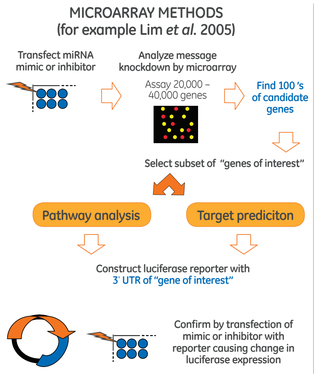
Identification of microRNA targets using microRNA modulation techniques and gene expression arrays

Emily M. Anderson, Maren Mayer, Kevin Sullivan, Barbara Robertson, Žaklina Strezoska, Annaleen Vermeulen, and Devin Leake
Dharmacon, now part of GE Healthcare, 2650 Crescent Drive, Suite #100, Lafayette, CO 80026, USA

Abstract

MicroRNAs (miRNAs) have been shown to regulate gene expression through both translational attenuation and degradation of messenger RNAs (mRNAs). Though these small noncoding RNAs are predicted to play a signicant role in development, differentiation, and disease etiology, validation of miRNA targets remains a challenge. miRNA mimics and inhibitors are valuable tools for elucidating the roles of miRNAs. Here we have transfected a human liver cell line alternately with a miRNA mimic and inhibitor to miR-122 and analyzed mRNA expression changes by whole genome microarray. By examining the overlap of messages down-regulated by miRNA mimics and up-regulated by miRNA inhibitors, we robustly identify miRNA-regulated messages, many of which have canonical seed matches and some which are not identied by standard target prediction programs.

Current methods for identification of miRNA targets



ALTERNATIVE METHODS

Direct detection of protein knockdown
(Baek *et al.* 2008, Selbach *et al.* 2008)

- Transfect miRNA mimic or inhibitor
- Detect knockdown of protein levels by SILAC (stable isotope labeling with amino acids in cell culture)
- Test 2000-5000 proteins
- Find hundreds to thousands of potential targets

Immunoprecipitation for all microRNAs or specic microRNAs (Easow *et al.* 2007, Zhang *et al.* 2007)

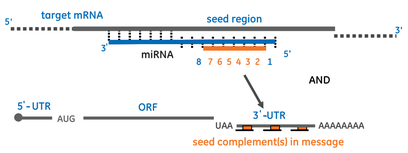
- IP a native/tagged component of RISC (Ago1,2 or other), identify co-precipitated RNAs
- Introduce tagged Ago2 and a miRNA mimic (Karginov *et al.* 2007), or a biotin-labeled microRNA (Ørom and Lund 2007, Ørom *et al.* 2008)
- IP by tag
  Identify co-precipitated mRNAs

HITS-CLIP
(Chi *et al.* 2009)

Microarrays provide one of the easier methods to screen the largest number of potential target genes.

Hypothesis: Messages observed to be modulated in an anti-correlated fashion with miRNA mimic and inhibitor treatments give a higher condence list of candidate target genes than mimic-only experiments.

miRNAs exert effects primarily in a target's 3' UTR



Types of Seeds
(Lewis *et al.* 2005):

6-mer seed, AS nucleotides 2-7
7-mers, adding either A1 or additional Watson-Crick match (M8)
8 mer (A1 and M8)

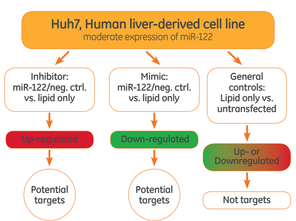
Methods

Cell Culture, cytotoxic analysis, and quantitation of target message knockdown:

- Huh7 cells plated at 10,000 cells/well in a 96-well plate: can modulate miR-122 both UP and DOWN
- Transfected after 24 hr with DharmaFECT 1 0.2 uL/well
  - 50 nM Dharmacon miRIDIAN mimics, miR-122 and negative control (NC)
  - 20 nM miRIDIAN Hairpin Inhibitor, miR-122 and negative control (NC)
- Harvested mimic 3 days, inhibitor 6 days after transfection
- Toxicity measured by resazurin metabolism assay
- Quantitative mRNA detection using Quantigene branched DNA assay (bDNA, Panomics/Affymetrix)

