APPLICATION NOTE

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Reciprocal microRNA modulation identifies gene targets in a biological context

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Abstract
Through their effect in down-regulation of gene transcripts, microRNAs play an important role in a range of biological processes such as development, differentiation, and disease. Although many methods have been proposed to discover messenger RNA (mRNA)-microRNA interactions, most are either time consuming or lack accuracy. Here, we present an empirical method for identifying direct microRNA targets using reciprocal mRNA expression response to mimic and inhibitor treatments. The method is supported through analysis of the enriched presence of microRNA seed complement sequences. Using reverse transcription quantitative real-time PCR (RT-qPCR) for confirmation, empirically determined miR-122 targets were compared to targets predicted by widely used microRNA target prediction programs. Overall, we conclude empirical detection is a more time efficient way to identify mRNA-microRNA interactions than selecting targets from lists generated by microRNA-target prediction programs.

Introduction
microRNAs are a class of small non-coding RNAs, 22 nucleotides (nt) in length, involved in post-transcriptional gene regulation of up to 50% of protein-coding genes[1-3]. Mammalian mature microRNAs down-regulate gene expression primarily by binding to the 3′ UTR[4-7]. The mRNA-microRNA binding occurs through the seed region of the microRNA (minimally positions 2-7 from the 5′ end of the mature strand)[1]. It is estimated that each microRNA family member may regulate hundreds of gene targets[8]. Even though some rules for mRNA-microRNA target recognition have been elucidated, prediction programs do not yet identify all targets[9-11].

Current methods for microRNA target discovery utilize one or more of the following approaches: microRNA target prediction algorithms[1], the anti-correlation of microRNA and gene target expression within large datasets[12] or immunoprecipitation of RISC[13-15]. Techniques involving cell-based crosslinking and immunoprecipitation of the Ago complex have also been developed, which are analyzed by deep sequencing and are intended to not only identify targets, but also to isolate specific regions of mRNA-microRNA binding[16, 17]. While it has been demonstrated that novel targets can be identified with such enrichment techniques, they present technical challenges and are still being adopted by the broader community. Each method has limitations with regard to accuracy and specificity as well as the long time commitment and technical expertise required to perform each experiment.

Target prediction algorithms search for seed complements in target mRNA 3′ UTRs and use sequence conservation to predict microRNA binding sites. These predictions are confirmed experimentally by modulating the levels of a microRNA using mimics and inhibitors[18, 19] and measuring effects on the predicted targets. A common approach is to systematically examine genes from target prediction lists based on the biology of interest[20] where only 30-60% of the predictions are confirmed experimentally[21-25]. This indicates that there are additional parameters affecting microRNA target regulation which limit the accuracy of current prediction algorithms. Additional experimentally determined targets are also missed by predictions that do not account for the biological context of each system[1, 19].

An empirical approach allows one to discover new targets and new biology in addition to confirming predicted targets.

Since high throughput gene expression microarray analysis remains a widely used, fast, and cost-effective approach, we used this as a basis for a robust method to efficiently identify microRNA targets. Expression changes of a direct gene target should be anti-correlated when treated with mimics and inhibitors independently. Mimics typically have stronger, more robust effects while inhibitors have more subtle effects[26]; therefore, when using inhibitors alone, many researchers have been unable to distinguish primary

effects from background noise in small experiments. Previous studies using gene expression microarrays have focused on use of microRNA mimics alone[12, 27-29] with few exceptions where both mimics and inhibitors were used in a specific context[30, 31].

Here, we investigate whether combining the use of Dharmacon™ miRIDIAN™ microRNA Mimics and Hairpin Inhibitors to modulate microRNA levels would allow for identification of a smaller and more biologically relevant set of candidate targets. As a model system we utilize miR-122, a prominent microRNA in liver cells that is involved in cholesterol metabolism and Hepatitis C viral replication[32, 33]. Empirically measured microRNA targets are bioinformatically probed for potential direct mRNA-microRNA interactions by determining enrichment of seed complement sites[1] in their 3' UTR regions. Finally, we perform individual evaluation of proposed targets by RT-qPCR, comparing these empirically detected targets to those predicted by common microRNA targeting algorithms. Overall, we conclude that empirical detection using modulation of microRNA by miRIDIAN Mimics and Hairpin Inhibitors leads to identification of high confidence targets in a more efficient manner than one-by-one follow up of targets from publicly available microRNA target prediction algorithms.

