Methods for Analyzing MicroRNA Expression and Function During Hematopoietic Lineage Differentiation

Hyeyoung Min and Chang-Zheng Chen

Summary

MicroRNAs (miRNAs), an abundant class of approx 22-nucleotide (nt) small RNAs that control gene expression at the posttranscriptional level, may play important roles during normal hematopoiesis and leukemogenesis. This chapter focuses on the methods and strategies for dissecting miRNA function during hematopoietic lineage differentiation. We describe a modified miRNA cloning method and expression analysis approach for determining miRNA expression during hematopoietic lineage differentiation. We illustrate a retroviral vector and a general strategy for the ectopic expression of miRNAs in hematopoietic stem/progenitor cells. We discuss in vitro and in vivo functional assays that can be used to examine the roles of miRNAs during hematopoietic lineage differentiation. The methods and principles described here should also be applicable to study the roles of miRNAs in the differentiation and function of nonhematopoietic cell types.

Key Words: MicroRNA; noncoding RNA; hematopoiesis; retroviral expression; bone marrow transplantation; hematopoietic lineage differentiation.

1. Introduction

The discovery of the *lin-4* small noncoding RNA (ncRNA) revealed the posttranscriptional genetic programs that are controlled by small ncRNAs and the importance of such genetic programs in controlling the timing of worm development (1,2). Now, nearly a decade later with the identification of a large number of miRNAs—an abundant class of approx 22-nt *lin-4*-like endogenous small ncRNAs—from animals and plants, it is apparent that miRNA-mediated gene regulatory programs may represent a fundamental layer of genetic regulation that is conserved throughout the animal and plant kingdoms (3–5).

Hundreds of miRNAs have been identified through experimental and computational approaches and many of them are expressed in high copy numbers in cells, suggesting that miRNAs are an abundant class of gene regulatory molecules in animal and plant genomes (6–20). More importantly, some miRNAs have been shown to play important
functional roles in plants and animals. In plants, some miRNAs control leaf and flower development (21–24). In Caenorhabditis elegans, the lin-4 and let-7 miRNAs control the timing of larval development (1,2,25,26), and the lsy-6 and mir-273 miRNAs act sequentially to control neuronal asymmetry (27,28). In Drosophila, the Bantam miRNA regulates fly growth by controlling cell proliferation and cell death (29,30), and the mir-14 miRNA represses apoptosis and affects fat metabolism (31). In mammals, some miRNAs have been shown to regulate hematopoietic lineage differentiation, insulin secretion, and adipocyte differentiation (32–34). Furthermore, as elegantly exemplified by the genetic studies of lin-4 and lin-14 interactions, animal miRNAs are likely to control gene expression at the posttranscriptional level through imperfect base pairing to the complementary site(s) in the 3' untranslated region of their target gene(s) (1,2). It has been predicted that each miRNA can potentially regulate large numbers of target genes (35–43). Therefore, miRNAs seem to have diverse functional roles and regulate a broad spectrum of protein-coding genes. This suggests that miRNA-mediated gene regulation represents a fundamental mode of posttranscriptional gene regulation that is potentially involved in all biological processes. Nevertheless, the biological functions and relevant target genes of the majority of known miRNAs remain elusive.

In an earlier study, we illustrated a systematic approach to identify and examine miRNA functions during hematopoietic lineage differentiation processes (32). The approach we used can be summarized in three basic steps:

1. The identification of candidate miRNAs.
2. The generation of miRNA expression constructs.
3. The characterization of miRNA function during hematopoietic lineage differentiation (Fig. 1).

In this chapter, we describe detailed protocols for identifying and testing the function(s) of miRNAs in mouse hematopoiesis. We discuss methods for analyzing the expression of miRNAs in mouse hematopoietic tissues or lineage-specific hematopoietic cells. We describe a strategy to generate viral constructs expressing miRNAs and also explain the use of in vitro and in vivo assay systems to assess the roles of miRNAs during hematopoietic stem/progenitor cell differentiation. The methods and principles we describe here can be applied to study the roles of miRNAs in the differentiation and function of nonhematopoietic cell types.

2. Materials

2.1. Common Reagents for Total RNA Preparation

- Rnase-free plastic bottles, Eppendorf tubes, and pipet tips.
- Trizol reagent (Invitrogen, Carlsbad, CA; cat. no. 15596-018).
- Chloroform.
- RNase free water.
- 100% Isopropyl alcohol.
- 100% Ethanol.
- 75% Ethanol (see Note 1).

2.2. Radiolabeling of Oligonucleotide Probes and Markers

1. 6000 Ci/mmol [γ-32P] adenosine triphosphate (ATP) (NEN Life Sciences, Boston, MA; cat. no. BLU502Z).
2. 3000 Ci/mmole [γ-33P] ATP (NEN Life Sciences; cat. no. NEG602H).
4. 10X kinase reaction buffer (New England Biolabs).
5. G-25 MicroSpin columns (Amersham Biosciences; cat. no. 27-5325 01).
6. 100 ng/µL Decade marker RNA (Ambion, Austin, TX; cat. no. 7778).
7. 10X cleavage reagent (supplied in the Decade marker system kit).

2.3. miRNA Cloning (see Note 2)
1. Carrier oligonucleotides: 5’-UGUCAGUUUGUUAAUUAACCAA-3’.
2. Restriction enzymes from New England Biolabs: PacI (cat. no. R0547S), BanI (R0118S).

