Published in final edited form as:

Curr Protoc Pharmacol. ; 79: 9.26.1–9.26.15. doi:10.1002/cpph.30.

In Vitro Validation of miRNA-Mediated Gene Expression Linked to Drug Metabolism

Botros B. Shenoda¹, Sujay Ramanathan¹, and Seena K. Ajit^{1,2}

¹Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania

²Corresponding author (seena.ajit@drexelmed.edu)

Abstract

Pharmacogenomic approaches used to investigate how genes affect drug responses are critical for designing personalized therapies aimed at maximizing efficacy and minimizing adverse effects. Drug efficacy is often dependent on the sequence and expression levels of drug target genes or those involved in the metabolism and transport of the therapeutic agent. Expression of these genes, in turn, is negatively regulated by small noncoding miRNAs. The levels of miRNAs in bodily fluids have been studied extensively as potential diagnostic and prognostic biomarkers. Studies have shown that miRNAs regulate multiple genes and sequence homology is used to predict which genes are subject to regulation by a particular miRNA. Once a gene is identified as a potential target for an miRNA of interest, experiments are undertaken to confirm that the miRNA interacts with the target gene and can alter its level of expression and/or its activity. For example, the differential expression of miRNAs in whole blood obtained from good and poor responders to ketamine has been reported both prior to, and following treatment for complex regional pain syndrome. In this case, hsa-miR-548d-5p was significantly lower in poor responders relative to good responders. This miRNA was predicted to target UDP-glucuronyl transferase 1A1 (UGT1A1), a key drug metabolizing enzyme. Described in this unit are protocols used to confirm miR-548d-5p-mediated UGT1A1 regulation. The approaches described can be employed broadly for the validation of miRNA-mediated negative regulation of any gene. Determining miRNA-mediated regulation of enzymes and transporters affecting drug metabolism is a critical step in designing personalized therapy and for understanding the mechanisms responsible for variations in the responses to therapeutic agents.

Keywords

drug metabolism; MicroRNA; ketamine; Hsa-miR-548d-5p; UDP-glucuronyl transferase; glucuronidation

Conflicts of Interest

The authors declare that they have no competing interests.

Internet Resources

Bio-Rad DC Protein Assay protocol: <https://www.biorad.com/webroot/web/pdf/lsr/literature/LIT448.pdf>.

INTRODUCTION

Experimental validation of the role of a particular miRNA in regulating target gene expression is performed sequentially. The first step is to confirm the ability of the selected miRNA, in this example hsa-miR-548d-5p, to bind the 3' untranslated region (3' UTR) of target mRNA (UGT1A1). This is described in Basic Protocol 1. If the results are positive, the next steps entail the evaluation of the changes in mRNA and protein expression or function of the target gene by overexpressing miRNA. Detailed in Basic Protocol 2 is the procedure for determining mRNA alteration and in Basic Protocol 3 the procedure for assessing UDP-glucuronyl transferase (UDP-GT) activity in microsomes purified from liver cells overexpressing hsa-miR-548d-5p. The information provided below describes procedures used for validating whether a selected miRNA regulates the expression or activity of a drug metabolizing enzyme.

BASIC PROTOCOL 1 CONFIRM BINDING OF miR-548d-5p TO 3' UTR OF UGT1A1 USING LUCIFERASE REPORTER ASSAY

To validate target gene expression, conventional molecular biology techniques are used for determining transcript levels, including quantitative RT-PCR, northern blot, microarrays, and RNA sequencing combined with immunoblot and enzyme-linked immunosorbent assay (ELISA) for assessing changes in protein levels. However, before confirming that a selected miRNA negatively regulates the target gene expression at mRNA and protein levels, it is imperative to determine whether the miRNA can bind directly to the mRNA of the candidate gene. While bioinformatic tools are used to predict miRNA target binding sites in the 3' UTR of mRNAs, this interaction must be validated experimentally to demonstrate the binding based on 6 to 8 nucleotides seed sequence complementarity between miRNA and the 3' UTR of the gene of interest. This is accomplished using a reporter assay. In the example detailed below, the 3' UTR of the mRNA of interest (UGT1A1) is cloned downstream of the luciferase reporter gene for expression in mammalian cells. A chimeric mRNA is transcribed consisting of the reporter gene in frame with the 3' UTR target sequence. Binding of co-transfected miRNA (e.g., miR-548d-5p) to the 3' UTR is indicated by a decrease in luciferase activity (Figure 9.26.1 and Table 9.26.2).

Materials

HEK-293 cells (ATCC #CRL-1573)

Trypsin/EDTA solution (Sigma, cat. no. T3924)

Complete cell culture medium [DMEM (Sigma, cat. no. D5796) with 10% fetal bovine serum (FBS; Corning, cat. no. 35-010-CV) and 1% Penicillin streptomycin (Cellgro, cat. no. 30-002-CI)]

Lipofectamine 2000 (Invitrogen, cat. no. 11668019)

Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia, cat. no. LPFR-P010)

Precursor miRNA clone (e.g., hsa-mir-548d Precursor miRNA clone; GeneCopoeia, cat. no. HmiR0132)

3' UTR clones (e.g., human UGT1A1; GeneCopoeia, cat. no. HmiT013615-MT01) miRNA target control clone vector (GeneCopoeia, cat. no. CmiT000001-MT05)

Phosphate-buffered saline (PBS; Lonza, cat. no. 17-517Q)

DMEM (Sigma, cat. no. D5796)

Fetal bovine serum (FBS; Corning, cat. no. 35-010-CV)

75-cm² flask

15-ml conical tubes

Centrifuge

Hemocytometer

96-well cell culture plates

37°C incubator

Rocking platform or orbital shaker

GloMax[®]-96 Microplate Luminometer (Promega)

