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Research Paper

Rational Probe Optimization and Enhanced Detection Strategy for MicroRNAs Using Microarrays

Loyal A. Goff[1]
Maocheng Yang[2]
Jessica Bowers[3]
Robert C. Getts[3]
Richard W. Padgett[2]
Ronald P. Hart[1,*]

[1]W.M. Keck Center for Collaborative Neuroscience; [2]Waksman Institute; Department of Molecular Biology and Biochemistry; and Cancer Center of New Jersey; Rutgers University; Piscataway, New Jersey USA

[3]Genisphere Inc..; Hatfield, Pennsylvania USA

*Correspondence to: Ronald P. Hart; W.M. Keck Center for Collaborative Neuroscience; Rutgers University; 604 Allison Road Room D251; Piscataway, New Jersey 08854 USA Tel.: 732.445.1783; Fax: 732.445.2063; Email: rhart@rutgers.edu

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NOTE

Supplementary material can be found at:
http://www.landesbioscience.com/journals/rnabiology/supplement/goffRNA2-3-sup1.xls
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ABSTRACT

MicroRNAs (miRNAs) are post-transcriptional regulators participating in biological processes ranging from differentiation to carcinogenesis. We developed a rational probe design algorithm and a sensitive labelling scheme for optimizing miRNA microarrays. Our microarray contains probes for all validated miRNAs from five species, with the potential for drawing on species conservation to identify novel miRNAs with homologous probes. These methods are useful for high-throughput analysis of micro RNAs from various sources, and allow analysis with limiting quantities of RNA. The system design can also be extended for use on Luminex beads or on 96-well plates in an ELISA-style assay. We optimized hybridization temperatures using sequence variations on 20 of the probes and determined that all probes distinguish wild-type from 2 nt mutations, and most probes distinguish a 1 nt mutation, producing good selectivity between closely-related small RNA sequences. Results of tissue comparisons on our microarrays reveal patterns of hybridization that agree with results from Northern blots and other methods.

INTRODUCTION

miRNAs represent a class of small (~18–25 nt), endogenous, non-coding RNA molecules that function in post-transcriptional regulation of specific target mRNAs.[1-5] While several hundred miRNAs have been identified to date, the functions of only a few have been described in detail. This has been hindered in part by their small size and imperfect base pairing to target mRNAs, although several computational methods have been proposed to identify miRNA-target mRNA interactions.[6-9] The functions of miRNAs that have been elucidated indicate that these miRNAs influence a wide range of biological activities and cellular processes. miRNAs have been implicated in developmental patterning and timing,[1] restriction of differentiation potential,[10,11] maintenance of pluripotency, hematopoietic cell lineage differentiation,[10] regulation of insulin secretion,[12] adipocyte differentiation,[11] proliferation of differentiated cell types,[13] genomic rearrangements,[14] and carcinogenesis.[14-17]

The recent discovery of miRNAs has led to the development of several species specific, high-throughput detection methods. In several reports, spotted oligonucleotide microarray technology has proven to be effective.[11,15,16,18-26] However, design of spotted oligonucleotide probes for mature miRNAs presents several challenges. Most approaches have made use of traditional reverse transcription labelling reactions. This does not seem optimal for producing efficient labelling with such short templates. In addition, strong conservation between miRNA family members makes it difficult to design probes that are specific at the level of a single nucleotide out of a 20 nucleotide sequence, and most reports have not assessed this possibility. We report here the development of miRMAX (MicroRNA MicroArray X-species), a cross-species, sensitive, and specific microarray platform for the detection of mature miRNAs.

Furthermore, we have developed a technique to sequence-tag mature miRNAs directly so that they may be detected with high specific-activity fluorescent dendrimers.[27] Using these techniques, we identify and validate selected tissue-specific differences in miRNA expression in rat liver and brain tissues, as well as a limited number of embryonic and neural stem tissues.

METHODS

Probe oligo design. A local MySQL database was developed and populated with mature miRNA sequences obtained from miRBase (http://microrna.sanger.ac.uk, formerly known as the Sanger Registry) version 5.0. All known and categorized sequences for *H. sapiens, M. musculus, R. norvegicus, C. elegans, and D. melanogaster* were utilized to create reverse-complementary microarray probes. The database is available for searching at: http://cord.rutgers.edu/mirmax/index.php.

Probe sequences were trimmed as described in Results to balance the T_m of each of the sequences. Several negative control probes were created for each species, with C → A or G → C mutations introduced to create mismatches. A 1 nt mismatch, a 2 nt mismatch, a random sequence, a shuffled sequence, and a monomer probe were generated for each selected control spot to serve as control. Shuffled sequences were randomized using the same base composition and tested for a lack of matches in GenBank by BLAST.[28] Artificial miRNAs were synthesized (IDT, Inc., Coralville, IA) for each of the 20 miRNAs to act as positive controls.

Probe sequences were synthesized by IDT, Inc., and suspended in Pronto Glymo Buffer (Corning Life Sciences, Acton, MA) at a concentration of 30 µM. Each control spot was printed in duplicate onto the array using an OmniGrid 100 (Genomic Solutions, Ann Arbor, MI) and Stealth SMP2 pins (Telechem, Inc., Sunnyvale, CA). Probes were arranged by species into different sub-arrays and were printed on Corning Epoxide slides. Slides were dried overnight in nitrogen, and then placed in a humid chamber for 3 hours to complete coupling. Slides were then washed sequentially in 0.1% Triton-X100, 0.1 M HCl, and 0.1 M KCl, water, and then unreacted groups were blocked with 50 mM ethanolamine in 100 mM Tris-HCl pH 9.0 and 0.1% SDS, followed by water washes. The arrays were then allowed to dry overnight prior to hybridization.

