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TITLE:

The Ago2-miRNA-co-IP Assay to Study TGF-β1 Mediated Recruitment of miRNA to the RISC in CFBE Cells

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SUMMARY:

Here, we describe the Ago2-miRNA-co-IP assay designed to quantify an active pool of specific miRNAs induced by TGF-β1 in human bronchial epithelial CFBE41o- cells. This assay provides functional information on the recruitment of miRNA to the RNA induced silencing complex, quantified by qRT-PCR using specific miRNA primers and TaqMan hydrolysis probes.

ABSTRACT:

Micro(mi)RNAs are short, non-coding RNAs that mediate the RNA interference (RNAi) by post-transcriptional mechanisms. Specific miRNAs are recruited to the cytoplasmic RNA induced silencing complex (RISC). Argonaute2 (Ago2), an essential component of RISC, facilitates binding of miRNA to the target-site on mRNA, followed by cleaving the miRNA-mRNA duplex with its endonuclease activity. RNAi is mediated by a specific pool of miRNAs recruited to RISC, and thus is referred to as the functional pool. The cellular levels of many miRNAs are affected by the cytokine Transforming Growth Factor-β1 (TGF-β1). However, little is known about whether the TGF-β1 affects the functional pools of these miRNAs. The Ago2-miRNA-co-IP assay, discussed in this manuscript, is designed to examine effects of TGF-β1 on the recruitment of miRNAs to RISC and it helps to determine whether changes in the cellular miRNA levels correlate with changes in the RISC-associated, functional pools. The general principles of the assay are as follows. Cultured cells treated with TGF-β1 or vehicle control are lysed and the endogenous Ago2 is immunoprecipitated with immobilized anti-Ago2 antibody, and the active miRNAs complexed with Ago2 are isolated with a RISC immunoprecipitation (RIP) assay kit. The miRNAs are identified with quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using miRNA-specific stem-looped primers during reverse transcription, followed by PCR using miRNA-specific forward and reverse primers, and TaqMan hydrolysis probes.

INTRODUCTION:

Transforming Growth Factor-β1 (TGF-β1) is a multifunctional cytokine that can change the expression of many micro(mi)RNAs[1-3]. The total cellular level of a particular miRNA does not correlate with its inhibitory potential because only a specific fraction of the miRNA is incorporated into RNA induced silencing complex (RISC) to perform RNA interference (RNAi)[3]. Only up to 10% of each miRNA is RISC-associated and participates in RNAi[4,5]. Next, the RNAi process involves binding of the RISC-associated miRNA to the target mRNA recognition sequence(s)[6]. The RISC association is influenced by the availability of the target mRNA and the miRNA complementarity to the binding site, usually present at 3' untranslated region (UTR) of the mRNA[4]. The Argonaute2-miRNA-co-immunoprecipitation (Ago2-miRNA-co-IP) assay, described in this manuscript, is designed to examine the effect of TGF-β1 on the recruitment of specific miRNAs to RISC by detecting differences in the RISC-associated miRNAs after TGF-β1 treatment, compared to the vehicle control. Examining the RISC-associated functional pool of a specific miRNA is much more informative about the miRNA effects than examining the total cellular level of the miRNA. RISC consists of proteins that scan the binding site on the target mRNA and cleave the miRNA-mRNA duplex. Argonaute2 (Ago2) is the main component of RISC. Out of the five Ago isoforms (Ago1-Ago5), Ago2 is the only one that has endonuclease activity and participates in RNAi in human cells[7-10]. The Ago2-miRNA-RISC complex is the functional unit for miRNA-mediated post-transcriptional mRNA repression[11]. The Ago2-associated miRNA represents the native state of miRNA in response to intracellular or extracellular signaling. Thus, immunoprecipitation of the endogenous Ago2 provides an excellent opportunity to detect the active, RISC-associated fraction of a specific miRNA as well as the functional assessment of its targets. This assay is superior to the pull-down of endogenous target mRNA with biotinylated miRNA mimics because of unpredictable efficiency of the cellular uptake of biotinylated nucleic acid molecules and their off-target effects.

The Ago2-miRNA-co-IP assay, discussed in this manuscript, was optimized to determine the effects of TGF-β1 on RISC recruitment of miRNAs in immortalized human bronchial epithelial CFBE41o- cells[3]. Components of the RIP assay kit were used to perform Ago2-miRNA-co-IP assay with modifications in the protocol provided by the manufacturer. A separation method was used to isolate small and large RNA, in which small RNA was used to quantify miRNA with the help of quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using miRNA-specific stem-looped primers during reverse transcription, followed by PCR using miRNA-specific forward and reverse primers, and TaqMan hydrolysis probes.

PROTOCOL:

1. Preparation before experiment

1.1. Seeding cells

1.1.1. Prepare 10% collagen I solution in Minimal Essential Medium (MEM) and add 500 µL to each 24 mm cell culture filter in a 6-well plate. Distribute to cover the entire surface of the filter by rotating gently by hand. Incubate filters under the UV light in the laminar flow hood at room

temperature for 30 minutes (min), followed by incubation in cell culture incubator at 37 °C for 1 h.

1.1.2. Prepare cell culture medium (MEM gassed with CO_2 for 20 min, 10% Fetal Bovine Serum (FBS), 50 U/mL penicillin, 50 U/mL streptomycin, 2 mM L-glutamine, 0.5 µg/mL puromycin).

1.1.3. Bring the collagen-coated filters (step 1.1.1) from the incubator and suction off the excess collagen. Add 1.5 mL of the cell culture medium (step 1.1.2) to the basolateral side and 0.5 mL to the apical side (onto the filter) in the 6-well plate.

1.1.4. Seed 1 x 10^6 of CFBE41o- cells[12] suspended in 500 µL of the cell culture medium. Rotate the plate gently by hand to distribute the cells evenly on the filters and incubate in the cell culture incubator at 37 °C with 5% CO_2.

1.1.5. Remove medium from the apical side one day after seeding cells and continue culturing cells in air-liquid-interface (no medium on the apical side). Change the basolateral medium daily and perform the experiment on the 8^{th} day.

1.2. Treatment of Cells with TGF-β1 or Vehicle Control

1.2.1. Prepare FBS-free cell culture medium (FBS-free medium): Gas MEM with 5% CO_2 for 20 min, add 50 U/mL penicillin, 50 U/mL streptomycin, and 2 mM L-glutamine and filter sterilize the medium.

1.2.2. Prepare vehicle control: Add 20 µL of 1 M HCl and 5 mg of BSA to 5 mL of double distilled water (4 mM HCl with 1 mg/mL BSA) to obtain 1 ng/µL stock solution. Filter sterilize the mixture before use.

1.2.3. Prepare TGF-β1 solution: Reconstitute 1 µg of lyophilized TGF-β1 in 1 mL of sterile-filtered vehicle (4 mM HCl and 1 mg/mL BSA) to obtain 1 ng/µL stock solution. Aliquot in small volumes and store at -20 °C.

