CHAPTER EIGHTEEN

Exploration of Self-Renewal and Pluripotency in ES Cells Using RNAi

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Contents

Abstract

Embryonic stem cells (ESCs) have the ability to expand indefinitely *in vitro* and give rise to cells of all three germ layers as well as germ cells. For these reasons, ESCs hold great promise for biomedicine. In order to harness the potential of pluripotent cells, it is necessary to first understand the molecular mechanisms that control the pluripotent state. The discovery of RNA interference has made

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such functional analysis, even at high(er) throughput, possible. Here, we describe the methods used for high-throughput siRNA screening by high-content microscopy to identify gene products that regulate mouse ESC fate decision. In addition, we will describe the application of lentivirus-based shRNA knockdown to explore or validate the role of candidate genes in ESC pluripotency.

1. Introduction

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the ability to self-renew and differentiate into cells of all three germ layers. These characteristics make them invaluable for studying the molecular mechanisms of self-renewal and lineage differentiation processes. Over the last several years many groups, including ours, have analyzed and cataloged the gene-expression profiles of pluripotent stem cells. In order to investigate the function of the genes assembled in these lists, one can either pursue the classical strategy of gene deletion or use RNA silencing approaches. Although gene deletion will result in complete null phenotypes, the strategy is hampered by the fact that it is rather time-consuming and not feasible for high-throughput analyses. The discovery of RNA interference (RNAi), a natural process through which the expression of target genes can get silenced by either suppressing translation or by site-specific cleavage followed by degradation of the target mRNA (Fire *et al.*, 1998; Meister and Tuschl, 2004), has opened the door for high(er)-throughput functional genomics analyses in organism previously inaccessible to such systemic genetic perturbations. Several reagents have been developed to induce RNAi. In mammalian cells, the most common are chemically synthesized short double stranded (ds) RNA known as small interfering RNAs (siRNAs), which share 100% sequence complementarity to their target mRNA (Elbashir *et al.*, 2001). siRNA libraries targeting the entire genome, or specific functional gene subsets thereof, can be obtained from several commercial vendors. These libraries/sets contain mostly two to four siRNAs per gene thereby minimizing off-target effects and maximizing knockdown efficiency. As an alternative to these chemical libraries, long dsRNAs, generated by *in vitro* transcription of full-length cDNA (libraries), can be enzymatically cleaved *in vitro* by Dicer or RNase III into multiple endo-RNase prepared siRNAs (esiRNA) per gene target (Buchholz *et al.*, 2006; Yang *et al.*, 2002). Once introduced into cells, these (e)siRNAs are unwound by the activated RNA-induced silencing complex (RISC) in an ATP-consuming process. Only one strand of the (e)siRNA, which is selected based on thermodynamic rules, is then incorporated into RISC. This antisense strand then directs recognition and cleavage of the target mRNA sequences by RISC (Hannon and Rossi, 2004). The cleaved

(e)siRNA target mRNA is subsequently degraded resulting in a loss of gene function (Hannon and Rossi, 2004). Both siRNA- and esiRNA-type silencing approaches can be delivered into cells using a variety of standard transfection protocols and allow for transient, 3–5 days knockdown of the target genes. For stable, long-term silencing of target mRNAs systems encoding for short hairpin RNAs (shRNAs), mimicing microRNAs, the endogenous triggers of the RNAi pathway, have been developed (Brummelkamp *et al.*, 2002; McCaffrey *et al.*, 2002; McManus *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Xia *et al.*, 2002; Yu *et al.*, 2002). The shRNAs can be expressed from polymerase II or III promoters, using constitutive or inducible systems, vary in length from 19 to 29 nucleotides with diverse stem loops structures, and with various degrees of similarity to natural microRNAs. Because these shRNA triggers are encoded by DNA plasmids, they can be delivered to cells in many ways, including standard transient transfection (not feasible for long-term silencing), stable transfection, and transduction using viruses including adenoviruses and retroviruses.

In this chapter, we briefly describe the experimental procedures to study gene function in mouse ESCs by siRNA transfection and/or lentiviral-based shRNA methods.

