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Application of MicroRNA in Cardiac and Skeletal Muscle Disease Gene Therapy

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Abstract

MicroRNAs (miRNAs) are a class of small ~22 nt noncoding RNAs. miRNAs regulate gene expression at the posttranscriptional levels by destabilization and degradation of the target mRNA or by translational repression. Numerous studies have demonstrated that miRNAs are essential for normal mammalian development and organ function. Deleterious changes in miRNA expression play an important role in human diseases. We and others have previously reported several muscle-specific miRNAs, including miR-1/206, miR-133, and miR-208. These muscle-specific miRNAs are essential for normal myoblast differentiation and proliferation, and they have also been implicated in various cardiac and skeletal muscular diseases. miRNA-based gene therapies hold great potential for the treatment of cardiac and skeletal muscle disease(s). Herein, we introduce the methods commonly applied to study the biological role of miRNAs, as well as the techniques utilized to manipulate miRNA expression.

Keywords

miRNA; Muscle; Cardiac; Gene expression; Posttranscriptional regulation; Gene therapy

1. Introduction

Cardiac and skeletal muscle disorders are a group of diseases caused by different mechanisms, including defective structural proteins, disorganized sarcomeres, and perturbed regulation of growth/maturation signaling pathways (¹). In cardiac muscles, these diseases can be classified into congenital heart defects such as hypoplastic left/right heart syndrome and cardiac septum defects, as well as cardiomyopathies such as dilated cardiomyopathy and ischemic cardiomyopathy. In skeletal muscle, these diseases can be classified as neuromuscular such as multiple sclerosis or musculoskeletal such as Duchenne muscular dystrophy and myotubular myopathy. In the adult heart, pathological cardiac hypertrophy is the end-term consequence of stresses such as hypertension, ischemia, and neurohormone disorders (²) and is often accompanied by myocardial alterations such as fibrosis and necrosis. As a result, pathological cardiac hypertrophy diminishes cardiac output and pumping efficiency, which in many cases eventually leads to heart failure. Despite the fact that cardiovascular disease is the leading cause of death worldwide, the underlying molecular mechanisms for these and other muscle-related diseases are not completely understood.

Recently, a large class of ~22 nt noncoding RNAs has been discovered and is collectively referred to as microRNAs (miRNAs). To date, thousands of miRNA genes have been identified in multiple organisms from nematodes and plants, to fish and mammals ⁽³⁾. Similar to protein encoding genes, expression of a miRNA begins with the transcription of the miRNA gene by RNA polymerase II. Though many miRNAs are under the control of their own promoter, some miRNA genes are found in clusters sharing a single promoter ⁽⁴⁾, while others are encoded within an intron and coexpressed with their host gene ⁽⁵⁾. After transcription, the primary miRNA transcript is processed in the nucleus by the microprocessor complex (Drosha/DGCR8) into a hairpin intermediate commonly referred to as a pre-miRNA. However, a small subgroup of miRNAs found within short introns is known to bypass this step ⁽⁶⁾. Pre-miRNAs are then exported from the nucleus by exportin-5 ⁽⁷⁾, where they are further processed into miRNA duplexes by the cytosolic RNase III enzyme Dicer ⁽⁸⁾. Finally, the functional strand of the miRNA duplex is loaded into the RNA-induced silencing complex (RISC) to facilitate target mRNA degradation and/or translational repression ⁽⁹⁾.