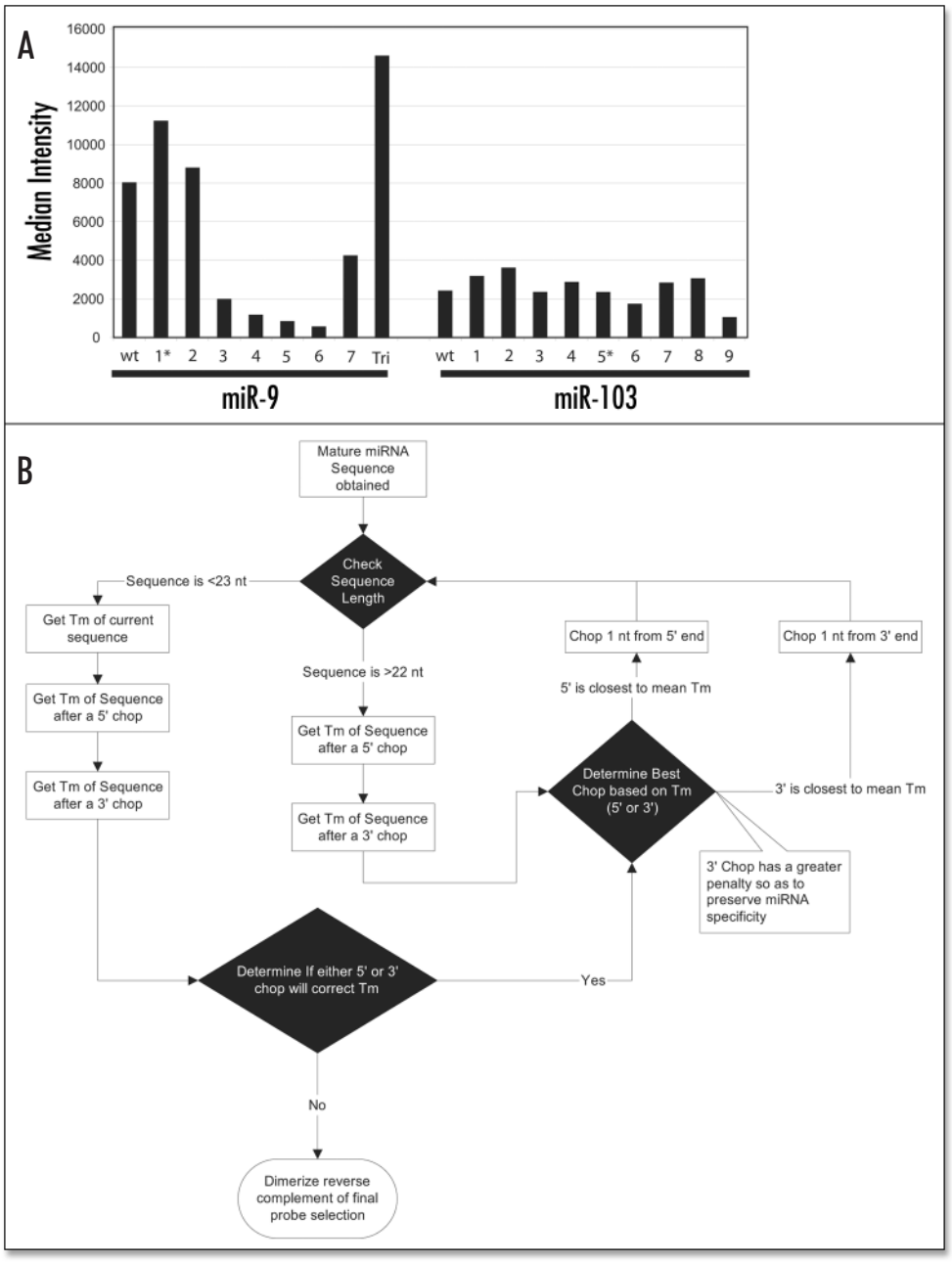

Figure 1. Probe design algorithm. (A) Evaluation of probe design algorithms. Test microarrays were printed with various versions of oligonucleotide probes to compare hybridization signals (sequences of numbered probes are shown in Table 1). Results show the median intensity values of hybridization to synthetic miR-9 and miR-103, for each of several different probe design truncation patterns. The numbers are codes for various versions of the probe using different design strategies. The patterns chosen by our final probe design algorithm are indicated with an asterisk and show hybridization levels equivalent to or, in most cases, stronger than that of the wt (unaltered) probe sequences while retaining appropriate hybridization results. (B) Selected probe design algorithm. A flow chart shows the steps in the selected design algorithm.

RNA preparation and labelling. Individual liver and brain tissue samples were obtained from three adult Long-Evans rats. Low molecular weight (LMW) RNA was extracted from each sample using the mirVana™ miRNA extraction kit (Ambion, Austin, TX). LMW RNA was quantified using the RiboGreen™ kit (Invitrogen, Carlsbad, CA) high-range assay. 100 ng of LMW RNA was typically used as input for the labelling reaction. Quality of LMW RNA was judged indirectly by running the high molecular weight fraction from the same preparation on an Agilent Bioanalyzer. We observed that low quality high molecular weight RNA produced poor hybridization results on arrays (not shown).

miRNAs were labeled using the Array900 miRNA Direct kit (Genisphere Inc, Hatfield, PA). Briefly, 100 ng of enriched miRNA was polyadenylated using poly(A) polymerase (2 U) and ATP (8 µM final concentration) in the provided reaction buffer (1X reaction buffer: 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$) in 25 µl for 15 minutes at 37°C. Polyadenylated miRNAs were sequence tagged by adding 6 µl of 6X Cy3 or Cy5 ligation mix and 2 µl of T4 DNA Ligase (1 U/µl) and incubating at 20°C for 30 min in a final volume of 36 µl. For these experiments, 6X Ligation Mix consists of two prehybridized oligonucleotides, a Cy3 or Cy5 capture sequence tag and the appropriate bridging oligonucleotide, in 6X concentrated ligation buffer diluted from10X Ligation Buffer (Roche). The capture sequence tag is a 31 base oligonucleotide complementary to an oligonucleotide attached to a 3DNA dendrimer labeled with either Cy3 or Cy5. The bridging oligonucleotide (19 nt) consists of 9 nt that are complementary to the capture sequence tag and 10 nt complementary to the added poly A tail

