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A Biochemical Approach to Identify Direct MicroRNA Targets

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Abstract

We have recently developed a biochemical approach to isolate miRNA-bound mRNAs and have used this method to identify the genome-wide mRNAs regulated by the tumor suppressor miRNA miR-34a. This method involves transfection of cells with biotinylated miRNA mimics, streptavidin pulldown, RNA isolation, and qRT-PCR. The protocol in this chapter describes these steps and the issues that should be considered while designing such pulldown experiments.

Keywords

MicroRNA; Pre-miRNA; MicroRNP; RISC; Native agarose gel; Native PAGE; Argonaute; Dicer; Ribonucleoprotein; RNA-protein

1 Introduction

MicroRNAs (miRNAs) are an abundant class of small (~22 nucleotides) regulatory RNAs that play crucial roles in diverse cellular processes [1, 2]. Each miRNA can regulate the expression of hundreds of genes by binding to the 3' untranslated region (UTR) of target mRNAs to inhibit mRNA translation and/or reduce mRNA stability [3–5]. MiRNA-mediated gene silencing is executed by the RNA-induced silencing complex (RISC) consisting of several proteins including the Argonaute (AGO) proteins which bind to miRNAs and mediate target mRNA recognition. Identifying the targets of a miRNA is essential to understand its biological function. However, identifying miRNA targets is not straightforward mainly due to partial complementarity between a miRNA and its target

We recommend including a known miRNA or previously published miRNA (miR-34a or miR-519) as a positive control for the PD experiment. Optimizing the transfection conditions is important for the success of the PDs. We usually determine the transfection efficiency by transfecting the cells with a Cyclophilin B siRNA or control siRNA (Thermo Scientific) and measuring Cyclophilin B mRNA levels by qRT-PCR. For a majority of cell lines including HeLa, HCT116, DLD1 and H1299, we observe the 75 % knockdown of Cyclophilin B mRNA after 48 h of transfection. To ensure that the Bi-miRNA is functionally similar to the non-biotinylated miRNA, we recommend (1) testing the effect of the Bi-miRNA mimics and non-biotinylated miRNA mimics on the 3' UTR of known targets by performing 3' UTR luciferase reporter assays, (2) determining the effect of the Bi-miRNA mimics and nonbiotinylated miRNA mimics on the mRNA and protein levels of known target mRNAs. Once the conditions have been optimized and select known targets of the candidate miRNA are consistently enriched in the PDs, genome-wide mRNAs bound to the candidate miRNA can be identified by microarrays or RNA sequencing from the PD material. Because a significant proportion of miRNA targets are also down-regulated at the mRNA level [5, 24], microarrays should also be performed from the input samples. Performing microarrays from the input samples as well as the PD samples may help identify the mRNAs that are bound and also down-regulated. The mRNAs which are not down-regulated but are enriched in the PDs may be regulated at the level of mRNA translation.

mRNA [6, 7]. Base-pairing between a miRNA and its target mRNA involves a small stretch of six to eight nucleotides (seed region) at the 5'-end of the miRNA, and as a result, in silico tools predict thousands of miRNA-regulated genes. Moreover, some miRNA targets can also be regulated through the coding region or 5' UTR [8, 9]. We and others have recently shown that some targets of miRNAs are regulated through noncanonical binding sites [10–13]. The rules of miRNA-mediated gene silencing are therefore complex and emphasize the need to develop experimental approaches to identify direct miRNA targets.

Although bioinformatic algorithms are useful in identifying miRNA targets, even the best algorithms such as TargetScan (<http://www.targetscan.org>) have a high false-positive rate, typically predicting thousands of target genes for each miRNA. Moreover, the overlap of predicted targets between different algorithms is relatively small and these in silico predictions are not cell type or context-specific. Combining bioinformatics with genome-wide approaches such as microarrays or proteomics upon miRNA overexpression or knockdown have been helpful in identifying miRNA targets [5, 12, 14–16]. Recently, biochemical approaches such as immunoprecipitation (IP) of Argonaute proteins have been used to identify direct miRNA targets by performing microarray analyses from Argonaute IPs [17, 18]. More recently, high-throughput sequencing coupled with UV crosslinking and immunoprecipitation (HITS-CLIP) was employed to identify the sequences in endogenous RNAs that are targeted by miRNAs in living cells [19]. An improved CLIP approach, called photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) has also been recently developed, in which a photo-activatable ribonucleoside analog (such as 4-thiouridine) is incorporated into transcripts of cultured cells and miRNA target sites can be identified by scoring for thymidine to cytidine transitions in the sequenced cDNA [20]. These biochemical approaches have provided genome-wide data sets of endogenous miRNA targets, miRNA-targeting rules, and biological roles of miRNAs. These technologies are continuing to evolve and are being applied to unique questions of miRNA biology.