2. Maintenance of Mouse Embryonic Stem Cells

The maintenance of undifferentiated, healthy ESCs is of critical importance to any successful screen. For large-scale screens, it is detrimental that enough cells of the same passage number are frozen away and enough reagents of the same lot number are available to last through the entire experiment from screen setup through optimization to the actual screen. We maintain our ESCs as follows:

1. Precoat 10-cm cell culture plates with 5–7 ml EmbryoMax ESC qualified 0.1% gelatin solution (Millipore; ES-006-B) for at least 15 min prior to use.
2. Mouse CCE and NG4 (CCE cells carrying a green fluorescent protein (GFP) expression cassette driven by the Nanog regulatory loci; Schaniel *et al.*, 2006, 2009) cells are maintained in feeder-free tissue culture condition. ESC culture medium consists of Dulbecco's modified Eagle medium with high glucose (DMEM high glucose; Invitrogen; Cat. no. 11965) supplemented with 15% qualified fetal bovine serum (FBS; we currently use Stasis stem cell FBS (Gemini Bio-Products; Cat. no. 100-125)), 2 m*M* L-glutamine (Invitrogen; Cat. no. 25030), 1 m*M* sodium pyruvate (Invitrogen; Cat. no. 11360), MEM nonessential amino acids (MEM NEAA; Invitrogen; Cat. no. 11140), 100 U/ml penicillin, and 100 μg/ml streptomycin (Pen/Strep; Invitrogen;

Cat. no. 15140), 1× β-mercaptoethanol (BME; preparing 100× BME stock by adding 37 μl BME to 100 ml Dulbecco's phosphate-buffered saline (DPBS); Sigma; M7522), and 1000 U/ml recombinant mouse LIF (ESGRO; Millipore; ESG1107).

3. Aspirate medium from the cell culture dish and rinse cells with DPBS (Invitrogen; Cat. no. 14287072).
4. Aspirate DPBS and harvest cells by incubation with 1 ml trypsin for 3–5 min to allow detachment of cells from the coated tissue culture plates.
5. Add 10 ml ESC culture medium w/o LIF to inactivate trypsin, pipet gently, and transfer to a 15-ml BD Falcon conical tube (BD Biosciences; Cat. no. 252097).
6. Centrifuge at 300×*g* for 3–5 min, discard supernatant, and resuspend cells in 10 ml ESC culture medium.
7. Remove gelatin from 10-cm tissue culture plates and replace with 10 ml of ESC culture medium.
8. Add 1 ml of resuspended cells per gelatin-precoated tissue culture dishes with 9 ml ESC culture medium and distribute cells evenly over the plate. Alternatively, count cells using a hemocytometer and add 2–2.5 × 10^6 cells per 10-cm plate. Cells are maintained at 37 °C in a humidified environment containing 5% CO_2.
9. Change medium every day and split cells every two days.

3. siRNA-Mediated Gene Knockdown

RNAi using (e)siRNAs is a powerful tool to study gene function. However, the analysis is limited to 3–5 days due to the transient inhibitory nature of (e)siRNAs. In 2009, two genome-wide RNAi screens—one using siRNA and the other using esiRNA—identified several novel regulators defining mouse ESC identity (Ding *et al.*, 2009; Hu *et al.*, 2009). Both these studies relied on flow cytometric analysis of Oct4 promoter-driven GFP expression. Before any screening can proceed, that is, irrespective whether a single gene, several genes, gene families, or the entire genome is to be analyzed, several parameters need to be established and optimized. This part of the screening procedure is the most time-consuming and can take several months to complete. The parameters to establish are (1) choice of (reporter) cell line and read-out, (2) appropriate cell plating density for your particular assay, (3) positive and negative controls to be used, (4) most optimal transfection reagent (depends on siRNA library used and the cell type to be transfected), (5) concentration of siRNA to be used, and (6) concentration of transfection reagent to be used with the optimal concentration of siRNA. Once these parameters are set even a genome-wide screen can be completed within a few weeks.

