Chapter 18

Efficient Gene Knockdowns in Mouse Embryonic Stem Cells Using MicroRNA-Based shRNAs

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Abstract

RNA interference (RNAi) is a powerful gene-knockdown technology that has been applied for functional genetic loss-of-function studies in many model eukaryotic systems, including embryonic stem cells (ESCs). Application of RNAi in ESCs allows for dissection of mechanisms by which ESCs self-renew and maintain pluripotency, and also specifying particular cell types needed for cell-replacement therapies. Potent RNAi response can be induced by expression of an microRNA-embedded short-hairpin RNA (shRNA^{mir}) cassette that is integrated in the genome by virus infection or site-specific recombination at a defined locus. In this chapter, I will provide detailed protocols to perform shRNA^{mir}-mediated RNAi studies in mouse ESCs using retrovirus infection and loxP site-directed recombination for efficient constitutive and inducible gene knockdown, respectively.

Key words: microRNA, RNA interference, microRNA-embedded short-hairpin RNA (shRNA^{mir}), embryonic stem cells.

1. Introduction

Embryonic stem cells (ESCs) are derived from epiblast cells within the inner cell mass of blastocysts (1, 2) and uniquely endowed with unlimited self-renewal (3) and multilineage differentiation capacity (4–6). Murine embryonic stem cells (mESCs) have become an indispensable tool for investigating genetic function both in vitro and in vivo and provided a platform to study the molecular regulation of stem-cell self-renewal and lineage commitment and cellular differentiation. The discovery of RNA interference (RNAi) (7) has provided an attractive alternative

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to traditional homology recombination-based gene knockout strategy for loss-of-function assays. Applications of RNAi in ESCs should provide valuable tools for the study of general stem-cell biology (8) as well as directed differentiation of ESCs for replacement cells/tissues in regenerative medicine (9–11). An improved understanding of pluripotency at the molecular level has led to generation of induced pluripotent stem (iPS) cells from both mouse (12, 13) and human somatic cells (14–16).

RNAi is an evolutionarily conserved, sequence-specific gene-silencing mechanism that is induced by dsRNA. MicroRNAs (miRNAs) are a class of endogenous dsRNAs that exert their effects through the RNAi pathway. Understanding the biology of the RNAi and miRNA pathways has led to the development of miRNA-based shRNA (shRNA^{mir}) RNAi strategy (17) that yields a higher level of siRNA and more efficient knockdown than a simple shRNA expression vector (18). The potent RNAi response and the ability to be regulated by Pol II promoters have made shRNA^{mir} vectors the basis for second-generation shRNA libraries in the mouse and human genomes (17). Built on these discoveries, we have adapted the shRNA^{mir}-based strategy for efficient knockdown in mESCs using retrovirus transduction (19) and also developed a site-directed, virus-free, and inducible RNAi (SDVFi) system in mESCs (20). A similar nonviral, inducible RNAi approach has also been developed independently by others (21). In this chapter, I will first provide detailed procedures for a quick and easy assay to screen for a functioning shRNA^{mir} cassette for a particular gene of interest (**Sections 3.1**, **3.2**, **and 3.3**); then I will describe the method for setting up the SDVFi system in mESCs to provide a more refined and controlled experimental tool for interrogating gene function.

2. Materials

2.1. Cloning and Plasmid Preparation

1. LMPIG plasmid (*see* **Fig. 18.1**).
2. pCR2.1 TOPO cloning kit (Invitrogen).
3. pLox (ATCC cat. no. MBA-276).
4. pSalkcre.
5. EcoPak.
6. PfuUltra® PCR kit (Stratagene).
7. Rapid DNA ligation kit (Roche).
8. Plasmid miniprep and maxiprep kits (Qiagen).
9. QIAquick gel extraction kit (Qiagen).