Recently, we developed a biochemical approach to isolate miRNA-bound mRNAs and used it to identify the genome-wide mRNAs regulated by the tumor suppressor miRNA miR-34a [21]. To do this, modified miR-34a mimics (RNA duplexes consisting of miR-34a and the passenger strand), in which a biotin molecule was covalently attached to the 3' end of the mature miR-34a strand (also called antisense strand) were introduced into HeLa (human cervical cancer cells) by transient transfection and Biotin-miR-34a-mRNA complexes were isolated from cytoplasmic extracts using streptavidin beads (Fig. 1). To develop the pull-down (PD) method, we measured the abundance of known miR-34a target mRNAs (CDK4, CDK6, and MYB) in the Biotin-miR-34a (Bi-miR-34a) pulldowns by qRT-PCR. The abundance of these mRNAs was also determined in control pull-downs performed from HeLa cells transfected with the control (Bi-CTL) biotinylated *C. elegans* miRNA (cel-miR-67) mimics. As shown in Fig. 2a, miR-34a target mRNAs CDK4, CDK6 and MYB were significantly enriched in the Bi-miR-34a PD but not in the control PD. Transcripts encoding housekeeping mRNAs UBC and SDHA were not enriched in the PDs demonstrating the specificity of the PD assay. Introduction of Bi-miR-34a in HeLa cells also resulted in down-regulation of CDK4, CDK6 and MYB mRNAs (Fig. 2b) as measured by qRT-PCR from the input samples. The PD method was therefore able to isolate miR-34a-

target mRNAs, even though these mRNAs were destabilized by Bi-miR-34a. Recently, we performed microarrays from PD samples to identify the genome-wide targets of miR-34a, miR-21, and miR-519 [21–23]. This strategy enabled us to identify key targets and pathways regulated by these miRNAs. For example, the PDs enabled us to identify a novel role of miR-34a in regulation of growth factor signaling [21]. We believe that the PD method is a straightforward strategy and can be used to discover miRNA targets (a) to provide mechanistic insights about how a miRNA regulates a specific phenotype, and/or (b) to identify phenotypes that a miRNA may regulate.

The PD method can be divided into the following steps: (1) transfection of cells with biotinylated miRNA mimics, (2) beads preparation, (3) lysate preparation, (4) streptavidin pulldown, (5) RNA isolation, and (6) qRT-PCR. The protocol in this chapter describes these steps and the issues that should be considered while designing the pulldown experiment.

2 Materials

2.1 Cell Culture

HeLa (Human cervical cancer) cells.

2.2 Transfection Reagents

Lipofectamine 2000 (Invitrogen, cat# 11668–030), Opti-MEM (Invitrogen cat# 31985–062), and 3′-Biotin-miRNA mimics (Dharmacon/Thermo Scientific). Note that the 3′-Biotin-miRNA mimics are custom-made and contain a single biotin molecule covalently attached to the 3′-end of the mature strand (also called antisense strand).

2.3 1× Binding and Washing Buffer (Store at Room Temperature)

Prepare 50 ml 1× Binding and Washing buffer by adding 250 µl of 1 M Tris-HCl (pH 7.5), 50 µl of 0.5 M EDTA, and 10 ml of 5 M NaCl to 39.7 ml nuclease-free H₂O. Prepare all reagents in nuclease-free H₂O (Ambion cat# AM9932).

2.4 Solution A and Solution B (Store at RT)

Solution A: To prepare 50 ml solution A dissolve 200 mg NaOH in 20 ml nuclease-free H₂O (final concentration 0.1 M), add 500 µl of 5 M NaCl solution (final concentration 0.05 M) and adjust the volume to 50 ml with nuclease-free H₂O.

Solution B: Prepare 50 ml solution B by adding 1 ml 5 M NaCl to 49 ml nuclease-free H₂O.

2.5 Lysis Buffer (Store at 4 °C)

Prepare 50 ml lysis buffer by adding 1 ml 1 M Tris-HCl (pH 7.5), 2.5 ml 2 M KCl, 250 µl 1 M MgCl₂, 150 µl NP-40 and 46.1 ml nuclease-free H₂O. Before the pulldowns, prepare 1.5 ml lysis buffer containing 7.5 µl RNaseOUT (Invitrogen, cat# 10777–019) and 60 µl 25× EDTA-free protease inhibitor cocktail (Roche, cat# 11873580001).

