW_047369.1|Rn12_1890 Rattus norvegicus chromosome 12 WGS
               supercontig

Mouse: 1       agtcatgggggctccaaagtgctgttcgtgcaggtagtgtaattacctgacctactgctg 60
               |||||||||||||||||||||||||||||||||||||||| | || ||||||||||||||
Rat:   5549656 agtcatgggggctccaaagtgctgttcgtgcaggtagtgca-ttgcctgacctactgctg 5549598

Mouse: 61      agctagcacttcccgagcccccaggaca 88
               ||||||||||||||||||||||||||||
Rat:   5549597 agctagcacttcccgagcccccaggaca 5549570
```

mmu-mir-99a *Mus musculus* miR-99a precursor RNA

```
>gi|34933964|ref|NW_047354.1|Rn11_1874 Rattus norvegicus chromosome 11 WGS
                supercontig

Mouse: 1        cataaacccgtagatccgatcttgtggtgaagtggaccgcgcaagctcgtttctatgggt 60
                |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Rat:   16397552 cataaacccgtagatccgatcttgtggtgaagtggaccgcacaagctcgtttctatgggt 16397611

Mouse: 61       ctgtg 65
                |||||
Rat:   16397612 ctgtg 16397616
```

mmu-mir-99b *Mus musculus* miR-99b precursor RNA

```
>gi|34854887|ref|NW_047555.1|Rn1_2077 Rattus norvegicus chromosome 1 WGS
               supercontig

Mouse: 1       ggcacccacccgtagaaccgaccttgcggggccttcgccgcacacaagctcgtgtctgtg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1805285 ggcacccacccgtagaaccgaccttgcggggccttcgccgcacacaagctcgtgtctgtg 1805344

Mouse: 61      ggtccgtgtc 70
               ||||||||||
Rat:   1805345 ggtccgtgtc 1805354
```

mmu-mir-103-1 *Mus musculus* miR-103-1 precursor RNA

```
>gi|34872015|ref|NW_047334.1|Rn10_1854 Rattus norvegicus chromosome 10 WGS
               supercontig

Mouse: 1       ttcttactgccctcggcttctttacagtgctgccttgttgcatatggatcaagcagcatt 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   7519813 ttcttactgccctcggcttctttacagtgctgccttgttgcatatggatcaagcagcatt 7519872

Mouse: 61      gtacagggctatgaaggcattgagac 86
               |||||||||||||||||||||||||
Rat:   7519873 gtacagggctatgaaggcattgagac 7519898
```

mmu-mir-103-2 *Mus musculus* miR-103-2 precursor RNA

```
>gi|34859757|ref|NW_047658.1|Rn3_2180 Rattus norvegicus chromosome 3 WGS
               supercontig
```

```
Mouse: 1       gtcttcgtgctttcagcttctttacagtgctgccttgtagcattcaggtcaagcagcatt 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   9173953 gtcttcgtgctttcagcttctttacagtgctgccttgtagcattcaggtcaagcagcatt 9174012

Mouse: 61      gtacagggctatgaaagaaccaagaa 86
               |||||||||||||||||||||||||
Rat:   9174013 gtacagggctatgaaagaaccaagaa 9174038
```

mmu-mir-124a-1 *Mus musculus* miR-124a-1 precursor RNA

```
>gi|34875912|ref|NW_047454.1|Rn15_1976 Rattus norvegicus chromosome 15 WGS
               supercontig

Mouse: 1        aggcctctctctccgtgttcacagcggaccttgatttaaatgtccatacaattaaggcac 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   15806503 aggcctctctctccgtgttcacagcggaccttgatttaaatgtccatacaattaaggcac 15806562

Mouse: 61       gcggtgaatgccaagaatggggctg 85
                |||||||||||||||||||||||||
Rat:   15806563 gcggtgaatgccaagaatggggctg 15806587
```

mmu-mir-124a-2 *Mus musculus* miR-124a-2 precursor RNA

```
>gi|34855621|ref|NW_047624.1|Rn2_2146 Rattus norvegicus chromosome 2 WGS
               supercontig

Mouse: 1       atcaagatcagagactctgctctccgtgttcacagcggaccttgatttaatgtcatacaa 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   7685115 atcaagatcagagactctgctctccgtgttcacagcggaccttgatttaatgtcatacaa 7685174

Mouse: 61      ttaaggcacgcggtgaatgccaagagcggagcctacggctgcacttgaa 109
               |||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   7685175 ttaaggcacgcggtgaatgccaagagcggagcctacggctgcacttgaa 7685223
```

mmu-mir-124a-3 *Mus musculus* miR-124a-3 precursor RNA

```
>gi|34861141|ref|NW_047667.1|Rn3_2189 Rattus norvegicus chromosome 3 WGS
              supercontig

Mouse: 1      ctctgcgtgttcacagcggaccttgatttaatgtctatacaattaaggcacgcggtgaat 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   505484 ctctgcgtgttcacagcggaccttgatttaatgtctatacaattaaggcacgcggtgaat 505543

Mouse: 61     gccaagag 68
              ||||||||
Rat:   505544 gccaagag 505551
```

mmu-mir-125a *Mus musculus* miR-125a precursor RNA

```
>gi|34854887|ref|NW_047555.1|Rn1_2077 Rattus norvegicus chromosome 1 WGS
               supercontig

Mouse: 1       ctgggtccctgagacccttaacctgtgaggacgtccagggtcacaggtgaggttcttgg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1805905 ctgggtccctgagacccttaacctgtgaggacgtccagggtcacaggtgaggttcttgg 1805964

Mouse: 61      gagcctgg 68
               ||||||||
Rat:   1805965 gagcctgg 1805972
```

mmu-mir-125b-2 *Mus musculus* miR-125b-2 precursor RNA

```
>gi|34933964|ref|NW_047354.1|Rn11_1874 Rattus norvegicus chromosome 11 WGS
               supercontig

Mouse: 2        cctagtccctgagaccctaacttgtgaggtattttagtaacatcacaagtcaggttcttg 61
                |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Rat:   16443677 cctagtccctgagaccctaacttgtgaggtattttagtaacatcacaagtcaggctcttg 16443736

Mouse: 62       ggacctaggc 71
                ||||||||||
Rat:   16443737 ggacctaggc 16443746
```

mmu-mir-125b-1 *Mus musculus* miR-125b-1 precursor RNA

```
Mouse: 1        tgcgctcccctcagtccctgagaccctaacttgtgatgtttaccgtttaaatccacgggt 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   14189379 tgcgctcccctcagtccctgagaccctaacttgtgatgtttaccgtttaaatccacgggt 14189438

Mouse: 61       taggctcttgggagctg 77
                |||||||||||||||||
Rat:   14189439 taggctcttgggagctg 14189455
```

mmu-mir-127 *Mus musculus* miR-127 precursor RNA

```
>gi|34935858|ref|NW_047762.1|Rn6_2286 Rattus norvegicus chromosome 6 WGS
                supercontig