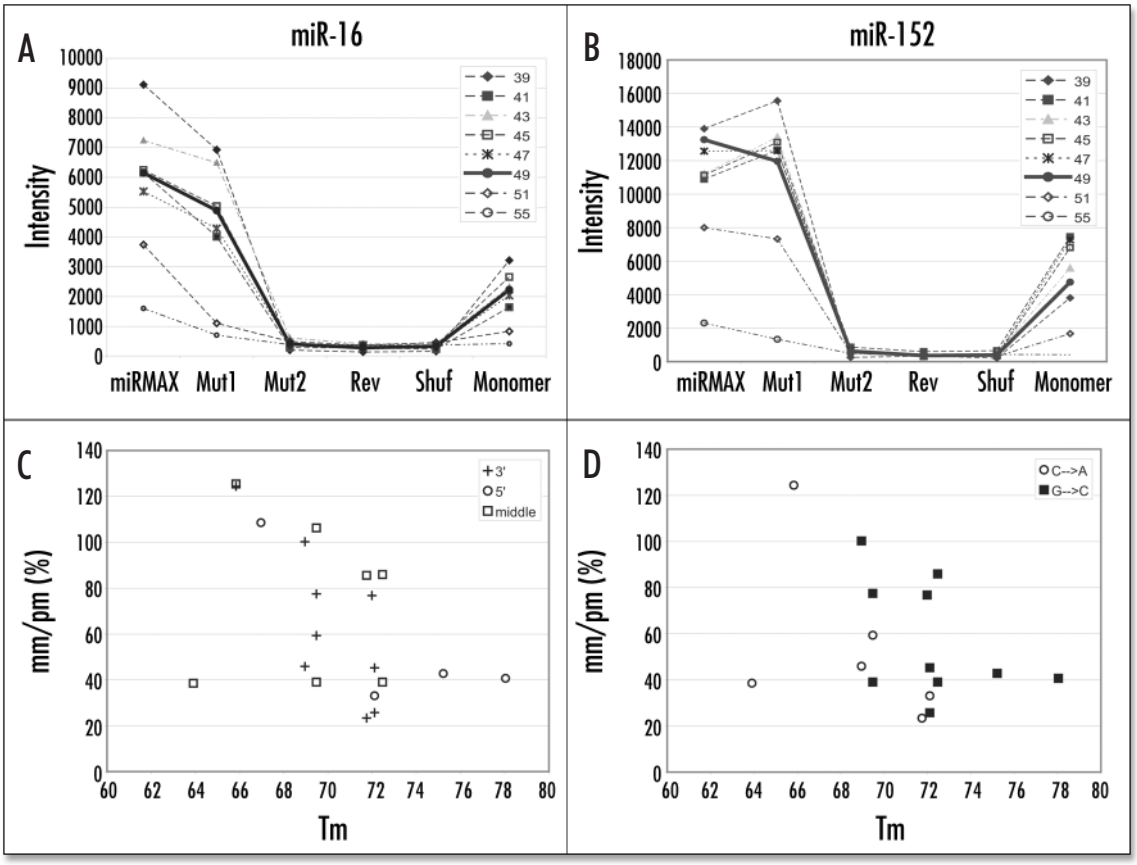

Figure 2. Sequence selectivity by hybridization temperature. Control probe median intensity values (background subtracted) were obtained from hybridization to a pool of synthetic miRNAs, each ~700 pg. Probes spotted onto the microarray for each control set included a wild-type, anti-sense monomer oligo (Monomer), a designed probe (miRMAX), the designed probe with one nucleotide mismatch (Mut1) or two nucleotides of mismatch (Mut2), a reverse complement probe (Rev) and a randomly shuffled sequence (Shuf). Individual lines indicate values obtained at various hybridization temperatures (see legend). The two predominant patterns of results obtained are demonstrated by the hybridization of (A) miR-16, in which the Mut1 intensities are decreased regardless of hybridization temperature, and (B) miR-152 in which the Mut1 probe showed comparable or slightly greater hybridization to the synthetic miRNA. This greater hybridization was almost entirely removed if more stringent hybridization temperatures were utilized. The full dataset from these hybridizations is available as Supplemental Material 1. In an attempt to find if specific mutation types affect the selective hybridization to our designed probes, we plotted the percentage ratio of Mut1 median intensities (mm; mismatch) to probe (pm; perfect match) intensities against the calculated melting temperatures of the miRNA:probe dimer. Individual points are keyed by type of mutation (see legend). While a general trend was observed for all data, no obvious patterns emerged when comparisons were made between relative position of the mutation within the miRNA sequence (C) or type of nucleotide change that was made (D).

(dT_{10}). After terminating the ligation reaction by adding 4 µl of 0.5 M EDTA, the tagged miRNAs were purified a MinElute PCR Purification kit (Qiagen) according to the manufacture's protocol for DNA cleanup.

Array hybridization. Sequence-tagged LMW RNA was hybridized to the miRNA microarrays using the Ventana Discovery System (Ventana Medical Systems, Tuscon AZ) using a custom protocol designed by us. Tagged miRNA samples were hybridized for 12 hours in ChipHyb buffer (Ventana) containing 8% formamide. After 12 hours, slides were washed with 2X SSC at 37°C for 10 min; and then with 0.5X SSC at 37°C for 2 min. After this initial hybridization, a mixture of Cy3 and Cy5 labelled 3DNA dendrimers was applied to each microarray and a second hybridization proceeded for 2 hours at 45°C. Arrays were washed with 2X SSC at 42°C for 10 min and then removed from the hybridization system. Slides were then manually washed (1 min each) twice in Reaction Buffer (Ventana) and a final, room temperature wash in 2X SSC. Arrays were dried and coated with DyeSaver (Genisphere) to preserve Cy5 intensities. Arrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices, Union City, CA) and median spot intensities collected using Axon GenePix 4.0 (Molecular Devices). Data analysis and manipulation were conducted in either GeneSpring 7.0 (Agilent, Redwood City, CA), or GeneTraffic Duo (Stratagene, La Jolla, CA).