Results and conclusions

Validation of miRIDIAN miR-122 Mimic and Hairpin Inhibitor effects

In order to optimize experimental parameters such as dose, time point, and transfection method for use with whole genome microarray analysis, we tested the effects of miRIDIAN Mimics and Hairpin Inhibitors on a previously reported endogenous target of miR-122, *ALDOA*[34, 35]. Huh-7 cells were selected because the endogenous microRNA is expressed at a moderate level and can be both up- and down-regulated in the same cell line. Message levels of *ALDOA* are reduced by introduction of a miRIDIAN miR-122 Mimic and are conversely increased by introduction of a miR-122 Hairpin Inhibitor in a dose- and time-dependent manner (Figure 1). The mimic effect is up to two-fold stronger than the inhibitor effect, while the inhibitor effect takes longer to appear, becoming significant between 3 and 7 days. Optimal conditions for observation of *ALDOA* gene modulation were 50 nM and 3 days for mimics and 20 nM and 6 days for inhibitors.

Discovery of additional miR-122 gene targets by whole genome expression microarray analysis

Using the optimal transfection conditions determined above, genome-wide expression profiling was applied to determine regulation of miR-122 gene targets (Figure 2). To increase confidence in the data, we performed four biological transfection replicates of both miR-122 mimic and inhibitor. The microarray ratio data was limited to transcripts that correspond to records in RefSeq 39, as hits are required to have an annotated 3' UTR for subsequent analysis. For each set of transfections, transcript sequences were selected as significantly modulated, relative to the lipid-treated control, if they had a p-value of ≤ 0.01 (Rosetta error model) in a specified number of arrays in that group. Since the expression of directly-targeted transcripts are expected to be anti-correlated with microRNA expression, transcripts were removed if the direction of their expression change was the same as microRNA modulation. Intersections of these lists were generated at various stringencies and using increasing numbers of replicates (Tables 1 and 2). No transcripts passed this analysis workflow when negative control mimic and inhibitor molecules were analyzed (Figure 1).

We observed that when using only a fold-change cutoff, the subtle effect of inhibitors was not discerned, indicating a large number of false negatives (data not shown), while use of a p-value threshold resulted in enrichment for targets that contain seed complement sites. Examining the intersection of mimic-and inhibitor-affected genes is important for several reasons. The number of genes in the target list for either experiment alone is prohibitively large and cannot be thoroughly validated using lower throughput methods (Table 1), whereas an intersection list results in a smaller list for validation (Table 2). Transfection replicates are used to narrow this list even further and allow one to select a number of genes that can be more thoroughly studied given the time and resource constraints of any given project. For example, the intersection of the significant anti-correlated genes from two of the four miR-122 mimic-treated samples and one of the four inhibitor-treated samples narrowed down the potential target list from approximately 750 or 1,750 genes in either experiment alone (Table 1) to a much smaller list of 22 in this more stringent intersection set (Table 2 and Figure 3A). Due to the subtle effects of inhibitors and the longer time points required for their action, false negatives are a great concern due to the overall fluctuation in gene expression over the course of the experiment. Taking this into account, we relaxed the criteria for inhibitors to require significant modulation in only one or two out of the four replicates while maintaining more stringent reproducibility criteria for mimics. This yields a slightly larger and yet still high-confidence target list as evidenced by RT-qPCR assessment of target modulation.

Table 1. Transcripts significantly modulated by miR-122 per number of biological replicates passing threshold. The number of transcripts down-regulated by miRIDIAN miR-122 Mimic (50 nM, 3 day time point) or up-regulated following treatment with the miRIDIAN Hairpin Inhibitor (20 nM, 6 day time point) all passing a p-value ≤ 0.01 versus number of biological replicates passing threshold.