Mouse: 1        ccagcctgctgaagctcagagggctctgattcagaaagatcatcggatccgtctgagctt 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   29527445 ccagcctgctgaagctcagagggctctgattcagaaagatcatcggatccgtctgagctt 29527504

Mouse: 61       ggctggtcgg 70
                ||||||||||
Rat:   29527505 ggctggtcgg 29527514
```

mmu-mir-128a *Mus musculus* miR-128a precursor RNA

```
>gi|34933508|ref|NW_047394.1|Rn13_1916 Rattus norvegicus chromosome 13 WGS
                supercontig

Mouse: 1        gttggattcggggccgtagcactgtctgagaggtttacatttctcacagtgaaccggtct 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1019804 gttggattcggggccgtagcactgtctgagaggtttacatttctcacagtgaaccggtct 1019863

Mouse: 61       cttttcagc 70
                |||||||||
Rat:   1019864 cttttcagc 1019873
```

mmu-mir-128b *Mus musculus* miR-128b precursor RNA

```
>gi|34866469|ref|NW_047802.1|Rn8_2326 Rattus norvegicus chromosome 8 WGS
                supercontig

Mouse: 1        cagtgggaaggggggccgatgcactgtaagagagtgagtagcaggtctcacagtgaaccg 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   3171003 cagtgggaaggggggccgatgcactgtaagagagtgagtagcaggtctcacagtgaaccg 3170944

Mouse: 61       gtctctttccctactg 76
                ||||||||||||||||
Rat:   3170943 gtctctttccctactg 3170928
```

mmu-mir-130a *Mus musculus* miR-130a precursor RNA

```
>gi|34857850|ref|NW_047657.1|Rn3_2179 Rattus norvegicus chromosome 3 WGS
                supercontig

Mouse: 1        gagctcttttcacattgtgctactgtcta-acgtgtaccgagcagtgcaatgttaaaagg 59
                |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Rat:   9350850 gagctcttttcacattgtgctactgtctacacgtgtaccgagcagtgcaatgttaaaagg 9350791

Mouse: 60       gcatc 64
                |||||
Rat:   9350790 gcatc 9350786
```

mmu-mir-132 *Mus musculus* miR-132 precursor RNA

```
>gi|34873416|ref|NW_047336.1|Rn10_1856 Rattus norvegicus chromosome 10 WGS
                supercontig

Mouse: 1        gggcaaccgtggctttcgattgttactgtgggaaccggaggtaacagtctacagccatgg 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   3479949 gggcaaccgtggctttcgattgttactgtgggaaccggaggtaacagtctacagccatgg 3480008

Mouse: 61       tcgccc 66
                ||||||
Rat:   3480009 tcgccc 3480014
```

mmu-mir-136 *Mus musculus* miR-136 precursor RNA

```
>gi|34935858|ref|NW_047762.1|Rn6_2286 Rattus norvegicus chromosome 6 WGS
               supercontig

Mouse: 1        gaggactccatttgttttgatgatggattcttaagctccatcatcgtctcaaatgagtct 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   29530106 gaggactccatttgttttgatgatggattcttaagctccatcatcgtctcaaatgagtct 29530165

Mouse: 61       tc 62
                ||
Rat:   29530166 tc 29530167
```

mmu-mir-138-1 *Mus musculus* miR-138-1 precursor RNA

```
>gi|34866724|ref|NW_047804.1|Rn8_2328 Rattus norvegicus chromosome 8 WGS
              supercontig

Mouse: 1      ctctagcatggtgttgtgggacagctggtgttgtgaatcaggccgttgccaatcagagaa 60
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   156597 ctctggcatggtgttgtgggacagctggtgttgtgaatcaggccgttgccaatcagagaa 156656

Mouse: 61     cggctacttcacaacaccagggccacactgcactgca 97
              ||||||||||||||||||||| | ||||||||||||
Rat:   156657 cggctacttcacaacaccagggtctcactgcactgca 156693
```

mmu-mir-138-2 *Mus musculus* miR-138-2 precursor RNA

```
>gi|34851309|ref|NW_047531.1|Rn19_2053 Rattus norvegicus chromosome 19 WGS
               supercontig

Mouse: 1        cagctggtgttgtgaatcaggccgacgagcagcgcatcctcttacccggctatttcacga 60
                |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Rat:   11119560 cagctggtgttgtgaatcaggccgacgagcaacgcatcctcttacccggctatttcacga 11119501

Mouse: 61       caccagggttg 71
                |||||||||||
Rat:   11119500 caccagggttg 11119490
```

mmu-mir-139 *Mus musculus* miR-139 precursor RNA

```
>gi|34860310|ref|NW_047562.1|Rn1_2084 Rattus norvegicus chromosome 1 WGS
              supercontig

Mouse: 1       gtgtattctacagtgcacgtgtctccagtgtggctcggaggctggagacgcggccctgtt 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1586714 gtgtattctacagtgcacgtgtctccagtgtggctcggaggctggagacgcggccctgtt 1586773

Mouse: 61      ggagtaac 68
               ||||||||
Rat:   1586774 ggagtaac 1586781
```

mmu-mir-142 *Mus musculus* miR-142 precursor RNA

```
>gi|34873416|ref|NW_047336.1|Rn10_1856 Rattus norvegicus chromosome 10 WGS
               supercontig

Mouse: 1        acccataaagtagaaagcactactaacagcactggagggtgtagtgtttcctactttatg 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   17049244 acccataaagtagaaagcactactaacagcactggagggtgtagtgtttcctactttatg 17049303

Mouse: 61       gatg 64
                ||||
Rat:   17049304 gatg 17049307
```

mmu-mir-145 *Mus musculus* miR-145 precursor RNA

```
>gi|34932227|ref|NW_047514.1|Rn18_2036 Rattus norvegicus chromosome 18 WGS
               supercontig

Mouse: 1       ctcacggtccagttttcccaggaatcccttggatgctaagatggggattcctggaaatac 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   3665639 ctcacggtccagttttcccaggaatcccttggatgctaagatggggattcctggaaatac 3665580

Mouse: 61      tgttcttgag 70
               ||||||||||
Rat:   3665579 tgttcttgag 3665570
```

mmu-mir-146 *Mus musculus* miR-146 precursor RNA

```
>gi|34872015|ref|NW_047334.1|Rn10_1854 Rattus norvegicus chromosome 10 WGS
              supercontig

Mouse: 1        agctctgagaactgaattccatgggttatatcaatgtcagacctgtgaaattcagttctt 60
                |||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||
Rat:   15301237 agctctgagaactgaattccatgggttatagcaatgtcagacctgtgaagttcagttctt 15301178

Mouse: 61       cagct 65
                 ||||
Rat:   15301177 tagct 15301173
```

mmu-mir-150 *Mus musculus* miR-150 precursor RNA

```
Mouse: 1       ccctgtctcccaacccttgtaccagtgctgtgcctcagaccctggtacaggcctggggga 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   4552248 ccctgtctcccaacccttgtaccagtgctgtgcctcagaccctggtacaggcctggggga 4552307