Northern blots. For each Northern blot, 3 µg of LMW rat brain or rat liver RNA was electrophoretically separated in a 15% urea-polyacrylamide gel. RNAs were again electroblotted onto Hybond-N^+ membrane, UV-crosslinked and baked for one hour at 80°C. StarFire probes[29] against miR-93 (5'-CTACCTGCACGAACAGCACTTT-3'), miR-16 (5'-CGCCA-ATATTTACGTGCTGCTA-3'), and miR-191 (5'-AGCTGCTTTTGGGA-TTCCGTTG-3') were radio-labelled with [α-P^{32}]-dATP at 6000 Ci/mmol. Membranes were probed with one of the StarFire Probes overnight for 50°C.

For the dot blot series of Northern hybridizations, 2 ng of either synthetic wt miR-191 RNA (5'-caacggaaucccaaaagcagcu-3'), a 1 nt mismatch miR-191 RNA (5'-caacgCaaucccaaaagcagcu-3'; mismatch underlined), or a 2 nt mismatch miR-191 (5'-caacgCaaucccaaaagAagcu-3'), was spotted to Hybond-N^+ membrane followed by UV-crosslinking and baking at 80°C for 1 hour. The quantity of synthetic miRNA was determined by comparing a serial dilution to 3 µg of LMW RNA (not shown). The membranes were then probed with StarFire probes (IDT) for either the miRMAX probe sequence for miR-191 or the mut-1 control probe for miR-191 that were radioactively labelled with [a-P^{32}]-dATP 6000 Ci/mmol following the vendor's recommendation. The membranes were probed overnight at 55°C. Dot intensities were recorded using a PhosphorImager (GE Biosciences, Niskayuna, NY) and dot