Evolutionarily conserved miRNAs have been identified in multiple eukaryotes from *Caenorhabditis elegans*, to *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*. The *C. elegans* genome contains a single mammalian miR-1 ortholog ⁽¹⁰⁾, whereas in higher eukaryotes there are multiple genes encoding miR-1 (identical coding sequences of *miR-1-1* and *miR-1-2*). miR-1 differs from miR-206 by only four nucleotides ⁽¹¹⁾. Several mammalian miRNAs, including the miR-1/206 and miR-133 families, and miR-208a/b, are specifically expressed in cardiac and skeletal muscle ^(12–14). miR-1 is known to regulate the *C. elegans* neuromuscular junction ⁽¹⁵⁾ as well as skeletal muscle differentiation and proliferation in C2C12 myoblasts (*M. musculus*) ⁽¹³⁾. In addition, miR-1 expression is dependent upon serum response factor in both the *D. melanogaster* ⁽¹⁶⁾ and *M. musculus* ⁽¹²⁾ model systems. Together, these data strongly suggest that both the function and regulation of miR-1 are conserved throughout eukaryotic evolution.

miRNAs play important roles in cell proliferation, differentiation, migration, and apoptosis during both normal development and disease progression ^(17, 18). miR-1 and miR-133 are clustered on the same chromosomal locus and transcribed together as a single transcript. However, they represent two distinct miRNAs, each with its own biological function ⁽¹³⁾. miR-1 overexpression has been shown to negatively regulate skeletal muscle differentiation in the *Xenopus laevis* model system ⁽¹³⁾. miR-1 also significantly impairs normal cardiac development in both the *X. laevis* and *M. musculus* model systems ^(12, 13). Further, miR-1 is overexpressed in individuals with coronary artery disease and miR-1 overexpression induces arrhythmogenesis through negative regulation of *Kcnj2* and *Gja1* in mouse model ⁽¹⁹⁾. Cardiac-specific miR-208a is known to potentiate stress-dependent cardiac hypertrophy and remodeling by downregulating the expression of thyroid hormone receptor-associated protein 1 ⁽¹⁴⁾. In addition, the expression of many miRNAs is altered under pathological conditions. Subsets of miRNAs are found to be both positively and negatively regulated in clinical human samples and animal models of cardiac and skeletal myopathies ^(20–23). In the *M. musculus* model system, overexpression of miR-195 in cardiomyocytes in vivo is sufficient to drive dilated cardiomyopathy and heart failure ⁽²¹⁾. In addition, dystrophin-deficient mice are found to have significantly decreased expression of miR-133a

and miR-206⁽²⁴⁾. Together, these results indicate that the proper expression of miRNAs is necessary for both normal development and function of skeletal and cardiac muscles.

As proper miRNA expression is critical for normal development and homeostasis, it is necessary to better understand the biological function of miRNAs. Strategies commonly used to investigate the function of a particular miRNA are the gain-of-function and loss-of-function studies. Gain-of-function studies are commonly performed in vitro, where cells can be transiently transfected with an expression construct encoding the pre-miRNA. Alternatively, synthetic miRNA duplexes and virus-based miRNA expression systems may also be employed. In vitro loss-of-function studies can be accomplished with either 2'-*O*-methyl miRNA antisense oligonucleotides or locked nucleic acid (LNA)-miRNA antisense oligonucleotides which will block the function of an endogenous miRNA. The in vivo determination of a miRNA's function is best examined utilizing conventional transgenic and gene knockout strategies. Recently, a lentivirus targeting strategy that overexpresses short RNA fragments containing multiple miRNA target sequences has been shown to phenocopy a genetic miRNA knockout mouse⁽²⁵⁾. In addition, intravenous delivery of cholesterol-modified miRNA antisense oligonucleotides (antagomiRs) can inhibit miRNA function in vivo⁽²⁶⁾. These molecular approaches are invaluable in elucidating the biological function of miRNAs and may potentially lend themselves to future gene-based therapies.

In this chapter, we are going to discuss how to determine the expression of miRNAs by Northern blotting analysis. Then, we will discuss the method of studying the regulation of miRNAs on their targets in vitro by luciferase reporter assays. Finally, we will talk about how to manipulate the function level of muscle miRNAs in C2C12 myoblast cell line and determine their biological function on muscle differentiation.

