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Gene Manipulation with Micro RNAs at Single-Human Cancer Cell

Andres Stucky, Xuelian Chen, and Jiang F. Zhong

Abstract

Micro RNAs (miRNAs) are small RNAs processed from longer precursor RNA transcripts that can fold back on themselves to form Watson-Crick paired hairpin structures. Once processed from the longer molecule, the small RNA is much too short to code for proteins but can play other very important roles, like gene regulation. The phenomenon of RNA interference was initially observed by Napoli and Jorgensen in transgenic petunia flowers, where gene suppression was observed after introducing a transgene of chalcone synthase (CHS) belonging to the flavonoid biosynthesis pathway. miRNAs were first discovered for their roles in development but it has quickly become evident that they have causal roles in cancer as well. miRNA can also be used to manipulate genes for the investigation of carcinogenesis. Single-cell transcriptome profiling studies in our laboratory suggest that carcinogenesis often is the result of the malfunction of multiple members of a molecular pathway. Here, we describe a protocol to manipulate multiple cancer-related genes in a single human cell to investigate how multiple genes interact during carcinogenesis.

Keywords

Gene manipulation; Cancer; Single-cell

1 Introduction

In 2006, the Nobel Prize in Medicine was jointly awarded to Craig Mello and Andrew Fire for the discovery of RNA interference or gene silencing by double-stranded RNA in *Caenorhabditis elegans*. Since then, a variety of miRNA families have been discovered in almost all metazoan organisms and have been well studied in a variety of model organisms including *C. elegans* and *Arabidopsis thaliana*.

The phenomenon of RNA interference was initially observed by Napoli and Jorgensen in transgenic petunia flowers, where gene suppression was observed after introducing a chalcone synthase (CHS) transgene belonging to the flavonoid biosynthesis pathway [1]. This resulted in white petunia petals, instead of the expected dark blue coloration. Later in 1992, a similar phenomenon known as “quelling” was observed in *Neurospora crassa* while attempting to enhance orange pigmentation by the all gene of this fungus [2].

Micro RNAs (miRNAs) are small RNAs processed from longer precursor RNA transcripts that can fold back on themselves to form Watson-Crick paired hairpin structures. Once processed from the longer molecule, the small RNAs are too short to code for proteins but they play other important roles, like gene regulation.

Much like other genes, miRNAs can be highly conserved among various species with very similar orthologs in humans, flies, and worms. There can also be several miRNA homologs within a single species that can produce highly similar, if not identical copies, of the same miRNA. In humans, there are 277 miRNA-coding genes constituting 153 families of miRNAs conserved in mammalian species. Of the 153 families conserved in mammals, 87 of them are shared with zebrafish and up to 33 are shared with flies and worms. Scientists rapidly recognized the potential uses of miRNAs in the treatment of disease and the silencing of any gene of interest upon demand. Moreover, the discovery of RNA interference added another layer of complexity to our understanding of gene regulatory networks.

RNA interference is a process inside eukaryotic and prokaryotic cells by which mRNA sequences are selected and targeted for destruction so that no protein is produced from the message. Interfering RNAs can come in three different flavors: small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), and micro RNAs (miRNAs). All three types perform similar gene silencing functions, but they vary with respect to the way they are produced, the extent of gene silencing they accomplish, and the process through which they achieve it.

In eukaryotes, the three key proteins required for the manufacture of miRNAs are Argonaut (AGO) and two endonucleases, Drosha and Dicer. The first step in the process is the initial transcription of a larger 100–120 nt primary RNA (pri-miRNA) transcript by Pol2 polymerase. While still in the nucleus, pri-miRNAs are bound by a Drosha/Pasha (DGCR8) binding complex that recognizes a specific motif in the hairpin molecule, which is then cleaved about one helical turn from the base of the hairpin. This releases a 70 nt pre-miRNA hairpin which exits the cell nucleus with the aid of the exportin 5 complex [3]. Once in the cytoplasm, the miRNA encounters Dicer, which then cleaves off the miRNA loop, leaving only a 15–21 nt pre-miRNA duplex with a 2 nt overhang at the 3' region and a 5' monophosphate. Following cleavage, the miRNA:miRNA*duplex strand is loaded onto the RNA binding pocket in the Argonaut (AGO) protein, the major component of the RNA-Interfering Silencing Complex (RISC). It is not yet clear which strand of the miRNA duplex is loaded, but there are indications that it is the strand with the least stable pairing at its 5' end which has a higher binding probability. The passenger strand is then released and the active miRNA complex migrates to the targeted mRNA and forms a nuclear hybrid structure with the target structure. Once nucleated, Argonaut recognizes the dsRNA and cleaves the mRNA, leaving the bound miRNA intact.