1.2.4. Prepare the working concentration of TGF-β1 or vehicle control at 15 ng/mL of FBS-free medium (add 15 µL of the TGF-β1 or vehicle control stock solution per mL of FBS-free medium). Suction off the cell culture medium from the basolateral side and add the TGF-β1 or vehicle control containing medium 24 h before the experiment.

1.3. Preparation of buffers for cell lysis and immunoprecipitation

1.3.1. Prepare mi-Lysis buffer (+) for cell lysis. Add 1 tablet of protease inhibitor mini and 15 µL of 1 M DTT per 10 mL of volume into the mi-Lysis buffer provided in the RIP-Assay Kit.

1.3.2. Prepare mi-Wash buffer (+) for washing protein G agarose beads. Add 52.5 µL of 1 M DTT per 35 mL of volume into the mi-Wash buffer provided in the RIP-Assay Kit.

1.4. Conjugation of Anti-Ago2 antibody with protein G agarose beads

1.4.1. Wash separately two 30 µL aliquots of protein G agarose bead slurry (50%) by repeating the following procedure three times: add 100 µL of ice-cold PBS, mix gently, centrifuge at 2,000 x *g* for 1 min, and suction off the supernatant with 200 µL pipette.

1.4.2. Wash the beads once with 100 µL of ice-cold mi-Wash Buffer (+), suction off the supernatant with 200 µL pipette and add 500 µL of mi-Wash Buffer (+) to the beads and mix gently. Keep the tubes on ice.

1.4.3. Add 7.5 µg of mouse monoclonal (IgG2) anti-human EIF2C2 (Ago2) antibody (see **Table of Materials**) or non-specific mouse IgG2 (negative control) to the protein G beads slurry in the mi-Wash Buffer (+).

1.4.4. Incubate the tubes overnight, rotating at 8 rotations per minute (rpm) in a cold room.

2. Immunoprecipitation of Ago2

2.1. Lysis of cells

2.1.1. Bring the plate containing cells cultured on 24 mm filters from the cell culture incubator and quickly transfer the filters to a plate on ice filled with ice-cold PBS. Allow PBS to overflow into the apical side of the filters to cover cells.

2.1.2. One side at a time, suction off PBS and wash the apical and basolateral side with ice-cold PBS twice.

2.1.3. Suction off all PBS, add 300 µL of ice-cold mi-Lysis buffer (+) to the cells, scrape the cells and transfer to 1.5 mL tube marked for respective treatment condition (TGF-β1 or vehicle control).

2.1.4. Add 200 µL of mi-Lysis buffer (+) to each tube and vortex thoroughly.

2.1.5. Incubate the tubes on ice for 15 min.

2.1.6. Centrifuge the tube at 14,000 x *g* for 10 min at 4 °C, collect the supernatant as the cell lysate, and keep on ice.

2.2. Preclearing of cell lysate with unconjugated protein G agarose beads

2.2.1. Wash 30 µL of fresh protein G agarose bead slurry (50%) in a 1.5 mL tube 3 times with 100 µL of PBS (step 1.4.1) and remove excess PBS.

2.2.2. Wash beads with 500 µL of mi-Wash Buffer (+) once, and once with 500 µL of mi-Lysis Buffer (+). Remove the excess buffer.

2.2.3. Add cell lysate from step 2.1.6 to the tubes containing unconjugated protein G beads.

2.2.4. Rotate (8 rpm) the tubes for 1 h in the cold room to preclear the cell lysates.

2.2.5. After preclearing, centrifuge the tubes at 2,000 x *g* for 1 min at 4 °C. Collect the precleared lysate as supernatant in fresh tubes and keep on ice.

2.2.6. Prepare pre-immunoprecipitation whole cell lysate (WCL) for detection of Ago2 protein by western blotting. Take 10 µL of precleared lysates and add 10 µL of sample buffer (with 10% DTT), heat at 85 °C for 4 min, cool down on the bench, and store at -20 °C.

2.3. Immunoprecipitation of Ago2 complexes with anti-Ago2 antibody immobilized on protein G agarose beads

2.3.1. Centrifuge the anti-Ago2 antibody conjugated to protein G beads prepared in step 1.4.4 at 2,000 x *g* for 1 min at 4 °C, remove the supernatant and discard. Wash beads once with 500 µL of mi-Lysis Buffer (+), centrifuge as before, and discard the supernatant again.

2.3.2. Mix 500 µL of the precleared cell lysates from step 2.2.6 with anti-Ago2 antibody conjugated with protein G beads and incubate for 3 h rotating (8 rpm) in the cold room.

2.3.3. Bring tubes from cold room on ice to the bench top and centrifuge at 2,000 x *g* for 1 min at 4 °C. Then remove and discard the supernatant.

2.3.4. Wash the protein G agarose beads with immobilized anti-Ago2 antibody-Ago2 complexes containing the co-immunoprecipitated miRNAs with 1 mL of mi-Wash buffer (+), centrifuge at 2,000 x *g* for 1 min at 4 °C, and discard the supernatant. Repeat this process twice.

2.3.5. Resuspend the beads with 1 mL of mi-Wash Buffer (+) and take 100 µL of the slurry to a new tube for the post-immunoprecipitation protein samples for western blotting.

2.3.6. Centrifuge the tubes at 2,000 x *g* for 1 min at 4 °C and discard the supernatant.

2.3.7. Add 20 µL of sample buffer (10% DTT), heat for 5 min at 85 °C, mix, and centrifuge at 2,000 x *g* for 1 min at room temperature. Store samples at -20 °C.

2.3.8. Centrifuge the tubes with the remaining 900 µL of immobilized anti-Ago2 antibody-Ago2 complexes from step 2.3.5 at 2,000 x *g* for 1 min at 4 °C, suction off and discard the supernatant. Keep the samples on ice.

3. RNA Isolation

3.1. Label two 1.5 mL tubes per sample for isolation of small RNA and large RNA in upcoming steps and add 2 µL of mi-solution IV to each tube, to be used in step 3.5 and 3.8.

3.2. Prepare the master mix solution by adding 10 µL of mi-solution I and 240 µL of mi-solution II from the RIP-assay kit, per sample, to a 1.5 mL tube and keep the tube at room temperature.

3.3. Add 250 µL of the master mix to each tube containing immobilized anti-Ago2 antibody-Ago2 complexes (prepared in step 2.3.8), vortex, and centrifuge at 2,000 x *g* for 1 min at room temperature.

3.4. Add 150 µL of mi-solution III in the same tube and mix well. Then centrifuge at 2,000 x *g* for 2 min at room temperature. The supernatant at this step contains large RNA in addition to small RNA (i.e., total RNA).

3.5. Carefully transfer the supernatant to the tube containing 2 µL of mi-solution IV, prepared in step 3.1. Avoid contaminating the supernatant with beads to interfere with qRT-PCR.

3.6. Add 300 µL of ice-cold 2-popanol to each tube containing total RNA, vortex, spin down, and incubate at -20 °C for 2 h for optimal precipitation of large RNA.