Replicates	Mimics down	Inhibitors up
1 of 4	1617	1757
2 of 4	753	747
3 of 4	401	346
4 of 4	183	65

Table 2. Number of transcripts both down-regulated by mimic and up-regulated by inhibitor combined with the number of biological replicates passing threshold. The intersection of up-and down-regulated transcripts as a function of the stringency of replicate threshold. Number of replicates designated in bold headings.

		# of mimicreplicates			
		4	3	2	1
# of inhibitor replicates	4	2	3	3	6
	3	4	6	7	12
	2	8	13	15	35
	1	10	15	22	60

Seed complement occurrence analysis of potential microRNA targets

A bioinformatic method to assess whether differentially expressed genes are likely to be direct targets of microRNAs involves determining the occurrence of seed complement sequences in their 3' UTRs[1]. We looked at miR-122 seed complement enrichment in the 3′ UTRs of the 22 most strongly regulated transcripts for three sets of data: treatment with miRIDIAN mimic-only alone, inhibitor-only alone, and an intersection of mimic-only and inhibitor-only treatment (Figure 3). This analysis demonstrates that the intersection set has the highest seed complement enrichment (compared to the expected seed complement enrichment of a random length-matched set of the same number of 3′ UTRs); there is at least one 6mer 3' UTR seed complement in 20 of the 22 transcripts (Table 3).

Although both the mimic-only and inhibitor-only datasets have some 3' UTR seed complement enrichment (1.6-fold and 1.2-fold, respectively), the magnitudes of enrichment are smaller than for the intersection set which indicates 3.75-fold enrichment (Figure 3B).

RT-qPCR confirmation of targets and comparison to prediction programs

RT-qPCR was used as a method to support array-detected targets as well as to compare this discovery strategy to a list of targets generated by commonly used prediction programs. Two empirical gene lists were selected to test with RT-qPCR: 22 transcripts down-regulated in two of four mimic experiments and up-regulated in one of four inhibitor experiments, and 60 transcripts that were directly targeted in one of four mimic experiments and up-regulated in one of four inhibitor experiments (Table 2, Supplemental Tables 1, 2). We compared RT-qPCR results of these array targets to a list of miR-122 targets generated by the overlap of widely used microRNA target prediction programs; TargetScan, Pictar, and Miranda, which contained 24 genes (Supplemental Table 3).

Confirmation criteria for RT-qPCR experiments included an expression change in the appropriate direction (negative for mimic and positive for inhibitor) and a multiple test corrected p-value of less than or equal to 0.05. For the smaller, more stringent set of empirical targets (22) we found 16 (73%) transcripts confirmed response to both mimic and inhibitor, four (18%) confirmed with mimic treatment only, two (9%) confirmed with inhibitor treatment only, and no transcripts were unresponsive (Figure 4A, Supplemental Table 4).

Comparing the members of the two array-based target lists (of 22 and 60 targets, Supplemental Tables 1 and 2) to their target ranking in prediction programs demonstrates that many high-confidence targets in the array-based list are not predicted by any program. The few that are predicted

