

Modulating endogenous microRNA targets with miRIDIAN microRNA Mimics and Inhibitors: miR-122 in hepatocarcinoma cells (Huh-7).

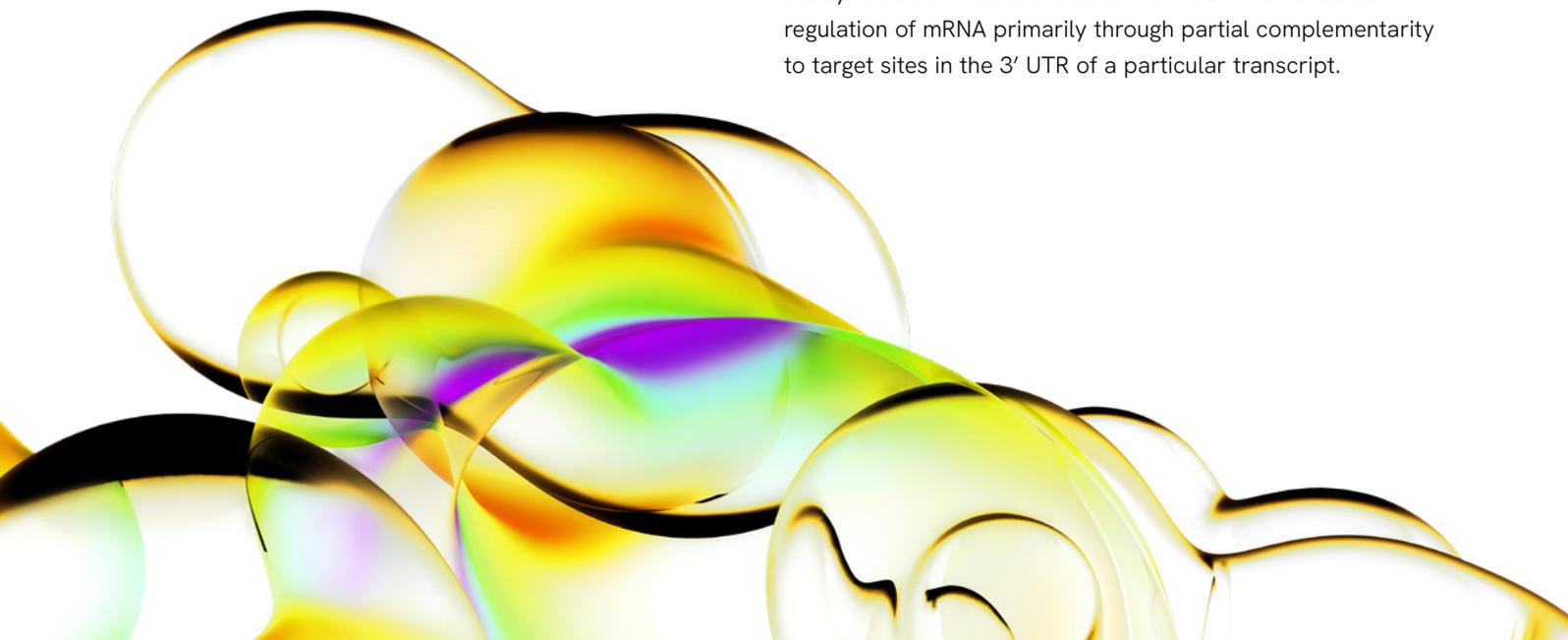
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Introduction

MicroRNAs (miRNAs) have been shown to regulate gene expression through both translational attenuation and messenger RNA (mRNA) degradation. These small non-coding RNAs typically target multiple genes simultaneously, inducing subtle but reproducible shifts in target gene expression. Synthetic miRNA mimics and inhibitors designed for potency and specificity can be extremely valuable tools for probing the function of target genes, thus helping to reveal the role of miRNAs in development, differentiation, and disease. Major challenges for researchers investigating miRNA function include the confirmation of subtle phenotypic changes associated with miRNA over-expression and inhibition, as well as identification of miRNA gene targets. The identification of a miRNA of interest can be performed using microarray expression profiling, by RT-qPCR, or with reporter constructs that register the status or changes to a specific biological pathway and/or phenotype (for example, target gene expression, cell growth or differentiation). Another emerging approach is the identification of miRNAs by screening with synthetic reagents that mimic or inhibit function to demonstrate a specific phenotypic effect.