Mouse: 61      taggg 65
                ||||
Rat:   4552308 caggg 4552312
```

mmu-mir-154 *Mus musculus* miR-154 precursor RNA

```
Mouse: 1        gaagataggttatccgtgttgccttcgctttattcgtgacgaatcatacacggttgacct 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   29762799 gaagataggttatccgtgttgccttcgctttattcgtgacgaatcatacacggttgacct 29762858

Mouse: 61       attttt 66
                ||||||
Rat:   29762859 attttt 29762864
```

mmu-mir-185 *Mus musculus* miR-185 precursor RNA

```
>gi|34869997|ref|NW_047358.1|Rn11_1878 Rattus norvegicus chromosome 11 WGS
              supercontig

Mouse: 1       agggattggagagaaaggcagttcctgatggtcccctcccaggggctggctttcctctgg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   2687772 agggattggagagaaaggcagttcctgatggtcccctcccaggggctggctttcctctgg 2687831

Mouse: 61      tcctt 65
               |||||
Rat:   2687832 tcctt 2687836
```

mmu-mir-191 *Mus musculus* miR-191 precursor RNA

```
>gi|34866469|ref|NW_047802.1|Rn8_2326 Rattus norvegicus chromosome 8 WGS
             supercontig

Mouse: 1     agcgggcaacggaatcccaaaagcagctgttgtctccagagcattccagctgcacttgga 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   58055 agcgggcaacggaatcccaaaagcagctgttgtctccagagcattccagctgcacttgga 58114

Mouse: 61    tttcgttccctgct 74
             |||||||||||||
Rat:   58115 tttcgttccctgct 58128
```

mmu-mir-213 *Mus musculus* miR-213 precursor RNA

```
>gi|34880444|ref|NW_047396.1|Rn13_1918 Rattus norvegicus chromosome 13 WGS
              supercontig

Mouse: 1       ggttgcttcagtgaacattcaacgctgtcggtgagtttggaattcaaataaaaaccatcg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   1976067 ggttgcttcagtgaacattcaacgctgtcggtgagtttggaattcaaataaaaaccatcg 1976126

Mouse: 61      accgttgattgtaccctatagctaacc 87
               |||||||||||||||||||||||||||
Rat:   1976127 accgttgattgtaccctatagctaacc 1976153
```

mmu-mir-300 *Mus musculus* miR-300 precursor RNA

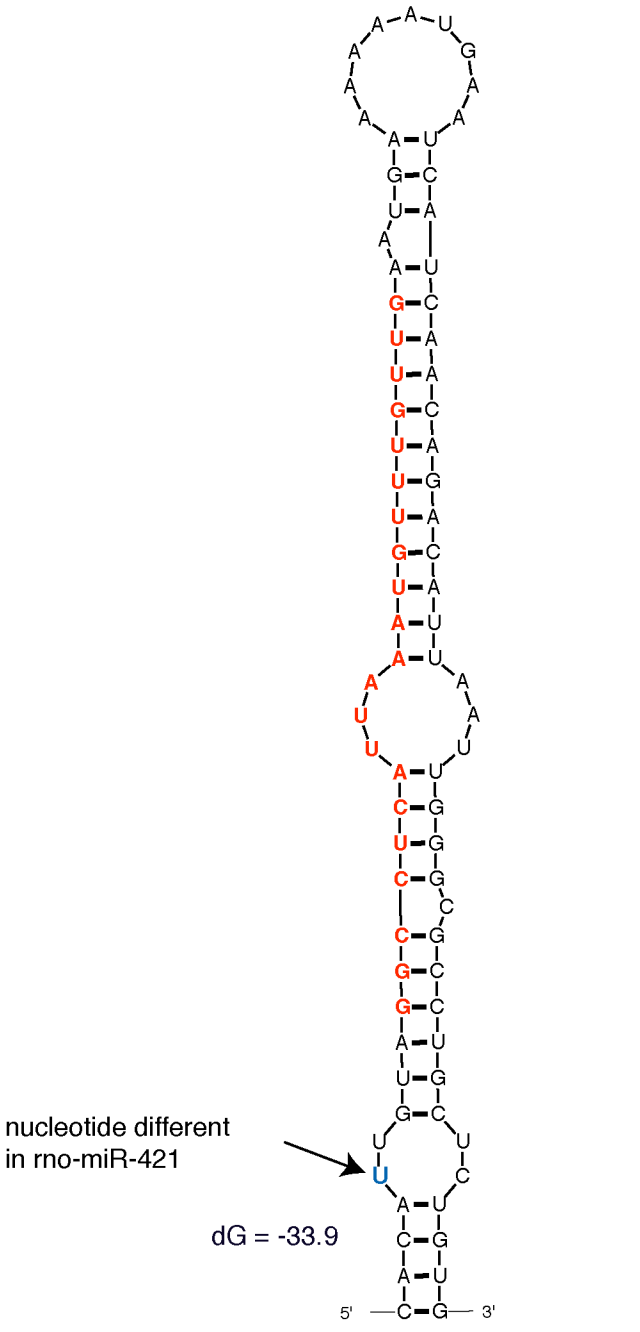
```
>gi|34935858|ref|NW_047762.1|Rn6_2286 Rattus norvegicus chromosome 6 WGS
                supercontig

Mouse: 1        gctacttgaagagaggttatcctttgtgtgtttgctttacgcgaaatgaatatgcaaggg 60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat:   29748356 gctacttgaagagaggttatcctttgtgtgtttgctttacgcgaaatgaatatgcaaggg 29748415