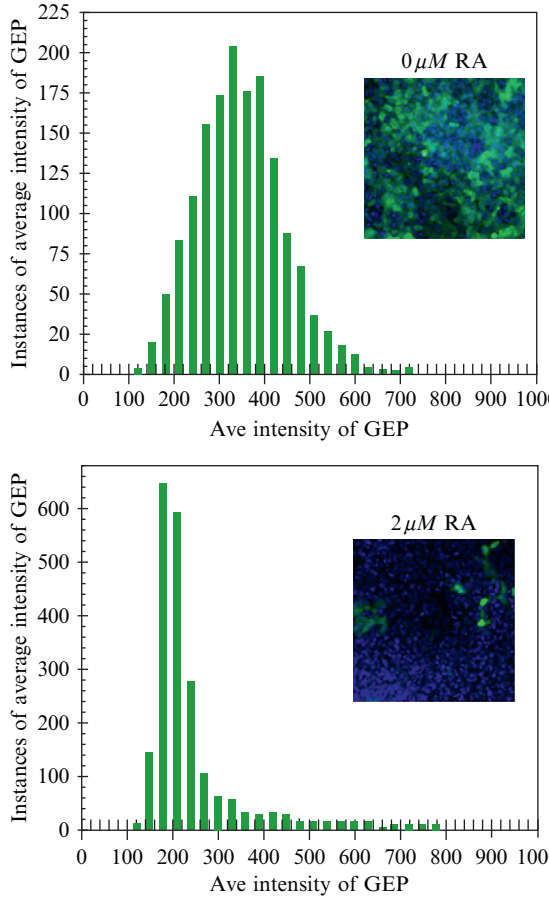

Figure 18.1 Detection of GFP expression using an ArrayScan IV by Cellomics. Control siRNA transfected NG4 cells are treated with 2 μM RA for 2 days. Cells were further fixed with 4% paraformaldehyde, stained with an anti-GFP antibody and FITC-labeled secondary antibody, scanned, and analyzed using Cellomics software.

Here we present a high-throughput, 384-well plate format screening method using NG4 (Nanog-GFP reporter) cells (Schaniel *et al.*, 2006, 2009) to analyze the effects of siRNA treatments on Nanog-promoter activity (see Fig. 18.1 for representative images). We monitor GFP expression, our read-out for Nanog-promoter activity, using fully automated high-content and high-throughput microscopy, such as the Arrayscan VTI (Cellomics, Thermo Scientific) or the Image Xpress Ultra Confocal Imaging System (Molecular Devices). For high-throughput analysis, the scanning system should be combined with a plate-moving robot and plate capacity hotel(s) that can accommodate storage of multiple plates to allow for fully automated unattended plate feeding into the imaging device.

3.1. siRNA transfection

It should be noted that dispersion of any liquid, including those containing cells, is best accomplished using automatic or semiautomatic liquid handlers and dispensers such as the Perkin Elmer JANUS eight-channel liquid handler, the Thermo Combi liquid dispenser or the WellMate microplate dispenser (Thermo Scientific). A critical point to determine before starting the screen is the capacity of daily plate handling. This will depend largely on the number of siRNAs to be screened, the number of total plates (including replicas), and the capacity of the liquid handler/dispenser and imaging system. One should not forget to take into account the number of new siRNA transfections one is to do the following day(s), the daily media change on plates previously transfected, the time required for preparation of plates for imaging and the image scanning itself.

We have established the following protocol that allows us to screen for genes involved in both the activation of the Nanog promoter (surrogate for the maintenance of ESCs) or the repression of Nanog (surrogate for genes required for ESC differentiation).

1. Maintain mouse NG4 ESCs in regular mouse ESC medium as described above. The day before use, harvest NG4 cells and plate at a 1:10 dilution onto gelatin-coated 10-cm tissue culture plates. Grow overnight.
2. On the day of transfection, precoat 384-well tissue culture plates (Corning Life Sciences) with 50 μl/well of EmbryoMax ESC qualified 0.1% gelatin solution at 37 °C for 15 min. Aspirate gelatin.
3. Add 5 pmol siRNAs/well. This process is done by automated liquid handlers such as the JANUS MDT (Perkin Elmer) workstation.
4. Dilute 0.15 μl of Lipofectamine 2000 transfection reagent (Invitrogen; Cat. no. 11668-019) in 25 μl DMEM high glucose medium per siRNA, mix and incubate at room temperature for 5 min.
5. Add Lipofectamine 2000 mixture to each well. *Note*: We use a different tubing to dispense the transfection reagents. Vortex plate gently and centrifuge at 300×*g* for 1 min, followed by incubation at room temperature for 15 min to allow for formation of siRNA-liposome complexes.
6. In the meantime, harvest mouse ESCs as described above, and resuspend in mouse ESC medium containing 2× FBS but no antibiotics. Count cell number and prepare a cell stock of 30 cells/μl.
7. Dispense 25 μl of cell stock—a total of 750 cells—into each well, vortex gently, centrifuge at 300×*g* for 1 min, and incubate at 37 °C in a humidified environment containing 5% CO_2.
8. Twenty-four hours after transfection, carefully aspirate the medium and replace it with fresh medium. (*Note*: We have used ESC medium minus LIF, supplemented with 0.5 μM retinoic acid (ESC medium + RA). This allows us to identify genes required for both Nanog-promoter activation as well as repression.)
9. Replace medium again with fresh medium after 24 h.