2. Materials

2.1. Detecting the Expression of Muscle miRNAs by Northern Blotting Analysis

1. Hoefer SE 400 vertical slab gel electrophoresis unit (Amersham Biosciences, Piscataway, NJ, USA).
2. Hoefer TE77 Semidry transfer unit (Amersham Biosciences).
3. UV stratalinker 1800 (Stratagene, Cedar Creek, TX, USA).
4. Trizol reagent (Invitrogen, Carlsbad, CA, USA).
5. 40% acrylamide: AccuGel 29:1 (National Diagnostics, Atlanta, GA, USA).
6. 10× TBE buffer: 0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA (pH 8.0), autoclave for 20 min.
7. Urea (molecular biology grade, Sigma-Aldrich, St. Louis, MO, USA).
8. 10% (w/v) Ammonium persulfate solution (APS). Aliquot and store at −20°C.
9. *N,N,N,N*-Tetramethyl-ethylenediamine (TEMED, Fisher Bioreagent, Fair Lawn, NJ, USA).
10. Formamide (Fisher Bioreagent).

11. Bromophenol blue solution: 10% (w/v) bromophenol blue.
12. Filter paper, sheet, grade 3, 460 × 570 mm (Whatman, Clifton, NJ, USA).
13. Zeta-Probe GT genomic-tested blotting membranes (Bio-Rad, Hercules, CA, USA).
14. T4 polynucleotide kinase (Promega, Madison, WI, USA).
15. Mini Quick Spin Oligo Columns (Roche, Indianapolis, IN, USA).
16. Adenosine 5'-triphosphate (γ - ^{32}P), 3,000 Ci/mmol (PerkinElmer, Waltham, MA, USA).
17. Anti-miRNA probe: the synthetic antisense oligonucleotide of the target miRNA.
18. Diethylpyrocarbonate (DEPC)-treated water: 1 mL DEPC in 1 L double distilled H_2O . Stir at room temperature for 1 h and autoclave.
19. 1 M phosphate buffer: 71 g of anhydrous Na_2HPO_4 , 4 mL of 85% H_3PO_4 . Add DEPC-treated water to 1 L.
20. Hybridization buffer: 0.5 M phosphate buffer; 1 mM EDTA at pH 8.0; 7% (w/v) of sodium dodecyl sulfate (SDS); 1% (w/v) of bovine serum albumen (BSA); in DEPC-treated water.
21. 20× SSC: 3 M sodium chloride and 300 mM Trisodium citrate dihydrate, pH 7.0.
22. Wash buffer: 1× SSC supplemented with 0.1% SDS.
23. Stripping buffer: 0.1× SSC supplemented with 0.1% SDS.
24. Storage phosphor screen (Amersham Biosciences).

2.2. Studying the Regulation of Muscle miRNAs on Their Targets by Luciferase Reporter Assays

1. Mouse genomic DNA.
2. Primers for reporter construction: HDAC4-UTR-F, 5'-ATCGGAGCTCCAGCACTGGTGATAGACTTGG-3'; HDAC4-UTR-R, 5'-GTCTTATTGAACTTATTCTTAAGCTCGAGATCG-3'; HDAC4-Mut-F, 5'-GTTTCTTTCCTCAGATTTTAAATCTTCACTGGTCACAGCCACG-3'; HDAC4-Mut-R, 5'-GTGACCAGTGAAGAATTTTAAATCTGAGGAAAGAAACACAACC-3'.
3. PfuTurbo DNA Polymerase (Stratagene).
4. pGL3cM vector (modified by Chen JF and Wang DZ). The backbone is the pGL3-Control vector (Promega).
5. Sac I restriction endonuclease (New England Biolabs, Ipswich, MA, USA).
6. Xho I restriction endonuclease (New England Biolabs).
7. T4 DNA ligase (New England Biolabs).