Mouse: 61       caagctctcttcgaggagc 79
                |||||||||||||||||||
Rat:   29748416 caagctctcttcgaggagc 29748434
```

Rat microRNA	Predicted folding Energy(kcal/mole)	Predicted stem loop precursor
rno-let-7a-2	dG = -38.9	10 20 30 40 --| UGC CU G U UAGAGUUACA GCA UCCCAGG GAG UAG AGGUUGUAUAGUU \ CGU AGGGUUC UUC AUC UCCGACAUGUCAA A CA^ UC- CU G C UAGAGGGAAC 90 80 70 60 50
rno-let-7b	dG = -44.0	---| U - ----- CA A GGG GAGGUAGUAGGUUGUGUGGUU UC AGGG GUG U CCC UUCCGUCAUCCAACAUAUCAA AG UCCC CGC G AGU^ - U AAGCC -- U
rno-mir-7-1	dG = -46.8	U--| U U A U A A U -- A UGGA GU GGCCU GU CUGUGUGG AGACU GUGAUUU GUUGUU UUUAG U AUCU CA CCGGA CA GGUAUACC UCUGA CACUAAA CAACAG GAAUC A GAC^ C - - C G - - CA A
rno-mir-7-2	dG = -29.1	---| C C A AGU U UG C CCAGC CUGU UGG AGACU GAUUU GUUGUUG U U GGUUG GGUA ACC UCUGA CUGAA CAACAAC G G ACU^ U C G CC- - CU U
rno-mir-16-2	dG = -36.9	----| UC CU UA C AG AAU CUUGU CGCU AGCAGCACG AAUAUUGG GU UGA A GGACA GUGA UCGUCGUGU UUAUAACC CA AUU A AUAG^ GU UU UA A A- AUA
rno-mir-24-2	dG = -53.5	-| U- CU--- CCGC G A AA UG U GCC CUCC GGGCU CUCCUGU CCU CUGAGCUGA CAGU AU C CGG GAGG CCCGA GAGGACA GGA GACUUGACU GUCA UG C A^ UC AUACC CCU- A C CG CG A
rno-mir-26b	dG = -42.6	U| - GA - U UC UG G GC CCGG CCC AGU CAAGUAAU AGGAUAGGU UGGU C CG GGCC GGG UCG GUUCAUUA UCUUGUCCG ACCG U C^ U GG C - CC -- G
rno-mir-28	dG = -41.2	| C A GCA UU-- U- CUU GGU CCU CCC AGGAGCUCACAGUCUA GAG UC \ UCA GGA GGG UCCUCGAGUGUUAGAU CUC AG U ^ C C AGG CACC UU UCU
rno-mir-30b	dG = -28.1	| U - -- U AUGUAAACAUCC ACA CUCAGCUG UCA A UGCAUUUGUAGG UGU GGGUCGGU AGU C ^ - A UG A
rno-mir-30c-2	dG = -28.0	GAGUGA| UACU U ACA GUGAAA CAGA GUAAACA CCU CUCUCAGCU A GUCU CAUUUGU GGA GAGGGUCGA G UUCC--^ CUCU C A-- AAGAAU
rno-mir-92-2	dG = -36.5	UGCCCA| A A G U CAU GUGUU UUC UCC CAGGU GGGAU AGUGC UACUU A AAG AGG GUCCG CCCUG UCACG AUGAA G A-----^ A A G U UU- AAAUA
rno-mir-93	dG = -48.2	A| A CA- - U G UG UU GUC UGGGGGCUC AAGUGCU GUUCG GCAG UAG CA \ CAG ACCCCCGAG UUCACGA CGAGU CGUC AUC GU G A^ G CCC U - - CA CC

rno-mir-99a	dG = -29.5	| A UC U G AAG CAUA ACCCGUAGA CGA CUUGUG UG U GUGU UGGGUAUCU GCU GAACAC GC G ^ C UU C - CAG
rno-mir-125b-2	dG = -33.3	-| UC UG C A GG- U CCUAG CC AGA CCU ACUUGUGA UAU U GGAUC GG UCU GGA UGAACACU AUG U C^ CA GU C C ACA A
rno-mir-130a	dG = -24.7	GA-| C UG A GUC A GCUCUUUU ACAUUG CU CU UAC C CGGGAAAA UGUAAC GA GA AUG G CUA^ U GU C GCC U
rno-mir-138-1	dG = -50.6	CUCUG| UG U AG UCA GCCAA GCA GUGU GUGGGAC CUGGUGUUGUGAA GGCCGUU \ CGU CACG CACUCUG GACCACAACACUU UCGGCAA U A----^ -- U G- CA- GAGAC