In humans, the Argonaut family is composed of four distinct proteins, human Argonaut 1–4 (hAgo1–hAgo4), all of which share similar architectural domains. The Mid, N, and PIWI domains form a base upon which the PAZ domain rests. This structure is critical for the binding and slicing of interfering RNAs. Consistent with the biochemistry of the slicer function, the PIWI domain of Argonaut has been shown to resemble a RNase H enzyme. A binding site in the Mid domain anchors the phosphate backbone of the guide strand at the 5' end [4], whereas the 3' end attaches to the PAZ domain [5]. A highly conserved DEDH catalytic tetramer in the PIWI active site is the critical slicing component in Argonaut [6, 7]. The remaining components needed for targeted mRNA cleavage are the RNA guide strand and a Mg^{2+} molecule [8].

In most cases, miRNA-mRNA pairing is not fully complementary; thus, the key target site of miRNA is a set of 6 nt, called the seed of the miRNA, that extends from nucleotides 2 through 7 at the 5' end. This sequence can be often complemented by a pair in nucleotide 8 or an A in the position 1 complementary site, so either or both of these complementary sites would form a seed of 8 pairing nucleotides which are most frequently conserved in the untranslated regions (UTRs) [9]. Interestingly, pairing at the 3' end in the center of the miRNA appears to play very little role in target recognition or binding, though occasionally some pairing at the 3' end will supplement the seed pairing, even compensating at times for a certain teeter due to mismatch in the seed region, but these events are very rare and only account for about 1–5% of functional miRNA [10]. The length of the actual seed pairing site ensures that a single miRNA can have numerous mRNA targets and indicates that mammalian mRNAs are under powerful selective pressure to preserve their pairing to miRNA in their UTRs. It has even been observed that on average there are a roughly 400 conserved targets per miRNA family and up to 4 or 5 conserved miRNA binding sites per conserved target. Therefore, a single target can be monitored by several different miRNAs, depending on the cellular context, and often multiple miRNAs are working together to orchestrate the magnitude and timing of gene silencing [9]. A single miRNA can have multiple targets. Conversely, a single miRNA might not be sufficient to silence a single target, so genetic engineering kits for gene silencing provide a set of up to three miRNAs to ensure strong silencing and target specificity. For these reasons, miRNAs are ideal tools to manipulate multiple genes in a molecular pathway, such as those responsible for carcinogenesis.

Here, we describe a protocol for gene manipulation with miRNA. These techniques hold potential for the development of miRNA cancer therapy. For instance, loss of the miR-34 tumor suppressor leads to faulty regulation of its downstream mytotic (MET, WNT, MYC) [11–13] and anti-apoptotic (BCL-2, SIRT-1 and Survivin) targets and cell cycle regulators (CyclinD, CDK6, and CDK4) [14]. Pathway inhibition can be restored by miR-34 supplementation, or with the addition of a CDK4-targeting siRNA, thereby repressing the associated pathway [15]. Although miRNA therapeutics have been found to suppress gene expression effectively compared to anti-sense oligonucleotides, they are still limited by their high biological instability and off-target effects, and the difficulty of delivering them to the desired cell system. To a certain extent, some of these hurdles have been overcome by using chemically synthesized molecules with special modifications such as cholesterol conjugates, morpholinos, cationic lipids, and nanoparticles [16]. Current progress in developing miRNA-based anticancer therapies has been most promising in the modulation of cancer cell miRNA by various chemoprophylactic agents.

2 Materials and Methods

All the solutions should be prepared beforehand, with buffers using ultrapure RNase-free water and molecular-grade reagents. Follow proper sterile and safe laboratory techniques, as well as all chemical and biological waste disposal regulations.