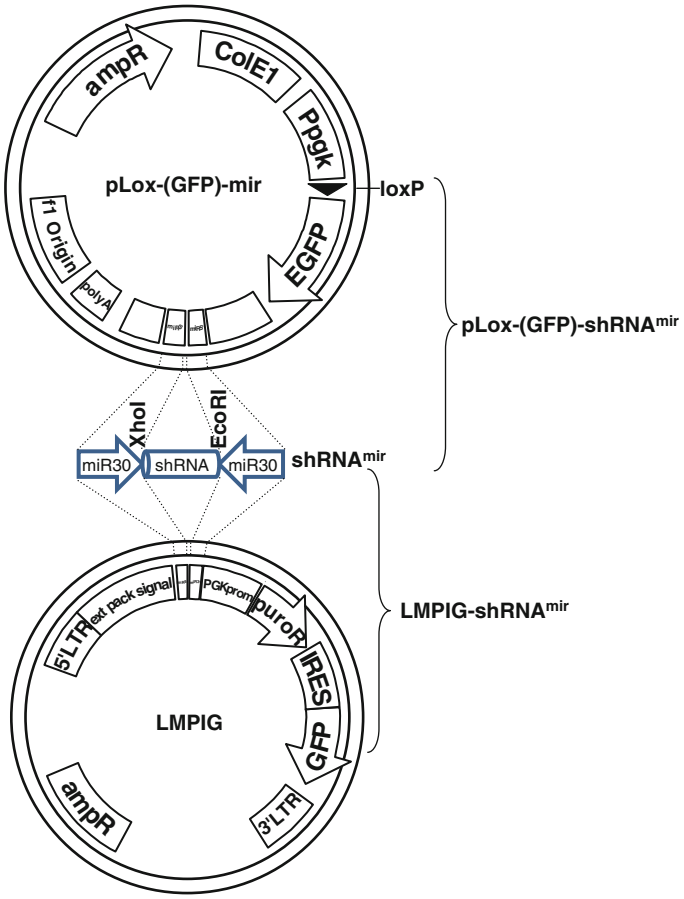

Fig. 18.1. Plasmids used in this study. (*Top*): pLox-(GFP)-shRNA^{mir} used in the SDVFi system; (*bottom*): LMPIG-shRNA^{mir} used for retrovirus-mediated, constitutive gene knockdown experiment, which also serves as a screening tool for functioning shRNAs; (*middle*) The shRNA^{mir} cassette with restriction sites *XhoI* and *EcoRI*. The shRNA can be replaced with any shRNA of the gene of interest by simple cloning into *XhoI* and *EcoRI* sites.

10. DH5α competent cells (Invitrogen).
11. Ampicillin (Sigma).

2.2. Cell Culture

1. ESGRO®/Leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA).
2. Geneticin®/G418 (GIBCO cat. no. 11811): It is an aminoglycoside antibiotic that blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred to mammalian cells genetically engineered to express a protein product encoded by the neomycin phosphotransferase gene (i.e., neomycin-resistance gene).