rno-mir-138-2	dG = -29.1	CAG--| UCA AC--- CAAC CUGGUGUUGUGAA GGCCG GAG \ GACCACAGCACUU UCGGC CUC G GUUGG^ UA- CCAUU CUAC
rno-mir-146	dG = -29.3	| CU C -- G AGCU GAGAACUGAAUU CAUGGGUU AUA C UCGA UUCUUGACUUGA GUGUCCAG UGU A ^ U- A AC A



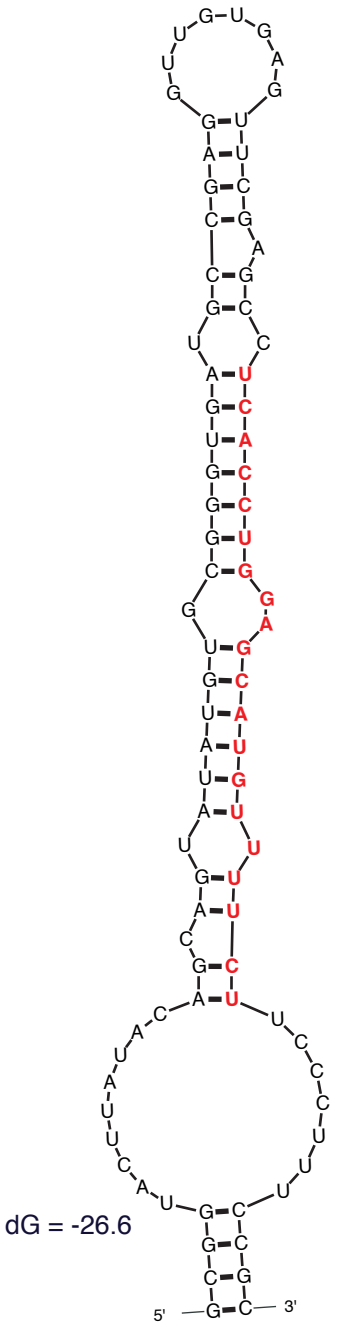
mmu-miR-421, hsa-miR-421

Figure AF2b

Figure AF2a - Alignment of precursor sequences for microRNA mir-421

ref|NW_048043.1|RnX_2568 *Rattus norvegicus* chromosome X WGS supercontig

R. norvegicus	NW_048043.1	3077508	CACACTGTA**GGCCTCATTAAATGTTTGTTG**AATGAAAAAATGAATCATCAACAGACATTAATTGGGCGCCTGCTCTGTG	3077429
H. sapiens	AC004386.1	5060	CACATTGTA**GGCCTCATTAAATGTTTGTTG**AATGAAAAAATGAATCATCAACAGACATTAATTGGGCGCCTGCTCTGTG	4982
M. musculus	AL683845.15	39424	CACATTGTA**GGCCTCATTAAATGTTTGTTG**AATGAAAAAATGAATCATCAACAGACATTAATTGGGCGCCTGCTCTGTG	39346
			*	



smallRNA-1

Figure AF3

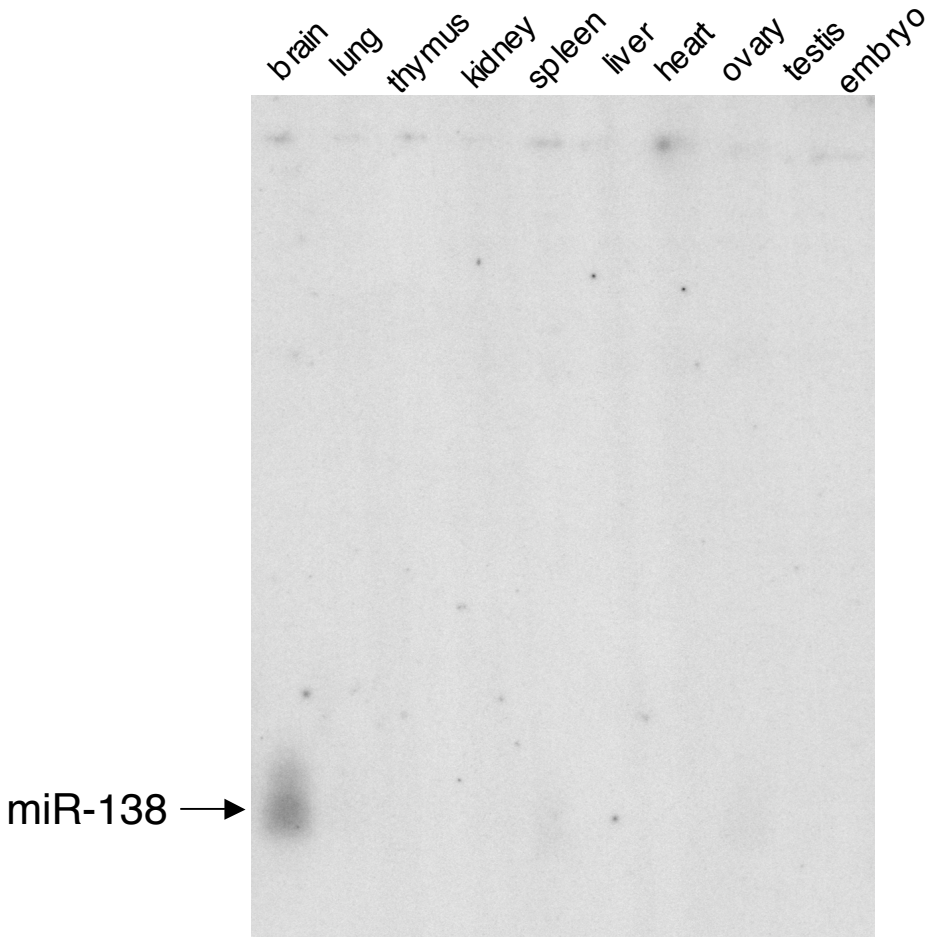

brain
lung
thymus
kidney
spleen
liver
heart
ovary
testis
embryo
miR-138

Figure AF4

Additional file 5 – Microarray design and data analysis (AF 5)

Samples/Hybridizations

Samples were processed as shown in Figure AF5a. For each sample, two independent ligations were performed. The products of the two ligations were split, and two independent reverse transcription/amplifications/hybridizations were performed. Thus, for each sample data were collected from four independent array hybridizations.

Arrays/Normalization

Glass slides were arrayed using quadruplicate spots for each of the 138 microRNA probes, 19 small RNA probes and control probes (Figure AF5a and Additional file 6). A sample microarray scan is shown in Figure AF5b. The microarray consists of 16 squares, of eight by seven spots each. Quadruplicates can be identified as rows of four spots (every other spot) in the individual squares. A TIFF file of the scanned array is used for subsequent array analysis (Digital Genome System Suite, MolecularWare). Normalized spot intensities were used for all data analysis. For inter-array comparisons, all data were scaled based on total array intensities (scaling factors ranged from 0.4 to 2.6), and data for each sample and each gene were averaged and the standard error of the mean (SEM) was calculated. Total array intensity was calculated as the sum of the normalized spot intensity for all spots in the microarray. Analysis of variance (ANOVA) performed out using Spotfire DecisionSite (Spotfire). Hierarchical clustering was performed using CLUSTER 3.0/TreeView software. For CLUSTER 3.0/TreeView output see Figure