2.6 Pulldown Reagents

Streptavidin-Dynabeads M-280 (Invitrogen, Cat# 65001), BSA (Ambion, Cat# AM2616), yeast tRNA (Ambion, Cat# AM7119) and Magnetic Separation Stand (Promega, Catalog # Z5332).

2.7 RNA Isolation

Acid-phenol:chloroform (Ambion, AM9720), Glycoblue (Ambion, cat# AM9516), Ethanol, 10 % SDS (Ambion, AM9822), sodium acetate pH 5.2 (Quality Biological, Inc. cat# 351-035-721), and Proteinase K (Invitrogen, 25530015).

2.8 Reverse Transcription and Real-Time PCR

iScript cDNA synthesis kit (BIO-RAD, cat # 170–8896) for reverse transcription. SYBR Green master mix (Applied Biosystems cat# 4309155) and StepOnePlus real-time PCR system (Applied Biosystems) for real-time PCR.

3 Methods

3.1 Transfection

Seed HeLa cells in a six-well plate (2.5×10^5 cells/well) and after 16 h transfect the cells with a 3'-Biotinylated control miRNA (celmiR-67) mimic (Bi-CTL) or 3'-Biotinylated miR-34a mimics (Bi-miR-34a) using Lipofectamine 2000 as per the manufacturer's instructions. Final concentration of each biotinylated miRNA mimic is 20 nM.

3.2 Beads Preparation

1. After 24 h of transfection, activate the Streptavidin-Dyna beads by adding 100 μ l beads (50 μ l/pulldown) to a 1.5 ml eppendorf tube. Wash three times with 1 ml binding and washing buffer by holding the tube on the magnetic stand for 2 min.
2. Wash the activated beads twice with 1 ml Solution A followed by a wash with 1 ml Solution B. Resuspend the beads in 1 ml lysis buffer.
3. To reduce nonspecific binding, the beads should be coated with RNase-free BSA and yeast tRNA. Add 10 μ l yeast tRNA (10 mg/ml stock), 10 μ l BSA (10 mg/ml stock) and 480 μ l lysis buffer to the beads and incubate at 4 °C for 30 min with rotation.
4. After the 30 min incubation, spin the beads at $500 \times g$ at 4 °C for 1 min to remove beads that adhere to the caps of the tube.
5. Wash the beads twice with 1 ml lysis buffer on the magnetic stand. The beads are now ready for the pulldowns.

3.3 Lysate Preparation

1. Twenty-four hour after transfection wash the cells twice with PBS (room temperature) and add 100 μ l Trypsin-EDTA to each well. After 5 min incubation at 37 °C, add 500 μ l cell culture medium to each well and transfer the cell

suspension to a 15 ml falcon tube. Add 14 ml PBS to the cell suspension and spin at $500 \times g$ for 5 min. Discard the supernatant and wash the cell pellet one more time with 15 ml PBS. Decant the supernatant and remove any remaining PBS with a p200 pipette.

2. To prepare cytoplasmic extract for the pulldowns, add 700 μ l lysis buffer supplemented with protease inhibitor cocktail and RNaseOUT to the cell pellet and transfer the lysate to a 1.5 ml eppendorf tube. Mix the lysate 20 times with a p1000 pipette and incubate on ice for 20 min.
3. Spin the lysate at $10,000 \times g$ at 4 °C for 15 min and collect the supernatant (cytoplasmic lysate) in a 1.5 ml eppendorf tube. Transfer 50 μ l of the cytoplasmic lysate (input) into a fresh 1.5 ml tube, add 200 μ l lysis buffer followed by 750 μ l Trizol LS. Mix by inversion and store at -80 °C.

3.4 Streptavidin Pulldowns

1. Incubate the cytoplasmic lysate (~600 μ l) to the pre-coated beads and incubate (with rotation) at 4 °C for 4 h.
2. After the 4 h incubation, spin at $500 \times g$ for 1 min to remove beads that adhered to the cap of the tube. Place the tubes on the magnetic stand for 2 min and discard the supernatant. Add 1 ml lysis buffer to the beads and discard the supernatant after keeping the tubes on the magnetic stand for 2 min. Repeat the washing procedure four times to remove unbound material.
3. After the final wash, add 100 μ l lysis buffer containing 5 μ l of RNase-free DNase I (2 U/ μ l). Incubate at 37 °C for 10 min and discard the supernatant. Add 500 μ l lysis buffer and discard the supernatant. This step will ensure degradation of genomic DNA contamination.
4. To the DNase-treated beads add 100 μ l lysis buffer containing 5 μ l Proteinase K (10 mg/ml) and 1 μ l 10 % SDS. Incubate at 55 °C for 20 min, spin at $500 \times g$ for 1 min and collect the supernatant (~100 μ l) on a magnetic stand. Add 200 μ l of lysis buffer to the beads and collect the supernatant (200 μ l). Combine the supernatants (~100 and 200 μ l) and add 300 μ l acid-phenol:chloroform. Proceed to RNA isolation.