2.4. Northern Blot
1. Vertical slab gel electrophoresis apparatus, glass plates, gel spacers, binder clips, combs, and aluminum plates. Aluminum plates are used to evenly distribute the heat generated during electrophoresis and eliminate the “smile” effects.
2. Acrylamide gel system (National Diagnostics Sequagel or equivalent): 10X gel buffer (8.3 M urea in 1 M Tris-borate and 20 mM ethylenediaminetetraacetic acid [EDTA]; 10X TBE buffer, pH 8.3; National Diagnostics cat. no. EC-835), gel concentrate (19% acrylamide, 1% bis acrylamide, and 8 M urea; National Diagnostics, Atlanta, GA; cat. no. EC-830), gel diluent (8 M urea; National Diagnostics cat. no. EC-840).
3. 5X TBE buffer: dissolve 54 g of Tris-base and 27.5 g of boric acid in H₂O. Add 20 mL of 0.5 M EDTA, pH 8.0. Adjust final volume to 1 L.
4. 10% ammonium persulfate; store at −20°C.
5. N,N,N′,N′-tetramethylethylene diamine (National Diagnostics; cat. no. EC-503).
6. 4 µg/mL ethidium bromide (EtBr) in 0.5X TBE.
7. 2X gel-loading buffer: 8 M urea, 20 mM EDTA, pH 8.0, 1 mg/mL xylene cyanol FF, and 1 mg/mL bromophenol blue. To make 1 L of 2X gel-loading buffer, mix 480 g of urea, 40 mL of 0.5 M EDTA, 2 mL of 1 M Tris-HCl, 1 g of xylene cyanol FF, and 1 g of bromophenol blue.
8. Nylon membrane (GeneScreen Plus, NEN Life Science; cat. no. NEF1017).
9. 20% sodium dodecyl sulfate (SDS).
10. 20X standard sodium citrate (SSC): 3 M NaCl and 0.3 M sodium citrate, pH 7.0. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL H2O, adjust pH to 7.0 with 1 M HCl, and adjust the volume to 1 L with H2O.
11. 1 M phosphate buffer (pH 7.2): dissolve 268.07 g of NaPO4 in 800 mL H2O, add 4 mL of 85% phosphoric acid, and adjust the volume to 1 L.
12. Prehybridization/hybridization (prehyb/hyb) solution: 5X SSC, 20 mM Na2HPO4, pH 7.2, 7% (w/v) SDS, and 2X Denhardt solution in H2O.
13. Nonstringent wash solution: 3X SSC, 25 mM Na2PO4, pH 7.5, 5% SDS, and 10X Denhardt solution in H2O.
14. Stringent wash solution: 1X SSC and 1% SDS in H2O.
15. Phosphoimager screen.

2.5. Preparation of Hematopoietic Cells for RNA Isolation and Immunocytochemistry
1. Bone marrow washing medium: Dulbecco’s Modified Eagle Medium (DMEM), 2% fetal bovine serum (FBS), and 10 mM HEPES, pH 7.2.
2. 1X phosphate-buffered saline (PBS): dissolve 8 g NaCl, 0.2g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 in 800 mL of H2O. Adjust pH to 7.2 and add H2O to a final volume of 1 L.
3. Turk’s staining solution: 0.01% (w/v) crystal violet in 3% (v/v) acetic acids (see Note 3).

2.6. Reagents for Magnetic Activated Cell Sorting and Fluorescence Activated Cell Sorting (see Note 4)
1. Magnetic-activated cell sorting (MACS) labeling buffer: PBS, pH 7.2, and 2 mM EDTA.
2. MACS separation buffer: PBS, 2 mM EDTA, and 0.5% bovine serum albumin.
3. Antibodies for MACS: purified anti-mouse CD16/32 antibody, biotinylated antibodies against lineage-specific surface antigens: anti-CD3e, anti-Mac1, anti-Gr-1, anti-Ter-119, and anti-B220 (lineage panel; BD Biosciences Pharmingen, San Diego, CA; cat. no. 559971).
4. MACS streptavidin microbeads (Miltenyi Biotec, Auburn, CA; cat. no. 30-048-101), anti-Sca-1 microbeads (Miltenyi Biotec; cat. no. 120-001-503), or microbead-conjugated antibodies against lineage-specific antigens.
5. Fluorescence-activated cell sorting (FACS) buffer: PBS with 2% FBS.
7. 1 mg/mL propidium iodide (PI) stock.

2.7. Generation of miRNA Expression Constructs
1. Lysis buffer: 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl. Sterilize the solution by filtering through a 0.45-µm nitrocellulose filter. Store the sterile
solution at room temperature. Adjust the proteinase K concentration to 0.2 mg/mL before use.
2. Proteinase K: 20 mg/mL stock solution; 0.2 mg/mL final concentration.
3. Isopropanol.
4. Nuclease-free water.
5. Sequencing primer for MDH1–3-phosphoglycerate kinase promoter (PGK)–green fluorescent protein (GFP) 2.0: 5’-GGATCCCAATATTTGATGATGTCGC-3’.

2.8. Generate Retrovirus by Transient Transfection
1. Culture medium for 293T and BOSC 23 cells: DMEM, 10% FBS, and penicillin/streptomycin (Pen/Strep).
2. FuGene 6 (Roche Diagnostics; cat. no. 1 815 091).
3. pCLeco packaging vector (44).