AF5c: Profile of microRNA expression in the developing mouse brain. Colors indicate relative signal intensities. The microRNA expression profile was sorted using a hierarchical clustering method (see above). Only data from 66 probes that changed at least two-fold over the developmental time course (ANOVA, $P < 0.001$) are shown. The data used for the analysis are available as Additional file 6.

Control probes

Control probes were either negative controls or mismatch controls. The negative controls were either a synthetic $(GCAT)_n$ oligonucleotide (EAM1100) or sequences derived from mouse mRNA sequences (EAM1101-1104):

oligo ID	oligo sequence	mRNA
EAM1100	GCATGCATGCATGCATGCATG	Synthetic
EAM1101	GTGGTAGCGCAGTGCGTAGAA	beta-tubulin
EAM1102	GGTGATGCCCTGAATGTTGTC	histone H4
EAM1103	TGTCATGGATGACCTTGGCCA	glyceraldehyde dehydrogenase
EAM1104	CTTTTGACATTGAAGGGAGCT	laminin alpha 4

The mismatch controls were probes with two mismatches to a specific microRNA probe. Two central Cs were replaced by two Gs; if this change was not possible, one C was replaced with a G and one T with an A. Averaged, normalized spot intensities for these negative controls ranged from –0.8 to 1.2, as compared with 90 to 14,250 for microRNA

probes that we scored as signals. Negative values were the consequence of the subtraction of the local background signal surrounding each spot on the arrays.

Expression levels and microarray correlations

We employed two methods to distinguish signal versus noise. First, we used correlation analysis among the four hybridizations for a given time point to assess reproducibility. Second, we used a set of negative control probes (see above) to measure noise. As an example, Figure AF5d shows the correlations (scatter plots) among the four hybridizations for time point E12.5. For each graph the axes show averaged mean spot intensities for all probes from a given data set, as indicated. Arbitrarily, we chose a cut-off of 90 for our analysis. Expressed relative to background values, a microRNA was identified as being present only if the signal was at least 75-fold over that of the negative controls for at least one timepoint. Figure AF5e shows the correlations (scatter plots) of the data for E12.5 with the data from each other timepoint (each averaged over four hybridizations). As expected, the correlation between E12.5 and E17.5 is highest, and the correlation decreases with samples from more distant developmental stages.

Specificity index