2.1 Selection of siRNA Sequences

1. Select a DNA sequence, preferably more than 50–100 nt downstream of the start codon within the open reading frame of your cDNA of choice. 5′ or 3′ untranslated regions (UTRs) as UTR-binding proteins could restrict RISC binding to the target RNA [17].
2. Search for a sequence 19–21 nt between a 5′ AA and a 3′ UU (5′-AA(N19-21)UU), in an mRNA sequence that shows an approximate 50% G/C content. High-G-content sequences should be avoided as they are likely to form G-quartet structures. Synthesize the sense siRNA as 5′-(N19)TT, and the sequence of the antisense siRNA as 5′-(N′19)TT, where N′19 denotes the reverse complement sequence of N19. N19 and N′19 indicate ribonucleotides; T indicates 2′-deoxythymidine.
3. Perform a basic local alignment search (BLAST) of the selected siRNA sequences against expression sequence tags (EST) mRNA libraries for your organism of interest to confirm that only a single gene is targeted. Preferred sites for BLAST are NCBI (www.ncbi.nlm.nih.gov/BLAST) and Ensembl (www.ensembl.org).
4. Synthesis of several siRNA duplexes might be required to control for knock-down target specificity; effective siRNA duplexes should produce the same phenotype. A scrambled nonspecific siRNA duplex should be designed as a negative control. Most RNAi kits will provide this control duplex. The use of an unrelated target gene that is absent from the selected model can be used as a positive RNAi control. If the siRNA does not work, make sure that the mRNA sequence used for the selection of the siRNA duplexes is reliable; it could contain sequencing errors, mutations, or polymorphisms.
5. Most predesigned DNA oligos purchased from a vendor will come already annealed. Otherwise, dsDNA should be incubated for 1 min at 90 °C, followed by 1 h at 37 °C in annealing buffer (4 mM magnesium acetate, 200 mM potassium acetate, 60 mM Hepes–KOH, pH 7.4). To prepare 100 µL of a 20 µM siRNA duplex solution, combine 50 µL 2× annealing buffer, sense siRNA to a 20 µM final concentration, antisense oligonucleotide to a 20 µM final concentration, and sterile RNase-free water.
6. Store the RNA suspension solution at –20 °C. The solution may be frozen and thawed several times without requiring any further heat-shock treatments. During use, it is recommended that the RNA solutions be thawed over ice and kept on ice as much as possible to minimize the hydrolysis of RNA.
7. The MW of the siRNA may be assessed by loading 0.5 µL 20 µM of sense, antisense, and duplex siRNA onto 4% agarose gels for loading of the samples. First, dilute the samples with a few microliters of 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM Na₂ EDTA) and sucrose loading buffer. Run the gel in 0.5× TBE buffer at 80 V for 1 h. The RNA bands can be visualized under UV light after ethidium bromide staining. Preferably, the ethidium bromide

should be added to the 4% gel/0.5× TBE solution at a concentration of 0.04% mg/L prior to casting of the gel.

8. Culture the cell line of interest in a humidified incubator with 5% CO₂ and 95% humidity at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (in the case of embryonic or progenitor stem cells, use the cell type-specific medium). Passage cells when they reach 90% confluency to maintain exponential growth. For a general reference for cell culture, please see Freshney 2016 [18]. The number of passages could affect DNA and siRNA transfection efficiencies as well as the experimental outcome. As a rule, do not exceed 30 passages for transformed cells or the 3rd or 4th passage in the case of embryonic or progenitor stem cells. Freeze and store low-passage number cells in liquid nitrogen, so they can be thawed and used when convenient.
9. To prepare for the experiment, passage cells 24 h before siRNA transfection. Trypsinize 90% confluent cells with enough trypsin–EDTA to cover the bottom of the culture plate for 5 min or until the cells have detached completely. Dilute the cell suspension 1:5 with fresh DMEM medium, transfer to a 15 or 50 ml tube and spin at 600 × *g* to pellet the cells. Wash and spin the cells twice with PBS, then add enough medium to transfer 500 µL aliquots of 5 × 10³ cells/cm² into each well of a 24-well plate.
10. If an immunofluorescent reporter is used to visualize transfection efficiency [19], cells should be plated on round coverslips and placed at the bottom of the 24-well plates prior to addition of cell suspension. Transfection should be performed according to cell confluence indicated by the manufacturer of the transfection reagent that will be used.
11. Transfection of duplex RNAs into tissue culture cells can usually be achieved using cationic lipids. Testing a variety of lipid agents with each cell type might be necessary to identify the optimal reagent to introduce the RNA duplex into the cells with the highest efficiency and least toxicity. A fluorescently labeled transfection control duplex may be added to the transfection mix (10 nM) to visually assess the transfection efficiency. The cells should show both fluorescent puncta and cytoplasmic diffusion while still appearing healthy. In cases where the cells are resistant to cationic lipid transfection, introduction of the siRNA can be achieved by electroporation.
12. On the day of transfection, replace old medium with 0.5 mL of fresh DMEM. To prepare the transfection mixture, add 2 µL Lipofectamine (or preferred transfection reagent, according to the manufacturer's recommendation) to 50 µL serum-free medium. Mix by gently inverting the tube and incubate the suspension for 5 min at room temperature without movement.
13. In a separate tube, add 2 nM of your siRNA duplex and 10 nM fluorescent probe to 50 µL serum-free medium and vortex, then spin down briefly on a tabletop centrifuge. Allow incubating at room temperature for 5 min.