3.2. Preparation of plates for image acquisition

The process of image acquisition is more time-consuming than the process of siRNA transfection or the daily medium changes. The time needed mainly depends on the imaging system used, the sparsity of cells, and the amount of parameters to be collected (see also below). We have found that the scanning of one 384-well plate can take up to 2 h. Therefore, cells ought to be fixed. Fixation allows for plate storage, scanning and rescanning should something go wrong during the original acquisition without loss in cellular phenotype. The following describes the process of cell fixation.

1. Seventy-two hours after initial transfection, aspirate culture medium and carefully wash cells three times with 50 μl of DPBS.
2. Add 30 μl of 4% paraformaldehyde (Electron Microscopy Sciences; Cat. no. RT-15710) (prepared in DPBS) and fix cells at room temperature for 30 min.
3. Aspirate paraformaldehyde solution and wash cells again three times with 50 μl of DPBS.
4. Permeabilize and block cells with 50 μl of blocking buffer (0.1% Triton X-100, 1% BSA, 10% normal donkey serum in DPBS) at room temperature for 30 min.
5. If needed, the GFP signal-to-background/noise ratio can be increased by staining the cells with an anti-GFP antibody. Otherwise go to step 9. Dilute anti-GFP rabbit antibody (Invitrogen; Cat. no. A-6455) 1:1000 in blocking buffer, add 50 μl/well, and stain at 4 °C overnight.
6. Remove primary antibody and wash wells three times with DPBS at room temperature.
7. Dilute anti-rabbit IgG-Alexa488 antibody (from any commercial vendor) 1:1000 in blocking buffer, add 50 μl/well, and incubate at room temperature for 1 h.
8. Remove secondary antibody and wash wells three times with DPBS at room temperature.
9. Add either 50 μl of DAPI (1 μg/ml) or Hoechst 33342 (5 μg/ml) at room temperature for 5–15 min (accurate time needs to be determined; this varies on the cells used for the screen and the imaging system) followed by a wash with DPBS.
10. Add 50 μl DPBS:glycerol (50%:50%) per well, seal plates, and store plates at 4 °C until image scanning.

3.3. Automated image scanning and quantitative image analysis

Image scanning can be performed on a number of devices equipped to handle high-throughput and high-content microscopy, such as the Arrayscan VTI (Cellomics, Thermo Scientific) or the Image Xpress Ultra

Confocal Imaging System (Molecular Devices). However, before high-throughput and high-content image scanning of any screen trade-offs need to be made regarding the number of optical planes, number of fields per well, channels acquired per exposure, the number of cells required to reasonably determine a significant effect on the cell population, and the time required for acquisition. Since image data are extremely information rich adequate storage capacity in the terabyte range, data backup and database tools are a crucial prerequisite.

Automated image scanning is key to reproducible and reliable quantitative measurements in high-throughput and high-content microscopy. Quantitative image analysis is typically done either by image segmentation, by supervised learning algorithms or a combination of the two methods. Most commercial image analysis software, such as ImageXpress (Molecular Devices), MetaXpress (Molecular Devices), or BioApplications (specific modules for distinct applications, Cellomics, Thermo Scientific), come with various modules that allow for image segmentation and pattern recognition, including determination of cell and nuclear boundaries and quantification of fluorescence in predetermined regions. For a more sophisticated discussion of ''computational image analysis for quantitative phenotyping'' and ''statistical methods for analysis of high-throughput RNAi'' read the reviews by Conrad and Gerlich, The RNAi Global Initiative, and references therein (Birmingham *et al.*, 2009; Conrad and Gerlich, 2010).

Although the rigorous parameter evaluation and optimization process should eliminate that cell boundaries of neighboring cells cannot be easily determined and distinguished, we have found the following method quite useful to work around such a problem. Nuclei, which are surrounded by cytoplasm, can easily be distinguished and marked, therefore identification of cytoplasmic regions around the nucleus can be inferred by using a fixed radius/pixel size spanning from the nuclear boundary. This approach allows for the gathering of fluorescent information of cytoplasmic reporters/markers used for subsequent quantification and analysis. This, however, comes at the cost of reduced signal since one excludes fluorescence in cytoplasmic regions outside this arbitrarily set radius.

4. Lentivirus-Based shRNA Knockdown System

The lentiviral-based shRNA technology has two important advantages over siRNA or esiRNA approaches. It allows for long-term gene repression as well as transduction of relative difficult to transfect cells, such as quiescent hematopoietic stem cells or primary neurons. Here, rather than describing large-scale shRNA screening approaches, we focus on shRNA as a tool for functional analysis on a gene-per-gene basis and as a confirmation strategy to validate or disproof hits obtained during siRNA or esiRNA screenings (Ivanova *et al.*, 2006).