2.9. Hematopoietic Lineage Differentiation Assays
1. 30 mg/mL 5-fluorouracil in saline.
2. S17 culture medium: Minimum Essential Medium Eagle, α-Modification (α-MEM), 20% FBS, and Pen/Strep.
3. B cell growth medium: α-MEM supplemented with 20% FBS, Pen/Strep, 2 mM L-glutamine, 10 ng/mL interleukin (IL)-3, 10 ng/mL IL-6, 10 ng/mL IL-7, and 50 ng/mL stem cell factor (SCF).
4. 0.05% Trypsin–EDTA.
5. 4 mg/mL polybren (hexadimethrine bromide).
6. 0.5 M EDTA, pH 8.0.
7. PBS in 5 mM EDTA.
8. C57BL/6J (CD45.2 or Ly5.2) mice (Jackson Laboratory, Bar Harbor, ME).
9. B6.SJL-CD45a-Pep3b mice (CD45.1 or Ly5.1) (Jackson Laboratory).
10. Hematopoietic stem cell infection medium: α-MEM supplemented with 20% FBS, Pen/Strep, 2 mM L-glutamine, 10 ng/mL IL-3, 10 ng/mL IL-6, 10 ng/mL IL-7, and 100 ng/mL SCF.
11. Heparinized capillary tube.
12. 2 U/mL heparin.
13. Ammonium chloride potassium (ACK) red blood cell lysis buffer (10X): to 500 mL of H2O, add 40.15 g NH4Cl, 5.0 g KHCO3, and 185.1 mg Na2–EDTA. Adjust the pH to 7.2 to 7.4. Make 1X buffer by diluting with H2O before use.

3. Methods
3.1. Identification of Candidate miRNAs for Functional Analyses
The spatial and temporal patterns of miRNA expression provide clues regarding their biological functions. Many approaches have been developed to examine miRNA expression, including miRNA cloning (6–8), Northern blot analysis (1), miRNA microarray analysis (45–51), the invader assay (52), and the ribonuclease protection assay. Although multiple miRNA array platforms have been reported, these platforms all have certain limitations. Many miRNA array platforms use probes or amplification methods that simultaneously measure the mature and precursor (pre)-miRNA forms rather than the functional mature miRNAs only. Furthermore, because miRNAs are short and have
a wide-range of melting temperatures, it is intrinsically difficult to design antisense probes that have similar hybridization kinetics that also ensure the sensitivity and specificity of the arrays. Lastly, these arrays may be incomplete because additional miRNA genes in human and mouse genomes are yet to be identified (53). Thus, to design a miRNA array with probe sets relevant to the cells of interests, it may first require systematic cloning to identify the miRNAs expressed in these cell populations. Because of limited space, we only provide detailed protocols for a modified cloning method and Northern Blot analysis in this chapter.

3.1.1. Cloning of miRNAs From Hematopoietic Progenitor Cells

Direct cloning of miRNAs from tissues or enriched cell populations offers a sensitive approach to identify new miRNAs. One can also use the cloning frequency, the number of times a miRNA is identified from a miRNA library, as a reliable indicator for the relative abundance of the cloned miRNA (54). Because most miRNA cloning experiments were carried out using cell lines and tissues, miRNAs expressed in rare progenitor cell populations are likely to be underrepresented or missed in previous studies. Therefore, the cloning approach may offer an alternative method for identifying new miRNAs and determining miRNA expression in purified hematopoietic progenitor cell populations. The drawback, however, is that the cloning method is relatively tedious and costly.

The original cloning methods from the Ambros, Bartel, and Tuschl laboratories all require a large amount of starting material (~100 µg of total RNA) (6–8). To increase the sensitivity of the cloning procedure, we made a slight modification to the original method (6–8) and successfully cloned miRNAs from as few as 100,000 purified progenitor cells (see Note 5). In this chapter, we describe only the modifications we made to the protocol. A detailed description of the original cloning protocol can be found in an early publication by Nelson et al. (7) and on the Bartel lab website (http://web.wi.mit.edu/bartel/pub).

In brief, we extract the total RNA from sorted hematopoietic stem/progenitor cell populations (>100,000 cells) using the Trizol reagent and following the manufacturer’s instructions (Invitrogen). To isolate the small RNAs, of 18- to 26-nt in length, from the purified cell populations, we spike 1 pmol of a 32P-labeled 23-mer carrier oligo (5’-UGUCAGUUUGUAUAAACCCAA-3’) into the total RNA samples and carry out RNA size fractionation on a 15% acrylamide/8 M urea gel. The radiolabeled oligo serves as a carrier and tracer during the subsequent precipitation, ligation, and gel purification steps. The carrier also contains a PacI restriction site that allows for the subsequent removal of the ligation product containing the carrier in later cloning stages. Following the original protocol, we ligate size-fractionated small RNAs first to a 3’ adaptor oligonucleotide (5’ preadenylated) without the presence of ATP, then to a 5’ adaptor oligonucleotide, and carry out reverse transcriptase polymerase chain reaction (PCR) to amplify the final ligation products. After reverse transcriptase PCR amplification, we digest the PCR product with PacI to eliminate the carrier sequences, then purify the undigested products on a 15% non-denaturing acrylamide gel, and amplify the PacI-digested PCR products with 10 to 20 rounds of PCR. Typically, one round of PacI diges-
tion can remove more than 90% of the carrier-containing amplification products. One can carry out another round of digestion to further reduce the carrier-containing PCR products, but no gel purification is necessary. We then digest the PCR products with the BanI restriction enzyme, concatermerize the BanI-digested products, ligate them into TOPO vectors (Invitrogen), and submit the miRNA libraries for sequencing analyses. Bioinformatics analyses will then be carried out to identify new miRNAs and to determine miRNA abundance (6–8).