Day 1: Preparation of cells for assay

1. Grow 1 million HEK293 cells to ~70% confluency in a 75-cm² flask in complete medium in 37°C incubator.
Other cell lines that can be easily transfected include HeLa, CHO, and NIH/3T3 cells.
2. Aspirate the medium and add 2 to 3 ml fresh warm trypsin/EDTA solution.
3. After 5 min, gently tap the side of the flask to dislodge the cells.
4. Quench the trypsin reaction by adding 5 to 6 ml complete cell culture medium.
5. Transfer the cells into sterile 15-ml conical tubes.
6. Pellet the cells by centrifugation for 5 min at 300 × *g*, room temperature.
7. Aspirate and discard the supernatant.
8. Wash the cells by pipetting 10 ml complete cell culture or PBS medium into a 15-ml conical tube and resuspending the pellet. Collect the cells by centrifugation for 5 min at 300 × *g*, room temperature.
9. Resuspend the washed cells in 2 to 5 ml complete cell culture medium and determine the cell density using a hemacytometer.

10. Plate 30×10^3 cells in 100 μ l complete cell culture medium per well of the sterile 96-well cell culture plate. Incubate the cells overnight at 37°C.

Day 2: Transfection of precursor miRNA, 3' UTR, or control clone

11. For each well, 100 ng DNA and 0.5 μ l Lipofectamine 2000 in 100 μ l culture medium are needed. For 6 wells, prepare amounts equivalent to 7 wells (Table 9.26.1) to account for the loss in pipetting.
12. Three different experimental conditions are required: (1) untransfected cells as a negative control and for establishing background values in the luciferase assay, (2) cells transfected with miR-548d-5p and 3' UTR UGT1A1, and (3) cells transfected with scrambled miRNA control and 3' UTR UGT1A1 (Table 9.26.1).
13. Prepare mixtures A and B as detailed on Table 9.26.1. Mix A and B together gently to form a complex mixture C. Allow mixture C to stand for 30 to 40 min at room temperature.
14. Aspirate and discard medium and wash the cells with 100 μ l of $1 \times$ PBS.
15. Add 100 μ l of mixture C to each well and incubate for 24 hr at 37°C. Remove 50 μ l of the transfection medium after 6 hr and replace with complete cell culture medium without antibiotics (DMEM with 10% FBS).

Day 3: Measurement of luciferase activity

16. The luciferase assay can be performed 24-48 hr post-transfection.
17. Aspirate the medium from the cultured cells, wash the cells gently with 100 μ l of $1 \times$ PBS and completely remove the rinse solution.
18. Dispense 20 μ l of lysis buffer into each well and place the culture plates on a rocking platform or orbital shaker with gentle shaking for 10 to 15 min at room temperature.
19. Pipette the cell lysates into a 96-well white opaque plate that is compatible with the luminometer being used.
20. Add 100 μ l of firefly luciferase assay working solution to each well and gently mix by gently tapping on the side of the plate.

Working solution refers to the buffer containing substrate and is stable for 1 to hr at room temperature. Prepare only the required amount just before use. This solution should be capped and covered with foil to protect from light. These reagents are included in the Luc-Pair Duo-Luciferase assay kit.

21. Incubate the samples for 3 to 5 min at room temperature and then measure luciferase activity. We used GloMax[®]-96 Microplate Luminometer at a wavelength of 560 nm for firefly luciferase (measurement 1).
22. Add the Renilla luciferase working solution (100 μ l per well) and gently mix by swirling the plate by hand.

23. Incubate for 3 to 5 min at room temperature and then measure Renilla luciferase activity at 480 nm (measurement 2).

Data analysis

24. Calculate the mean blank firefly ($\bar{X}$ firefly) and Renilla ($\bar{X}$ Renilla) luciferase measurements for experimental condition 1 shown in Table 9.26.1.
25. Calculate corrected firefly and Renilla luciferase values by subtracting the mean blank firefly and Renilla luciferase measurements (obtained from the previous step) from individual firefly and Renilla luciferase measurements, respectively for experimental condition 2 and 3.
26. Calculate relative luminescence for each well from experimental conditions 2 and 3 (Table 9.26.1) by dividing corrected firefly luciferase measurement over Renilla luciferase measurement:

$$\begin{aligned} \text{Relative luminescence (for each well)} \\ = (\text{firefly reading} - \bar{X} \text{ firefly}) / (\text{Renilla reading} - \bar{X} \text{ Renilla}) \end{aligned}$$

27. Analyze relative luminescence values in experimental condition 2 relative to that of experimental condition 3 using Student's-t test.

See representative data and results on Table 9.26.2 and Figure 9.26.1.

BASIC PROTOCOL 2 CONFIRM THE ROLE OF miRNA IN REGULATING THE EXPRESSION LEVELS OF TARGET GENE mRNA

A number of factors, including the location and number of miRNA target binding sites in the 3' UTR, can influence gene expression. It is known that miRNAs regulate gene expression by inhibition of translation initiation, translation elongation, or by targeting mRNAs for degradation. The type of regulation can vary depending on the 3' UTR and miRNA combination being tested. Typically, for in vitro studies, cell lines with high levels of endogenous target gene expression that are amenable to transfection are chosen for study. In the example below HepG2 liver cells are used because they express high levels of UGT1A1 and are easily transfected (Figure 9.26.2 and Table 9.26.4).