3.7. After the incubation at -20 °C, centrifuge the samples at 12,000 x *g* for 10 min at 4 °C to separate the small and large RNAs, contained in the supernatant and pellet, respectively. Keep the pellets on ice until step 3.11.

3.8. Transfer the supernatant with small RNAs to the tube prepared in step 3.1 (containing 2 µL of mi-solution IV) and add 500 µL of ice-cold 2-propanol, vortex, and spin down.

3.9. Incubate the supernatants overnight at -20 °C for optimal precipitation of small RNAs.

3.10. The next day, take out the tubes containing small RNAs from -20 °C freezer and centrifuge at 12,000 x *g* for 10 min at 4 °C. Aspirate the supernatant, without disturbing the pellet, and wash the pellet containing most of the small RNA following step 3.11.

3.11. Rinse the pellet containing large RNA (step 3.7) and small RNA (step 3.10) with 500 µL of ice-cold 70% ethanol, each. Slowly mix the pellet with ice-cold 70% ethanol and then centrifuge at 12,000 x *g* for 3 min at 4 °C. Carefully aspirate the supernatant, without disturbing the pellet. Repeat the process of washing once again.

3.12. Aspirate remaining ethanol with pipette of smaller volumes (10 µL or 20 µL) and allow to air dry for 30 min at room temperature in RNase free environment.

3.13. Reconstitute the small RNA and large RNA pellets by adding 50 µL of nuclease free water and heating at 65 °C for 5 min. Then store the RNA at -80 °C till further use after checking the

quality and concentration of RNA by nanodrop.

4. Quantification of miRNA by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

4.1. cDNA Preparation through Reverse Transcription of miRNA with miRNA Specific Stem-looped Primers

NOTE: RNA detection by standard qRT-PCR requires a template of at least two times the length of the forward or reverse primer, each approximately 20 nucleotides long. Thus, the minimum length is at least 40 nucleotides, making small RNAs too short for detection. The protocol describes miRNA-specific RT-primers (**Table of Materials**) containing highly stable stem-loop structure that lengthens the target cDNA. The forward PCR primer adds additional length with nucleotides that optimizes its melting temperature and enhances assay specificity. The reverse primer disrupts the stem loop[13]. The following protocol describes RT of RNA to cDNA for miR-145-5p, miR-143-5p, and miR-154-5p using the miRNA reverse transcription Kit (**Table of Materials**).

4.1.1. Prepare a master mix for each miRNA by adding 100 mM dNTPs (0.3 µL), Reverse Transcriptase (2 µL), 10x RT buffer (3 µL), RNase Inhibitor (0.38 µL), miRNA-specific looped primer (6 µL), and nuclease free water (8.32 µL). The combined volume of master mix is 20 µL.

4.1.2. Add 10 µL of small RNA from step 3.13 (50-100 ng) to three separate 200 µL tubes for each cDNA of miR-145-5p, miR-143-5p, and miR-154-5p.

4.1.3. Add the master mix to small RNA, mix gently, and spin to the bottom of tubes. Keep the tubes on ice until loading into the thermal cycler.

4.1.4. Set the program in thermal cycler for single cycle as hold at 16 °C for 30 min, hold at 42 °C for 30 min, hold at 85 °C for 5 min, and hold at 4 °C. Set the reaction volume to 30.0 µL. See step 4.1.6.

4.1.5. Load the reaction tubes into the thermal cycler. Start the RT run. See step 4.1.7.

4.1.6. Switch **ON** the thermal cycler and place the tubes into the tray. It will show that “System is booting, please wait”. Click on **Browse/New Methods**. Save the method and use in another experiment by browsing through the menu. Either select from the list of methods saved before and start run or create “new” method. The “edit run” method or “new” method screen will appear.

4.1.6.1. Edit temperature and cycles as stated in step 4.1.4. Add or remove stages and steps by clicking on particular stage or step and then clicking on add or delete button. Click on **Save** or directly run the program. In **Save Run Method**, name the “run” method, set the reaction volume (30 µL), set cover temperature to 105 °C, save, and exit.

4.1.6.2. When “browse run method” screen appears, select the saved method from the “run method” list. Click **Start Run** and again confirm the reaction volume and cover temperature. Click on **Start Run Now**. The screen will show sample temperature, cover temperature, and time remaining. Record the start and stop time after completion and remove the tubes from the thermal cycler.

NOTE: The tubes containing cDNA can be stored in -20 °C till their further use for qPCR of miRNA.

4.2. qPCR with the miRNA-specific forward/reverse primers and hydrolysis probes

NOTE: The following protocol is for qPCR of miR-145-5p, miR-143-5p, and miR-154-5p using miRNA-specific cDNAs prepared in step 4.1. The single tube miRNA Assay (20x) contains miRNA specific forward/reverse primers and hydrolysis probe (**Table of Materials**). The forward primer adds nucleotide to increase length and optimize melting temperature, while the reverse primer disrupts the stem loop of cDNA[13]. The Universal PCR Master Mix (**Table of Materials**) provides additional components required for qPCR. The reaction (20 µL) is done in triplicate for each miRNA.

4.2.1. Prepare separate master mix for miR-145-5p, miR-143-5p, and miR-154-5p in triplicates using respective miRNA assay (20x) (1 µL), 2x Universal PCR Master Mix (No AmpErase UNG; 10 µL), and nuclease free water (7.5 µL). The total volume for one reaction is 18.5 µL.

4.2.2. Add miRNA-specific cDNA (1.5 µL) prepared in 4.1 to to each well of the PCR plate in triplicates.

4.2.3. Add the miRNA-specific master mix (18.5 µL) prepared in step 4.2.1 to the wells of PCR plate containing respective cDNA (1.5 µL).

4.2.4. Seal plate with appropriate sealer and run qPCR for 45 cycles on a qPCR thermocycler (**Table of Materials**). Set cycle temperature as hold at 95 °C for 10 min, followed by 45 repeats of 95 °C for 15 seconds (s) and 60 °C for 60 s. See step 4.2.6.

4.2.5. Set the qPCR curve baselines manually per plate and standardized thresholds for all qPCR runs at a point at which amplification becomes logarithmic for all samples.

4.2.6. Switch **ON** the thermal cycler and place the PCR plate into the tray. The thermal cycler machine operates with the software installed on the accompanying computer. Click on the icon of the system software.

4.2.6.1. Click on **Create New Document** or **Open Existing Document** (if there is a previously saved document). New document wizard will appear. The assay will be “standard curve (Absolute quantification)”. Click on **Next**.

4.2.6.2. Add a new detector. Name the detector as miR-143, miR-145, and miR-154 one by one by clicking **Create Another**. Select reporter dye as "FAM" for all, and click **Okay**. Then find the name as miR-143, miR-145, and miR-154 and add them into detector's document by clicking **Add**. Then click **Next**.

4.2.6.3. "Set up sample plate" will appear. Select the wells for a detector and then click on **Use** to select the corresponding detector. Then click on **Finish**. The system initializing will appear.