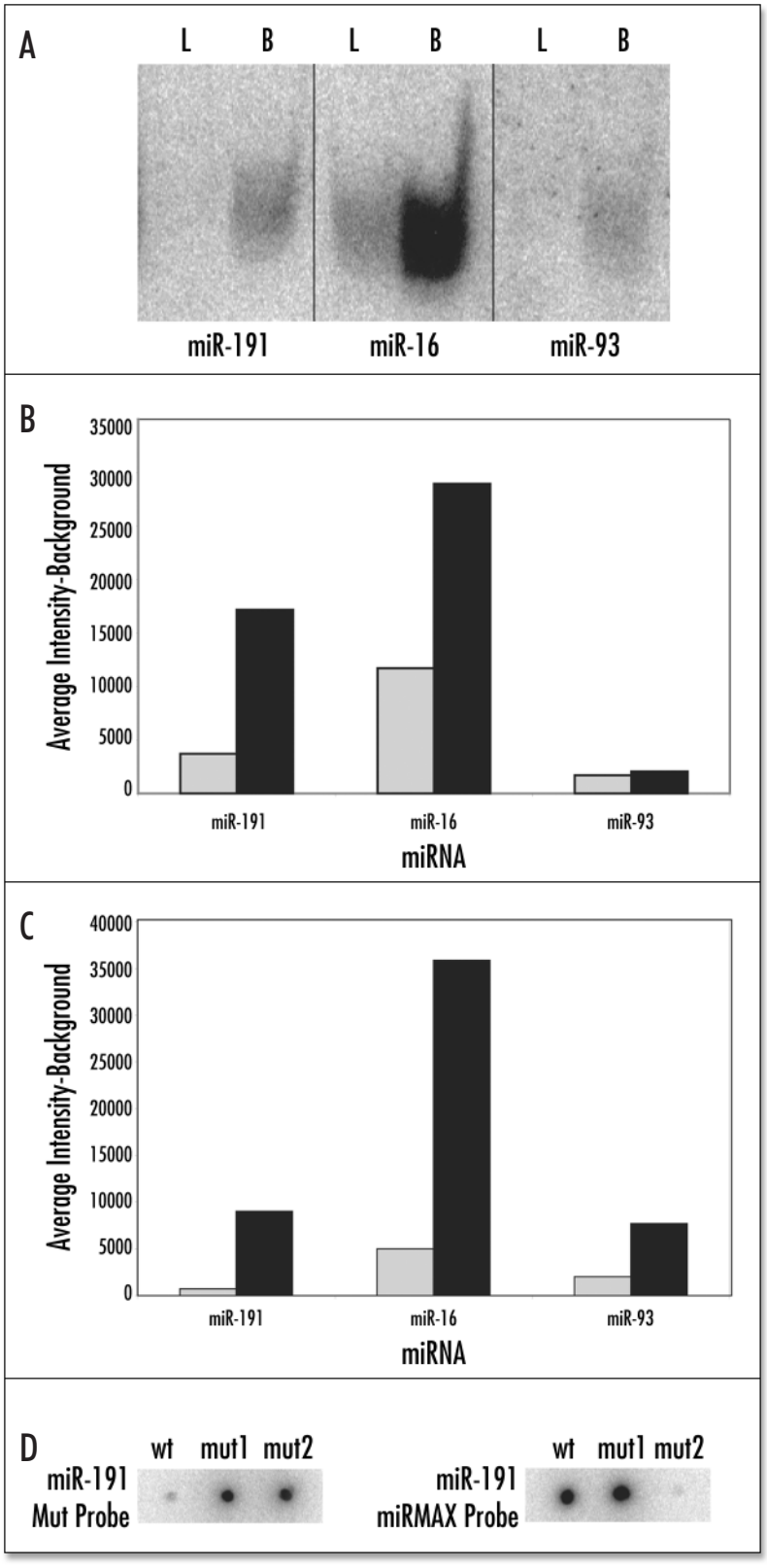

Figure 3. Northern validation of microarray results. (A) Northern blots of three mature miRNA species, miR-191, miR-16, and miR-93, from liver (L) and brain (B) LMW RNA samples are shown. Probes for Northern and dot blots consisted of traditional antisense oligo probes coupled with StarFire detection sequences (IDT). Mean intensity values from the three liver/brain microarray hybridizations are shown in (B) for liver (grey) and brain (black). The integrated volume for each of the Northern images (C) shows similar patterns of relative miRNA levels between the two tissues for each of the three miRNAs. (D) Dot blots compared sequence specificity of synthetic miRNAs spotted on nylon membranes using traditional oligo probes. Synthetic miR-191 miRNA (wt), or a single mutation (mut1) or double-mutation (mut2) RNAs were spotted and detected with probes matching mut1 or wt sequence. Each probe detected its perfect complement as well as a 1 nt mismatch. Interestingly, the mut1 probe hybridized primarily with mut2 RNA over wt RNA, even though both synthetic RNAs were 1 nt different from probe.

volume was measured using ImageQuant (GE Biosciences) software.

Neural stem cell culture. Neural stem cell cultures were created and maintained as described previously.[30,31] The N01 NS clone was prepared from rat fetal blood and grown as neurospheres using similar methods (D. Sun, unpublished). For comparison, tissues were prepared from adult rat olfactory bulb, brain or liver.

RESULTS

Probe oligo design. The initial probe design incorporated several concepts, including: (1) trimming of miRNA sequences to adjust for an inherently wide variance in melting temperatures, (2) constructing reverse-complement probes to allow direct hybridization to labelled miRNAs, and (3) comparing monomer, dimer, and trimer probe sequences to maximize sensitivity.

We decided to truncate miRNA sequences in an attempt to reduce the large range of T_m values across all known miRNA sequences. Several different miRNA truncation algorithms were evaluated to determine the effect on hybridization to a labelled extract. Initially, we judged hybridization intensity with reverse-complement dimer probes using several variations in probe sequence content. Initial truncation algorithms removed 1 nt from 3' or 5' ends in alternating succession from probes with high T_m. Further refinement of our approach involved calculating which end of the miRNA allowed for the most precise adjustment of T_m during truncation. Additionally, it has been shown that the 5' "seed" region of a miRNA is conserved among miRNA family members.[7,32-34] Additional weight and preference was therefore given to truncation at the 5' end, so as to preserve the more variable 3' sequence, and allow for better discrimination between closely related miRNAs. The final adopted design algorithm created probe sequences with a mean T_m of 66.72°C with a 95% CI ranging from 66.47 to 66.97°C, as compared to the wider distribution of the original miRNA sequences (mean 68.07°C, 95% CI 67.75 to 68.39°C). This adjustment in melting temperature is expected to allow more uniform hybridization among different probe sequences with minimal loss of selectivity.

Previous methods for spotting probes for miRNAs have demonstrated the efficacy of constructing multimeric probe sequences to maximize the availability of a complementary sequence for hybridisation.[18,20] One potential method would be to add a terminal amine group for attachment to epoxy groups on the glass slides, but since all oligos also contain internal amine groups that would compete for this reaction, we chose to eliminate the use of terminal amines. Using unmodified oligos also greatly reduces the cost of manufacture. We reasoned that multimers of probe sequence would covalently attach to epoxy groups via internal bases with primary amines without significantly affecting hybridization efficiency. With this in mind, we constructed monomer, dimer, and trimer probe sequences for comparison. While both dimer and trimer probes showed enhanced hybridization signal intensity as compared to the monomer sequence, there was no significant advantage to trimer sequences over dimer

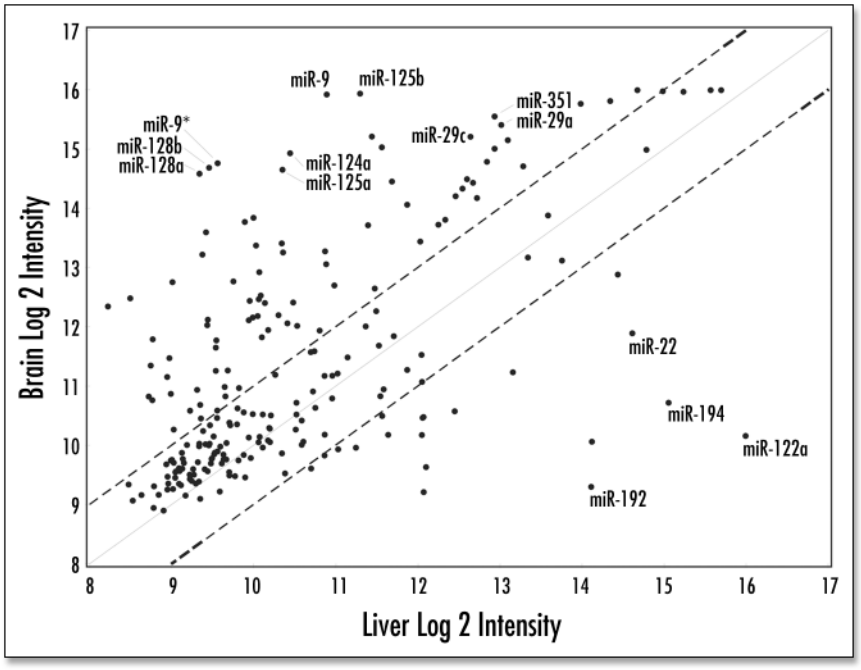

Figure 4. Tissue-specific hybridization. Scatterplot depicts average $\log_2$ fluorescence intensity values for each rat and mouse miRNA probe for three liver and brain miRMAX hybridizations. A table of results, designated by tissue specificity, can be found in Supplemental Materials 2.