3. Puromycin (Sigma cat. no. P8833).
4. 0.05% (wt/vol) trypsin (Mediatech cat. no. 25-052-CI).
5. 0.25% (wt/vol) trypsin (Mediatech cat. no. 25-053-CI).
6. DMEM with low and high glucose (Invitrogen).
7. Nucleoside mix (100X, Chemicon cat. no. ES-008-D).
8. Penicillin/Streptomycin (GIBCO cat. no. 15070-063).
9. Fetal calf serum (FCS) (Hyclone cat. no. SH30071.03).
10. Plat-E cell-culture medium: DMEM with high glucose, 10% FCS, 2% Pen-Strep, 1% L-Glutamine; add puromycin (1 μg/mL) and blasticidin (10 μg/mL) before use.
11. ES cell-culture medium: Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, 15% (vol/vol) fetal calf serum (FCS)*, 0.1 mM ß-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acid, 1% (vol/vol) nucleoside mix, 1000 U/mL recombinant leukemia inhibitory factor (LIF), 50 U/mL Penicillin/Streptomycin. Store at 4°C. *FCS needs to be prescreened batch to batch for supporting optimal ES cell growth.
12. Irradiated mouse embryonic fibroblast (iMEF) cell-culture medium: Same as ES cell-culture medium except that LIF can be omitted. MEF cells are used in the culturing of ESCs. They provide a substrate for the ESCs to grow on and secrete many factors necessary for ESCs to maintain pluripotency. Feeders are MEF cells that have been mitotically inactivated by treatment with mitomycin C or by γ-irradiation. A unique quad-resistant DR4 feeder cell line can be purchased (Open Biosystems cat. no. MES3948) or prepared from DR4 mouse embryos [JAX® mice strain STOCK Tg (DR4)1Jae/J; The Jackson Laboratory] as previously described (22).
13. Gelatin (Bacto®, DIFCO cat. no. 0143-15-1): Dissolve 5 g of gelatin in 500 mL distilled water and autoclave (1% stock). Store at room temperature indefinitely. Before use, dilute 1:10 (to make 0.1% working solution) with sterile dH_2O and filter through 0.45- μ filter apparatus.
14. Ainv15 cells (ATCC cat. no. SCRC-1029).
15. J1 ESCs (ATCC cat. no. SCRC-1010).
16. 10-cm tissue culture plate (Falcon cat. no. 35-3003).
17. 24-well plate flat-bottom (Falcon cat. no. 35-3047).
18. Culture incubator (37°C, 5% CO_2 and 100% humidity).
19. 15-mL conical tubes (Corning cat. no. 430791).
20. 50-mL conical tubes (Corning cat. no. 430829).

2.3. Plasmid Transfection and Viral Infection

1. FuGENE 6 (Roche).
2. Lipofectamine 2000 (Invitrogen).
3. Polybrene (Hexadimethrine bromide, Sigma#H9268): Make a stock solution 4 mg/mL, use it 1:1000.
4. Syringe filters (0.45 μ) (Millipore).
5. Dulbecco's Modified Eagle's Medium (DMEM) with high or low glucose (Invitrogen).

2.4. RNA Isolation and Quantitative Real-Time PCR

1. Trizol® (Invitrogen).
2. Water (Nuclease free, Ambion cat. no. am9937).
3. iCycler and SYBR Green PCR Master Mix (Biorad).
4. Primers for your gene of interest.

2.5. Total Protein Isolation and Western Blotting

1. RIPA buffer (Boston BioProducts cat. no. BP-115).
2. Protease inhibitor cocktail (Sigma cat. no. P8340).
3. Western blotting apparatus (Biorad).

2.6. FACS Sorting for GFP-Positive Cells

1. Falcon tube (#2063).
2. PBS (with calcium and magnesium) (Sigma).
3. Cell strainer (70–100 μM Nylon; BD Falcon cat. no. #352360).
4. Gentamicin (Invitrogen).

2.7. Inducible RNAi

1. Doxycycline (Sigma).
2. Genomic DNA extraction kit (Gentra Systems, Inc.).

3. Methods

3.1. Designing and Cloning shRNA Hairpins Against Your Favorite Gene

1. Go to http://codex.cshl.edu/scripts/newmain.pl Website and search for the predesigned 97-mer hairpin oligos against your favorite gene (*see* **Note 1**).
2. Order 4–5 oligos (if available) targeting different regions (5′UTR, CDS, 3′UTR) of your favorite gene (*see* **Note 2**).
3. PCR amplify the fragment using PfuUltra® PCR master mix (Stratagene) with the primers (pSM2c-Forward and pSM2c-Reverse) (*see* **Table 18.1**) and the ordered shRNA oligos (100 ng/reaction) as template. These primers will add XhoI and EcoRI sites (*see* **Table 18.1**) to the ends of your hairpin for cloning into the LMPIG vector (*see* **Section 3.2**) (see **Note 3**).