Endogenous miRNAs are transcribed as stem-loop structures and subsequently processed by several enzymes, including the RNA-induced silencing complex (RISC), into functional, mature miRNAs with one or two mature (targeting) strands. miRIDIAN™ miRNA Mimics are synthetic duplexes representing mature miRNAs as recorded in miRBASE (www.mirbase.org/). Like the naturally occurring miRNAs, the synthetic mimics are loaded into RISC which enables regulation of mRNA primarily through partial complementarity to target sites in the 3' UTR of a particular transcript.



miRIDIAN Mimics have chemical modifications that are incorporated into a given strand of the molecule to establish strand bias and ensure that the mature (targeting) miRNA strand is preferentially loaded into RISC. Thus, by introducing these molecular mimics into a cell type of interest, the researcher can enhance or supplement endogenous miRNA activity representing a gain-of-function assay.

Conversely, the addition of miRNA inhibitors into a biological system results in a loss-of-function assay with a predicted decrease in endogenous miRNA function. In general, inhibitors comprise a non-hydrolyzable, single-strand reverse complement to the mature miRNA strand. The mechanism of inhibition is likely mediated by irreversible binding of the inhibitor to mature miRNA thus preventing binding of the mature miRNA to its endogenous targets¹. Inhibitor potency can be improved by either enhancing the binding of the inhibitor to the mature miRNA through chemical modification and/or by incorporating additional attributes, such as structure, into the molecule. The newest generation of inhibitors, miRIDIAN miRNA Hairpin Inhibitors incorporate terminal stem-loop structures and chemical modifications to further enhance potency and extend inhibitory activity². The combination of these design elements in loss-of-function assays increases the likelihood of observing otherwise subtle phenotypes often associated with miRNA activity.

To demonstrate the utility of miRIDIAN miRNA Mimics and Hairpin Inhibitors in assays for miRNA function in an endogenous system, we chose a well-studied miRNA:target-pair, miR-122:Aldolase-A (*ALDOA*). The *ALDOA* message has a conserved miR-122 target site in its 3' UTR and is normally repressed in hepatocytes (Figure 1). Previous studies suggested that a primary function for miR-122 was to maintain the adult liver phenotype by suppressing expression of "non-liver" genes like *ALDOA*. This model is supported by work in mice where inhibition of miR-122 led to up-regulation of *ALDOA*, a decrease in hepatic fatty acid and cholesterol synthesis, and reduced plasma cholesterol levels³⁻⁵. Here we describe an application for miRIDIAN miRNA Mimics and Inhibitors that further reinforces a regulatory role for miR-122 in liver tissue identity (phenotype and function), and more importantly highlights a practical utility for these novel reagents.

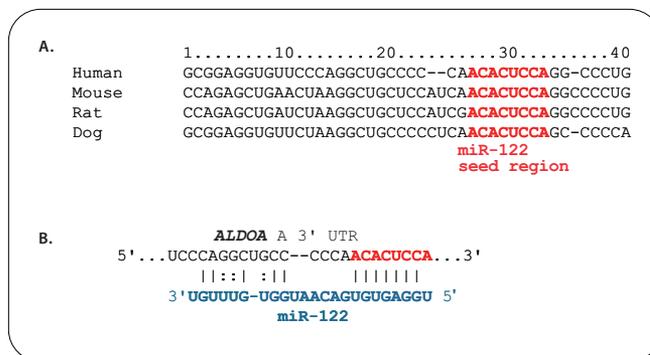


Figure 1: Three miRNA target prediction programs predict the same site for miR-122 in the *ALDOA* 3' UTR (PicTar, TargetScan, miRBASE). A. The site is in a highly conserved region of the 3' UTR of several species. B. The predicted binding of the miR-122 (blue) to the *ALDOA* target is through a 8mer seed match (red).

Materials and methods

Cell culture

Huh-7 cells were obtained from ATCC and cultured under recommended media conditions. The cells were plated in serum-containing medium without antibiotics in 96-well plates (10,000 cells/well) approximately 24 hours before transfection.

miRNA mimics and inhibitors

Targeting and negative control miRIDIAN miRNA Mimics and Inhibitors were synthesized by our researchers. Three different inhibitor designs were synthesized: (1) a fully 2' O-methylated inhibitor reverse complement to the mature miRNA (RC), (2) a first generation inhibitor design and (3) the current miRIDIAN miRNA Hairpin Inhibitor containing flanking hairpin structures.