Table 1 **Sequences of oligo probes used in Figure 1A**

Target miRNA	Variant	Printed Probe
miR-9	wt	TCATACAGCTAGATAACCAAAGATCATACAGCTAGATAACCAAAGA
	1	TCATACAGCTAGATAACCAAAGTCATACAGCTAGATAACCAAAG
	2	CATACAGCTAGATAACCAAAGCATACAGCTAGATAACCAAAG
	3	TCATACAGCTAGATAACCAATCATACAGCTAGATAACCAA
	4	CATACAGCTAGATAACCAAACATACAGCTAGATAACCAAA
	5	TCATACAGCTAGATAACCATCATACAGCTAGATAACCA
	6	TCATACAGCTAGATAACCTCATACAGCTAGATAACC
	7	TCATACAGCTAGATAACCAAATCATACAGCTAGATAACCAAA
	Tri	TCATACAGCTAGATAACCAAAGATCATACAGCTAGATAACCAAAG ATCATACAGCTAGATAACCAAAGA
miR-103	wt	TCATAGCCCTGTACAATGCTGTCATAGCCCTGTACAATGCTG
	1	TCATAGCCCTGTACAATGCTTCATAGCCCTGTACAATGCT
	2	CATAGCCCTGTACAATGCTGCATAGCCCTGTACAATGCTG
	3	CATAGCCCTGTACAATGCTCATAGCCCTGTACAATGCT
	4	TCATAGCCCTGTACAATGCTCATAGCCCTGTACAATGC
	5	ATAGCCCTGTACAATGCTGATAGCCCTGTACAATGCTG
	6	ATAGCCCTGTACAATGCTATAGCCCTGTACAATGCT
	7	TCATAGCCCTGTACAATGTCATAGCCCTGTACAATG
	8	TAGCCCTGTACAATGCTGTAGCCCTGTACAATGCTG
	9	TCATAGCCCTGTACAATTCATAGCCCTGTACAAT

All sequences are 5′ to 3′, left to right

sequences as both yielded comparable intensities (not shown). For this reason, dimer probe sequences were utilized in our final design.

Low molecular weight (LMW) rat brain RNA extracts, hybridized to microarrays with probes of various truncation patterns (Table 1), indicated that our final probe design algorithm provides comparable intensities to wt (full-length, reverse-complement dimer) probe sequences (Fig. 1). In all but a few test cases, the designed probe showed an intensity equal to or greater than that of the wild-type probe. Those with weaker intensities than the wt probe showed only slight variation across different truncation patterns as well, indicating a minimal threshold of intensity for that given miRNA. We conclude that our probe design algorithm produces hybridization results that are indistinguishable from unaltered sequences. Furthermore, dimer probes produce improved hybridization over monomer probes and are similar to trimer probes. Probes were created for each mature miRNA from *Homo sapiens, Rattus norvegicus, Mus musculus, Caenorhabditis elegans*, and *Drosophila melanogaster* in the Sanger miRNA Registry[35] release 5.1. We designed a total of 457 unique probe sequences targeting 225 human, 198 rat, 229 mouse, 85 fly and 117 worm miRNAs.

As compared with traditional microarrays, the miRNA labelling method faces unique limitations and challenges. Importantly, mature miRNAs are not normally polyadenylated, so traditional methods of priming with oligo d(T) will not work. Furthermore, since miRNAs are so small, either reverse transcription into labelled cDNA or direct coupling of fluorescent dyes to miRNAs would be expected to produce relatively low specific activities and also tend to interfere with sequence-specific hybridization. Finally, reverse transcription might label precursors to miRNAs with more dye molecules, enhancing hybridization signals disproportionately from non-mature species.

Parallel to the testing of our probe design algorithm, a direct miRNA labelling reaction was developed at Genisphere, Inc. In this reaction, LMW RNA is 3' extended with poly(A) polymerase and then ligated to a "capture" sequence tag via a bridging oligo. The sequence-tagged miRNA is hybridized directly to the anti-sense oligo probes and detected by hybridization to a complementary capture sequence on a fluorescent dendrimer. This protocol allows detection of a single molecule of miRNA with as many as 900 molecules of fluorescent dye, greatly amplifying the signal. While this protocol is designed to label mature miRNA we did not evaluate relative labelling efficiency of mature miRNA versus precursor species. After testing a series of diluted RNA samples, we chose to routinely begin with 100–200 ng of LMW RNA per sample, corresponding to 1 μg of total cellular RNA or less, since this gave median hybridization intensities near the center of our fluorescence detection range (not shown). Using 50-fold less input RNA produced essentially undetectable hybridization, and using 50-fold more RNA produced strong hybridization signals for mismatch probes. Other miRNA microarray labelling methods require 5–7 μg[16,19,21] or much more.[22,36]

Optimization of hybridization. After validation of our probe design algorithm, we examined the ability to select specific miRNA sequences over different hybridization temperatures. Of the probes designed, a

subset of 20 was chosen and additional control probes were designed to test sequence selectivity. The control probes included a 1 nt mismatch, 2 nt mismatch, reverse complement, shuffled sequence and monomer probe. The 1 and 2 nt mismatch control probes allowed for determination of the specificity and selectivity of our probes. An equimolar mix of synthetic miRNAs corresponding to the 20 control probe miRNAs was labelled and hybridized to the array. Median signal intensities were calculated for each of the wt probes, 1 nt mutant, 2 nt mutant, reverse complement, shuffled, and monomer sequences and compared for each of the 20 control miRNAs (example results in Fig. 2A and B; full results in supplemental data). As anticipated, signal intensities for the 2 nt mismatch, reverse complement, and shuffled control probes were all but abolished in each case. As in earlier results, monomer probe sequences were also significantly less intense than the dimer sequence. Two distinct patterns emerged from the 1 nt mismatch results. In the majority of the 1 nt mismatch sequences, the intensity was only slightly reduced compared to the miRMAX probe (Fig. 2A). In a few instances however, at less stringent hybridization temperatures, the 1 nt mismatch probe yielded a slightly greater intensity than that obtained from the miRMAX probe (Fig. 2B). This signal was always, however, completely abolished in the 2 nt mutant probe.