Table 18.1
Primers used in this study

Primer name	Sequence (5′→3′)
M13 reverse	CAGGAAACAGCTATGAC
T7	TAATACGACTCACTATAGGG
pSM2C forward	GATGGCTGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG
pSM2C reverse	GTCTAGAGGAATTCCGAGGCAGTAGGCA
pSM2cseq-F	GTCGACTAGGGATAACAG
pSM2cseq-R	AGTGATTTAATTTATACCA
Loxin-F	CTAGATCTCGAAGGATCTGGAG
Loxin-R	ATACTTTCTCGGCAGGAGCA

4. Clone the PCR products directly into pCR2.1 TOPO vector from Invitrogen following manufacturer's instruction (*see* **Note 4**).
5. Pick 8 white colonies for miniprep (Qiagen MiniPrep kit).
6. Verify the shRNA hairpin sequences by sequencing the miniprep DNA with primers M13 reverse and T7 (**Table 18.1**; for both + and − strands) (*see* **Note 5**).

3.2. Cloning the shRNA^{mir} Constructs

1. Digest the LMPIG vector and the miniprep TOPO plasmid (from **Section 3.1**, step 6) with *XhoI* and *EcoRI*. Excise the vector DNA band [Vector] (−8 kb) and the shRNA hairpin inserts [Insert] (−120 bp).
2. Purify digested LMPIG vector and hairpin inserts with QIAquick gel extraction kit (*see* **Note 6**).
3. Ligate the Vector and the Insert using Rapid DNA ligation kit (Roche).
4. Transform half of the ligated products into 100 μL DH5α competent cells (Invitrogen), and plate them on Luria agar ampicillin plates.
5. Inoculate 8–16 colonies for plasmid miniprep; screen the colonies by digestion of the plasmid minprep DNA with *XhoI* and *EcoRI* for correct insertion (you should see a band of −120 bp in addition to the vector band).
6. Maxiprep the positive clones. These are the LMPIG-shRNA^{mir} retroviral vectors (**Fig. 18.1**) for subsequent RNAi studies (*see* **Section 3.3**).
7. Verify the shRNA^{mir} sequence with primers pSM2cseq-F and pSM2cseq-R. (**Table 18.1**) (*see* **Note 5**).

3.3. Retroviral Delivery of shRNA^{mir} for RNAi in mESCs

3.3.1. Preparation of PLAT-E Cells and mESCs

1. Seed Plat-E cells in a 10-cm tissue culture dish with Plat-E media containing puromycin (1 μg/mL) and blasticidin (10 μg/mL) (*see* **Note 7**).
2. When they reach near 100% confluence, split 1:5 onto new tissue culture dishes. Depending on the number of samples, another round of expansion of Plat-E cells may be needed.
3. On the day of transfection, Plat-E cells should reach 60–70% confluence. Replace with fresh medium without puromycin and blasticidin immediately before transfection.
4. A day or days prior to transfection of Plat-E cells, thaw out J1 or mESCs of your choice and seed them on top of a layer of irradiated mouse embryonic fibroblasts (iMEFs) in a 10-cm dish. The iMEFs (-1×10^6 cells) should be seeded a few hours or a day before thawing mESCs (*see* **Note 8**).

3.3.2. Transfection of Plat-E Cells with LMPIG-shRNA^{mir} Plasmids

1. Prepare plasmids: Mix 20 μg DNA (10 μg LMPIG-shRNA^{mir} plasmid + 10 μg Ecopak); leave them at room temperature (*see* **Note 7**).
2. Prepare diluted FuGENE 6: Per transfection, add 1 mL of DMEM with low glucose to a 15-mL Falcon tube, then add 60 μL FuGENE 6 reagent directly to medium in tubes (avoid contacting wall of the tube). Incubate at RT for 5 min (*see* **Note 9**).
3. Add 1 mL of the diluted FuGENE 6 (Step 2) into the DNA mix (Step 1). Incubate at RT for 15 min.
4. Add mixed DNAFuGENE 6 complex dropwise onto the Plat-E cells (from **Section 3.3.1**, step 3). Return to incubator and culture O/N.
5. After 24 h, gently aspirate the medium and add 5 mL of ES medium. Return to incubator and culture O/N.
6. Split ESCs in a ratio such that the number of ESC plates should be equal to or larger than that of Plat-E cells (*see* **Section 3.3.1**, step 2) and ESCs should reach 60–70% confluence by the next day for use in **Section 3.3.3**, step 3.