3.1.2. Northern Blot to Analyze miRNA Expression

Northern blotting is a routine lab method for analyzing miRNA expression. It reveals information regarding miRNA size and the relative abundance of both the mature and pre-miRNAs. However, it has limited sensitivity (at least 5 µg of total RNA is required for detection) that precludes its application in detecting miRNA expression of many rare hematopoietic stem/progenitor cell populations.

3.1.2.1. Prepare a Single-Cell Suspension From Hematopoietic Tissues

Isolate bone marrow cells by flushing femurs and tibias with bone marrow washing medium (see Note 6). Pipet bone marrow cells up and down several times to produce a single-cell suspension and filter through a 70-µm nylon mesh cell strainer. For the preparation of thymocyte or splenocyte suspensions, place the thymus or spleen in a 35-mm Petri dish containing PBS (cut tissues into several pieces if necessary), and mince by gently pressing the thymus or spleen between the frosted ends of two glass slides. Wash glass slides with cold PBS and filter through a 70-µm nylon mesh cell strainer (see Note 6). Wash cells with cold PBS. Count enucleated hematopoietic cells by staining with Turk’s solution. Cells are now ready for immunochemistry or RNA preparation.

3.1.2.2. Isolate Hematopoietic Progenitor Cell Populations by MACS or FACS

Label cells with fluorescence-conjugated antibodies against lineage-specific antigens. For example, use anti CD45R or CD19 for B-lineage cells; Mac1 or Gr-1 for myeloid lineage cells; and CD3 or Thy-1.2 (CD90.2) for T-lineage cells. Determine the appropriate dilution for each antibody before use. For MACS separation, add micro-bead-conjugated antibodies against lineage-specific antigens (see Subheadings 3.3.1.3. and 3.3.2.4. for detailed protocols for MACS separation and immunofluorescence staining). Proceed to MACS or FACS cell separation.

3.1.2.3. Total RNA Preparation

Trizol reagent (Invitrogen) was used to isolate total RNA from hematopoietic tissues and cell populations. Compared with other commercial column- or resin-based RNA purification kits, the Trizol protocol gives a better yield and is more reliable in isolating small RNAs. To prepare RNA samples for gel electrophoresis, dissolve RNA samples (5 to 20 µg of total RNA) in 15 µL of water (or less) and add an equal volume of 2X gel-loading dye. Heat samples at 80°C for 5 to 10 min and centrifuge.
3.1.2.4. Preparation of Radiolabeled RNA Marker

Label the Decade marker (Ambion,) with $\gamma^{33}$P ATP according to the manufacturer’s instructions. $^{33}$P-labeled marker can be stored at $-20^\circ$C and used for a month. Heat at $95^\circ$C for 5 min before use.

3.1.2.5. Electrophoresis of RNA Samples

Prepare a 15% acrylamide/8 M urea gel with the Sequagel sequencing system solutions (National Diagnostics). Prerun the gel at 25 W for 15 min in 0.5X TBE. Load the RNA samples and $^{33}$P-labeled RNA ladder and run the gel at 25 W for approx 1 h, until the bromophenol blue band reaches the end of the gel. Separate the gel onto plastic wrap and stain the gel with 4 $\mu$g/mL EtBr in 0.5X TBE for 5 to 10 min. Examine the EtBr-stained image to evaluate the RNA quality and loading consistency. Discrete transfer RNA (78-nt) and 5S ribosomal RNA (120-nt) bands indicate a good quality of RNA preparation. The 5S ribosomal RNA bands can be used as loading controls in publication. Take a digital picture and save the EtBr-stained image.

3.1.2.6. Gel Transfer

Reduce the gel size by cutting off the loading wells and empty lanes. Cut the nylon membrane (GenScreen Plus) and six sheets of 3M Whatman filter paper to the size of the gel. Soak the nylon membrane and filter papers in 0.5X TBE. Assemble the transfer sandwich on the surface of a semi-dry transfer unit in the following order: three sheets of soaked filter paper, nylon membrane, gel, and three sheets of soaked filter paper. Make marks on the RNA side of the nylon membrane with pencil. Remove possible air bubbles within the transfer sandwich by gently rolling a plastic serological pipet over the sandwich. Place the top on the semidry transfer unit, and run the transfer at a constant current (3.3 mA/cm$^2$) for 35 min. Place the filter with the RNA side up on a dry piece of filter paper and crosslink with ultraviolet light at 1000 $\mu$J of energy. Bake the filter at $80^\circ$C for 1 h. Store it at $-20^\circ$C or proceed to the prehybridization step.

3.1.2.7. Preparation of Radiolabeled miRNA Probes

Design and synthesize the antisense oligonucleotide probes against the mature miRNA targets. Prepare a 50-$\mu$L reaction mixture by mixing 2 $\mu$L of oligonucleotides (20 pmol/ $\mu$L), 5 $\mu$L of 10X T4 polynucleotide kinase buffer, 26 $\mu$L H$_2$O, 15 $\mu$L of [$\gamma^{32}$P] ATP, and 2 $\mu$L of T4 polynucleotide kinase (New England Biolabs). Incubate at 37°C for 1 h. Heat the mixture at 68°C for 10 min. Use a G-25 column (Amersham) to remove unlabeled radionucleotides.