Materials

HepG2 cells (ATCC® #HB-8065™)

Eagle's Minimum Essential Medium (EMEM; ATCC, cat. no. 30-2003) Penicillin/streptomycin (Corning, cat. no. 30-002-CI)

Precursor miRNA clone (e.g., hsa-mir-548d Precursor miRNA clone GeneCopoeia, cat. no. HmiR0132)

miRNA scrambled control clone (GeneCopoeia, cat. no. CmiR0001)

Xtreme gene HP DNA transfection reagent (Roche, cat. no. 06366244001) Phosphate-buffered saline (PBS; Corning, cat. no. 21-040)

RNase decontamination solution (e.g., Ambion RNaseZap[®] Solution) *or* RNase Zap wipes (Ambion, cat. no. AM9786 and AM9788)

0.25% Trypsin-EDTA solution (Sigma, cat. no. T4049) mirVana miRNA isolation kit (Life Technologies, cat. no. AM1561) Ice

Acid Phenol:Chloroform (Ambion, cat. no. AM9722)

Ethanol 200 Proof for molecular biology (Decon Laboratories, cat. no. 3916EA)

RNase-free DNase set (Qiagen, cat. no. 79254)

Water (nuclease-free) (Ambion, cat. no. AM9937)

Maxima First-Strand cDNA Synthesis Kit (Thermo Scientific, cat. no. K1642)

Taqman Fast Universal PCR master mix (Life Technologies, cat. no. 4352042)

Taqman gene expression assay mix for target gene (e.g., UGT1A1 Life Technologies, cat. no. Hs02511055_s1)

Taqman gene expression assay mix for housekeeping mRNA (e.g., 18sRNA Life Technologies, cat. no. 4319413E)

6-well plates

RNase-free barrier pipette tips (for transferring solutions)

Cell scrapers

Centrifuge

Vortex mixer

Microcentrifuge tubes

Filter cartridge (Invitrogen, cat. no. 10051G2)

Nanodrop Spectrophotometer

Thermal cycler

MicroAmp fast optical 96-well plate reaction plate (Life Technologies, cat. no. 4344906)

Optical adhesive films (Life Technologies, cat. no. 4311971)

7900HT Fast Real-Time PCR System (Applied Biosystem)

Transfection of HepG2 cells

1. Plate HepG2 cells (density of $3-4 \times 10^5$) in EMEM with 10% FBS and 1% Penicillin/Streptomycin in a 6-well plate (2.5 ml medium per well).
2. Prepare the transfection mixtures (Table 9.26.3) and incubate for 30 min before transfection at room temperature.
3. Wash the HepG2 cells once using 10 ml $1 \times$ PBS.
4. Gently add the prepared transfection complex to 3 ml of antibiotic-free EMEM medium and add the mixture to HepG2 cells.

RNA isolation 24 hr post-transfection

5. Laboratory bench and all the instruments, including pipettes, must be cleaned with an RNase decontamination solution (e.g., Ambion RNaseZap[®] Solution). Use RNase-free barrier pipette tips for transferring solutions.
6. Collect HepG2 cells from the 6-well plates either by scraping or upon trypsinization by adding 0.5 ml of 0.25% trypsin-EDTA for 2 to 3 min at 37°C, and then washing the cells once with 10 ml cold PBS.

Steps 7 to 15 are performed following instructions from the mirVana kit.

7. Centrifuge the cells for 5 min at $300 \times g$, 4°C, and disrupt the cell pellet in 350 μ l Lysis/Binding buffer (mirVana miRNA isolation kit) by vortexing for 15 sec.
8. Add 35 μ l of miRNA Homogenate additive, incubate 10 min on ice, and mix well by vortexing or inverting the tube several times.
9. Add 350 μ l (or volume equal to the volume of lysis buffer) of acid phenol:chloroform (withdraw from the bottom phase in the bottle because the upper phase consists of an aqueous buffer) and vortex for 60 sec. Centrifuge for 5 min at $12,000 \times g$, room temperature, to separate the aqueous and organic phases.
10. Without disturbing the lower phase, withdraw the aqueous (upper) phase, placing it into a new microcentrifuge tube and then adding 1.25 vol of 100% ethanol. Mix thoroughly by pipetting and pass the lysate/ethanol mixture through a filter cartridge included in the mirVana kit. Centrifuge the tubes with their filter cartridge for 15 to 60 sec at $10,000 \times g$, room temperature, discard the flow-through, and reuse the collection tube for the washing steps.
11. Wash the filter with 350 μ l miRNA wash solution 1 (provided with mirVana miRNA isolation kit) applied to the filter cartridge and then centrifuge for 15 to 45 sec at $12,000 \times g$, room temperature. Discard the flow-through from the collection tube.
12. Add DNase (10 μ l of reconstituted RNase-Free DNase + 70 μ l of RDD buffer (RDD included in the RNase-free DNase set) to the filter. incubate for 30 min at room temperature.

13. Wash the filter with 350 μ l miRNA wash solution 1, applied to the filter cartridge and centrifuge for 15 to 45 sec at $12,000 \times g$, room temperature. Discard the flow-through from the collection tube.
14. Wash the filter twice, each time with 500 μ l wash solution 2/3 (provided with mirVana miRNA isolation kit). Apply 500 μ l wash solution 2/3 (working solution mixed with ethanol) and draw it through the filter cartridge as in the previous step. Centrifuge for 1 min at $12,000 \times g$, room temperature, to remove residual fluid from the filter.
15. Elute the RNA with 100 μ l 95°C elution buffer or nuclease-free water. For this, transfer the filter cartridge into a fresh collection tube and apply 100 μ l of preheated (95°C) elution buffer or nuclease-free water to the center of the filter without touching, and centrifuge for 20 to 30 sec at maximum speed, room temperature, to recover the RNA. Collect the eluate (which contains the RNA) and store up to 3 to 6 months at -80°C .
16. Measure RNA quantity and purity using a Nanodrop spectrophotometer.