3.3.3. Infection of mESCs with Retroviruses

1. Collect the medium from dishes (in **Section 3.3.2**, step 5) after 24 h (designated 1° virus supernatant) and add another 5 mL of fresh ES medium. Return plates to incubator.
2. Dilute 1° virus supernatant 1:2 by adding 5 mL of ES medium (total 10 mL), filtered with a 0.45-μm syringe filter (*see* **Note 10**).

3. Gently aspirate the medium from ESCs (from **Section 3.3.2**, step 6) and add the 10 mL of filtered virus-containing medium to ESCs.
4. Add 10 μL polybrene (final 4 μg/mL) to each dish. Return to incubator.
5. On second day, collect the 5 mL virus medium (designated 2° virus supernatant) from Plat-E cells (in **Section 3.3.3**, step 1); rinse each dish with another 5 mL of ES medium. Pool total 10 mL ES medium and filter with a 0.45-μm syringe filter.
6. Gently aspirate the old medium from ESCs and add the 2° supernatant (i.e., 10 mL filtered virus-containing media) to ESCs. The cells should now reach near 90% confluence.
7. Add 10 μL of polybrene (final 4 μg/mL) to each dish. Return to incubator and culture O/N.
8. Replace old medium with fresh medium containing 1–2 μg/mL puromycin daily for the next few days (*see* **Note 11**).

3.3.4. Verification of Gene Knockdown

3.3.4.1. Harvest Cells Directly for RNA and Protein Extractions

1. On day 3 (i.e., 48 hr after initiation of puromycin selection) or later, remove ES medium by aspiration, and rinse ESCs once with 0.05% trypsin. Then, add a sufficient amount of 0.25% trypsin to cover the ESCs and incubate at 37°C for 35 min. The ESCs become detached from the vessels and can be collected after neutralization of the trypsin with 3 × vol of ES cell medium (pipetting up and down to mix them).
2. Split cell suspension into halves, one for RNA, the other for total protein. Centrifuge at 200 × *g* for 5 min to harvest cells.
3. Resuspend the cell pellets with 10 mL of PBS and centrifuge at 200 × *g* for 5 min.
4. Resuspend the cell pellets in Trizol for RNA extraction, and in RIPA buffer for total protein extraction.
5. Perform standard Q real-time PCR and Western blotting to verify gene knockdown (*see* **Note 12**).

3.3.4.2. Harvest Cells by Sorting GFP-Positive Cells for RNA and Protein Extractions

1. On day 3 (i.e., 48 h after initiation of puromycin selection) or later, remove ES medium by aspiration, and rinse ESCs once with 0.05% trypsin. Then, add a sufficient amount of 0.25% trypsin to cover the ESCs and incubate at 37°C for 3–5 min. The ESCs become detached from the vessels and can be collected after neutralization of the trypsin with

3 × vol of ES medium (pipetting up and down to mix them) (*see* **Note 13**).

2. Centrifuge at 200 × *g* for 5 min to harvest cells.
3. Aspirate and resuspend cell pellets in 10 mL of PBS (with calcium and magnesium) containing 5% FCS. Wash twice with 10 mL of the same buffer.
4. Resuspend cell pellets with appropriate amount of the same buffer to make 2–5 × 10^6/mL cells for sorting.
5. Filter cell suspension with 70–100 μm cell strainer to remove cell clumps. Have collection tubes prefilled with ~1 mL of 100% heat-inactivated FCS containing Pen/Strep (50 U/mL), gentamicin (50 μg/mL), and puromycin (1–2 μg/mL) (*see* **Note 14**).
6. Sort cells for high, medium, and low GFP intensity. Collect cells in a Falcon tube prepared in Step 5.
7. After sorting, you can either harvest all the cells for RNA or total protein extraction following **Section 3.3.4.1**, step 4. (if you have enough cells) or reculture them for expansion or single-colony formation (*see* next step).
8. Add directly 5–10 mL of ES medium to the sorted cells and transfer them to a 10-cm iMEF dish or 6-well iMEF plate depending on cell numbers after sorting. Return to incubator and culture for a few more days under 37°C/5% CO_2 (*see* **Note 15**).
9. Perform RNA and protein preparation for real-time PCR and Western blotting for gene knockdown at RNA and protein levels, respectively.