3.1.2.8. Hybridization

Wet the membrane with water, place it in a hybridization tube, and add 20 mL warm prehyb/hyb solution (~50°C). Rotate at 50°C for 1 to 2 h. Replace with 20 mL fresh prehyb/hyb solution and add the denatured radiolabeled probe. Cap the tube tightly and rotate in the hybridization oven at 50°C overnight. Recover probes and store at $-20^\circ$C (probes can be reused two to three times within a week). Wash three times with 25 mL of nonstringent wash solution in the hybridization oven at 50°C for 30 min. Repeat the
wash two more times with 25 mL of nonstringent wash solution in the hybridization oven at 50°C for 1 h. Finally, wash with stringent wash solution in the hybridization oven at 50°C for 5 min. Wrap the membrane with Saran wrap and expose the membrane to a phosphorimager screen at room temperature overnight.

3.2. Ectopic Expression of miRNAs in Hematopoietic Stem/Progenitor Cells

Gain-of-function analysis has been the most fruitful approach for identifying many of the key protein players in hematopoietic lineage differentiation (55,56). In human leukemias, chromosomal translocations often result in aberrant gene expression and can reveal gene function in normal hematopoiesis (57). The ectopic expression of an active Notch in hematopoietic stem/progenitor cells revealed the key role of Notch signaling in T-cell and B-cell fate determination, which is consistent with Notch loss-of-function studies in mice (55,58). Furthermore, ectopic expression seems to be quite effective in revealing miRNA function in animals and plants (21,29,31,32). The high degree of redundancy of miRNA genes may present a unique challenge for elucidating miRNA gene function using a loss-of-function approach in mice. Furthermore, many miRNAs may carry out very fine genetic controls in animals, as was elegantly illustrated in worms, in which lsy-6 and mir-273 miRNAs act sequentially to determine the neuronal patterning in merely two neurons (27,28). It would be difficult to discern these functions without well-informed functional guidance. Thus, miRNA gain-of-function analyses in hematopoietic stem/progenitor cells will provide a solid foundation and guidance for future loss-of-function studies in mice.

3.2.1. Design of Retroviral Constructs for miRNA Expression

To ectopically express miRNAs in primary hematopoietic stem/progenitor cells, we have developed a retroviral vector using the murine stem cell virus backbone (32). In one of the configurations, a polymerase (pol) III expression cassette that contains the human H1 promoter and a polyT (T5) termination sequence was placed in the U3 region of the 3’ long terminal repeat (LTR). This vector design is termed a “double-copy” configuration because the process of retroviral reverse transcription and integration leads to two copies of the expression cassette to be integrated into the host genome (Fig. 2A). We found that the double-copy configuration provides robust and consistent expression of the miRNA hairpins when infecting primary hematopoietic cells. In contrast, when the H1 expression cassette is placed after the 5’ LTR, the H1 promoter is silenced when infecting primary hematopoietic cells (data not shown). In addition, as a marker for infection, GFP is introduced under the control of the constitutive murine PGK promoter.

We also noted that functional miRNAs cannot be effectively processed from minimal pre-miRNA stem-loop precursors and that the genomic flanking sequences of pre-miRNAs are essential for miRNA processing and maturation (32). Based on these observations, we designed a general strategy for the ectopic expression of miRNA genes by placing approx 270-nt-long primary (pri)-miRNA transcripts into the H1 expression cassette of MDH1-PGK-GFP 2.0 (Fig. 2B). The approx 270-nt pri-miRNA transcript contains the approx 22-nt mature miRNA and the 125-nt corresponding genomic sequences flan-
ing both sides of the mature miRNA. It is now known that miRNA biogenesis consists of sequential steps that are essential for the production of functional miRNAs (59). The pri-miRNA transcripts are processed into the approx 60-nt pre-miRNA stem-loops by the nuclear RNase III, Drosha, with the facilitation of Parsha (59,60). The pre-miRNA is then actively transported into the cytoplasm by Exportin 5 in a Ran guanosine triphosphate-dependent manner (61,62) and further processed into a approx 21-nt duplex by Dicer in the cytoplasm (63–65). The information for the sequential processing and maturation of miRNA is likely to be stored in the sequences of pri-miRNAs (32,59).

3.2.2. Generation of miRNA Expression Constructs

3.2.2.1. DESIGN PCR PRIMERS TO AMPLIFY PRE–miRNA GENE FRAGMENTS

Obtain mature and pre-miRNA sequences from Rfam—“the miRNA Registry” (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl)—and search the genome database of relevant organisms using the pre-miRNA sequences to extract the pri-miRNA gene fragments containing the corresponding flanking sequences (see Note 7). Use Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to select PCR primers from pri-miRNA gene fragments for amplifying the approx 270-nt PCR products that include the 22-nt mature miRNAs and 125-nt of genomic sequences flanking the mature miRNAs. Add XhoI and EcoRI restriction enzyme sites to the ends of the PCR primers to facilitate cloning of the PCR products into the MDH1–PGK–GFP 2.0 vector (Fig. 2A). Use other restriction enzymes when the pri-miRNA gene fragments contain internal EcoRI or XhoI sites.