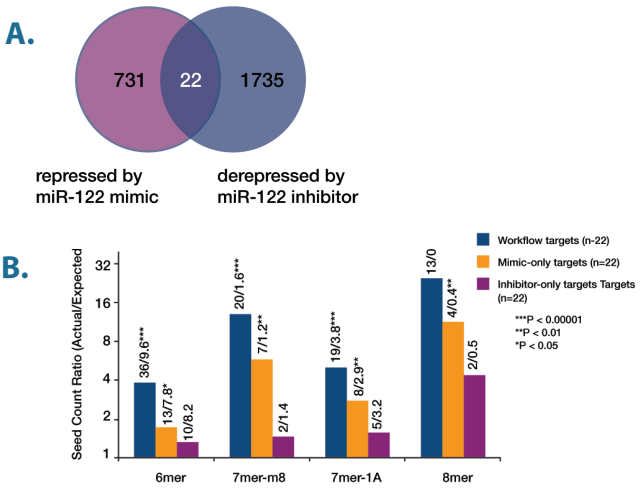

Figure 3. Analysis of potential microRNA targets. A. The Venn diagram of the data analysis performed in Rosetta Resolver yielding potential microRNA target lists. The repressed group is the set of messages significantly down-regulated in any 2 of 4 biological replicates of Huh-7 cells at the 50 nM mimic dose (72 hours). The derepressed group is the set of messages significantly up-regulated in any 1 of 4 biological replicates of Huh-7 cells at the 20 nM inhibitor dose (144 hours). **B.** miR-122 seed enrichment in proposed intersection workflow compared to targets identified by mimic and inhibitor effects alone. The top 22 most down-regulated targets (mimic-only targets) as well as the top most up-regulated targets (inhibitor-only targets) were identified among four microarray replicates and compared to 22 intersection workflow targets described in **A.** The number of seed complements were calculated in the target 3' UTRs (RefSeq 39) for each seed type[1]. Expected background counts were calculated by averaging seed counts in 150 trials of randomly chosen 3' UTR length-matched sets (sampled with replacement, excluding actual targets). Actual and expected seed counts are listed above each bar, followed by a p-value category for significance of enrichment.

Table 3. Top 22 miR-122 targets determined by microarray experiment and the number of seed complements counted in their 3' UTRs.

	Gene Symbol	Accession	6mer-2-7	7mer-2-8	7mer-1A-2-7	8mer-1A-2-8
1	AADACL1	NM_020792.4	2	1	1	1
2	ABR	NM_021962.2	2	1	2	1
3	C10orf118	NM_018017.2	4	3	3	2
4	C9orf86	NM_024718.3	3	1	1	1
5	FBXO21	NM_033624.2	2	0	2	0
6	FGF18	NM_003862.1	1	1	0	0
7	GALNT3	NM_004482.3	1	1	1	1
8	GBA3	NM_020973.3	0	0	0	0
9	GPR172A	NM_024531.3	1	1	1	1
10	KIAA0101	NM_014736.4	1	0	0	0
11	LCT	NM_002299.2	0	0	0	0
12	LMNB2	NM_032737.2	4	2	1	1
13	MASP1	NM_139125.2	5	2	1	1
14	MTHFD2	NM_006636.3	1	0	1	0
15	NPPB	NM_002521.2	0	0	0	0
16	PKM2	NM_182470.1	1	1	1	1
17	PLEKHB2	NM_017958.2	2	2	2	2
18	PRTFDC1	NM_020200.5	2	2	0	0
19	RAB42	NM_152304.1	1	0	1	0
20	SFT2D1	NM_145169.1	1	1	1	1
21	SH3BGRL3	NM_031286.3	1	1	0	0
22	SH3MD2	NM_020870.3	1	0	0	0

are relatively far down in their respective ranking, so that systematic, one-by-one testing of a list of predicted targets does not appear to be an efficient target confirmation strategy. In the highest confidence set of 16 targets determined by microarray and confirmed by RT-qPCR, only four targets were predicted by TargetScan (specifically C9 or F86 #33, GPR172A #44, LMNB2 #6, and PKM2 #40), two targets were predicted by Pictar (C9 or F86 #51 and GPR172A #22), and four targets were predicted by Miranda (GALNT3, LMNB2, PRTFDC1, and SH3BGRL3; Table 1). The remaining nine high confidence targets were not predicted by any of these three programs, illustrating that many novel microRNA targets can be discovered using this empirical anticorrelative method.