8. pShuttle-CMV-lacZ Vector (Stratagene).
9. NucleoBond plasmid Maxi kit (Macherey-Nagel, Bethlehem, PA, USA).
10. HEK293T cells (ATCC, Manassas, VA, USA).
11. CELLSTAR 12- and 24-well tissue culture plate (Greiner Bio-One, Monroe, NC, USA).
12. Cell culture medium: DMEM, high glucose with L-glutamine (Gibco-BRL, Langley, OK, USA).
13. Fetal bovine serum (FBS) (Hyclone, Logan, UT, USA).
14. Penicillin G-Streptomycin (PS): Penicillin 100 U/mL DMEM culture medium and Streptomycin 100 µg/mL DMEM culture medium (Gibco-BRL).
15. 1× Trypsin-EDTA: 0.25% Trypsin, 1 mM EDTA/4Na (Gibco-BRL).
16. Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA).
17. Opti-MEM I reduced-serum medium (Gibco-BRL, Langley).
18. miR-1 miRIDIAN miRNA mimic (Dharmacon, Lafayette, CO, USA).
19. 10× Phosphate-buffered saline (PBS) solution: 80.6 mM sodium phosphate, 19.4 mM potassium phosphate, 27 mM KCl and 1.37 M NaCl at pH 7.4.
20. Luciferase assay system (Promega).
21. LacZ buffer: 0.06 M of Na₂HPO₄, 0.045 M of NaH₂PO₄, 0.01 M of KCl, 2 mM of MgSO₄.
22. LacZ substrate: to 5 mL of LacZ buffer add 1 mL of o-Nitrophenyl-β-galactopyranoside (ONPG) stock solution (4 mg/mL), and 13 µL of β-Mercaptoethanol.
23. 1 M Na₂CO₃.

2.3. Overexpression and Knockdown of Muscle miRNAs In Vitro

1. Primers for miR-22 overexpression vector construction: miR22-F 5'-TAGCAGGTACCTTATTCAAGAACCCCTCATTAG-3', miR22-R 5'-GTATCTCTAGATTCCCTCCCATAAAGCCAT-3'.
2. pcDNA3.1(+) vector (Invitrogen).
3. Anti-miR-22 probe: antisense oligonucleotide to miR-22.
4. C2C12 cells (ATCC).
5. Kpn I restriction endonuclease (New England Biolabs).
6. Xba I restriction endonuclease (New England Biolabs).
7. 2'-O-methyl miR-133 antisense oligonucleotide (Dharmacon).
8. Growth medium for C2C12: DMEM medium with 10% FBS and 1% PS.

9. Differentiation medium for C2C12: DMEM medium with 2% horse serum and 1% PS.
10. Nonidet P-40 (NP-40) (Fisher Bioreagent).
11. 4% Paraformaldehyde (PFA) solution: 4% (w/v) PFA in 1× PBS.
12. Antibody buffer: 0.1% NP40 and 3% BSA in 1× PBS.
13. Anti-phospho-histone H3 antibody (Upstate, Lake Placid, NY, USA).
14. Alexa-488 or Alexa-495 conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA).
15. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes).

3. Methods

3.1. Detecting the Expression of Muscle miRNAs by Northern Blotting Analysis

1. Prepare total RNA from tissue or cultured cells with Trizol reagent according to manufacturer's protocol (see Note 1).
2. Prepare 15% denaturing gel for electrophoresis separation of miRNAs. Carefully wash, dry, and assemble the Hoefer SE 400 vertical slab gel electrophoresis unit. Prepare denaturing gel containing 18.75 mL of 40% acrylamide, 2.5 mL of 10× TBE buffer, 12.5 mL of DEPC-treated water, and 20 g of urea. Mixture may need to be gently heated in 37°C water bath in order for urea to completely dissolve. To polymerize, add 400 µL of 10% APS; 40 µL of TEMED, mix well, and quickly pour. Allow the gel polymerize for 1 h.
3. Prerun denaturing gel for 30 min at 200 V. Use 0.5× TBE for running buffer.
4. Prepare RNA samples for electrophoresis. Mix the RNA sample (40 µg) 1:1 (v/v) with formamide, and incubate at 65°C for 10 min. Chill RNA on ice for 3 min and add 2 µL of bromophenol blue solution. Mix well.
5. Load the sample(s) into the well(s) and run the gel at 250 V (see Note 2). Use 0.5× TBE for running buffer. Voltage can be stopped when the loading dye reaches the bottom of the plate.
6. Transfer the RNA from the gel to the membrane with Hoefer TE77 Semidry transfer unit. Soak the membrane and six pieces of filter papers in 0.5× TBE. Set up the transfer in the order from top (–) to bottom (+) as: three pieces of filter paper, gel, membrane, three pieces of filter paper (see Note 3). Transfer with constant current (0.8 mA/cm² of gel area) for 1 h.