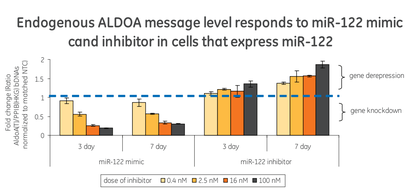
Microarray experiments:

- Total RNA puried using RNeasy columns withon-column DNase digestion (Qiagen)
- 650 ng total RNA amplied using Low Input RNA Fluorescent Linear Amplication Kit (Agilent)
- Whole Human Genome Microarray (Agilent)
- Hyb reference: mock (lipid) transfected cells in Cy3 channel
- Data analysis in Rosetta Resolver using visual scripts, P value cutoff of 0.01 used to determine signicance, clustered images used the agglomerative algorithm and cosine correlation similarity measure for message analysis [a collection of Agilent Human probes with NM annotation, present in the RefSeq 32 database (23,558 probes)]
- Random control gene lists were generated from an average of three sets



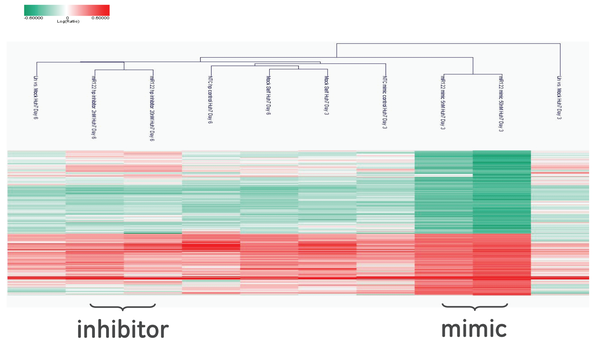
miR-122 is modulated by mimics and Inhibitors

Effects of mimic and inhibitor on endogenous target mRNA levels are subtle



In Huh7 cells, message levels of the endogenous miR-122-target ALDOA are reduced by introduction of a miR-122 mimic and are increased by introduction of a miR-122 inhibitor in a dose and time-dependent manner (bDNA assay, Panomics/Affymetrix)

Endogenous effects are stronger from miRNA mimic than from inhibitor



A heatmap demonstrating strongest gene expression effects of miR-122 miRIDIAN Mimics and Hairpin Inhibitors. A gene bioset was created from Huh7 cells transfected with miRIDIAN Mimic miR-122 (at 3 days and 50 nM) where the experiment met the gene criteria (2-fold cutoff and p-value < 0.01). A visual script used this bioset to mine data across the set of 10 experiments/arrays.

Modulation in both directions and multiple replicates are essential for robust results

A.

Genes signicantly modulated by miR-122 per number of biological replicates

	Mimics down	Inhibitors up
1 of 4	1698	1874
2 of 4	783	800
3 of 4	443	389
4 of 4	198	71

Chart that displays the number of genes down-regulated by miR-122 mimic or up-regulated by inhibitor (pval 0.01) in up to 4 biolgical replicates of transfection. Blue highlighting indicates data used in next panel.

B.

Intersection of genes both downregulated by mimic and upregulated by inhibitor combined with the number of biological replicates analyzed

# of inhibitor experiments analyzed	# of mimic experiments analyzed: 4	3	2	1
4	2	3	4	7
3	4	6	7	14
2	8	13	15	36
1	10	15	22	64

Chart showing the number of potential targets of miR-122 change depending on how many biological replicates are included in the analysis. Blue highlighting indicates data used in next panel.