For each of the 1 nt mutant probes, a ratio of median intensities of the mismatch/perfect match probes (MM/PM) was determined and analyzed to discover what effect, if any, specific mutation types (C → A or G → C; Fig. 2D) or positions within the miRNA sequence (Fig. 2C) had on observed signal intensity. No obvious correlations were identified between sequence transversions or mutation position and signal intensity between the miRMAX probe and the 1 nt mismatches, although a wide range of MM/PM ratios was observed. These observations indicate that our miRNA detection system was quite capable of distinguishing between miRNAs with as few as two different nucleotides, but is less reliable in discriminating between more closely related family members, such as the let-7 family of miRNAs. This is similar to the best of other, published miRNA microarray systems.

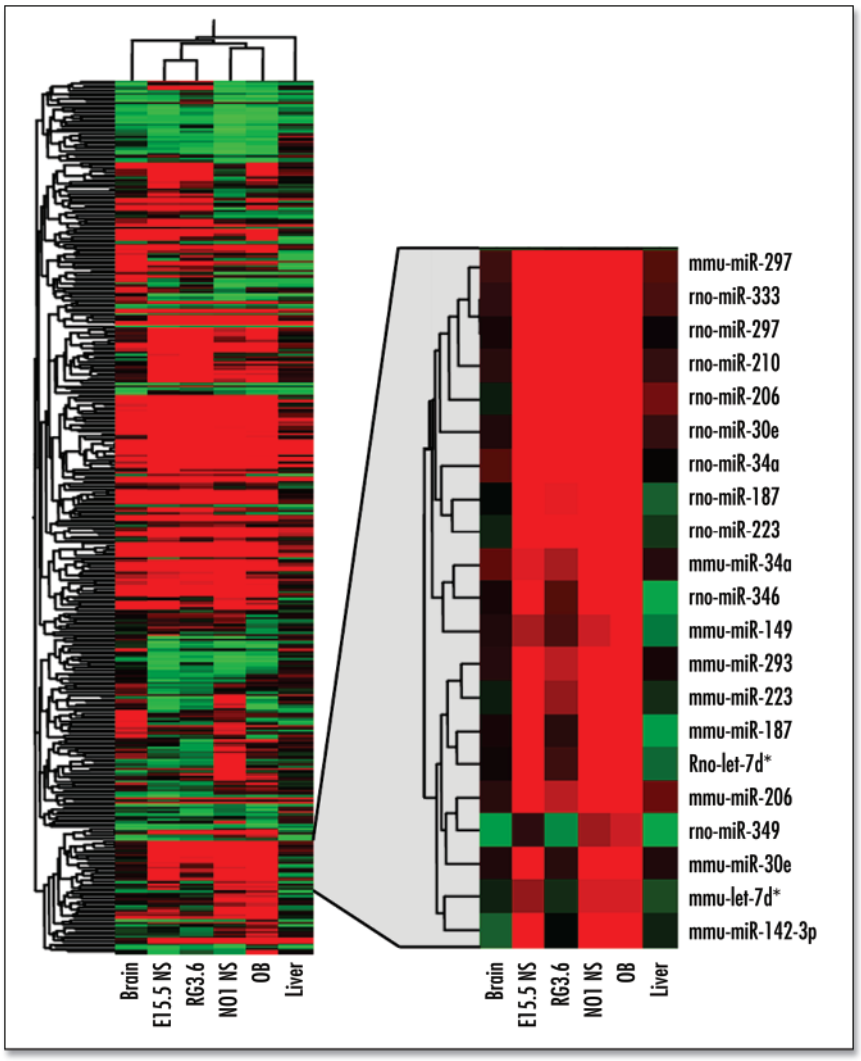

Figure 5. Hierarchical clustering of miRNA expression levels in neural stem cell clones. A hierarchical clustering heat map shows rat and mouse miRNA expression levels in various stem cell lines as well as in adult liver and brain LMW RNA. Several miRNAs appear to be expressed more intensely in the stem cell lines as compared to the adult tissue (expanded region), including members of a previously identified "ES-cell specific" miRNA cluster.[42]

Interpreting the temperature data for all control probes, we selected 47°C as the best trade-off between sequence specificity and signal intensity. Increasing the temperature to 49°C slightly reduced the mismatch hybridization signal, but immediately above 49°C the full-length probe intensity decreased substantially (by 35% from 49–51°C). We selected 47°C to reduce the chance of losing signal due to minor changes in temperature. All subsequent data were collected at 47°C.

Our design of control miRNA probes also provides methods for normalizing hybridization results between microarrays. If one sample is assayed per microarray, the second fluorescent channel can be used to label the mixture of 20 synthetic miRNAs as an internal standard. This standard can be used to adjust the fluorescence signal among different microarrays within an experiment. Alternatively, the use of many cross-reacting miRNA probes from other species increases the number of observed hybridization events so that Lowess normalization[37] can be applied to two-color experiments with a more valid number of spots. Experiments can therefore be designed to take advantage of internal standards (one sample per array) or more hybridization results for traditional two-color designs.[38]

Validation of miRNA expression. Northern blots were used to validate relative hybridization signals for three miRNAs, miR-191, miR-16, and miR-93. These miRNAs were chosen among the miRNAs for which control sequences had been made so as to facilitate analysis of sensitivity and selectivity (Fig. 3A). For Northern blots, probes were composed of complementary, monomer sequence modified to use the StarFire labelling system (IDT, Inc.). While none of these three miRNAs was expressed at high levels in either adult rat liver or brain, a similar order of hybridization signals was obtained from both Northerns and miRMAX microarrays. The background-subtracted median intensities from the microarray hybridizations matched the pattern observed for the Northern blots between liver and brain samples across all three miRNAs (Fig. 3B and C), indicating that our miRNA detection method was able to mimic results obtained via traditional Northern blot methods. In addition, observable signals of weakly-expressing miRNAs (miR-191 and miR-16 in liver as examples) were relatively greater (as compared to background levels) in the miRMAX system than in the Northern assay. Furthermore, Northern blots generally required 30-fold more input RNA than the microarrays.