4.2.6.4. Go to the **Instrument** and set stage, temperature and time as mentioned in step 4.2.4. Set the sample volume to 20 µL. Then start the run by clicking **Start**. Then save the plate with a name in a desired location. The estimated time will show on the screen near the start button. Note down the time.

4.2.6.5. After completion, go to **File** | **Export** | **Results**, and save results as CT values in desired location. The CT values will be in .csv files. Open it and copy the values into a spreadsheet file.

4.2.7. Subtract average of triplicate threshold cycle (Ct) of the vehicle control-treated samples from average Ct of TGF-β1-treated samples to calculate ΔCt. Finally, calculate fold change (FC) of miRNA levels using formula $2^{-\Delta Ct}$. The log2 transformation of the FC value can be used for graphical representation.

REPRESENTATIVE RESULTS:

We have previously shown that TGF-β1 increased the total cellular levels of miR-145-5p, miR-143-5p, and miR-154-5p miRNAs in CFBE41o- cells[3]. Next, we employed the Ago2-miRNA-co-IP assay to elucidate the functional effects of TGF-β1 on these miRNAs. The RISC recruitment of miR-145-5p, miR-143-5p, and miR-154-5p was studied in CFBE41o- cells stably expressing the wild type (WT)-cystic fibrosis transmembrane conductance regulator (CFTR) or mutant CFTR with the deletion of phenylalanine at 508 position (F508del)-CFTR[14,15]. The air-liquid interface cultures of WT- or F508del-CFBE41o- cells were treated with TGF-β1 or vehicle for 24 h. Cells were lysed and endogenous Ago2 was immunoprecipitated and detected by western blotting with the primary mouse monoclonal anti-Ago2 antibody at 1:3000 dilution followed by anti-mouse horseradish peroxidase secondary antibody. The quantification of Ago2 immunoprecipitation was performed by densitometry using ImageJ with exposures within the linear dynamic range of the film. The abundance of immunoprecipitated Ago2 was calculated after subtracting the background and normalizing to WCL Ago2. The co-immunoprecipitation of miR-145-5p, miR-143-5p, and miR-154-5p with Ago2 was detected by qRT-PCR using miRNA-specific stem-looped primers for cDNA preparation, followed by the PCR with the miRNA-specific forward and reverse primers, and TaqMan hydrolysis probes. The means between the groups were calculated by two-tailed student *t*-test. Data are presented as mean ± S.E.M. The Ago2 protein abundance and its immunoprecipitation efficiency were similar in TGF-β1 or vehicle treated cell lines (**Figure 1A–1C**). miR-145-5p and miR-143-5p were present in the complexes co-immunoprecipitated with Ago2 in the WT- and F508del-CFTR expressing cells (**Figure 1D-1E**). TGF-β1 increased co-immunoprecipitation of miR-145-5p and miR-143-5p with Ago2 in both cell lines, compared to

vehicle control. miR-154-5p co-immunoprecipitated with Ago2 but TGF-β1 did not affect the abundance of the active miR-154-5p pool, compared to vehicle control (**Figure 1F**). Taken together, the above results demonstrate that TGF-β1 can increase the total cellular miRNA level without affecting its functional pool. The Ago2-miRNA-co-IP assay increased our understanding of the distinct effects of TGF-β1 on miRNAs.

FIGURE AND TABLE LEGENDS:

Figure 1. Summary of the Ago2-miRNA-co-IP assay showing that TGF-β1 mediated selective recruitment of specific miRNAs to RISC in WT- and F508del-CFBE41o- cells. (**A**) Endogenous Ago2 was immunoprecipitation (IP) from whole cell lysates (WCL) of CFBE41o- cells expressing either WT- or F508del-CFTR with the anti-Ago2 antibody or non-immune IgG2 (a negative control) and detected by western blotting (WB). Shown are representative WB from WT-CFTR expressing cells. The non-specific band in IP samples is marked with an asterisk. Representative WB images (**B**) and summary of data (**C**) showing that TGF-β1 had no effect on the Ago2 abundance in WCL and did not change the efficiency of Ago2 IP. (**D**-**F**) qRT-PCR data showing the co-IP of miRNAs with endogenous Ago2. The Ct values of miR-145 (miR-145-5p) in vehicle-treated cells were subtracted from the Ct values of miR-145 in TGF-β1-treated cells to generate ΔCts. The fold change (FC) in miR-145 level between samples was determined using the equation $2^{-\Delta Ct}$ and expressed as Log2 FC *versus* vehicle. TGF-β1 increased the miR-145 (**D**) and miR-143 (miR-143-5p) (**E**) co-IP with Ago2 in WT- and F508del-CFBE41o- cells and did not affect the co-IP of miR-154 (miR-154-5p) (**F**). Error bars, S.E.M. N = 10/group. * $p < 0.05$. (This is a representative figure adapted from a previously published manuscript[3]).

DISCUSSION:

The Ago2-miR co-IP assay is designed to investigate the active pool of miRNAs in response to TGF-β1 treatment. The active or RISC-associated miRNAs are important to understand their inhibitory potential for the target mRNA[4]. Panshin et al. recently showed that the immunoprecipitation efficiency of Ago2 and miRNAs may depend on the protocol[16]. There are several differences between the protocol here and the above published data. The protocol here was optimized for CFBE41o- cells. By contrast, Panshin et al. studied Ago2 IP in human plasma or HEK293 cells. We immunoprecipitated Ago2 with mouse monoclonal, anti-human Ago2 antibody, subclass IgG2, conjugated with protein G agarose beads[17]. Panshin et al. used polyclonal anti-Ago2 antibody conjugated with protein A agarose beads, which can induce variability between immunoprecipitated miRNA pools[16]. The Protein A beads do not bind to all human IgG subclasses as efficiently as Protein G. We controlled for the IgG2 subclass of the anti-Ago2 antibody by using the non-specific IgG2 negative control. Using the same IP protocol, we examined the TGF-β1 effects on the RISC-associated miRNA pool.

We introduced several modifications to the RIP assay kit to optimize the immunoprecipitation and RNA isolation to study the TGF-β1 induced RISC recruitment of miRNAs. First, we used a larger volume of PBS for complete washing of protein G agarose bead. Second, we reduced the amount of anti-Ago2 antibody and the wash buffer by 50% to reduce the cost. Third, we performed all steps at unified cold temperature and added cold lysis buffer directly on the cells. Fourth, we have not isolated pre-immunoprecipitation RNA for miRNA quantification because we

already published the data on miRNA expression in whole cell lysate of CFBE41o- cells[3]. Other methods, such as the magnetic beads-based RNA binding protein immunoprecipitation can be used to examine the mRNA. However, the magnetic bead-based methods can not specifically target the component of RISC and thus may not be appropriate to evaluate active pool of miRNAs.