There are several strengths of this microRNA target identification workflow. In silico prediction models give no information as to expression level of predicted targets in a particular cell line or system of interest and they do not account for biological features or context (transcription/degradation rates, etc.). Using the whole genome expression approach, one can assay both predicted and novel targets in an individual context. While many studies look at mimic effects exclusively because they are generally of higher magnitude, we use the intersection of modulation in both directions to address false positive and false negative discovery. Modulation by mimics alone will produce a large yet artificial target list, since the cells are overloaded with a microRNA beyond its native expression level; many of the observed effects will be biological false positives. However, background variation in gene expression over time can obscure specific inhibitor effects upon the microRNA and thus produce false negatives. The method presented here is reliable, straightforward, and more efficient than a gene-by-gene RT-qPCR query of predicted target messages. We narrow down the list of microRNA targets for follow up to a reasonable size, in this case 22 high confidence targets. Starting with a bioinformatic prediction tool, the list of targets to individually test would be at least twice as long. While some may be concerned that this method surveys transcript levels alone, recent studies using mass spectrometry and ribosome profiling have shown excellent correlation between message and protein changes due to microRNA modulation[21, 22], making this a good starting point for follow up with a protein detection method. Reciprocal modulation can also be applied using other large scale expression readouts such as Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) or Next Generation Sequencing (NGS).

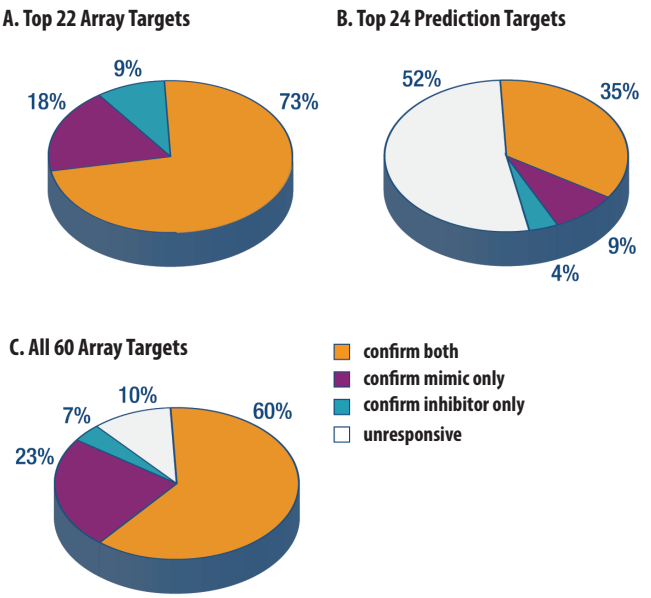

Figure 4. Diagrams illustrating RT-qPCR confirmation of microarray identified and bioinformatically predicted targets. A. Results from RT-qPCR of the top 22 microarray targets. **B.** Results from RT-qPCR of the top 24 prediction algorithm targets. **C.** Results from RT-qPCR with an expanded, less stringent list of 60 microarray targets.

Methods

Cell culture, cytotoxicity analysis, quantitation of mRNA by branched DNA assay

Huh-7 and HeLa cell lines were plated at 10,000 cells per well without antibiotics in 96-well plates and transfected after 24 hours using Dharmacon™ DharmaFECT™ 1 (0.2 µL/well; (Cat #T-2001-02) miR-122 miRIDIAN Mimic (Cat #C-300591-05-0010) and Hairpin Inhibitor (Cat #IH-300591-06-0010) were transfected at 50 and 20 nM, respectively. Five biological replicates of these transfections were completed over the course of 28 months. Cells treated with miRIDIAN Mimics were harvested at three days post-transfection; cells treated with miRIDIAN Hairpin Inhibitors were harvested at six days post-transfection with a medium change at day three. Cellular toxicity was measured by the resazurin metabolism assay[36]; all transfected wells showed ≥ 85% viability relative to untreated wells. *ALDOA* were measured using the Panomics™ Quantigene™ Branched DNA Assay.