14. Combine the transfection mixture with the siRNA suspension, gently mix, and allow the formation of liposome complexes by incubating for 20–25 min at room temperature. Be careful not to exceed a 30 min incubation time.
15. Add the 100 μ L liposome complexes to the well containing 0.5 mL of DMEM, mix gently for 30 s, and incubate the plate for 20–48 h at 37 °C. Cytoplasmic fluorescent diffusion should be visible 48 h following the transfection.

2.2 Fluorescent Visualization

1. Fix and permeabilize the cells by removing the medium and incubating the cells in a 10% formalin solution for 30 min. Wash the formalin-fixed cells with phosphate-buffered saline (PBS: 137 mM NaCl, 7 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH 7.1) remove excess PBS. If no further staining is required, mount cover slips on microscope slides using a mounting solution and seal the edges of the coverslip with a small amount of clear nail polish.
2. If further staining is required, place the coverslips in a covered chamber prepared by soaking filter paper in water and placing on the bottom of the chamber. It is important that the prepared cells do not dry out during the staining process. Spread 1 mL of primary antibody appropriately diluted in PBS and 10% FBS evenly over the entire surface of the coverslip and incubate at room temperature for 60 min. Wash the cells with PBS 3 $\times$ and add an appropriately diluted fluorescent-tagged secondary antibody. Incubate for 30 min, wash three times and coverslip as before. Visualize the cells under a fluorescent microscope (Fig. 1).

2.3 Detection of Protein by Western Blotting

1. Remove the tissue culture medium from the siRNA-treated cells. Rinse the cells twice with 200 μ L PBS and add 200 μ L trypsin–EDTA. Incubate for 5 min at room temperature and resuspend the cells in 800 μ L DMEM medium to quench trypsin.
2. Transfer the cells to a 1.5 mL tube. Collect the cells by centrifugation at 3000 rpm ($600 \times g$) for 4 min at 4 °C to pellet the cells. Resuspend the pellet in ice-cold PBS and centrifuge again. Remove the supernatant and add 25 μ L of 2 $\times$ concentrated Laemmli buffer with 10% concentration of sodium dodecyl sulfate (SDS) to the cell pellet obtained from one well of a 24-well plate. Incubate the sample for 10 min in a boiling water bath and vortex.
3. Load the samples onto minigels with an appropriate acrylamide concentration to resolve the corresponding molecular weight of the targeted protein [20]. Perform SDS–PAGE electrophoresis using a constant 20 mA current. Transfer proteins from the acrylamide minigels onto nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine, 0.01% SDS, 20% methanol), with a constant 333 mA current for 2 h in a cold room. Incubate the membrane in blocking solution (5% milk powder in TBST (0.2% Tween 20, 20 mM Tris–HCl, 150 mM NaCl, pH 7.4)) for 1 h at 37 °C.

4. Add the appropriate dilution of primary antibody and incubate for 2 h at room temperature or at 4 °C overnight. Wash the blot three times with TBST for 10 min. Incubate the blot with horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:2000. Proceed to ECL detection according to the vendor's instructions. Details of the Western blotting procedure were published previously [20].

3 Note

Unlike messenger RNAs, the precursor RNAs for interfering RNAs are double-strand. Primary or precursor miRNAs can be produced in several different ways. In the case of miRNA, a single miRNA stem loop transcript is produced from a gene. For shRNA, three individual hairpins are produced from the same transcript; each hairpin is a 20–25 nt sequence that fold backs onto itself to form a short hairpin structure. The hairpin is exported from the nucleus and Dicer cleaves both RNA strands to form two complementary sequences. Finally, in the case of siRNA, a large double-stranded mRNA transcript is synthesized from the same locus after Dicer processing. This system can produce a large amount of smaller RNA duplexes called small interfering RNAs (siRNAs). Other differences between miRNA and siRNA include the fact that miRNAs are single-stranded and come from endogenous noncoding RNA found within the introns of long RNA molecules, whereas siRNAs are usually found as exogenous double-stranded RNA. These siRNAs then guide the siRNA-AGO complex to silence the same loci from which they were produced, ensuring a very efficient feedback loop to silence transgenes, transposons, and viruses. This stands in contrast to miRNAs, which can inhibit many different mRNAs because their pairing is imperfect. siRNAs typically show perfect pairing with their mRNA target. Given the major roles that siRNA and miRNA play in the control of gene expression, it is not surprising they have both been extensively studied for therapeutic application in RNA-induced transcriptional silencing (RITS) [21].

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Fig. 1.

Fluorescent visualization of TYE563-tagged RNA inside cells. RNA tagged with red fluorescent dye TYE563 is inside the majority of cells