To assess the selectivity of our microarray probes, we performed a dot blot comparing hybridization of wt, 1 nt mutated, and 2 nt mutated miR-191 to both the miRMAX probe as well as a probe with a complementary mutation to the 1 nt mutated miR-191 sequence (Fig. 3D). As anticipated, the miRMAX probe for miR-191 strongly hybridized to the wt miR-191, was slightly weaker in hybridizing to the mut1 RNA, and showed

only minimal hybridization to the 2 nt mutated RNA. This indicates that the standard Northern assay is no more selective than our microarray assay in distinguishing between miRNA species with only 1 nt difference. Hybridization of the miR-191 mut1 probe to the dot blot array however, resulted in strong hybridization to both the mut1 RNA and the mut2 RNA, but curiously, not the wt miR-191 synthetic RNA.

The probe design has also been validated and demonstrated to be effective on other assay systems. The Luminex bead assay system has been used previously to detect miRNAs with a LNA labelling technology.[20] We synthesized several terminally-aminated probes, using sequences identical to those found on our microarrays. Using the Luminex assay system with the same labelling system as our microarrays, we were able to reproduce the rank order of detection of mir-1, mir-122 and mir-124a in rat heart, liver and brain LMW RNAs, respectively (not shown). These three probes were chosen from microarray results because of their clear tissue-specific expression patterns. Similarly, using these probes in an ELISA-like well-based hybridization system also replicated the microarray results (not shown). These alternative assays further demonstrate the utility of our probe design and sensitive detection system in methods that may be more applicable for high-throughput assay of limited numbers of miRNAs with optimized sequence selectivity.

Comparison of miRNA levels in rat brain and liver. To test and validate the new platform, we chose to examine miRNAs in rat brain and liver, where there exists data for comparison. Three adult rat brain LMW RNA samples (Cy3) and three liver LMW RNA samples (Cy5) were labelled and hybridized to our custom chips. A wide range of $\log_2$ ratios was observed (Fig. 4) indicating a distinct expression profile in each of the two tissues. Using a 2-fold expression level cutoff, it is interesting to note that there are more miRNAs preferentially expressed in brain than in liver (Supplemental data 2). Expression of brain and liver specific miRNAs was well correlated with previously published data. miR-124a, miR-125a and b, miR-128, miR-181, and miR-9, all previously shown to be enriched in brain tissue,[18,22,39,40] were also very highly expressed in the brain tissues in our assay. miR-122, miR-192, miR-194 and miR-337 were expressed at levels much higher in liver than brain in our study which again correlates with other studies.[19,26,39-41]

miRNA expression in neural stem cells. Several studies have indicated that miRNAs may play an important role in stem cell maintenance and differentiation.[10,11,42,43] As a broad comparative study, several available rat stem cell populations were assayed using the miRMAX microarray system (Fig. 5). While some miRNAs had similar profiles across all stem cell lines and adult tissues, the vast majority showed dramatic differences in expression between the stem cell lines and the adult tissues. Among the samples tested and clustered, the relationships appear to make sense. Liver is the least related sample. The most similar samples are E15.5 neurospheres and RG3.6 cells, which were derived from E15.5 neurospheres.[44] RG3.6 is transfected with v-myc to stabilize a radial glial phenotype. The next most similar samples were neurospheres of N01 clones, derived from rat fetal blood and olfactory bulb. Among the miRNAs that are enriched compared to brain or liver was a member of the "ES"-specific cluster,[42] mir-293. Others (mir-223 and 142s) have been identified for expression in hematopoietic cell lines.[10] Interestingly, none of these miRNAs correlates with a list found in human embryonic stem cells or embryonic carcinoma cells.[43] In many cases, homologous probes from the two selected species hybridized similarly across all samples. We conclude that rat neural stem cell preparations express distinct populations of miRNAs, as has been observed in other species.

DISCUSSION

We have developed an optimized miRNA microarray platform, including rationally-designed probes for multiple species printed on a single microarray as well as a high specific-activity labelling method. Our design reduced the predicted variability of miRNA melting temperatures, but retained hybridization intensities similar to unmodified sequence. Using a subset of probes with specific mutations, we find that all probes are specific within 2 nt, and many are detected selectively within 1 nt. Using a detailed hybridization temperature series, we selected the appropriate hybridization temperature (47°C), a step that is crucial for optimizing sequence specificity. The labelling method is straightforward, producing directly-labelled miRNA, which allows use of minimal quantities of input RNA and takes advantage of more stable RNA-DNA hybridization properties. Results are similar to Northern blots performed with 30-fold more RNA. Using this platform, we have performed hundreds of arrays with validated and reproducible results, including the detection of tissue-specific expression in rat brain vs. liver, characterization of miRNA expression in several stem cell clones available in our laboratory, and a comparison of brain-specific miRNAs across all five species present on our chip. The latter study (Yang et al., in preparation) highlights the value of including probes for multiple species on a single microarray. Furthermore, the validation of a rational probe design algorithm is expected to be important for extending miRNA assays to high-throughput experiments as the numbers of miRNAs per genome is predicted to increase from 200 up to 1,000.[34] Efficient miRNA microarray platforms will be valuable in identifying miRNAs regulating biological systems and in predicting interactions with specific target mRNAs.

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