Gene expression microarrays

Total RNA was purified using the RNeasy kit including on-column Qiagen™ DNase™ digestion, and concentration was measured with the Thermo Scientific™ NanoDrop™ 1000 UV-Vis spectrophotometer. All samples were diluted to a standard concentration of 100 ng/µL. Total RNA (650 ng) from each sample was amplified and labeled using the Low Input RNA Fluorescent Linear Amplification kit (Agilent). Samples were hybridized to the Agilent™ Whole Human™ Genome Microarray 4 × 44K. Experimental samples were Perkin Elmer Cy5™ labeled and reference samples (lipid-only transfectants) were Cy3 labeled. Slides were processed according to the Agilent protocol and scanned (model G2505B) at 5 micron resolution with XDR, data extracted (Feature Extraction 9.5.1), and further analyzed using the Rosetta Resolver™ Gene Expression Analysis software. For each transfection condition, sets of consistently and significantly responsive probes among replicate arrays were determined by applying two filters. First, probes showing inconsistent response directionality among replicates were removed; for mimic transfection conditions, only probes with negative response (Cy5/Cy3 < 1) were retained while for inhibitor conditions, only probes with positive response (Cy5/Cy3 > 1) were retained. Second, probes with significant response (p-value threshold < 0.01) in some fraction of the replicates (for example 3 out of 4) were retained. Unless otherwise noted, a p-value threshold of 0.01 was used. Only probes annotated with NM-prefixed accession numbers in RefSeq 39 were considered (23,558 sequences).

microRNA seed complement enrichment analysis

For each set of targets, complementary matches to miR-122 seeds (6mer, 7mer-m8, 7mer-1A, 8mer[37]) were counted using mRNA sequence data in RefSeq 39, limited to a region of the sequence (ORF or 3' UTR). Background counts were obtained by counting seed complements in corresponding length-matched sets, also limited by mRNA region. Random length-matched sets were constructed by ordering all possible sequences by region length, then for each actual target sequence (the anchor) randomly choosing a sequence within a defined neighborhood window around the anchor (sampled with replacement, excluding the anchor). The expected value of background counts was estimated by repeating counts of random length-

matched sets for a number of trials. We used a neighborhood window size of 600 with 150 trials. Set-wise seed enrichment was calculated as the ratio of actual counts to the expected value of background counts. Set-wise seed complement counts were modeled as a Poisson distribution, using the average counts over each random trial as an estimate of the Poisson parameter λ. Significance of set-wise seed enrichment was calculated by evaluating the cumulative distribution function for the actual counts.

Target prediction using publicly available tools

A list of predicted miR-122 targets was created from the overlap of three microRNA target prediction algorithms (TargetScan Release 5.1, Pictar 2006, and Miranda Sept 2008). The TargetScan database (targetscan.org) contained 124 conserved targets for hsa-miR-122. The Pictar database based on Lall et al. 2006 was accessed and queried for vertebrates with the dataset of predictions for all human microRNAs based on conservation in mammals (human, chimp, mouse, rat, dog) for hsa-miR-122a resulting in a list of 169 targets – 155 excluding replicates (pictar.mdc-berlin.de/). The Miranda database (microRNA.org, September 2008 release) contained a list of 1091 unique gene transcripts for miR-122. The overlap of all three lists is 24 transcripts.

High throughput RT-qPCR methods

RNA was isolated using Promega SV 96 Total RNA Isolation System (Promega). Total RNA from four replicate plates was pooled and 10 µL used for reverse-transcription utilizing the Verso cDNA Synthesis Kit (Thermo Scientific, Cat #AB-1453) with 3:1 (volume:volume) oligo dT to random hexamer primers in a 20 µL reaction. Gene expression analysis was performed using Thermo Scientific™ Solaris™ qPCR Gene Expression Assays (Supplemental Table 5) and Master Mix (Thermo Scientific, Cat #AB-4350); 4 µL cDNA (3-fold diluted) in a 16 µL reaction. Six replicate qPCR reactions were prepared manually in 96-well plates then condensed robotically into 384-well white plates (Thermo Scientific AB-1384/W) and run on an Applied Biosystems™ Prism™ 7900HT system
with the following cycling program: 15 minute activation at 95 °C, 40 cycles of 15 seconds denaturation at 95 °C and 60 seconds of annealing and extension at 60 °C. Relative expression was calculated using a ΔΔCq method, normalizing to GAPDH expression and then to the lipid-only control samples and reported as a ratio.

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