We would like to highlight several conditions critical to the success of the Ago2-miRNA-co-IP assay. The efficient conjugation of the anti-Ago2 antibody with the protein G agarose beads is the first critical step. Next, the efficient pull-down of Ago2 from WCL with the immobilized anti-Ago2 antibody is the next step. Lysed cells contain debris, which should be efficiently removed through centrifugation to prevent interference with the Ago2 pull-down. Care must be taken while collecting the supernatant containing cell lysate so that the pellet containing cellular debris would not dislodge and mix with the supernatant. The pre-clearing step prior to immunoprecipitation is also necessary to remove any non-specific binding of proteins to the empty beads (beads not conjugated with the antibody). The choice of pre-immunoprecipitation RNA isolation should be determined according to the experiment. The isolation of pre-immunoprecipitation RNA was omitted and here we examined the effect of TGF-β1 on the RISC recruitment of miRNAs. The co-immunoprecipitated miRNA FC after treatment with TGF-β1 *versus* vehicle control provides compelling evidence for the TGF-β1 recruitment of miRNAs to RISC. The working temperature has a crucial role and all steps during the immunoprecipitation of Ago2 must be performed on ice or at 4 °C to avoid degradation or denaturation of RISC associated proteins and RNA. All further steps involving elution of RNA from beads must be performed at similar temperature because the activity of RNase, an enzyme ubiquitous and responsible for rapid RNA degradation, is optimal at room temperature. The use of RNase free environment, including equipment, tips, tubes, pipettes, and bench top is highly recommended. Wearing the RNase free, powder free nitrile gloves, changed frequently, should be practiced during every step. It is recommended to immediately store RNA at -80 °C after elution. The contamination with agarose beads can cause problems during cDNA preparation and may affect the relative quantification of miRNA or mRNA during qRT-PCR. The cell culture requires special attention and should be monitored carefully. The tissue culture dishes should be coated with collagen to increase cell adherence and promote epithelial cell differentiation. We performed the Ago2-miRNA-co-IP assay in the CFBE41o- cell model.

The limitation of the protocol is that it is very sensitive to variation in the number of cells used. The cell number must be precisely controlled and the Ago2 immunoprecipitation has to be optimized according to the cell input to prevent saturation of the immunoprecipitated Ago2 with miRNAs. In the future, the assay could be applicable in several experimental settings, such as gene knockdown and to examine active pool of miRNAs in different disease models. The assay can be optimized for use with other epithelial and non-epithelial cell models, including primary cells or animal models.

ACKNOWLEDGMENTS:

We thank John Wakefield from Tranzyme, Inc. (Birmingham, AL) who generated the CFBE41o- cells, and J.P. Clancy from the CFFT who provided the cells. This research was funded by the National Institutes of Health grants R01HL144539 and R56HL127202 (to A.S.-U.), and the Cystic

Fibrosis Foundation grant SWIATE18G0.

DISCLOSURES:

The authors declare that, they have no competing financial interest, and they have nothing to disclose.

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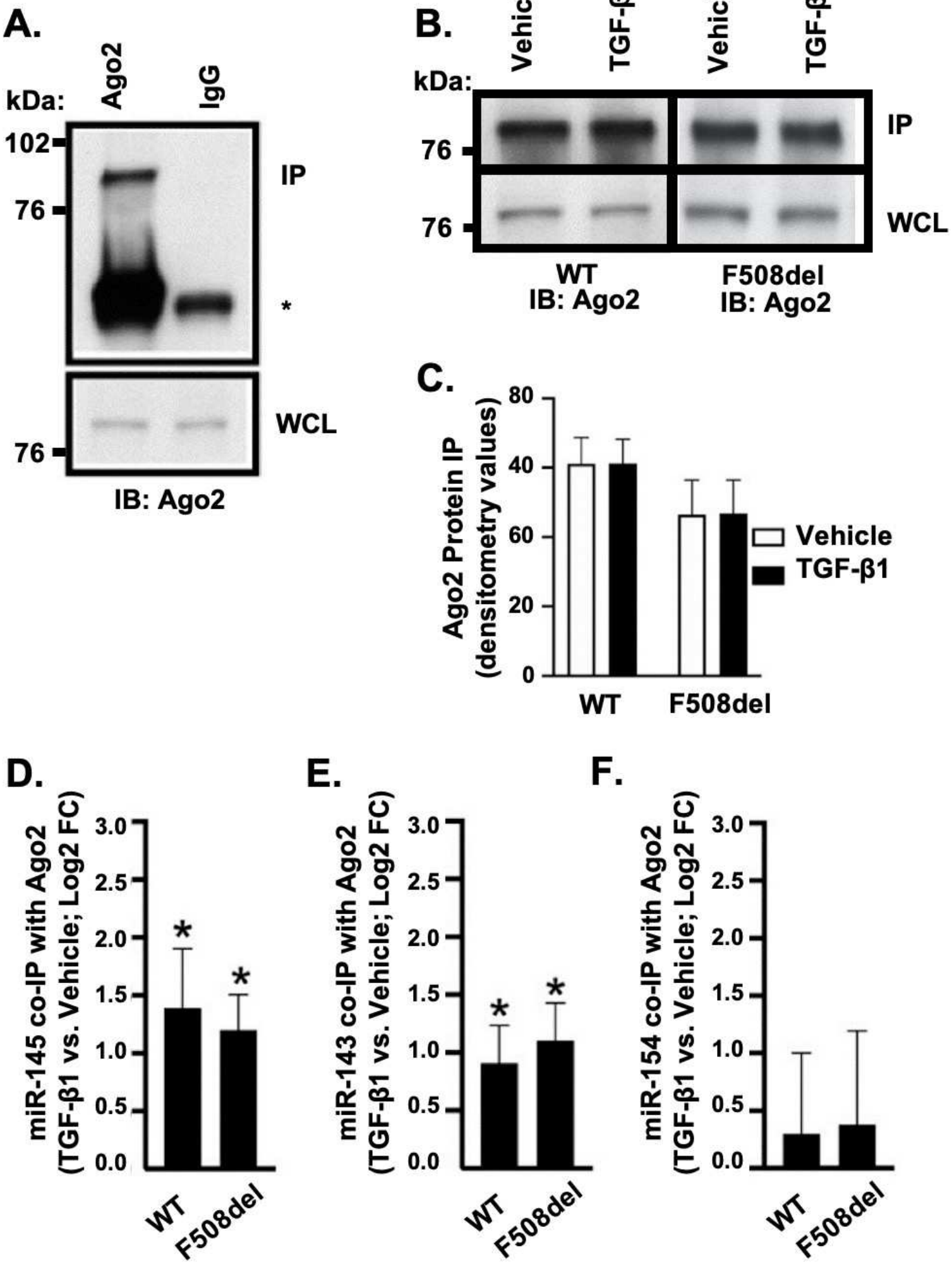