3.4. Site-Directed, Virus-Free, and Inducible Expression of shRNA^{mir} for RNAi in mESCs

3.4.1. Establishment of ESCs Expressing shRNA^{mir} in a Defined Locus

1. Digest the plasmid carrying the validated shRNA^{mir} (in **Section 3.3.4**) with *XhoI* and *EcoRI* to release the shRNA and clone it into the pLox-mir or pLox-GFP-mir (to mark the mir expression with GFP reporter) vector previously digested with *XhoI* and *EcoRI*. The resultant vector is pLox-(GFP)-shRNA^{mir} (**Fig. 18.1**) (*see* **Note 16**).
2. Make miniprep and maxiprep of pLox-(GFP)-shRNA^{mir}, and verify the plasmids by restriction digests and direct DNA sequencing.

3. Thaw and grow Ainv15 ESCs on a 6-well iMEF plate. When ESCs reach a density necessary for passage, split a single well into five to six similar wells. Incubate overnight or until cells reach 80–90% confluence.
4. Prepare master mixes in an Eppendorf tube or a 15-mL Falcon tube per sample: Add 10 μL Lipofectamine 2000 into 250 μL serum-free DMEM with high glucose (*see* **Note 17**).
5. In a 1.5-mL Eppendorf tube, add 5 μg of pLox-(GFP)-shRNA^{mir} and 5 μg of pSalkcre DNA into 250 μL serum-free medium.
6. Incubate the tubes from Steps 4 and 5 for 5 min at room temperature.
7. Aliquot 250 μL of Lipofectamine 2000 mix (Step 4) into 250 μL Eppendorf tubes containing DNA mix (Step 5), and mix gently by inverting the tubes. Incubate this mixture for 10 min at RT to allow the lipid and DNA complexes to form.
8. While the incubation is proceeding, aspirate ES cell medium from Ainv15 cells, wash each well with PBS, treat cells using trypsin, and harvest by centrifugation at 400 × *g* for 5 min in 15-mL Falcon tubes.
9. Resuspend cell pellet with DNA/lipid complexes (Step 7) and incubate at RT for 10 min.
10. Add DNA/lipid complexes/cells (from Step 9) dropwise to gelatin-coated wells of a 6-well plate, rock the plate back and forth to distribute the cell suspension, and incubate overnight under 37°C/5% CO_2 in tissue culture incubator. Prepare same number of 10-cm iMEF dishes for use the next day.
11. On the second day, the cells in the 6-well plate should be near 100% confluence. Trypsinize cells, harvest by centrifugation as in step 8, resuspend in 10 mL of fresh ES medium, transfer cells into 10-cm iMEF plates, and incubate under 37°C/5% CO_2.
12. On the fourth day (48 h later), add 10 μL of G418 (final 300 μg/mL) directly to each dish to start drug selection.
13. Replace the old medium daily with fresh medium containing 300 μg/mL G418 for the next 10–15 days (*see* **Note 18**). Pick the emerging G418-resistant clones, expand cells for genomic DNA extraction (*see* Gentra manuals), and freeze cell stocks in 90% fetal bovine serum/10% DMSO (designated Ainv/pLox-(GFP)-shRNA^{mir}).
14. PCR confirm the correct targeting in positive clones using primers Loxin-F/Loxin-R (*see* **Table 18.1**). PCR

amplification products are analyzed by agarose gel electrophoresis, and the presence of a ~500-bp band that is amplified across the loxP site indicates a correct targeting event.