cDNA synthesis

17. Mix 4 μ l of $5 \times$ reaction mix (containing random hexamers, dNTPs, and RNase inhibitors), 2 μ l of enzyme mix (Maxima First-Strand cDNA Synthesis Kit), template RNA (200 to 500 ng), and nuclease-free water to a final volume of 20 μ l. Centrifuge gently for a few seconds at $100 \times g$, room temperature.
18. Set the thermal cycler for 10 min at 25°C followed by 15 min at 50°C , 85°C for 5 min and terminate at 4°C . Store the synthesized cDNA up to 1 year at -80°C .

qPCR

19. Perform Taqman assays for UGT1A1 as follows: The 20 μ l reaction volume for an individual assay has 10 μ l Taqman Fast Universal PCR master mix (2 $\times$) no AmpErase UNG, 1 μ l Taqman gene expression assay mix (20 $\times$) (UGT1A1 Assay ID: Hs02511055_s1 Applied Biosystems), 2 μ l cDNA, and 7 μ l nuclease free water. No-cDNA template control reactions are run in parallel to ensure there was no contamination generating false-positive results.
20. Use 18S ribosomal RNA as the endogenous control or normalizer (Assay ID 4319413E). The PCR components are identical except for replacing UGT1A1 Taqman gene expression assay mix (20 $\times$) with 18S.
21. Run the 96-well plate covered by the optical adhesive film in the Fast 96-well block as recommended by the manufacturer using the default fast thermal cycler protocol (Stage 1: 95°C for 20 sec for 1 cycle, stage 2: 95°C for 1 sec and 60°C for 20 sec for 40 cycles). Relative expression of UGT1A1 is calculated from raw CT values using the 2^{-CT} method (Schmittgen & Livak, 2008).

See representative data and results in Table 9.26.4 and Figure 9.26.2.

BASIC PROTOCOL 3 FUNCTIONAL ASSAY TO VALIDATE THE REGULATORY ROLE FOR miRNA

A functional miRNA can downregulate protein expression of its target gene. This is confirmed in vitro by assessing protein levels by western blot analysis or ELISA and by a functional assay. Functional analysis is particularly informative because, in addition to confirming miRNA-mediated decrease in protein expression, it enables evaluation of functional significance. Described is an assay used for investigating the role of miR-548d-5p in regulating the function of an UDP-glucuronosyltransferase (UGT) isoform by testing its enzymatic activity. The results indicate that hsa-miR-548d-5p was significantly lower in those patients who respond poorly to ketamine as compared to good responders. Most metabolites, including those generated from ketamine, are rendered inactive and eliminated by UGT through glucoronidation (Turfus et al., 2009). The human UGT family is comprised of UGT1A and UGT2B. The UGT1A family has nine functional protein isoforms, all of which have in common fifth exon and 3' UTR (Mackenzie et al., 2005). Thus, even though only UGT1A1 is tested in the example provided below, because of the identical 3' UTR, miR-548d-5p binding can be a common attribute of all nine UGT1A family members. This protocol can be used to test other miRNAs thought to bind this 3'UTR.

Materials

10 mM Tris-Cl, pH 7.4 (Teknova, cat. no. T3456)

EDTA (Fermentas, cat. no. R1021)

Glycerol (Sigma-Aldrich, cat. no. G5516)

50 mM potassium phosphate buffer, pH 7.4 (Sivertsson et al., 2010)

Bio-Rad *DC*TM Protein Assay Kit I (BIO-RAD, cat. no. 5000111)

Albumin from bovine serum (Sigma-Aldrich, cat. no. A2934)

UGT-GloTM Assay kit (Promega, cat. no. V2081)

Ultracentrifuge tubes

Sonicator

Centrifuge

White opaque 96-well plates

37°C incubator

GloMax R-96 microplate Luminometer (Promega)

Additional reagents and solutions for transfecting HepG2 cells (see Basic Protocol 2)

Transfect HepG2 cells

1. Transfect HepG2 cells as described in Basic Protocol 2 with empty vector or selected miRNA (e.g., miR-548d-5p).
2. Harvest cells 36 hr post-transfection.

Isolate microsomes

3. Homogenize the cells in 10 mM Tris-Cl, pH 7.4, containing 1 mM EDTA and 20% glycerol, sonicate (20×1 sec pulses), and centrifuge for 10 min at $10,000 \times g$, 4°C .
4. Centrifuge the resulting supernatant for 1 hr at $100,000 \times g$ for 1 hr, 4°C .
5. Resuspend the microsomal pellet in 50 mM potassium phosphate buffer, pH 7.4 (Sivertsson et al., 2010). Ensure the protein is not too dilute by using a small volume (20 μl) to resuspend.
6. Determine protein concentration using Bradford or other suitable procedure, such as the Bio-Rad DC assay (online resource: <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT448.pdf>). Briefly, add 25 μl of working reagent A (20 μl of reagent S to each ml of reagent A) to 5 μl of standards or samples to a clean 96-well plate and then add 200 μl reagent B into each well. Gently agitate the plate to mix the reagents. The absorbance can be read at 750 nm after 15 min. Bovine serum albumin is used for protein standards. Use the online resource link for protocol details.