¹RNase(s) rapidly degrade RNA and are abundant in the environment. When extracting total RNA from samples, RNase-free tubes, DEPC-treated water, and solutions made with DEPC-treated water are highly recommended. RNA samples can be preserved in pellet for more than 1 year if stored in 100% ethanol at –80°C.

²Prior to loading the RNA sample into the denaturing gel, wash the well by flushing with 0.5× TBE running buffer. Excess urea in the well will prevent the RNA sample from sinking to the bottom of the well.

³Exclude air bubbles when assembling the “sandwich” for RNA transfer.

7. After transfer, wash the membrane with 0.5× TBE and perform UV crosslink using the auto crosslink option.
8. Mix 5 µL of Adenosine 5'-triphosphate [γ - 32 P], 5 µL of 1 µM anti-miRNA probe, 2 µL of 10× PNK buffer, 1 µL of T4 polynucleotide kinase, and 7 µL of double distilled water and incubate at 37°C for 1 h.
9. Purify the [γ - 32 P]-labeled probe using a mini Quick Spin Oligo Column according to manufacturer's protocol (see Note 4).
10. Prehybridize the membrane for 1 h at 37°C with 5–10 mL of hybridization buffer.
11. Add the labeled anti-miRNA probe into the hybridization buffer and incubate overnight at 37°C.
12. Remove the hybridization buffer and wash the membrane 3 times with wash buffer (10 min per wash).
13. Expose the membrane to the storage phosphor screen for 4–24 h. The length of exposure depends upon strength of signal and will vary with different miRNA probes (see Note 5).
14. Scan the screen with Typhoon phosphor-imager.
15. If you want to probe the membrane with a different miRNA probe or if you want to store the membrane for long term, add the membrane to heated stripping buffer (>95°C). Incubate for approximately 10 min while rocking.
16. After stripping, rinse membrane with fresh stripping buffer, and then allow to dry. The membrane may be reprobbed immediately following the steps outlined above. The membrane can also be stored at –20°C for future use.

3.2. Studying the Regulation of Muscle miRNAs on Their Targets by Luciferase Reporter Assays

Here we show an example using the luciferase reporter vectors which contain either the wild-type or the mutant HDAC4 3' UTR.

1. Generate the ~400 bp HDAC4 gene 3' UTR DNA fragment containing the seed sequence for miR-1 by PCR reaction using mouse genomic DNA as the template and the HDAC4-UTR-F and HDAC4-UTR-R primers. The Sac I and Xho I sites are introduced at the 5' and 3'-end, respectively, by the PCR primers. The UTR PCR products are cloned into the Sac I/Xho I sites of the pGL3cM vector (see Note 6). The resulting Luc-WT-UTR reporter contains the wild-type 3' UTR of the HDAC4 gene.

⁴It is important to have enough protection when conducting the isotope-related experiments. Always wear personal protective equipment when handling radioisotopes.

⁵Besides the phosphor-imager system, Northern blot can also be imaged with X-ray autoradiography. In general, the membrane needs to be exposed to film for 1 day to 1 week.

⁶To generate the pGL3cM vector, the multiple cloning sites (MCS) are removed from pGL3-control vector by Kpn I + Bgl II and filled in by Klenow. The 53 bp oligonucleotides containing the MCS are then introduced into the Xba I site.