To assess probe specificity, we compared the signal from oligonucleotides (probes) complementary to microRNAs (matched probe) and oligonucleotides with mismatches (mismatched probe, see above). Mismatched oligonucleotides were printed for the first 24 probes (EAM101, EAM103, … EAM147) and were named EAM102, EAM104, … EAM148. Mismatched oligonucleotides were spotted as nearest neighbors to microRNA

oligonucleotides. To calculate the specificity index (Figure 2*a*) we used datasets from two samples of each of the five time points from this study (5 time points × 2 independent samples = 10 hybridizations total). Calculations were based on cumulative signals from all experiments. EAM141, EAM143, EAM145 and EAM147 are *let*-7 family members and have very similar sequences. EAM117, EAM119 and EAM107 and EAM109 are also closely related. Therefore, there might be cross-reactivity within each of these groups. The matched/mismatched probe pair EAM135/EAM136 was excluded from Figure 2*a* as EAM136 did not give a signal above background at any of the five time points.

Summary of features on the microarray (probe set)

Mouse microRNAs (The miRNA Registry 3.2)	129
Other mammalian microRNAs (The miRNA Registry 3.2, rat and human)	9
Other unique small RNAs	18
Total	156

Of the 156 unique small RNA probes, we found that 116 (74 %) showed robust signals at at least one of the five timepoints. Of these, 83 changed significantly (ANOVA, $P < 0.001$) and 66 more than two-fold.

RNA signal quantification

The following three artifical RNAs were synthesized (Dharmacon): syn1 5'-P-CAGUCAGUCAGUCAGUCAGUCAG-3', syn2 5'-P-GACCUCCAUGUAAACGUACAA and syn3 5'-P-UUGCAGAUAACUGGUACAAG-3'. Sequences were chosen at random and were generated based on the following criteria: approximately 50% GC content, no self-annealing, no significant sequence similarity to any known mammalian microRNA and no significant sequence complementarity to any probe on the microarray. A microarray similar to the one described in this manuscript was printed with the addition of probes that are the reverse complement of these control RNAs. To quantify microRNA microarray signals the artifical RNAs were mixed with 50 μg of total RNA (an equal mix of total RNA from all five timepoints used in this study). RNA was labeled as described. The total amounts of each RNA used were 0, 0.025, 0.1, 0.375, 0.75, 2.5, 5 and 10 fmoles. Without the addition of these RNAs the normalized signal intensities were indistinguishable from background signals, which was 9 ± 6.5 (S.D.).

Analysis of base-bias of RNA ligation