Perform UDP-GT activity assay

The microsomes are assayed immediately for UGT activity using UGT-Glo™ assay (Promega). This system uses a luminescence method to measure UGT activity. First, the UGT enzymes, if present, attach a glucuronic acid moiety to the proluciferin substrate. The second step of the assay entails the incubation of the proluciferin with D-Cysteine (a component of the Luciferin detection reagent). This results in the conversion of the proluciferin into luciferin, which is necessary to produce light. Because a portion of the proluciferin substrate is glucuronidated during the incubation with the UGT enzymes, the luciferin produced from the glucuronidated proluciferin will not emit light. Therefore, the decrease in light output is proportional to the glucuronidation activity present in the first step. For all assay conditions (here, with miR-548d-5p and control miRNA), two glucuronidation reactions are established in parallel. Although both reactions contain a source of UGT and the proluciferin substrate, only one of them contains the uridine 5'-diphosphoglucuronic acid (UDPGA) cofactor. Any decrease in luminescence between the plus-UDPGA and the minus-UDPGA reactions is proportional to the glucuronidation activity for each condition.

Step 1

7. Set up the reactions in a white opaque 96-well plate as shown on Table 9.26.5. Sample prepared and analyzed are: Blank –, without UGT, without UDPGA; Blank +, without UGT with UDPGA; Control –, transfected with control

miRNA, without UDPGA; Control +, transfected with control miRNA, with UDPGA; Test –, transfected with miR-548d-5p, without UDPGA; Test +, transfected with miR-548d-5p, with UDPGA.

8. Add 10 μ l of 4 $\times$ test proteins (the concentration used is 1 μ g/ μ l) to each well followed by 10 μ l of 16 mM UDPGA to each *plus* (+) well and 10 μ l of water to each *minus* (–) well.
9. Initiate the reaction by adding 20 μ l of a UGT reaction mixture containing 2 $\times$ UGT-Glo™ Buffer, 2 $\times$ UGT substrate, and 2 $\times$ enzyme to each well.
10. Incubate for 3 hr at 37°C.

Step 2

11. Add to each well 40 μ l of reconstituted Luciferin detection reagent plus D-cysteine.
12. Allow the luminescent signal to stabilize for 20 min at room temperature.
13. Record luminescence using a luminometer (settings adjusted as per manufacturer guidelines).

Calculate relative substrate consumption

14. Calculate the difference in luminescence due to the presence of 4 mM UDPGA. Subtract the average of the blank (+) values from the average of the blank (–) values. Add this to all of the remaining (+) values i.e., Control (+) and Test (+) (background corrected + value).
15. Subtract each of the background-corrected (+) data points from the corresponding average (–) values to determine differences for each condition (relative luminescence). The magnitude of this difference is proportional to the UGT activity in the reaction.
16. Determine the percentage of substrate consumed in control samples, which is [relative luminescence for control/Average Control (–)] $\times$ 100 and for miRA-548d-5p transfected samples [relative luminescence for test/Average test (–)] $\times$ 100. Analyze the statistical significance of differences between samples using the Student's *t*-test.

See representative data and results in Table 9.26.6 and Figure 9.26.3.

COMMENTARY

Background Information

Epigenetic modifications can affect therapeutic responses by altering the expressions of genes involved in drug metabolism. Small noncoding miRNAs are an important epigenetic regulatory mechanism for modifying gene expression through binding to the 3' UTR of target mRNAs to induce translational repression or mRNA degradation. It is known that miRNAs are not only essential for physiological cellular homeostasis, but are also

implicated in many medical conditions (Esteller, 2011). It has been proposed that the functions of miRNAs are more prominent under stressful conditions or in disease states (Mendell & Olson, 2012). Moreover, miRNA pharmacogenomics is known to be crucial in mediating drug response (Rukov & Shomron, 2011; Shomron, 2010). For example, the polymorphism in the miRNA binding site on the dihydrofolate reductase (DHFR) gene is associated with reduced activity of methotrexate, a DHFR inhibitor, because of overexpression of the DHFR gene (Mishra et al., 2007). Also, lower levels of miR-27b are thought to be responsible for the higher protein and enzymatic activity of CYP1B1 in breast cancer tissues (Tsuchiya, Nakajima, Takagi, Taniya, & Yokoi, 2006). By their influence on drug-metabolizing enzymes, miRNAs can affect drug pharmacokinetics resulting in toxicity or a diminished therapeutic response.

Stable, circulating miRNAs present in bodily fluids have been extensively studied as possible biomarkers (De Guire et al., 2013). Mechanistic studies focusing on differentially expressed miRNAs can provide important insights on the physiological implications of miRNA changes. Circulating miRNAs can therefore be instrumental in determining changes associated with the expression of enzymes and transporters affecting drug metabolism and in personalizing therapy. For example, we investigated whether differential expression of miR-548d-5p could affect the clinical response to ketamine by affecting UGT and thereby influencing the glucuronidation of this analgesic. Ketamine, an NMDA receptor antagonist, is used as a treatment for chronic pain disorders, including complex regional pain syndrome (CRPS) (Schwartzman, Alexander, & Grothusen, 2011). However, heterogeneity in ketamine metabolism is known to be a limiting factor in the degree of ketamine-induced analgesia, with ~30% of patients experiencing little pain relief with this agent (Dahan et al., 2011). We previously identified differential expression of miRNAs in whole blood between good and poor ketamine responders both prior to and after therapy (Douglas et al., 2015). Differential expression of 33 miRNAs in pretreatment samples indicate that miRNA signatures are useful biomarkers for predicting treatment response (Douglas et al., 2015).

As a key drug-metabolizing enzyme, UDP-glucuronyl transferase 1A1 (UGT1A1) is a likely target for miRNA-548d-5p. In pretreatment samples, this miRNA was found to be significantly lower in poor responders as compared to good responders. It has also been hypothesized that patients with treatment-resistant depression metabolize ketamine differently than those who respond favorable to this agent (Zarate et al., 2012). We postulated that one factor contributing to ketamine resistance in CRPS patients is an altered pharmacokinetic profile caused by hsa-miR-548d-5p-mediated regulation of UGT1A1 that results in an enhanced elimination of active metabolites.