Name of Material/Equipment	Company
100 mM dNTPs (with dTTPs)	Applied Biosystems
10x Reverse Transcription Buffer (RT Buffer)	Applied Biosystems
2-propanol	Fisher BioReagents
2x Laemmli Sample Buffer	Bio-Rad
7300 Real Time PCR System	Applied Biosystems
Anti-Ago2 antibody (anti-EIF2C2), mouse monoclonal against human Ago2	Medical & Biological Laboratories Co. Ltd
Bovine Albumin Fraction V (7.5% solution)	Thermo Scientific
Collagen I (Purecol-Type I Bovie collagen solution)	Advanced Biometrix
DL-Dithiothreitol (DTT)	Sigma
DTT	Sigma-Aldrich
Ethanol	Deacon Laboratories
Fetal Bovine Serum	ATLANTA Biologicals
Goat Anti-Mouse IgG	Bio-Rad
L-glutamine (200 mM Solution; 29.20 mg/mL)	Corning
Mini cell scrapers United Biosystems	Thermo Fisher
Minimal Essential Medium	Thermo Fisher Scientific
miRNA specific stem looped RT primers	Applied Biosystems
Mouse IgG2 control	Dako, Glostrup, Denmark
MultiScribe Reverse Transcriptase, 50 U/µL	Applied Biosystems
Nano Drop ND-1000 Spectrophotometer	NanoDrop Technologies, Inc.
Nuclease-free water	Ambion
Opti-MEM (1x) Reduced Serum Medium	Gibco by Life Technologies
PBS	Gibco
Penicillin-streptomycin, Sterile	Sigma-Aldrich
Pierce Protease Inhibitor Tablets, EDTA-Free	Thermo Scientific
Protein G agarose beads (Pierce Protein G Plus Agarose)	Thermo Scientific
Puromycin	InvivoGen
RiboCluster Profiler RIP-Assay Kit for microRNA	Medical & Biological Laboratories Co. Ltd
RNase Inhibitor, 20 U/µL	Applied Biosystems
TaqMan 2x Universal PCR master mix without AmpErase UNG	Applied Biosystems

TaqMan miRNA single tube Assay (20x) containing miRNA specific forward/reverse primers and probe	Applied Biosystems
TGF-beta1	Sigma
Transwell filters (24 mm)	Corning Life Sciences Plastic
Veriti 96 Well Thermal Cycler (Model:9902)	Applied Biosystems

Catalog Number	Comments/Description
4366596	
4366596	
BP2618-1	
1610737	
4345240	
RN003M	
15260037	
50005-100mL	
646563-.5ML	
646563	
64175	
S10350	
1706516	
25-005-Cl	
MCS-200	
11095-080	
4427975	
A0424	
4366596	
E112352	
AM9937	
11058-021	
14190250	
P0781	
A32955	
22851	
ant-pr-1	
RN1005	
4366596	
4427975	

4427975 (assay ID #002278, #002146, and #000477)
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June 22, 2020

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE

Dear Ms. DSouza:

Enclosed please find our modified manuscript entitled: "***The Ago2-miRNA-co-IP assay to study TGF-β1 mediated recruitment of miRNA to the RISC in CFBE Cells***". We thank the reviewers for their insightful comments and suggestions. We made the modifications and provide answers below.

Editorial Comments Answer:

Comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors. **Answer:** The manuscript is proofread and checked for any spelling or grammatical error.

Comment: Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write the text on lines 79-87 to avoid this overlap. **Answer:** The text in line 79-87 is modified to avoid the overlap with previous published work.

Comment: Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples: 1) 1.1.3: mention culture environmental conditions; and 2) 1.4.1: mention centrifuge speed in g**. Answer:** The centrifuge speed, culture environment conditions, and other suggested details were added.

Comment: Protocol Numbering: There must be a one-line space between each protocol step. **Answer:** One-line space between each protocol step was added.

Comment: Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting. **Answer:** All relevant details required to perform the step are included in the highlights.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next. **Answer:** Highlighted steps are written in logical flow from one step to next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length. **Answer:** Complete sentences are highlighted. Total length of highlighted text is 2.75 pages including sub-headings and spaces.
4) Notes cannot be filmed and should be excluded from highlighting.

Comment: Results: mention statistical test performed. **Answer:** The statistical test performed was mentioned in the result section.

Comment: **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. **Answer:** We have modified discussion to cover the five areas.

Comment: **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are RiboCluster Profiler, MultiScribe™, Transwell, (Sigma, catalog # T1654, Dako, Thermo Fisher Scientific Cat# 4427975, TaqMan Universal Master Mix II (Applied Biosystems, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
Answer: All commercial language as well as registered trademark symbols (TM/R) have been removed from the manuscript. The registered trademark symbol is also removed from list of materials.

Comment: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]." **Answer:** We asked the publisher for permission to reuse the figure in the JoVe manuscript. As documented in the uploaded word file, we do not need a permission from an Open Access publisher. Per the publisher's request, the article, in which the figure was originally published has been referenced (Ref. #3).

Answer to the Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The authors describe a protocol for Ago2-miRNA IP in TGFb1 stimulated human bronchial epithelial cell line, CFBE. The authors utilize commercially available kits and lengthy procedures to isolate RNA without providing data on the efficacy of their method compared to others. Indeed, the results show a small enrichment of miRs bound to Ago2, arguing against the efficacy of this method. In addition, experiments are missing important controls that would help with the interpretation and biological meaning of results.

Major Concerns:

Comment: Ago2-miR co-IP is widely used and detailed methods have been published previously, including a study from the same authors. Therefore, the current protocol should demonstrate its efficacy over other procedures.
The authors need to demonstrate that their method is superior (at least for this type of cells) to other methods by comparing different IP procedures and RNA isolation methods. **Answer:** The protocol described in this manuscript is optimized to study the effects of TGF-β1 on recruitment of miRNAs to RNA induced silencing complex (RISC) in human bronchial epithelial cells CFBE41o- expressing wild type (WT)- or F508del-CFTR and the only variable is the use of TGF-β1 or vehicle control. The following control conditions have been used: (1) pre-clearing step in which protein G beads were incubated with the whole cells lysates to exclude any non-specific interactions between Ago2 and the beads; (2) non-specific IgG to control for immunoprecipitation (IP) of Argonaute-2 (Ago2; Fig. 1A); (3) comparing Ago2 IP efficiency in the presence of TGF-β1 and vehicle control; (Fig. 1B and 1C); and (4) examining IP efficiency in the WT- and F508del-CFTR expressing cells (Fig. 1B and 1C). To optimize the protocol for CFBE41o- cells, we introduced several modifications in the immunoprecipitation procedure and RNA isolation protocol compared to the original kit protocol. First, we have used larger volume of PBS for washing of protein G agarose bead conjugated with Ago2 antibody to decrease the non-specific binding. This modification was based on our expensive experience with immunoprecipitation protocols. Second, we reduced the amount of anti-Ago2 antibody and wash buffer use by 50% to make the process cost effective. Third, we performed cell lysis on ice and added ice-cold lysis buffer directly on the filters to prevent protein and RNA degradation that happens at room temperature to shorten the time of cell lysis. Fourth, we skipped pre-immunoprecipitation RNA for miRNA quantification because we already had expression data for

these miRNAs in whole cell lysate of CFBE41o- cells (Mitash N et al, 2019). The modifications are included in the revised Discussion.