2. Generate the Luc-Mut-UTR reporter using the same method described in step 1 except for the primers. Here, the HDAC4-Mut-F and HDAC4-Mut-R primers are used in the PCR reaction (Fig 1).
3. Prepare high-quality Luc-WT-UTR reporter, Luc-Mut-UTR reporter, and pShuttle-CMV-lacZ plasmid for reporter assays with NucleoBond Plasmid Maxi Kit. These plasmids will be used for HEK293T cell transfection.
4. At one day before transfection, plate HEK293T cells in a 24-well plate at 5×10^4 cells per well in 500 μ L of growth medium (see Note 7). This will yield 50–80% confluence at the day of transfection.
5. To generate the transfection complex for one well, add 25 ng reporter (either Luc-WT-UTR or Luc-Mut-UTR), 25 ng pShuttle-CMV-lacZ plasmid, and 0.5 μ L of 10 μ M miRIDIAN miRNA mimic to 100 μ L of Opti-MEM I reduced-serum medium and mix gently. To this mixture, add 0.5 μ L of PLUS reagent, mix gently, and incubate for 5–10 min at room temperature. Finally, add 1.25 μ L of Lipofectamine LTX Reagent, mix gently, and incubate for 30 min at room temperature.
6. Add the transfection complex (~100 μ L) to the well. Mix gently by rocking the plate back and forth.
7. At 24 h after transfection, remove cell culture medium and wash cells twice with $1 \times$ PBS.
8. Lyse cells with 100 μ L of cell lysis buffer and perform one freeze–thaw cycle (see Note 8).
9. Mix 40 μ L of cell lysate with 40 μ L of luciferase substrate and measure the signal with a scintillation counter (see Note 9).
10. Mix 20 μ L of cell lysate with 150 μ L of the LacZ substrate and incubate at 37°C with gentle rocking until the mixture turns yellow. Add 50 μ L of 1 M Na_2CO_3 to stop the reaction. The signal is measured by scintillation counter.
11. Normalize luciferase activity to LacZ activity.

3.3. Overexpression or Knockdown of Muscle miRNAs In Vitro

In this section, we describe steps to overexpress miR-22 in HEK293T cells and to knockdown miR-133 in C2C12 myoblasts.

1. For the overexpression study, use PCR to generate a ~350 bp DNA fragment containing the intact hairpin for the miR-22 precursor plus the flanking sequences on both ends (see Note 10). Use mouse genomic DNA as the template. The Kpn I and Xba I sites are introduced at the 5' and 3'-end, respectively, by the PCR

⁷At least 12 wells are needed for one experiment to examine four combinations of transfection reagents including Luc-WT-UTR reporter and miR-1 miRIDIAN mimic, Luc-WT-UTR reporter and control miRIDIAN mimic, Luc-Mut-UTR reporter and miR-1 miRIDIAN mimic, and Luc-Mut-UTR reporter and control miRIDIAN mimic. Each combination of transfection reagents is performed in triplicate.

⁸Cells are frozen at -80°C for 1 h and then thawed at room temperature by rocking for 30 min. Once thawed, incubate the lysate on ice to prevent proteolysis.

⁹The luciferase substrate should be preserved in -80°C and protected from light.

primers. Clone the PCR product into the Kpn I/Xba I sites in the pcDNA3.1(+) vector (see Note 11). The resulting construct is termed the miR-22 overexpression vector (Fig. 2a).