Described in this unit are the experiments to test this hypothesis and to determine whether miR-548d-5p can modulate the expression and function of UGT1A1. These assays can be used more generally to examine the regulation of other gene products, such as enzymes and transporters, by miRNA.

Detailed in Basic Protocol 1 is the reporter assay to confirm binding of miRNA to the 3' UTR of target mRNA. The experiment described in Basic Protocol 2 is used to determine whether the miRNA regulation of its target genes is through mRNA degradation or

translational repression. Multiple assays, including immunoblot analysis or ELISA, are used to investigate whether a particular miRNA can regulate protein expression of a proposed target gene. Detailed in Basic Protocol 3 are the experimental steps taken to define alterations in the enzymatic activity of UGT1A1 in liver cells following miRNA transfection. In this case, instead of a western blot analysis or ELISA to quantify changes in protein expression, a functional assay was employed to study the regulatory role of miRNA. As indicated in the protocol, microsomes isolated from hepatocytes are used to measure the enzymatic activity of UGT1A1 (Jones, Sun, Freeman, & Lazarus, 2012; Ma et al., 2014).

Critical Parameters

For Basic Protocol 1, any cell line that can be readily transfected can be used for the reporter assay. An experimentally validated microRNA-target interactions database, miRTarbase (Chou et al., 2016), is a useful resource to determine whether a reporter assay has been previously reported for the miRNA-mRNA target of interest. For Basic Protocols 2 and 3, it is important to use a cell line from the animal species of interest and with high endogenous expression of the target gene. Because differential expression of miR-548d-5p was identified in patients, a human cell line was used for investigating changes in mRNA and protein of the target gene. The miRNA can be expressed from a plasmid and if transfection is an issue a GFP marker in the same plasmid can help determine transfection efficiency. Upregulation of miRNAs in cells can also be accomplished by transfecting cells with synthetic pre-miRNAs. Depending on the gene and availability of crucial reagents such as antibodies, protein expression or enzymatic or functional assays can be employed to monitor miRNA-mediated changes in protein. For example, quantitative analysis of UDP-GT enzyme using immunoblotting or ELISA is utilized to demonstrate and confirm the changes in protein levels following miRNA dysregulation. The *in vivo* regulatory role of miRNA on UGT activity is indirectly reflected by the unconjugated: conjugated serum bilirubin levels (Douglas et al., 2015). However, this ratio might only be valid in individuals without comorbid hematological, hepatic or biliary dysfunctions.

Troubleshooting

The main challenge with these assays is determination of the amount of microsomes to be used per well in Basic Protocol 3. In our experience, it is essential to test the UGT-Glo assay using different concentrations of control microsomes. In general, a concentration producing 50% to 70% substrate consumption during the 2 hr incubation period is adequate.

Anticipated Results

The miRNA-target validation protocols detailed in this unit can be employed to study other miRNA families and their predicted targets. Such miRNA profiling studies are used to identify differential expression of a number of miRNAs between diseased and control groups. Understanding how these miRNAs reflects the disease state largely depends on predicting their targets. Typically, a large number of genes are thought to be targeted by miRNA by various bioinformatics approaches and genes that are of pathophysiological relevance to the disease process of interest are selected for confirmatory studies. The *in vitro* studies confirming the prediction are outlined here.

For Basic Protocol 1, if the miRNA under consideration (e.g., miR-548d-5p) binds to the 3' UTR of the predicted mRNA target, a reduction in luciferase gene expression, and thus luminescence, would be anticipated. Firefly luciferase measurements normalized to Renilla luciferase is used as the transfection control.

For Basic Protocol 2, the regulation of the target gene (e.g., UGT1A1) mRNA by the miRNA (e.g., miR-548d-5p) would result in lower expression of target gene following overexpression of the miRNA. However, the absence of a difference does not rule out the possibility of miRNA-mediated negative regulation of the target gene. That is, miRNA binding to 3' UTR does not always lead to mRNA degradation, even though it can result in translational repression (Valencia-Sanchez, Liu, Hannon, & Parker, 2006). In the latter case, the regulatory role of miRNA on the target gene can be confirmed by target protein quantification techniques.

The experiment described in Basic Protocol 3 is designed to define the effect of the miRNAs (miR-548d-5p) on protein function i.e., glucuronidation activity of UGT1A1. The relative enzymatic activity is measured by studying the relative substrate consumption by microsomes obtained from cells overexpressing miR-548d-5p relative to control. The lower the substrate consumption, the greater the effect of this miRNA on UGT1A1 expression.

Time Considerations

Each of the three protocols can take several days to complete. This is due primarily to the time necessary for preparing the cell culture and for transfections. All other manipulations are performed 24 to 48 hr post-transfection. The reporter assay (Basic Protocol 1) requires less than 3 hr to complete, with the RNA isolation, cDNA synthesis, and Taqman analysis (Basic Protocol 2) completed in a single day. Isolation of microsomes requires ~3 hr and the UGT-Glo assay 4 to 5 hr to complete.

Acknowledgements

This study was supported by funds from the NIH (NINDS 1R21NS082991-01), Rita Allen Foundation, and Drexel Clinical & Translational Research Institute to Seena Ajit. Botros Shenoda is a recipient of the Fulbright Foreign Student Program fellowship funded by the U.S. Department of State, Bureau of Educational and Cultural Affairs and Dean's Fellowship for Excellence in Collaborative or Themed Research, Graduate School of Biomedical Sciences and Professional Studies, Drexel University.