3.4.2. Inducible Gene Knockdown in Ainv/pLox-(GFP)-shRNA^{mir} Cells

1. Thaw Ainv/pLox-(GFP)-shRNA^{mir} cells and expand them onto 2 wells of a 24-well iMEF plate.
2. Grow cells until they reach 40–50% confluence.
3. Add doxycycline (1–2 μg/mL) to one of the wells.
4. Replace old medium with fresh medium daily (adding 1–2 μg/mL doxycycline to the well under selection) for the next 3–7 days. Split cells into several new wells of a 24-well iMEF plate when cells are near confluence.
5. Harvest cells from one well for RNA extraction, and another well of cells for total protein lysates as described in **Section 3.3.4.1**.
6. Perform quantitative real-time PCR and/or Western blotting to validate knockdown of the gene of interest at RNA and protein levels, respectively (*see* **Note 19**).

4. Notes

1. RNAi Codex provides a single database that curates publicly available RNAi resources including the Hannon-Elledge shRNA libraries (mouse and human) that are available through Open Biosystems. The Codex provides the most complete access to this growing resource, allowing investigators to access available clones and clones that are soon to be released. Independent of the optimal shRNA design by the RNAi Codex, control experiments are necessary to confirm the specificity of an RNAi phenotype (*see* **Note 2**).
2. If a pSM2-shRNA already exists in the Open Biosystems collection (http://www.openbiosystems.com/expression_arrest_shrna_libraries.php), and/or the oligos in http://codex.cshl.edu/scripts/newmain.pl search are marked as "released", you can order them from Open Biosystems as a bacterial stock. As mentioned in **Note 1**, control shRNAs should also be ordered and processed simultaneously. Open Biosystems offers pSM2 Retroviral shRNA^{mir} controls in glycerol stock (Catalog # RHS1705, RHS1706, RHS1707). The Firefly Luciferase pSM2 shRNA^{mir} is a positive control designed against pGL3 Firefly Luciferase

(Promega, cat. no. E1741). The eGFP pSM2 shRNA^{mir} is a positive control designed against the enhanced GFP reporter (Invitrogen, cat. no. v355-20; GenBank accession number: pEGFP U76561). The nonsilencing pSM2 shRNA^{mir} is a negative control containing a target sequence that does not match any known mammalian genes. Depending on your experimental setting, the Firefly Luciferase shRNA^{mir} and the eGFP shRNA^{mir} can also be used as negative controls against your gene of interest.

3. High-fidelity amplification PCR kit (e.g., Stratagene's PfuUltra®, Roche's High-Fidelity®, and NEB's Phusion®) is preferred for amplification of shRNA hairpin sequences. In any case, when using 100 ng/reaction of the oligo as template, 12–16 cycles are sufficient to generate enough PCR product for cloning. Over-amplification will increase errors in the final products.
4. TOPO cloning of PCR products amplified with high-fidelity polymerase such as PfuUltra® requires pretreatment of the PCR products with Taq polymerase to add T/A overhangs (*see* TOPO cloning kit for details).
5. When setting up sequencing reactions, you should include 5% (vol/vol) dimethyl sulfoxide (DMSO) to resolve certain compressions caused by strong shRNA^{mir} hairpin secondary structure.
6. When transferring *XhoI/EcoRI* hairpin fragments from one vector to another using a gel-purification step, "melt" agarose at lower temperature (42 instead of 50 degrees) to reduce probability of melting hairpins (resulting in snap back ss DNA).
7. A potent retrovirus packaging cell line named Platinum-E (Plat-E) was generated based on the 293T cell line. Plat-E cells have been engineered to stably express the *gag-pol* and *env* genes under the strong EF1α promoter for efficient virus packaging. The high titer of retroviruses derived from the Plat-E cells can be maintained by simply culturing the cells in the presence of selection drugs (puromycin and blasticidin) (23). EcoPak is a plasmid that expresses the *gag-pol-env* packaging functions as previously described by another group (24). The use of EcoPak in transfection of Plat-E cells is optional.
8. This step should be carefully planned so that you will have enough ESCs for infection (*see* **Section 3.3.3**) after splitting when they reach confluence (*see* **Section 3.3.2**, step 6).
9. If not using EcoPak, then mix 10 μg shRNA plasmid with 30 μL FuGENE 6 (dilute with 500 μL DMEM

with low glucose). FuGENE 6 as well as other lipid-based transfection reagent (e.g., Lipofectamine 2000) should be warmed up to ambient temperature (approximately 10–15 min at room temperature) prior to use and added directly to the media while avoiding contact with the walls of tubes/plastics. Chemical residues in plastic vials can significantly decrease the biological activity of the reagent. In addition, no drug should be present in DMEM (low glucose) during FuGENE 6 or Lipofectamine 2000 dilution.