3.2.2.2. PREPARATION OF GENOMIC DNA

Genomic DNA can be isolated from cultured cells or mouse tissues using the following protocol. Add 0.5 mL of DNA lysis buffer to mouse tissue or cells (~25 mg tissue
or $10^9$ cells per 1 mL lysis buffer) and digest overnight at 55°C while rotating in the hybridization oven. After overnight incubation, the tissue should no longer be visible. Centrifuge to remove debris, hair, and bone. Precipitate the DNA by adding an equal volume of isopropanol. Use a sterile pipet tip to transfer the precipitated DNA to a clean tube. Add 200 µL water and incubate overnight at 55°C to allow DNA to dissolve. Measure the DNA concentration.

3.2.2.3. Generation of miRNA Expression Construct

Amplify the pre-miRNA gene fragments from genomic DNA by PCR. Most of the pre-miRNA gene fragments we tried can be easily amplified from mouse genomic DNA. However, in some cases, it is necessary to optimize PCR conditions by varying the annealing temperature or using different Taq polymerases. For those that are extremely difficult to amplify, one can try to use bacterial artificial chromosome DNA clones containing the pre-miRNA gene fragments as templates. Digest the PCR products with restriction enzymes and clone into MDH1–PGK–GFP. Verify inserts by DNA sequencing analysis with the following primer (from the H1 promoter side): 5’-GGATCCCAATATTTGCATGTCGC-3’.

3.2.3. Viral Packaging by Transient Transfection

This protocol is designed for transfecting viral construct and packaging construct into cells on a 6-well plate. Adjust the amount of FuGene 6 reagent and DNA accordingly for larger scale transfections.

1. Prepare 50 to 80% confluent 293T (or BOSC23) cells by plating approx $5 \leftrightarrow 10^5$ cells per well in a 6-well plate the day before the transfection.
2. Add 100 µL of serum–free DMEM to a sterile 1.5-mL Eppendorf tube (Tube A) and aliquot 6 µL of FuGene 6 to Tube A (do not touch the pipet tip to the side of the tube because the FuGene 6 will stick to plastic). Mix and let stand for 5 min at room temperature.
3. Mix 2 µg of the miRNA expression vector and 1 µg of the pCLeco packaging vector (44) in a separate Eppendorf tube (Tube B).
4. Add diluted FuGene 6 solution (Tube A) to Tube B, mix well by tapping the tube, and incubate for 15 min at room temperature.
5. Remove the media from cells and replace it with 3 mL of fresh media.
6. Drop-wise, add the FuGene 6/DNA mixture (A and B mixture) to the cells and swirl to mix. Culture for 2 d.
7. Check GFP expression at 48 h after transfection and collect the supernatant. Store viral supernatant at −80°C.

3.3. Analysis of miRNAs’ Roles in Hematopoietic Lineage Differentiation

As one of the best-studied developmental systems, many in vitro and in vivo assays have been developed to examine the hematopoietic lineage differentiation processes. Some hematopoietic progenitor cells can be grown clonally in a semisolid culture. If provided with the right combination of colony stimulating factors, cultured progenitor cells will differentiate into mature forms, and can be enumerated and characterized based on the size and morphology of the colonies. A variety of myeloid, erythroid, and lymphoid progenitor cells at different stages of differentiation can be identified and
scored using this assay (66). Moreover, hematopoietic stem/progenitor cells can be maintained in vitro with some bone marrow stromal cell lines that provide a niche for stem cell maintenance and progenitor differentiation (67–69). With defined culture medium, this culture system can also be used to examine the differentiation potential of hematopoietic stem/progenitor cells. For example, S17, OP9, and MS-5 bone marrow stromal culture assays are commonly used to study B lymphopoiesis, whereas the fetal thymic organ culture and OP9–DL1 stromal culture assays are used to study T lymphopoiesis (70–72). Finally, the self-renewal and differentiation potentials of hematopoietic stem/progenitor cells can be measured by the bone marrow transplantation assay, in which transplanted stem cells are capable of rescuing lethally irradiated mice and repopulating the entire blood system (73,74). With these assays, one can alter the miRNA expression in hematopoietic stem/progenitor cells and then examine the effects of miRNA perturbations on stem cell self-renewal and lineage differentiation using in vitro and in vivo assays described in Subheadings 3.3.2. and 3.3.3. Here, we will only describe the S17 stromal culture assay and the bone marrow transplantation assay.

3.3.1. Enrichment of Lineage-Negative Cells From the Bone Marrows of 5-Fluorouracil-Treated Mice

3.3.1.1. Prime Hematopoietic Stem/Progenitor Cells With the Pyrimidine Analog 5-Fluorouracil

Inject mice (BS.SJL or C57BL/6J) with 5-fluorouracil (FU) (30 mg/mL stock concentration; 150 mg/kg body weight) via the lateral tail vein or retro-orbital vein. Injection of 5-FU kills the cycling hematopoietic cells, stimulates the quiescent stem cells into the cell cycle, and increases the retroviral infection of hematopoietic stem/progenitor cells (see Note 8).

3.3.1.2. Prepare Bone Marrow Cells for Immunohistochemistry and Lineage Depletion

Harvest bone marrow cells on day 3 or 4 after 5-FU treatment (see Subheading 3.1.2.1). Count the enucleated cells by staining with Turk’s solution (see Note 3). Wash the cells with labeling buffer and resuspend in labeling buffer to a final concentration of 10^8 cells/mL.