Specific comments:
Comment: 1) 1.4 The authors used a specific kit and procedures. Would a different kit work similarly? For instance, other laboratories have successfully used the Magna RIP RNA binding protein IP kit (Millipore), which uses magnetic beads. Please discuss this subject. **Answer:** The Magna RIP RNA binding protein IP kit (Millipore), mentioned by the reviewer, is designed to study protein-RNA interactions, specifically mRNA and long noncoding RNA, whereas the RiboCluster Profiler RIP-Assay Kit we used is designed to examine the interactions between miRNAs and the essential component of RISC, Ago2. Detection of miRNA recruitment into RISC helps to elucidate the active pool of miRNAs. The overview of different assays has been added to the revised Discussion.

Comment: 2) Similar to the point above, authors should discuss if a different approach to isolate RNA would be equally effective. The protocol would benefit from additional experiments showing results obtained with the described method compared to other (faster) methods (i.e. Qiagen). **Answer:** The approaches to isolate RNA have to be optimized for specific cell types. We have used the separation method mentioned in RiboCluster Profiler RIP-Assay Kit in CFBE41o- cells. For comparison, we have used the Zymo kit to isolate RNA. The yield (<2ng/μl) and the quality (A260/A280<1.5) of isolation were inferior to the separation method for RNA isolation modified from RiboCluster Profiler RIP-Assay Kit and mentioned in this manuscript.

Comment: 3) While I agree that Ago2-IP can be more informative on the function of miRs compared to the analysis of overall miR expression, I disagree with the authors that looking at miR expression in the pre-IP RNA is unnecessary when stimulating cells. The authors statement can be misleading, since information derived from both types of analysis, miR expression and Ago2-IP, is equally incomplete without following-up with evaluation of miR targets. **Answer:** Stating that "the isolation of pre-immunoprecipitation RNA is not necessary" is just to indicate that we have already seen the expression of these miRNAs in whole cell lysate in response to TGF-β1 that's why we omitted the step and stated that it depends on experiment. We have modified the sentence to avoid any confusion.

Comment: 4) Graphs in the figure should show standard deviation, as SEM does not describe the variability between experiments. Is the small difference (what is the fold change?) in the miR bound to Ago2 between WT and mutant biologically relevant?
Fig1 should show Ago2 levels in both, WT and mutant, stimulated and unstimulated cells in one blot. MiR levels should be measured in the control IgG sample, since RNA may bind non-specifically to the beads, in stimulated and unstimulated cells, as well as in the whole cell RNA extracts. **Answer:** The recruitment of miRNAs to RISC in response to TGF-β1 treatment was compared to the vehicle control. It is unknown which miRNAs are recruited to RISC after TGF-β1 treatment. Thus, for the quantification of miRNAs co-IP with Ago2, we do not have the option to normalize the expression to the endogenous control and minimize the variability between samples, unlike the miRNAs in whole cell lysate. Thus, in our opinion, S.E.M. is justified. We have added Fig. 1B to demonstrate the IP of Ago2 in CFBE41o- cells expressing WT- or F508del-CFTR in the presence of TGF-β1 or vehicle control. We would like to emphasize that the protocol is not intended to compare active pool of miRNA between WT- and F508del-CFTR expressing CFBE41o- cells. Instead, we are examining the TGF-β1 effect on miRNA co-IP with Ago2 in WT and F508del-CTRR expressing CFBE41o- cells (please, see the above response to the commend above use of controls).

Minor Concerns:
Comment: 1) Step 1.1.1, please specify the volume of collagen solution needed to cover the surface**. Answer:** The volume of collagen was specified.

Comment: 2) 1.1.3 Specify the volume**. Answer:** Volume was specified.

Comment: 3) 1.1.4 Specify the meaning of "feed daily". Does it mean replace the medium with..? If yes, what is the volume of medium? **Answer:** We have added the frequency of feeding and volume of medium used for feeding the cells.

Comment: 4) Although the authors used 1x106 cells in their experiments, they should mention that other cell types could require more cells. **Answer:** Number of cells and culture condition will be different for different cell types. We have mentioned this in the Discussion.

Comment: 5) 1.2.4 "treat cells" by replacing the medium or adding to the medium? Please specify. **Answer:** The treatment should be done by mixing TGF-β1 and vehicle control in appropriate volume of before experiment media and replacing the existing media with treatment media from basolateral side of the cells.

Reviewer #2:
Manuscript Summary:
This manuscript by Dr. Mitash and colleagues describes a procedure to isolate miRNAs in the RISC associated functional pool from cell cultures. The protocol is an immunoprecipitation assay using an anti-Ago-2 antibody followed by qRT-PCR to identify specific miRNAs of interest. The context of this experimental protocol in the paper is to determine the effect of TGFbeta1 treatment on the functional miRNA population in WT and F508del CFBE cells. The title and the abstract are appropriate for this methods article, and the protocol appears largely complete and logical for the described outcome. Some parts of the protocol are unclear, however, and will require careful editing and modification.

Major Concerns:
Comment: 1. There are numerous (but individually minor) ambiguities, omissions, and errors in this protocol. Together, they may impede understanding of this technique. The authors should review the text of the protocol carefully for accuracy and correct as needed. I have noted several as indicated by step:

-Step 1.4.1: Speed of centrifugation not indicated. **Answer:** The speed of centrifuge was added.

Comment: -Step 2.2.4: References cells from step 2.1.6, but there is no step 2.1.6. Authors likely mean step 2.1.5. **Answer:** The reference step is corrected to 2.1.5.

Comment: -Step 2.2.6: May want to note how to store precleared lysate sample (ie on ice/4 degrees). **Answer:** The precleared lysate sample should be on ice. This is mentioned in the step 2.2.6.

Comment: -Step 2.3.1: References beads from step 1.5.4, which does not exist. Likely should be step 1.4.4. **Answer:** The reference step is corrected to 1.4.4.

Comment: -Step 2.3.4: When "mix slowly with help of pipette" is stated, does this mean physically mix with tip of pipette or gently pipette up and down? Clarification would help. **Answer:** "mix slowly with help of pipette" means gently pipette up and down. We have made changes and to clarify this step.

Comment: -Steps 3.4 and 3.5: Does the supernatant at this step contain large RNA as stated in 3.4 or total RNA as stated in 3.5? There is some ambiguity here. It is possible that the final sentence in step 3.4 would be clearer as "The supernatant at this step contains large RNA in addition to small RNA", if this is indeed a true statement. It appears separation of small and large RNA does not occur until step 3.8/3.9. **Answer:** The supernatant contains large RNA as well as small RNA in step 3.4. We have corrected it and made changes in step 3.4, 3.5, and 3.6 to make it clear. Up to step 3.6 the supernatant contains total RNA i.e. large RNA and small RNA. In step 3.8 the large RNA will be in pellet and small RNA will be in supernatant.

Comment: -Step 3.8: Is the tube containing total RNA from step 3.6? If so, would recommend stating this here. **Answer:** We stated in step 3.8 that the total RNA is from step 3.6.