Exogenous dual luciferase assay for detection of miR-122 function

The dual-luciferase plasmid, psiCHECK-2 vector, containing both the synthetic Firefly Luciferase (Fluc) gene and the synthetic Renilla Luciferase (hRluc) gene, each with its own promoter and poly(A)-addition sites, was obtained from Promega (Cat #C8021). A fully complementary target site for miR-122 miRNA was inserted between the XhoI-NotI restriction sites in the multiple cloning region in the 3' UTR of the hRluc gene². For luciferase assays, cells were co-transfected with 100 ng/well of the reporter plasmid and a range of concentrations for the mimic or the inhibitor (1, 6, 16, and 40 nM) using 0.3 µL/well DharmaFECT™ Duo.

Cells were grown at 37 °C and harvested at multiple time points after transfection for luciferase and viability assays. Firefly and Renilla luciferase activities were measured using the Dual-Glo™ Luciferase Assay System (Promega, Cat #E2980)².

Endogenous ALDOA mRNA knockdown assay

For ALDOA mRNA knockdown assays, cells were transfected with a concentration range of either the miR-122 miRIDIAN miRNA Mimic or Inhibitor (1, 6, 16, and 40 nM). Cells were cultured at 37 °C and harvested 1, 3, 5 and 7 days post-transfection. mRNA knockdown was determined using the branched DNA assay QuantiGene™ Screen Kit (Panomics, Cat #QG-000-050)⁶.

Cell viability assay

For both luciferase and mRNA detection, cell viability was determined on a duplicate plate using the Thermo Scientific™ alamarBlue™. Cell viabilities for control and experimentally treated cells were always within 20% of untreated (or mock treated) cells.

Results

Exogenous dual luciferase assay with miR-122 mimic and inhibitors

Previous studies demonstrated that miR-122 targets ALDOA mRNA in the liver of animal models⁵. Microarray analysis performed in-house (data not shown) revealed that the hepatocellular carcinoma derived cell line, Huh-7, expresses moderate levels of miR-122 as well. For this reason, we chose the Huh-7 cell line as a good *in vitro* system for miRNA gain and loss-of-function experiments. To test mimic and inhibitor function, respectively, molecules were co-transfected with either a dual-luciferase reporter plasmid that detects miR-122 expression (see Materials & Methods) or an empty psiCHECK-2 reporter plasmid. The untreated and negative controls confirmed that miR-122 is expressed at moderate but readily detectable levels in Huh-7 cells (Figure 2). Cells transfected with the miR-122 reporter had decreased Renilla luciferase expression levels relative to cells transfected with the empty psiCHECK-2 reporter. When miRIDIAN miR-122 Mimics were co-transfected with the miR-122 reporter, expression of Renilla luciferase was further decreased (> 3 fold). Conversely, co-transfection with miR-122 hairpin inhibitor resulted in an increased (> 8 fold) expression of Renilla luciferase.

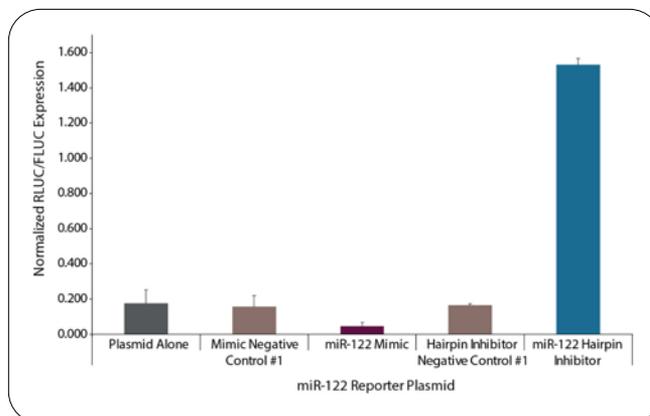


Figure 2: psiCHECK-2 dual luciferase assay in Huh-7 cells. Huh-7 were transfected with reporter plasmids alone or co-transfected with miRIDIAN products with DharmaFECT Duo (0.3 µg/well). Luciferase expression was measured 48 hours after transfection. Transfection efficiency was normalized to Firefly luciferase expression. Firefly/Renilla luciferase ratios were further normalized to psiCHECK-2 reporter alone.

Endogenous ALDOA assay

While reporter assays permit rapid assessment of functionality, monitoring the effect of miRNA mimics or inhibitors towards a biologically relevant target provides greater insight into their effectiveness on putative endogenous miRNA-target pairs. To explore how well the miRIDIAN miRNA Mimics and Inhibitors performed when monitoring an endogenous target, we transfected each class of regulatory molecules into Huh-7 cells at 1, 6, 16, and 40 nM per well and monitored mRNA expression of the endogenous target (ALDOA) over time. As expected, transfection of the miRIDIAN miRNA Mimic resulted in further downregulation of ALDOA mRNA levels (Figure 3). Addition of miRIDIAN miRNA Hairpin Inhibitor molecules to the cells resulted in increased ALDOA mRNA levels (Figure 3). While both the RC and first generation version of miRNA inhibitors resulted in increased ALDOA mRNA level, the inhibition was less pronounced compared to hairpin inhibitors.