3.3.1.3. Lineage Depletion by MACS

Add an antibody to CD16/32 to cells (<1 µg/10^6 cells) and incubate on ice for 5 min to block the Fc receptors (optional). Add 200 µL of each biotinylated mouse lineage panel antibody per 10^8 cells (anti-CD3ε, anti-B220, anti-Mac1, anti-Gr-1, and anti-Ter-119) and incubate cells on ice for 20 min (see Note 9). Wash twice with 10 mL of ice-cold labeling buffer and spin down in a refrigerated centrifuge. Resuspend cells to 10^7 cells/90 µL with labeling buffer. Add 10 µL of MACS streptavidin-microbeads per 10^7 cells and incubate on ice for 20 min. Wash twice with 10 mL of ice-cold labeling buffer and spin down in a refrigerated centrifuge. Resuspend cells in separation buffer (up to 10^8 cells in 500 µL) and proceed to the MACS separation system to isolate lineage-negative cells. Count the lineage negative (Lin−) cells with Turk’s solution.
3.3.2. S17 Stromal Culture Assay

3.3.2.1. PREPARE S17 STROMAL CELLS

Trypsinize the S17 stromal cells and seed 20,000 cells/well in 24-well plates on the day of 5-FU injection. The S17 stromal cells should be confluent by the time of culture initiation.

3.3.2.2. RETROVIRAL INFECTION OF LIN⁻ CELLS BY SPINOCULATION

Resuspend enriched Lin⁻ cells to $5 \times 10^5$ cells/mL in the B-cell culture medium. Mix 1 mL of Lin⁻ cells and 1 mL of virus (at least $10^6$ cfu/mL) in a 5-mL FACS tube. Add 2 µL of polybrene to a final concentration of 4 µg/mL. Cap the tube tightly and centrifuge at 900 g for 2 h at room temperature (see Note 10).

3.3.2.3. SEED THE INFECTED CELLS ONTO S17 STROMA

Remove the infection supernatant, resuspend cells in B-cell culture medium, and seed them onto the S17 stromal layer (20,000–40,000 infected Lin⁻ cells/0.5 mL/well). Prepare 12 culture replicates for each viral infection. Place the plate in a 37°C, 5% CO₂, humidified incubator. Feed the cells with fresh B-cell culture medium 5 d after initiating the culture.

3.3.2.4. PREPARE CELLS FOR IMMUNOCHEMISTRY

Collect adherent and nonadherent cells from the S17 stromal culture at day 10 after culture initiation. First collect the nonadherent cells from the culture medium into FACS tubes (or 1.2-mL cluster tubes) and centrifuge at 5000 g for 5 min. Carefully remove the supernatant and save the cell pellets on ice. While centrifuging the nonadherent cells, add 0.5 mL of PBS/5 mM EDTA to the remaining adherent cells and incubate on a rocking platform at room temperature for 5 to 10 min. Recover adherent cells by repetitive pipetting to generate a single-cell suspension. Add the adherent cells to the nonadherent cells in the same tube. Wash twice with cold FACS buffer and resuspend cells in 100 µL of FACS staining buffer. When multiple stainings are required for each culture, resuspend cells in a larger volume of FACS buffer and aliquot 100 µL of cells into multiple tubes. When analyzing a large number of culture assays, carry out staining in a 96-well plate.

3.3.2.5. IMMUNOCHEMISTRY AND FACS ANALYSIS OF S17 CULTURE

Add antibodies (anti-CD-19 PE and anti-Thy-1.2 APC) to the cells and incubate at 4°C to 8°C for 15 min (see Note 9). Spin down the cells in a refrigerated centrifuge and wash twice with cold FACS buffer. Resuspend cells in FACS buffer containing 1 µg/mL PI. PI is used for live/dead cell discrimination because PI cannot permeate the membrane of the live cells, but can enter the nucleus of dead cells and stain the chromatin. Analyze the stained cells by FACS. Viral-infected donor cells are GFP positive. Analyze FACS data with BD CellQuest™ to determine the lineage profiles of the viral-infected cells and compare the lineage profiles of cells that are infected with the control vector and the miRNA-expressing virus.
3.3.3. Bone Marrow Transplantation Assay

3.3.3.1. Retroviral Infection of Hematopoietic Stem Cells

Prepare Lin- bone marrow cells from 5-FU-treated B6/SJL mice and infect them with viruses by spinoculation (see Subheading 3.3.2.2.). After centrifuging for 2 h, culture the infected cells in hematopoietic stem cell infection medium at 37°C in humidified air with 5% CO₂ for 24 h. Repeat spinoculation and culture for another 24 h.

3.3.3.2. Prepare Sca-1-depleted Bone Marrow Cells

Isolate bone marrow cells from C57BL/6J mice. Stain the bone marrow cells with anti-Sca-1 microbeads following the instructions from Miltenyi Biotec. Proceed to MACS separation to isolate the Sca-1-depleted bone marrow cells. Sca-1-depleted bone marrow cells, which lack of long-term repopulating hematopoietic stem cells, will be used as supporting cells in the bone marrow transplantation assay.

3.3.3.3. Bone Marrow Transplantation

Irradiate recipient mice on the day of transplantation. Recipient C57BL/6J (Ly 5.2) mice of 6- to 8-wk old are subjected to lethal irradiation (10 Gy) 3 to 5 h before transplantation (see Note 11). About 20 to 40 recipients per group are used for each miRNA construct. Mix 2.5 × 10⁴ infected cells with 4 × 10⁵ C57BL/6J bone marrow cells depleted of Sca-1-positive cells and inject them into lethally irradiated C57BL/6J recipient mice via the retro-orbital route.