10. The virus supernatant should be filtered with a 0.45-μm syringe filter (recommended). However, if there are obvious big cell clumps floating in the supernatant, you should centrifuge the supernatant at 1500 rpm for 5 min to remove clumped cells before using the syringe filter, otherwise the syringe filter will be clogged by clumped cells.
11. The puromycin resistance is conferred on the infected cells by expression of the puromycin-ires-GFP expression cassette in the LMPIG-shRNA^{mir} vector (*see* **Fig. 18.1**). Selection of puromycin-resistant cells will enrich the infected cell population for downstream analyses.
12. Based on the variable expression of GFP-dependent fluorescence, we concluded that puromycin-selected cell populations are represented by cells that can be divided into those with low, moderate, and high levels of gene knockdown. Moreover, when genes important for stem-cell self-renewal were studied, we observed that the low-level knockdown cells have a growth advantage over the high-level knockdown cells, which leads them to outcompete the high-level knockdown cells. Consequently, prolonged puromycin selection should be avoided. A time course extending over a 3-day period from day 1 to day 3 (within 72 h post-puromycin selection) during which the ratios of low, moderate, and highly fluorescent cells are monitored may prove useful in estimating dynamic changes in the cell population. As an alternative procedure, **Section 3.3.4.2** provides a solution to this problem by sorting out the GFP high, medium, and low populations for downstream analyses.
13. Depending on the infection efficiency, the number of puromycin-resistant ESCs after several days of puromycin selection may not be enough for downstream RNA and protein analyses after GFP sorting. Therefore, if more cells are deemed necessary for downstream analyses, ESCs should be further expanded and grown onto 10-cm iMEF plates, provided that knockdown cells have not differentiated.

14. Pen/Strep, gentamicin, and puromycin were added to the collection tube to prevent potential contamination from bacteria and other irrelevant cells from sorting. This is only necessary if the sorted cells will be further cultured.
15. If a single cell colony is preferred, then collect 400–800 GFP+ cells, and culture them in a 10-cm iMEF dish to allow individual clones to form. Many cells will die due to the sorting stress, but enough cells survive and will grow out as single colonies after 10–14 days, provided that the knockdown of the gene does not result in cell death or differentiation.
16. The advantage of using pLox-GFP-shRNA^{mir} over pLox-shRNA^{mir} is that the expression of shRNA^{mir} will be marked by the GFP reporter upon doxycycline induction. In addition, it has been reported that an increased spacer between the promoter and the shRNA^{mir} cassette delivered by lentiviral vectors may enhance the knockdown levels (25), although the mechanism is unclear.
17. Transfection of Ainv15 cells with pLox and pSalkcre constructs has only been reported using electroporation (26), which often yields only a few G418-resistant homology recombinants. In this chapter, I have tested using Lipofectamine 2000 as transfection reagent to introduce pLox-GFP-shRNA^{mir} and pSalkcre into Ainv15 cells, and found that Lipofectamine 2000 transfection yields almost twice as many G418-resistant colonies than electroporation.
18. Most cells die after 3–4 days of selection using 300–350 μg/mL G418 in complete ES cell growth media. Culture and change the drug-containing medium daily until colonies appear around day 10–14.
19. Due to the single copy integration of the shRNA^{mir} in the SDVFi system, the knockdown of high-abundance genes may not be as efficient as that in the retroviral system, which often involves multicopy shRNA^{mir} random integration into the genome. In this case, a tandem, multiple shRNA^{mir}-cassette strategy may be attempted.

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