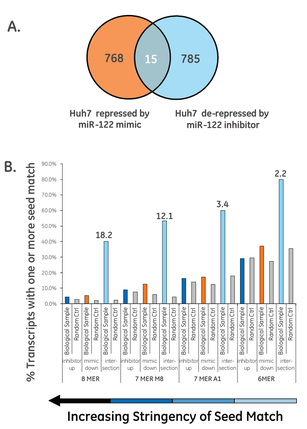
Combining mimic and inhibitor experiments greatly improves confidence in potential target list

Examining the overlap of targets from 4 biological replicates of mimic and inhibitor experiments in one cell line with moderate miR-122 expression yields a much smaller target set with signicantly higher seed enrichment in 3' UTRs

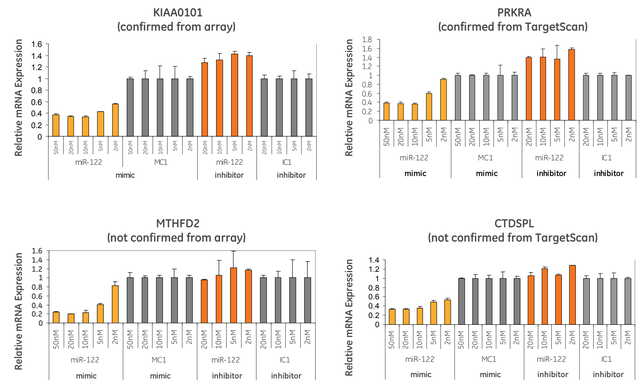
A. The number of messages differentially expressed in the intersection of miR-122 mimic- and inhibitor-treated Huh7 cells. The repressed group is the set of messages that are signicantly down-regulated in 2 of 4 mimic replicates. The de-repressed group is the set of messages that are signicantly up-regulated in 2 of the 4 inhibitor replicates.

B. The percentage of 3' UTRs with at least one miR-122 seed match compared to 3' UTRs of length-matched random controls. These are plotted by groups of messages taken from the Venn diagram (Figure A). The numbers above the gray bars are the ratio of the biological sample to the matched control.

With Increasing stringency of seed sequence (greater length), the enrichment of seed matches in 3' UTRs compared to a length-matched random set increases dramatically.



Confirmation of targets/predictions by qPCR analysis

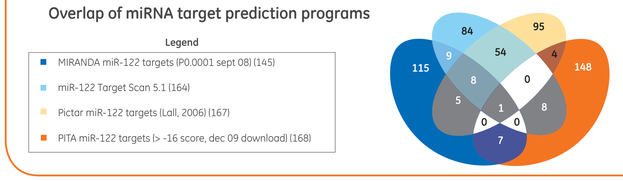


Huh7 cells were transfected with increasing concentrations of miR122 mimic or hp inhibitor and appropriate negative controls (MC1 =mimic control 1; IC1= inhibitor control 1). RNA was isolated at day 3 (mimic) or day 7 (inhibitor) and RT-qPCR was performed using Thermo Scientific™ Solaris™ qPCR Assays for expression detection on a Roche LightCycler480 (384-well) instrument. Expression of each gene was normalized to the PPIB reference gene using a ΔΔCq method. Expression levels were further normalized to negative control treatment (MC1 or IC1) at the appropriate corresponding concentration.

Our method identifies novel genes not identified by prediction programs

Lists		Target Scan (164 seq)		Pictar (167 seq)		Miranda (145 seq)		PITA (168 seq)	
Intersection Huh7 mimic and inhibitor	Total # genes	Genes in common	% experimental	Genes in common	% experimental	Genes in common	% experimental	Genes in common	% experimental
1 of 4 mimic, 1 of 4 inhibitor	64	8	12.5 %	7	10.9 %	2	3.1 %	2	3.1 %
2 of 4 mimic, 2 of 4 inhibitor	15	2	8.0 %	2	13.3 %	1	6.6 %	0	0.0 %

Overlap of miRNA target prediction programs



Conclusions

We have demonstrated that the combined use of mimic and inhibitor to search for miRNA targets with microarray analysis produces a restricted list of potential target genes that includes some overlap with predictive programs as well as unique genes of interest

Due to the subtle nature of inhibitor effects, multiple replicate experiments should be performed to detect modulation and guard against a potential false negative result

This empirical method results in a higher condence set of potential targets than the method of searching for targets based on seed site alone

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