3.3.3.4. Prepare Peripheral Blood Cells for Immunocohemistry

Bleed the recipient mice from the retro-orbital veins using heparinized capillary tubes at 1, 3, and 4 mo after transplantation. Collect 0.1 to 0.2 mL of peripheral blood into collection tubes containing 30 µL of heparin solution (2 U/mL). Mix the blood and heparin solution to prevent coagulation. Add 1 mL of ACK lysis buffer to the collection tubes and incubate at room temperature for 15 min. Centrifuge at 500 g at 4°C for 5 min and carefully aspirate out the lysis buffer. Repeat the ACK lysing step if there are still many red blood cells left. Wash cells twice with FACS buffer and resuspend in FACS buffer.

3.3.3.5. Immunohemistry and FACS Analysis (see Note 12)

To examine the influences of miRNAs on lineage differentiation, peripheral blood cells from the recipient mice are immunoreacted with lineage-specific antibodies to determine the lineage profiles of the viral-infected donor cells (marked by the GFP reporter). Enucleated peripheral blood cells from each recipient are immunoreacted with the following sets of antibodies:

1. Anti-CD4–PE and anti-CD8–APC.
2. Anti-CD19–PE and anti-Thy-1.2–APC.
3. Anti-Gr-1–PE and anti-Mac1–APC.

Add antibodies to the cells and incubate at 4°C to 8°C for 15 min. Spin down the cells in a refrigerated centrifuge and wash twice with cold FACS buffer. Resuspend
cells in the FACS buffer containing 1 µg/mL PI and subject to FACS analyses. Viral-infected donor cells are GFP positive. Analyze FACS data with BD CellQuest to determine the lineage profiles of the viral-infected cells and compare the lineage profiles of cells infected with the control vector and the miRNA-expressing virus.

4. Notes

1. New and unused plastic bottles, Eppendorf tubes, and pipet tips are generally RNase free. Designate a set of unopened chemicals and solutions for RNA preparation. Wear gloves to handle all RNase-free reagents.

2. A comprehensive list of reagents required for miRNA cloning can be found in the Bartel lab cloning protocol. Preadenylated 3' adaptor oligo is available from Integrated DNA Technologies.

3. Turk's solution is used to count enucleated cells in the peripheral blood. Acetic acid selectively lyses red blood cells. However, Turk's staining does not discriminate dead cells from the live cells. To count the liver cells, use Trypan blue dye staining.

4. Unless otherwise stated, buffers and solutions for MACS and FACS analyses are maintained on ice. Entire staining procedures are carried out on ice (0°C–4°C). Centrifugations are carried out in a refrigerated centrifuge or in the cold room (4°C–8°C).

5. The modified miRNA cloning method is now being tested for cloning miRNAs using as few as 10,000 hematopoietic stem/progenitor cells.

6. Use a 10-mL syringe and 21-gage needles for flushing femurs, and 23-gage needles for flushing tibias. Alternatively, place thymus or spleen on a 70-µm nylon mesh cell strainer, gently mash the thymus or spleen with the rubber end of a plunger from a 3-mL syringe, and wash with cold PBS to release thymocytes or splenocytes into a 50-mL conical tube.

7. Try to avoid a long stretch of Ts when designing primers to amplify the approx 270-nt pri-miRNA gene fragments because the H1 Pol III promoter will terminate at a long stretch of Ts (more than five Ts). miRNAs can also be expressed from a Pol II promoter. However, we noted that a longer pri-miRNA gene (~520-nt in length) containing the 22-nt mature miRNA and approx 250-nt of genomic sequences flanking the mature miRNA is required for higher expression using Pol II promoters.

8. Approximately 10 to 15 ↔ 10^6 cells can be isolated from the bone marrow of a 5-FU-treated mouse.

9. It is recommended that antibodies be titrated to determine their optimal concentration. Once the optimal concentrations are determined, make diluted antibodies stock with FACS staining buffer. If performing multicolor labeling, prepare the antibody mixture in a microcentrifuge tube and add the multiple antibodies simultaneously to the sample. Also, prepare single-color staining controls for adjusting the compensations.

10. Typically, more than 50% of the progenitor cells are infected with the miRNA vector as indicated by GFP expression, and this percentage does not substantially change during the 10-d assay.

11. Irradiation dose will vary depending on the strain and age of the animals and the source of irradiation. Therefore, a preliminary dose-titration experiment is required to determine the optimal irradiation protocol and dose. In general, lethal-dose irradiation can be delivered by exposing mice to two doses of 500 rad, 3 to 4 h apart, or to a single dose of 1000 rad.

12. One can follow the same animal over time by screening peripheral blood. Donor-derived cells will start to appear in the peripheral blood approx 2 wk after reconstitution, when bone marrow chimerism is about to be achieved. An alternative method is to set up multiple sets
of animals, kill animals at various time points, and analyze the lymphoid tissues as well as the peripheral blood. If mice are killed to harvest the lymphoid tissues, prepare a single-cell suspension as described in Subheading 3.1.2.1. Then, transfer 10^6 cells/100 µL of FACS buffer into FACS tubes and label with antibodies. For the labeling of bone marrow cells and splenocytes, red blood cells should be lysed by ACK lysis buffer before proceeding to the labeling step.

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**References**