Of note, modulation of miR-122 function with miRIDIAN miRNA Mimics and Inhibitors revealed that the optimal time for detecting effects on the ALDOA target may differ for the corresponding modulator. For example, in this study down-regulation of ALDOA mRNA levels is most pronounced at day 3 posttransfection whereas increased expression in response to miR-122 miRIDIAN miRNA Inhibitor was best observed at day 5 and 7 (Figure 3). Whether this is a consequence of these specific reagents or a general feature

of this methodology requires further investigation. Until there is a greater body of evidence, we would suggest a time course as part of assay optimization for miRIDIAN miRNA Mimic and Inhibitor studies.

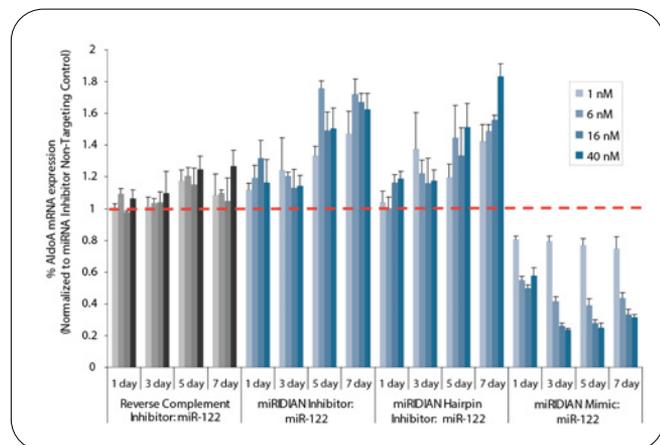


Figure 3: Modulation of *ALDOA* mRNA levels in Huh-7 cell line using miRNA mimics and inhibitors. Dashed red line indicates *ALDOA* expression level as a result of endogenous miR-122 regulation. *ALDOA* mRNA expression values > 1 indicates inhibition of miR-122, while values < 1 indicates additional down-regulation of *ALDOA* by miRNA. miRIDIAN Hairpin and first generation inhibitor performs well to inhibit miR-122, while RC inhibitor effect is slight.

Summary

miRNAs are non-coding RNAs that mediate post-transcriptional gene regulation via a mechanism that involves RISC-mediated binding to complementary sequences in the 3' UTR of target genes. While miRNAs are clearly involved in critical steps of animal development, cellular differentiation and disease, target identification remains challenging. Efforts to identify gene targets are confounded by unique attributes of miRNA regulation which include regulation mediated by partial complementarity of the miRNA: target pair, the potential for modulation of

hundreds of genes by a single miRNA and the potential for combinatorial regulation of a single gene. Furthermore, miRNA-mediated regulation and the resulting mRNA attenuation often result in subtle phenotypic effects which are difficult to detect. Consequently, there are currently only a few well-characterized direct targets of miRNAs. Recently, identification of miRNA-target relationships relied on labor intensive miRNA and gene expression profiling and/or surrogate reporter assays. Now with the availability of reliable miRNA-based mimics and inhibitors, researchers have the necessary tools to perturb specific miRNA activities and assess the phenotypic consequences in gain- or loss-of-function assays.

Here we describe the specific application of miRIDIAN miRNA Mimic and Inhibitors in an endogenous system for gain- and loss-of-function experiments. To demonstrate the power of these novel molecular tools, we selected a gene target for which evidence pointed to regulation by a defined miRNA, miR-122. Delivery of the corresponding miR-122 miRIDIAN miRNA mimic resulted in reduction of the endogenous target (*ALDOA*), while delivery of either of two inhibitor designs resulted in an increase in *ALDOA* mRNA level. The more recent inhibitor design includes terminal hairpin structures, and unlike the first generation of short RC inhibitors, exhibited highly functional and long-lasting activity. This work further reinforces the concept that secondary structure likely promotes sustained function of the inhibitor permitting detection of a potentially subtle endogenous phenotype². Therefore, the combination of gain-of-function (mimic-induced down regulation) and loss-of function (inhibitor-induced up regulation) experiments provided further support for direct miR-122-*ALDOA* target relationships. In summary, we have demonstrated that miRIDIAN miRNA Mimic and Hairpin Inhibitors can serve as complementary molecular tools providing critical phenotypic information and rapid confirmation of putative miRNA-target interactions.

References

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