Literature Cited

- Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, ... Huang HD (2016). miRTarBase 2016: Updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Research*, 44(D1), D239–D247. doi: 10.1093/nar/gkv1258. [PubMed: 26590260]
- Dahan A, Olofson E, Sigtermans M, Noppers I, Niesters M, Aarts L, ... Sarton E (2011). Population pharmacokinetic-pharmacodynamic modeling of ketamine-induced pain relief of chronic pain. *European Journal Of Pain*, 15(3), 258–267. doi: 10.1016/j.ejpain.2010.06.016. [PubMed: 20638877]
- De Guire V, Robitaille R, Tetreault N, Guerin R, Menard C, Bambace N, & Sapienza P (2013). Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: Promises and challenges. *Clinical Biochemistry*, 46(10-11), 846–860. doi: 10.1016/j.clinbiochem.2013.03.015. [PubMed: 23562576]
- Douglas SR, Shenoda BB, Qureshi RA, Sacan A, Alexander GM, Perreault M, ... Ajit SK (2015). Analgesic Response to Intravenous Ketamine Is Linked to a Circulating microRNA Signature in

- Female Patients With Complex Regional Pain Syndrome. *The Journal of Pain*, 16(9), 814–824. doi: 10.1016/j.jpain.2015.05.008. [PubMed: 26072390]
- Esteller M (2011). Non-coding RNAs in human disease. *Nature Reviews. Genetics*, 12(12), 861–874. doi: 10.1038/nrg3074.
- Jones NR, Sun D, Freeman WM, & Lazarus P (2012). Quantification of Hepatic UDP glucuronosyltransferase 1A splice variant expression and correlation of UDP glucuronosyltransferase 1A1 variant expression with glucuronidation activity. *The Journal of Pharmacology and Experimental Therapeutics*, 342(3), 720–729. doi: 10.1124/jpet.112.192658. [PubMed: 22661630]
- Ma G, Lin J, Cai W, Tan B, Xiang X, Zhang Y, & Zhang P (2014). Simultaneous determination of bilirubin and its glucuronides in liver microsomes and recombinant UGT1A1 enzyme incubation systems by HPLC method and its application to bilirubin glucuronidation studies. *Journal of Pharmaceutical and Biomedical Analysis*, 92, 149–159. doi: 10.1016/j.jpba.2014.01.025. [PubMed: 24525562]
- Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, ... Nebert DW (2005). Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet Genomics*, 15(10), 677–685. doi: 10.1097/01.fpc.0000173483.13689.56. [PubMed: 16141793]
- Mendell JT, & Olson EN (2012). MicroRNAs in stress signaling and human disease. *Cell*, 148(6), 1172–1187. doi: 10.1016/j.cell.2012.02.005. [PubMed: 22424228]
- Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GS, Banerjee D, & Bertino JR (2007). A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 104(33), 13513–13518. doi: 10.1073/pnas.0706217104. [PubMed: 17686970]
- Rukov JL, & Shomron N (2011). MicroRNA pharmacogenomics: Post-transcriptional regulation of drug response. *Trends in Molecular Medicine*, 17(8), 412–423. doi: 10.1016/j.molmed.2011.04.003. [PubMed: 21652264]
- Schmittgen TD, & Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, 3(6), 1101–1108. doi: 10.1038/nprot.2008.73. [PubMed: 18546601]
- Schwartzman RJ, Alexander GM, & Grothusen JR (2011). The use of ketamine in complex regional pain syndrome: Possible mechanisms. *Expert Review of Neurotherapeutics*, 11(5), 719–734. doi: 10.1586/ern.11.31. [PubMed: 21539489]
- Shomron N (2010). MicroRNAs and pharmacogenomics. *Pharmacogenomics*, 11(5), 629–632. doi: 10.2217/pgs.10.26. [PubMed: 20415550]
- Sivertsson L, Ek M, Darnell M, Edebert I, Ingelman-Sundberg M, & Neve EPA (2010). CYP3A4 Catalytic Activity Is Induced in Confluent Huh7 Hepatoma Cells. *Drug Metabolism and Disposition*, 38(6), 995–1002. doi: 10.1124/dmd.110.032367. [PubMed: 20233841]
- Tsuchiya Y, Nakajima M, Takagi S, Taniya T, & Yokoi T (2006). MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Research*, 66(18), 9090–9098. doi: 10.1158/0008-5472.CAN-06-1403. [PubMed: 16982751]
- Turfus SC, Parkin MC, Cowan DA, Halket JM, Smith NW, Braithwaite RA, ... Kicman AT (2009). Use of human microsomes and deuterated substrates: An alternative approach for the identification of novel metabolites of ketamine by mass spectrometry. *Drug Metabolism and Disposition*, 37(8), 1769–1778. doi: 10.1124/dmd.108.026328. [PubMed: 19448136]
- Valencia-Sanchez MA, Liu J, Hannon GJ, & Parker R (2006). Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & Development*, 20(5), 515–524. doi: 10.1101/gad.1399806. [PubMed: 16510870]
- Zarate CA Jr., Brutsche N, Laje G, Luckenbaugh DA, Venkata SL, Ramamoorthy A, ... Wainer IW (2012). Relationship of ketamine's plasma metabolites with response, diagnosis, and side effects in major depression. *Biological Psychiatry*, 72(4), 331–338. doi: 10.1016/j.biopsych.2012.03.004. [PubMed: 22516044]

Figure 9.26.1.

(A) Schematic representation for luciferase assay protocol. (B) Relative luminescence 24 hr after miRNA (e.g., miR-548d-5p) transfection. The 3' UTR of target gene (e.g., UGT1A1) was cloned downstream of the luciferase open-reading frame. Significance was determined using Student *t*-test, *p* value <0.05 .

Figure 9.26.2.