3.5 RNA Isolation

1. Vortex the tubes for 1 min and spin at $10,000 \times g$ at room temperature for 1 min. Collect 250 μ l of upper layer and transfer to a fresh 1.5 ml tube. To precipitate the RNA, add 5 μ l Glycoblue, 25 μ l sodium acetate pH 5.2 and 625 μ l 100 % prechilled ethanol. Mix by inversion and keep the tubes at -20 °C for 16 h. After 16 h, precipitate the RNA by centrifuging the tubes at $16,000 \times g$ for 30 min at 4 °C. Decant the supernatant, add 750 μ l 70 % ethanol to the RNA pellet and precipitate the RNA by spinning the tubes at $7,500 \times g$ for 5 min at 4 °C. Decant the ethanol and carefully remove any residual ethanol near the pellet. Allow the pellet to air dry at room temperature for 5 min (do not allow the RNA pellet to

over dry). Add 40 µl nuclease-free water to the pellet and dissolve the RNA by incubating on ice for 5–10 min.

2. To isolate RNA from input add 250 µl nuclease-free H₂O to the Trizol LS containing lysate (Subheading 3.3) and vortex for 1 min after adding 200 µl chloroform. Spin at 12,500 × *g* for 15 min and collect 400 µl upper layer in a 1.5 ml tube. Add 400 µl isopropanol, 5 µl Glycoblue and incubate at room temperature for 10 min. Spin the tubes for 15 min at 12,500 × *g* at 4 °C. Discard the supernatant and wash the pellet by adding 1 ml 70 % chilled ethanol. Spin at 7,500 × *g* for 10 min at 4 °C. Decant the ethanol and carefully remove any residual ethanol near the pellet. Allow the pellet to air dry at room temperature for 5 min (do not allow the RNA pellet to over dry). Add 40 µl nuclease-free water to the pellet and dissolve the RNA by incubating on ice for 5–10 min.
3. Quantitate the RNA using a Nanodrop spectrophotometer.
4. The isolated RNA is now ready for downstream analysis such as qRT-PCR or microarray.

3.6 Reverse Transcription and Real-Time PCR

Use 100 ng total RNA or 10 µl PD RNA for first-strand cDNA synthesis using iScript cDNA synthesis kit. Abundance of target mRNAs in the input or PD samples can be assessed by real-time PCR using SYBR Green master mix. We recommend measuring the levels of housekeeping mRNA such as GAPDH, SDHA, and UBC as negative controls. Primer sequences for qRT-PCR for these housekeeping genes have been previously described [21].

3.7 Concluding Remarks

The Biotin-miRNA pulldown method allows the experimental identification of direct miRNA targets. This approach can be used to validate putative targets of a miRNA and/or to discover novel miRNA targets.

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Fig. 1.

Overview of pulldown method. 3'-Biotinylated miRNA mimics are transfected into cells and cytoplasmic extracts are prepared after 24 h. The Biotin-miRNA-mRNA complexes are isolated from cytoplasmic extracts using streptavidin beads. RNA isolated from the pulldown material is used to determine the enrichment of known targets of the miRNA. Microarray analysis from the pulldown RNA can be used to identify the genome-wide mRNAs bound by the transfected Biotin-miRNA

Fig. 2.

The pulldown method captures miR-34a target mRNAs that are actively degraded. **(a)** HeLa cells were transfected with Bi-CTL or Bi-miR-34a for 24 h. Enrichment of 3 miR-34a target mRNAs (CDK4, CDK6, and MYB) in streptavidin pulldowns was assessed by qRT-PCR of PD RNA normalized to gApdH. **(b)** Effect of Bi-miR-34a on the levels of the miR-34a target mRNAs CDK4, CDK6 and MYB was determined by qRT-PCR from input RNA normalized to GAPDH. Housekeeping mRNAs UBC and SDHA were used as negative controls