Comment: -Step 3.12: The pellet with large RNA is not from step 2.4.8 as stated, but rather is likely from step 3.8. **Answer:** The large RNA is from step 3.8. We have corrected it.

Comment: -Steps 4.2.2 and 4.2.3: It would be useful to note the amount of cDNA and master mix to add to each individual well (ie, 1.5 uL and 18.5 uL respectively). **Answer:** Amount of cDNA and master mix is mentioned in steps 4.2.2 and 4.2.3 respectively.

Minor Concerns:
Comment: 1. I think an expanded discussion of other uses of this technique would be useful. For example, the authors could note that this procedure is applicable in a wide range of experimental contexts to investigate the influence of various treatments or conditions on functional miRNA pools. **Answer:** We have added the other uses of this technique to the Discussion.

Comment: 2. TGF is an abbreviation for "Transforming growth factor" not "transcription growth factor" **Answer:** The correction has been made.

Comment: 3. It may be useful to include the reference for CFBE cell line creation (Bebok et al J Physiol, 2005.) **Answer:** Reference was added (Ref. #12).

Comment: 4. Some ingredients in cell culture media (step 1.1.2) are not listed in supplies, e.g. antimicrobials. **Answer:** We have listed the ingredients.

Comment: 5. Mouse IgG as a negative control is mentioned in step 1.4.3, but is not further clarified. It may be helpful to include a brief description of relevant concentrations and use of Mouse IgG. **Answer:** We have used equal amount of mouse IgG2 as a negative control i.e. 7.5 µg/500µL. We mentioned this in step 1.4.3.

Reviewer #3:
Manuscript Summary:
The authors Mitash et al., have described a protocol for analyzing TGF-b-induced miRs through Ago2-miRNA-immunoprecipitation-based techniques.

Major Concerns
Comment: Authors used RiboCluster Profiler (RIP)-Assay Kit for their experiments, it's not clear what additional changes they made in the protocol to further enhancing th kit efficiency. What is new in their protocol? And different from the existing ones? **Answer:** We have added the description of all changes in the protocol in the revised Discussion. We introduced several modifications in immunoprecipitation and RNA isolation protocol and most importantly the assay is used as a tool to study TGF-β1 induced RISC recruitment of miRNAs. First, we have used larger volume of PBS for washing of protein G agarose bead conjugated with Ago2 antibody to decrease the non-specific binding. This modification was based on our expensive experience with immunoprecipitation protocols. Second, we reduced the amount of anti-Ago2 antibody and wash buffer use by 50% to make the process cost effective. Third, we performed cell lysis on ice and added ice-cold lysis buffer directly on the filters to prevent protein and RNA degradation that happens at room temperature to shorten the time of cell lysis. Fourth, we skipped pre-immunoprecipitation RNA for miRNA quantification because we already had expression data for these miRNAs in whole cell lysate of CFBE41o- cells (Mitash N et al, 2019). The modifications are included in the revised Discussion.

Comment: The figure they have shown in the current manuscript is same as they published before in 2019 (See Fig 9) of the link below
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6801718/ **Answer:** We have used the figure which we published in 2019 with small modification i.e. showing western blotting image of Ago2 in whole cell lysate and immunoprecipitation in both wild type and mutant cell line. We can use this with the permission of the previous publisher and current editor. Please see editorial comments.

Comment: Authors used Protein G agarose beads in their experiments, why not Protein A, or Protein A/G, which binds all of the subtypes to which Protein A and G individually bind. **Answer:** Protein G is an immunoglobulin (IgG) binding bacterial cell wall protein. The avidity for IgG binding was found greater for protein G than protein A (Akerstrom et al. 1985; PMID4031496). Protein A binds to all subclasses of human and mouse IgG as well as IgM and IgA of human whereas Protein G binds to only IgG subclasses of human and mouse which makes it more specific. Protein G is also for monoclonal antibody (we used monoclonal anti-Ago2). We reviewed this in revised Discussion.

Comment: The Authors focus is on microRNAs induced by TGF-b, there is no control miRNA analysis data in the IP samples i.e. not induced by TGF-b. **Answer:** We consistently used vehicle control to examine the effects of TGF-β1 on the miRNA co-IP with Ago2 (Figure 1). The values of vehicle control treatment were used to calculate the fold change after TGF-β1 treatment. We do not provide control miRNA analysis data because there are no known 'control' miRNAs recruited by TGF-β1 to co-IP with Ago2. As shown in Figure 1D-1F, TGF-β1 did not recruit miR-154. This data demonstrates that the effects of TGF-β1 on the recruitment of miR-145 and miR-143 were specific.

Minor concern:
Comment: Please make sure the representation of TGF-β1 is correct throughout the manuscript. For example, in the

abstract it is stated as Transcription Growth Factor-β1 (TGF-β1) similarly on Page 1 lane 29, 42. **Answer:** The corrections were made.

Reviewer #4:
Manuscript Summary:
In the present study the authors presented protocol for Ago2-miRNA-coIP assay in CFBE41o cells treated with TGFβ1. The coIP was performed modifying and adapting RIP assay kit protocol from manufacturer to isolate small and large RNA. Small RNA was used to quantify miRNA with qRT-PCR using miRNA-specific stem-looped primers in reverse transcription, followed by PCR using miRNA-specific forward and reverse primers, and TaqMan hydrolysis probes. Authors concluded that results obtained with Ago2-miRNA-coIP assay increased comprehension of the different effects of TGFβ1 on total cellular miRNAs level.
This is a well written report and the protocol is detailed and well described.

Major Concerns:
Comment: In "The efficiency of immunoprecipitation of microRNA/AGO2 complexes from human blood plasma is protocol dependent" Pashin et al, 2020 described the levels of Ago2 and miRNAs purification from plasma and from HEK293 cells using three different IP protocols. Pashin concluded that different protocols for immunoprecipitation of microRNA/Ago2 complexes favor different miRNAs.
Here, the authors discussed that the efficient conjugation of the anti-Ago2 antibody with the protein G agarose beads is the first critical step and the preclearing step is necessary to remove non-specific binding of proteins to empty beads. It could be useful discuss this point to exclude that the abundance of the active miR-154-5p pool after TGFβ1 treatment was not influenced by IP method, specifically used in this report, that efficiently worked with miR-145-5p and miR-143-5p. **Answer:** The approach of Panshin et al, 2020 is different from what we used in our protocol. First, the authors used Protein A beads that do not bind to all human IgG subclasses and second, they used polyclonal anti-Ago2, which can induce variability between immunoprecipitated miRNA pools. We have added this information to Discussion section to clarify that the abundance of the active miR-154-5p pool after TGFβ1 treatment was not influenced by IP method because the conditions were exactly the same except for the treatment with TGFβ1 or vehicle control. Our approach is more specific for immunoprecipitation of human Ago2 with monoclonal anti-Ago2 antibody (subclass IgG2) conjugated with protein G agarose beads.

Sincerely,

Nilay Mitash