	Terminal base composition of microRNAs that can be detected by the array		microRNAs expressed robustly (as defined above)	
Nucleotide	5' end	3' end	5' end	3'end
U	57	92	77% (44/57)	77% (71/92)
G	37	12	73% (27/37)	33% (4/12)
A	31	21	61% (19/31)	71% (15/21)
C	31	31	81% (25/31)	81% (25/31)

We calculated the terminal base composition of microRNAs detected robustly or not detected in our array and found that there does not appear to be a significant bias in base composition. This observation suggests that the ligation efficiency does not vary strongly depending on the identity of the 5' or 3' nucleotide and, as a consequence, that the method accurately reflects the microRNA content of the samples analyzed.

For the microRNAs that can be detected on the microarray, sequences with a U at the 5' end are slightly overrepresented (57 out of 157) while sequences with U at the 3' end are overrepresented (97 out of 157). Sequences with a G at the 3' are underrepresented (12 out of 157). When these biases are taken into account, the percentage of microRNAs that can be detected over background ranges between 61 and 81 for the four bases at the 5' end. While only 33% of microRNAs with G at the 3' end can be detected, the significance of this apparently low number is difficult to determine. First, this fraction is based only on a small number of oligonucleotides in the array (12). Second, we and others have observed that the 3' end of cloned microRNAs is variable. For example, in Table 1 we show that for microRNAs that we cloned more than once 14 of the 30 rat microRNAs and 14 of the 26 monkey microRNAs had variable 3' ends. Thus, it is difficult to assess the significance of a correlation between base composition at the 3'end and detection of expression.

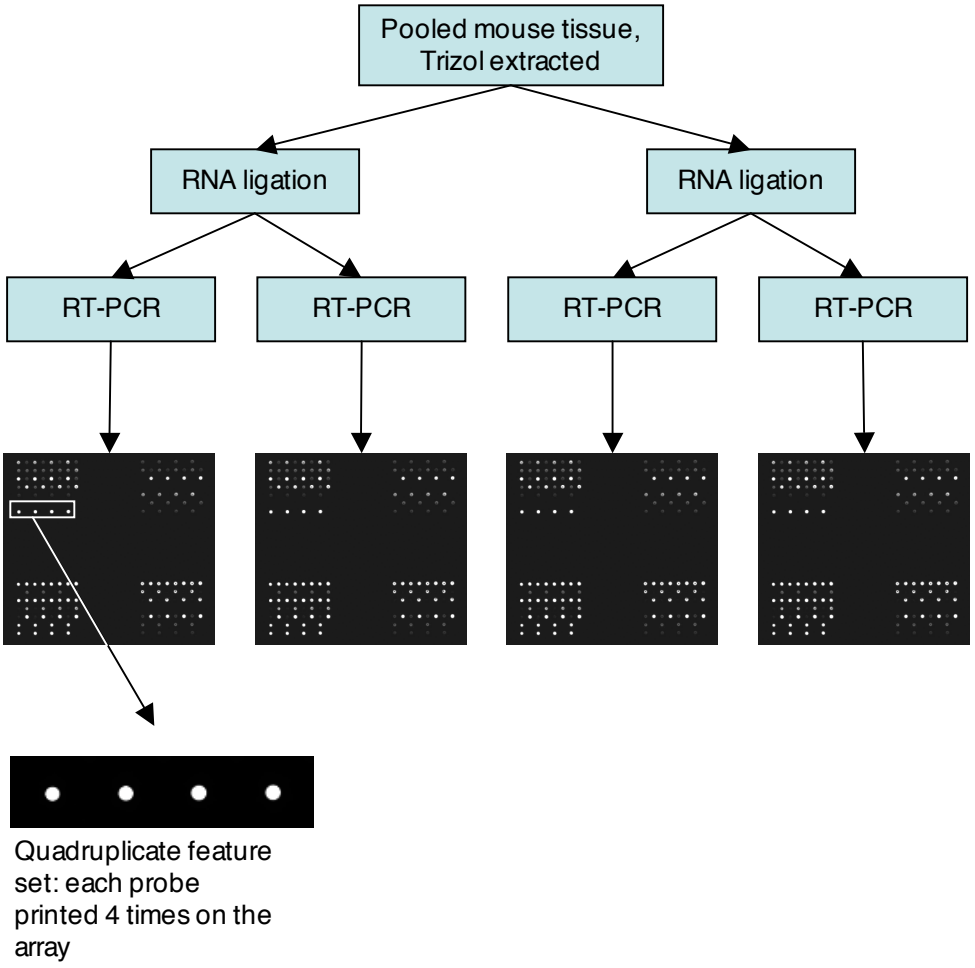

Pooled mouse tissue, Trizol extracted
RNA ligation
RNA ligation
RT-PCR
RT-PCR
RT-PCR
RT-PCR
Quadruplicate feature set: each probe printed 4 times on the array

Figure AF5a

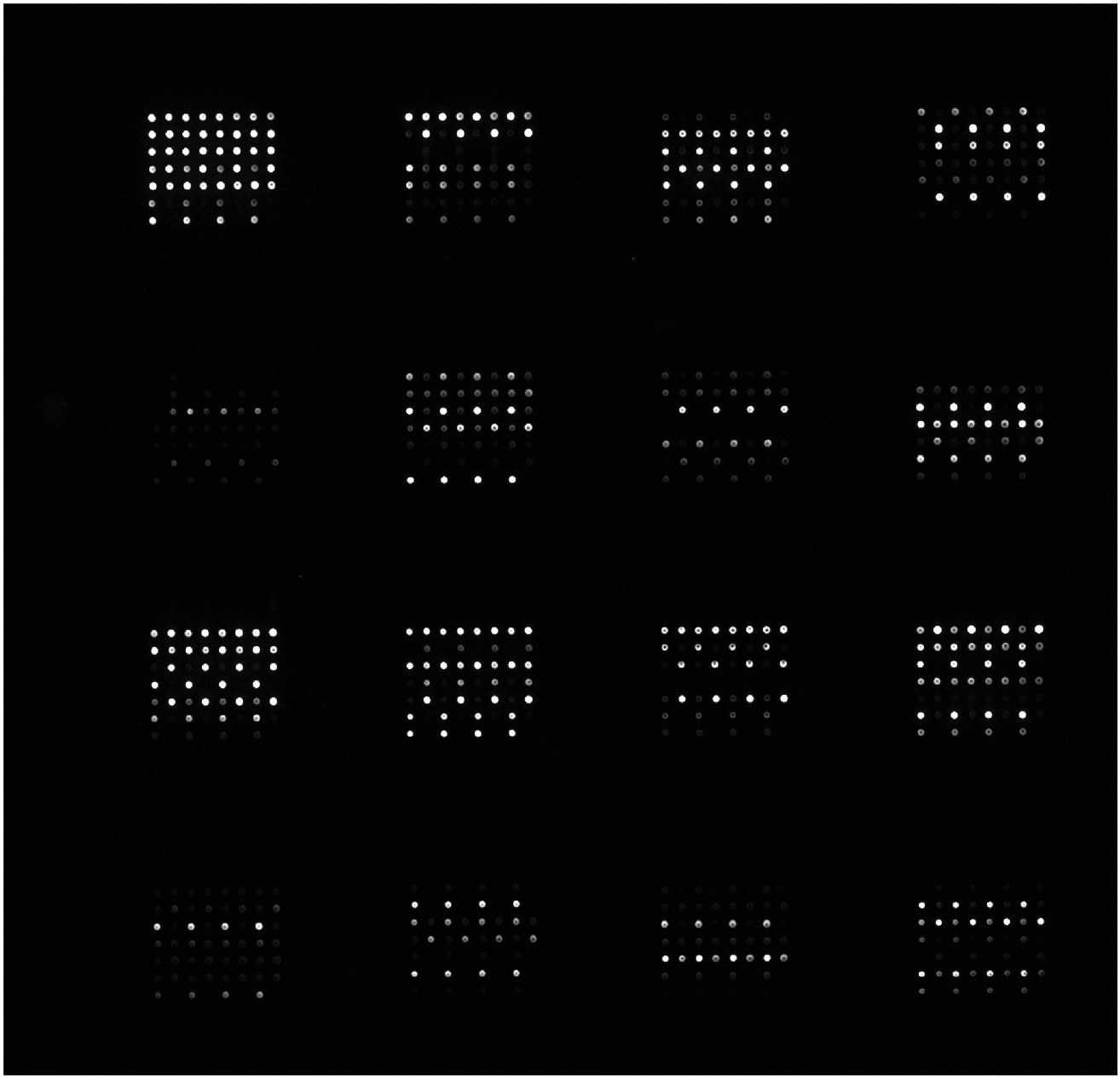


Figure AF5b. Hybridization Sample

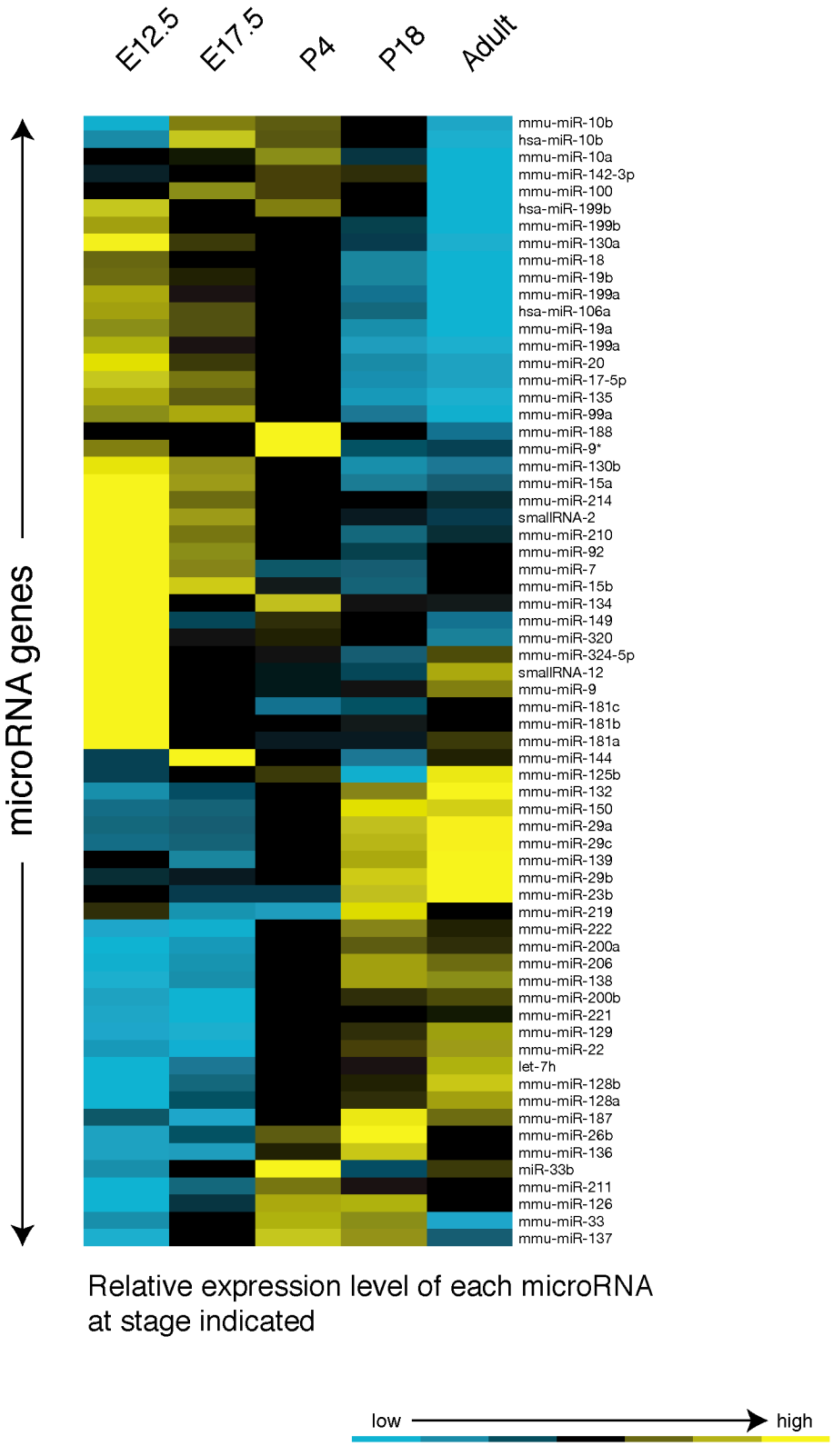


Figure AF5c. Microarray data analysis

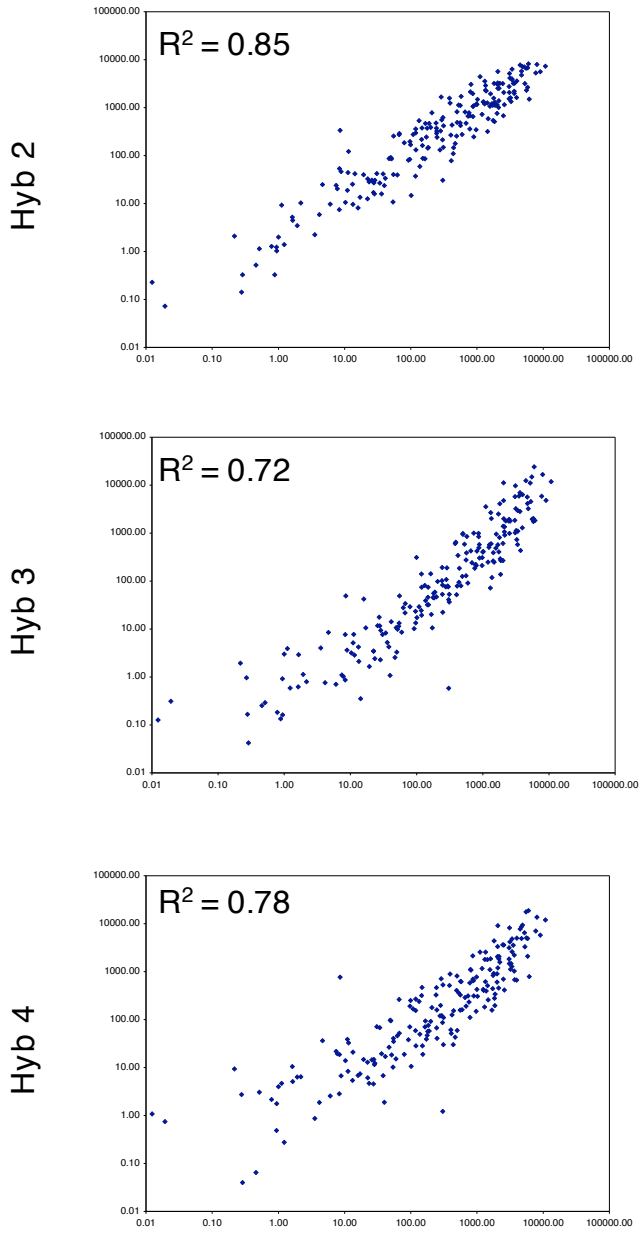

Hyb 2
$R^2 = 0.85$
Hyb 3
$R^2 = 0.72$
Hyb 4
$R^2 = 0.78$
100000.00
10000.00
1000.00
100.00
10.00
1.00
0.10
0.01
0.01
0.10
1.00
10.00
100.00
1000.00
10000.00
100000.00
Hyb 1

Figure AF5d

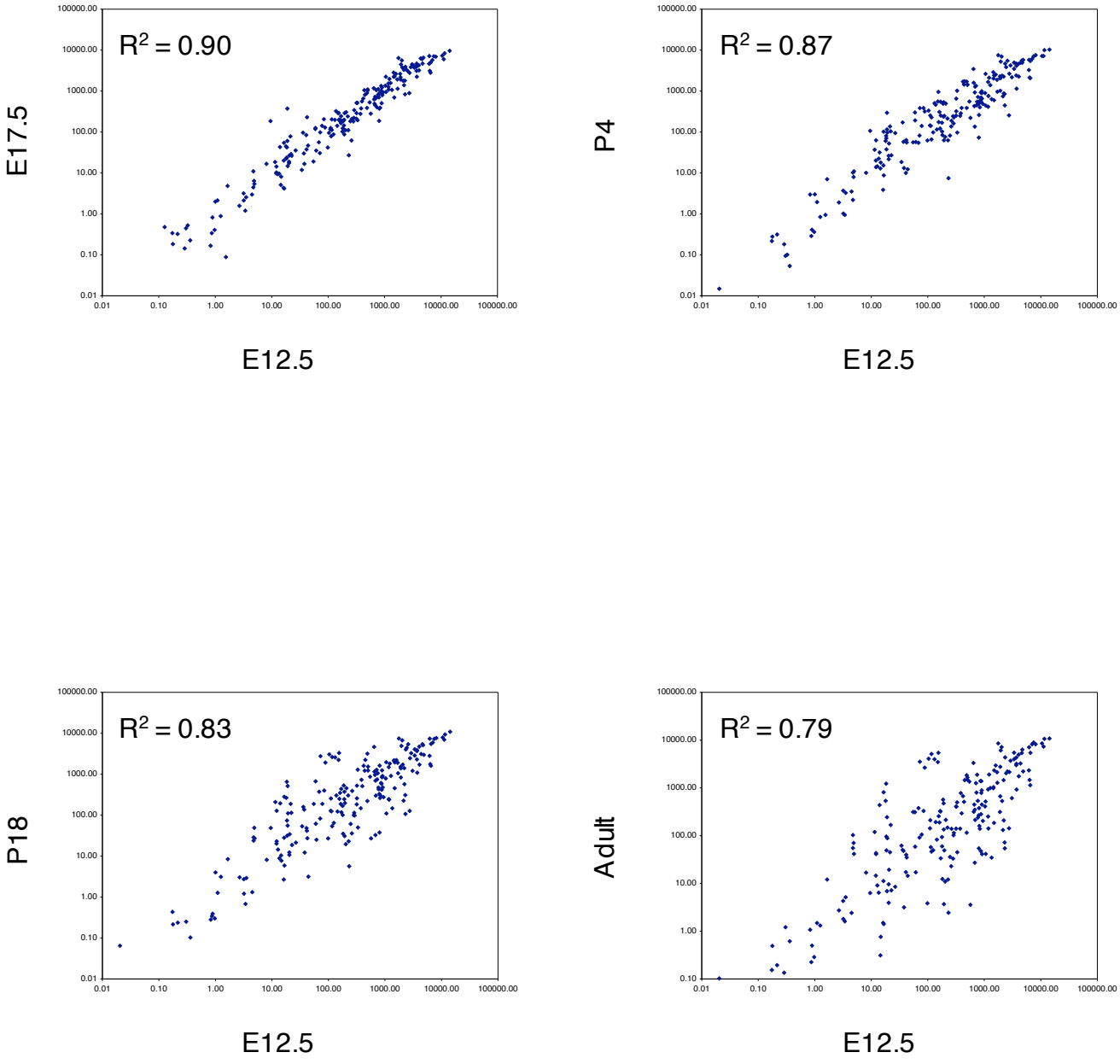

E17.5
R² = 0.90
E12.5
P4
R² = 0.87
E12.5
P18
R² = 0.83
E12.5
Adult
R² = 0.79
E12.5

Figure AF5e