(A) Schematic representation of steps to investigate transcriptional regulation of target gene (e.g., UGT1A1) by miRNA (e.g., hsa-miR-548d-5p) in liver cells. (B) Relative expression of the target gene 24 hr after miRNA transfection. HepG2 cells were transfected with hsa-miR-548d-5p and the relative expression of endogenous UGT1A1 mRNA was determined using qPCR; 18S rRNA was used as the normalizer. Significance was determined using Student *t*-test, *p* value <0.05 .

Figure 9.26.3.

(**A**) Schematic representation of steps to investigate the role of hsa-miR-548d-5p in regulating the UGT1A1 enzymatic activity. (**B**) UDP-GT activity in hsa-miR-548d-5p transfected liver cells. Using microsomes isolated from the HepG2 cells transfected with empty vector or miR-548d, the enzymatic activity was measured as a function of relative substrate consumption. Significance was determined using Student *t*-test, *p* value $* < 0.05$.

Table 9.26.1

Experimental Conditions for Transfections to Confirm miR-548d-5p Binding to the 3' UTR of UGT1A1 Using Luciferase Reporter Assay

	Condition 1	Condition 2	Condition 3
Mixture A (allow to stand for 5 min)	350 µl of serum free medium	350 ng of miRNA clone (e.g., hsa-miR-548d-5p) and 350 ng of 3' UTR target clone (e.g., UGT1A1) in 350 µl of serum free media	350 ng of scrambled miRNA control clone and 350 ng of 3' UTR target clone (e.g., UGT1A1) in 350 µl of serum free media
Mixture B	Add 3.5 µl of lipofectamine 2000 to 350 µl of serum free media	Add 3.5 µl of lipofectamine 2000 to 350 µl of serum free media	Add 3.5 µl of lipofectamine 2000 to 350 µl of serum free media

Table 9.26.2

Representative Data for the Luciferase Reporter Assay

Control	Replicates					Average
Firefly luciferase reading	827	1,205	1,291	1,290	889	1,100
Renilla luciferase reading	69,806	69,865	71,914	61,251	25,245	59,616
Firefly/renilla luminescence ratio	0.012	0.017	0.018	0.021	0.035	0.021
<i>Test miRNA</i>						
Firefly luciferase reading	426	895	730	674	335	612
Renilla luciferase reading	48,152	73,251	71,903	64,861	38,325	59,299
Firefly/renilla luminescence ratio	0.009	0.012	0.010	0.010	0.009	0.010

Table 9.26.3

Experimental Conditions for Transfections to Determine Alterations in UGT1A1 mRNA in Liver Cells by hsa-miR-548d-5p

	Condition 1 - Control	Condition 2 - miR-548d-5p
Reagents used per well (6-well plate)	Add 1.5 μ l of Xtreme gene HP DNA transfection reagent to 1.5 μ g of miRNA scrambled control clone diluted in 100 μ l serum-free medium	Add 1.5 μ l of Xtreme gene HP DNA transfection reagent to 1.5 μ g of miRNA clone (e.g., miR-548d-5p) diluted in 100 μ l serum-free medium

Table 9.26.4

Representative Data Showing the Effect of miRNA on the Expression of Its Target Gene mRNA

Transfection	Relative expression of target gene
Control (scrambled miRNA)	0.99
Control (scrambled miRNA)	1.73
Control (scrambled miRNA)	1.76
Average	1.49
miRNA (test miRNA)	0.86
miRNA (test miRNA)	0.51
miRNA (test miRNA)	0.76
Average	0.71

Table 9.26.5

Experimental Conditions for Functional Assay to confirm the Role of hsa-miR-548d-5p in Regulating the Enzymatic Activity of UGT1A1

	Microsomes from HepG2 cells	UDPGA (16 mM)	Water	2× control reaction mixture	2× UGT reaction mixture	Final volume
Blank (–)	–	–	20 µl	20 µl	–	40 µl
Blank (+)	–	10µl	10 µl	20 µl	–	40 µl
Control (–)	Microsomal proteins in 10 µl volume	–	10 µl	–	20 µl	40 µl
Control (+)	Microsomal proteins in 10 µl volume	10µl	–	–	20 µl	40 µl
Test (–)	Microsomal proteins in 10 µl volume	–	10 µl	–	20 µl	40 µl
Test (+)	Microsomal proteins in 10 µl volume	10 µl	–	–	20 µl	40 µl

Table 9.26.6

Relative Substrate Consumption as a Function of miRNA Regulation of Enzymatic Activity of UGT1A1

<i>Control samples</i>					
A Control +	B Control –	C Ave control–	A-C	(A-C)/C	(A-C)*100/C Relative substrate consumption
2987347	2767537	2630917	356430.1	0.135	13.55
2897483	2576045		266566.1	0.101	10.13
2846135	2612991		215218.1	0.082	8.18
3027632	2745877		396715.1	0.151	15.08
2881163	2618723		250246.1	0.095	9.51
2952990	2690675		322073.1	0.122	12.24
2916205	2658593		285288.1	0.108	10.84
2858548	2376894		227631.1	0.087	8.65
					Average=11.02
<i>Samples transfected with test miRNA e.g., miR-548d</i>					
A Test +	B Test –	C Ave Test –	A-C	(A-C)/C	(A-C)*100/C Relative substrate consumption
2949664	2819807	2641512.75	308151.3	0.117	11.67
2946483	2658363		304970.3	0.115	11.55
2904084	2736894		262571.3	0.099	9.94
2793614	2633869		152101.3	0.058	5.76
2819385	2582737		177872.3	0.067	6.73
2731175	2725212		89662.25	0.034	3.39
2856156	2605982		214643.3	0.081	8.13
2848672	2369238		207159.3	0.078	7.84
					Ave=8.13