2. Prepare high-quality plasmid for transfection with NucleoBond Plasmid Maxi Kit.
3. Transfect the miR-22 overexpression vector into HEK293T cells following the steps outlined in Subheading 3.2.
4. Extract total RNA from cells 48 h after transfection using the Trizol reagent according to manufacturer's protocol.
5. Evaluate the overexpression of miR-22 by Northern blot according to the protocol described in Subheading 3.1. Similarly, the miR-22 overexpression vector can also be evaluated in other cells such as C2C12 myoblasts (Fig. 2b).
6. For miR-133 knockdown study, plate C2C12 myoblasts in a 12-well plate at 6×10^4 cells per well in 1 mL of growth medium one day before transfection. This will yield 50–80% confluence at the day of transfection.
7. Transfect C2C12 myoblasts with 200 nM 2'-O-methyl miR-133 antisense oligonucleotides (see Note 12). Use the same protocol described in Subheading 3.2, except to double all volumes. The final volume should be 200 μ L per well.
8. Change growth medium 4–6 h after transfection and continue to culture the cells for additional 24 h.
9. 24 h after transfection, replace growth medium with differentiation medium and culture the cells for additional 12 h.
10. Confirm miR-133 knockdown by Northern blotting analysis according to the protocol described in Subheading 3.1.
11. Examine cell proliferation in C2C12 cells treated with 2'-O-methyl miR-133 antisense oligonucleotides. Wash the cells twice with $1 \times$ PBS, and then fix with 4% PFA for 5 min at room temperature. Wash the cells 3 times with $1 \times$ PBS containing 0.1% NP-40 (10 min each wash). Dilute the primary antibody, anti-phospho-histone H3, to 1:100 with the antibody buffer. Phospho-histone H3 signal indicates mitotic cells. Incubate overnight at 4°C. Wash the cells 3 times with $1 \times$ PBS containing 0.1% NP-40 (10 min each wash). Dilute the secondary antibody, Alexa Fluor 488 or Alexa Fluor 495-conjugated goat anti-rabbit IgG antibody, to 1:1,000 with the antibody buffer. Incubate at room temperature for 1 h. Wash the cells 3 times with $1 \times$ PBS containing 0.1% NP-40 (10 min each wash). Rinse the cells with $1 \times$ PBS. Dilute DAPI to 1:50,000 dilution in $1 \times$ PBS. Incubate at room temperature for 10

¹⁰Different cloning strategies can be applied to generate a miRNA overexpression vector. In this protocol, our strategy is to clone the fragment containing the whole hairpin (miRNA precursor) and a 100–150 bp flanking sequence on both the 5' and 3' ends of the miRNA precursor sequence. Alternatively, the full-length noncoding transcript can be cloned into the expression vector. However, this is only applicable for miRNAs generated from a nonprotein coding gene.

¹¹Besides pcDNA3.1(+), other expression vectors can be used to construct a miRNA overexpression plasmid. Virus-based expression vectors have already been reported for miRNA overexpression (²⁷).

¹²In this protocol, a 2'-O-methyl miRNA antisense oligonucleotide is used to knockdown the endogenous miRNA. Alternatively, LNA antisense oligonucleotides can be used to obtain similar effects.

min. Rinse with 1× PBS 3 times. Observe immunofluorescence signal using an inverted fluorescence microscope (see Note 13) (Fig. 3).

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¹³The immunofluorescence signal can be preserved in 1× PBS at 4°C for up to 1 month.

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Fig. 1.

The strategy for the construction of the Luc-Mut-UTR reporter. The primer pair (P_{mF}/P_{mR}) for mutation introduction contains complementary mutated nucleotides (see Subheading 2.2, sequences in bold) for miR-1 seed sequence mutant. Besides the mutated sequence, these primers contain 20–25 nt 3' end sequence and 10–15 nt 5' end sequence. PCR with P_F/P_{mR} and P_{mF}/P_R produces two DNA fragments with 30–40 bp overhang, containing the miR-1 seed sequence mutant. The Mut-UTR template is generated by one cycle of RCR reaction.

Fig. 2. (a) The strategy for the construction of miR-22 overexpression vector. *P*, miRNA hairpin precursor; *F*, flanking sequences. (b) Evaluate the overexpression of miR-22 by Northern blotting analysis. Lane 1, cells transfected with the miR-22 overexpression vector; Lane 2, cells transfected with a control vector. U6 snRNA serves as loading control.

Fig. 3. Detecting phospho-histone H3 by immunostaining after miR-133 knockdown using 2'-O-methyl miR-133 antisense oligonucleotide in C2C12 cells. 2'-O-methyl GFP antisense oligonucleotide serves as control. DAPI counterstained nuclei.