4.1. Generation of pLKO.puro-IRES-eGFP (pLKO.pig) lentiviral vector

The pLKO.puro cloning vector (Fig. 18.2A), generated by the Broad Institute of MIT and Harvard, has been tested in a broad range of cells (Moffat *et al.*, 2006). This vector contains (1) the human U6 promoter, which drives expression of the shRNA transcript; (2) the human phosphoglycerate kinase (hPGK) promoter, which drives expression of the puromycin resistance gene ($Puro^R$); (3) a central polypurine tract (cPPT), which facilitates nuclear import of the vector preintegration complex thereby increasing transduction efficiency. The shRNA duplex is cloned between *Age*I and *Eco*RI restriction sites (Fig. 18.2). This vector is a replication-incompetent lentiviral vector and can be used to knockdown gene expression by direct transfection or by infection after conversion into lentiviral particles. In order to easily evaluate infection efficiency and monitor the

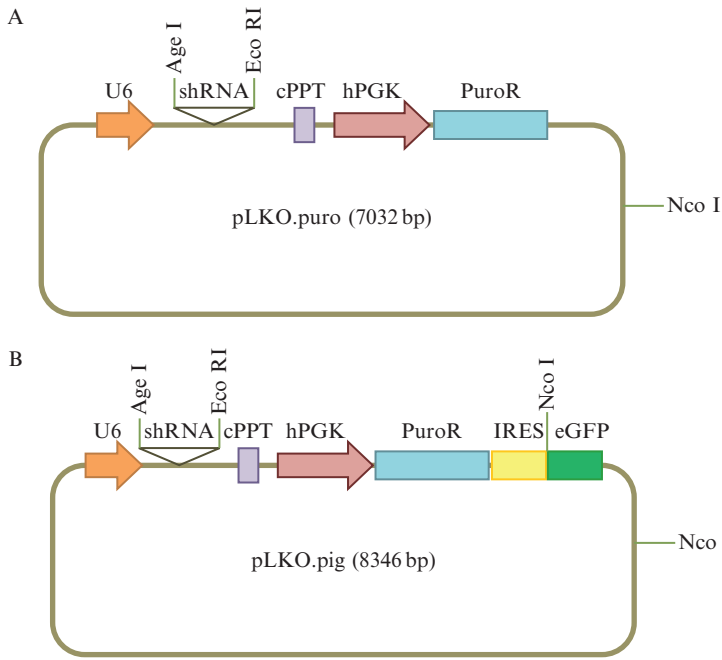

Figure 18.2 Schematic of pLKO.puro and pLKO.pig lentiviral vectors used in our studies. Both pLKO.puro (A) and pLKO.pig (B) vectors contain a U6 promoter driving shRNA, a cPPT, and two multiple cloning sites (*Age*I and *Eco*RI) for inserting annealed shRNA oligos (Fig. 18.1). While the hPGK promoter drives expression of $Puro^R$ in pLKO.puro (A), it drives expression of the $Puro^R$-IRES-eGFP cassette in pLKO.pig (B). The *Age*I, *Eco*RI, and *Nco*I restriction enzyme sites used for shRNA cloning and diagnostic analysis are indicated.

potential phenotypic changes after transfection or infection, we modified the original pLKO.puro plasmid and created pLKO.pig by inserting the internal ribosome entry site (IRES)-enhanced green fluorescent protein (eGFP) cassette after $Puro^R$ (Fig. 18.2B). This modification allows us to select transduced cells by $Puro^R$ selection and/or monitor eGFP expression.

4.2. Design and cloning of shRNA expression cassette into pLKO.pig lentiviral vector

There are several useful web sites to predict a suitable region in your interested gene for shRNA targeting. We opted for the shRNA tool designed by the RNAi consortium (TRC), based at the Broad Institute in Boston. TRC is a collaboration between six academic research institutions and five leading life sciences organizations aimed to generate two shRNAs/gene targeting all human and mouse genes. The following steps will guide through the design and cloning of shRNA targeting our gene of interest into pLKO.pig.

1. Design your shRNA forward and reverse oligos using the TRC shRNA library database (http://www.broadinstitute.org/rnai/public/gene/search). You can search either by gene or transcript IDs (NM_ or XM_ sequences) or by official gene symbols. For instance, if you search for Nanog (Official Gene Symbol) or NM_028016 (Reference Transcript ID) for the mouse Nanog gene, the database search will return five shRNAs. Each shRNA duplex consists of two different oligos, a forward oligo of 5′-CCGG-21 bp sense-CTCGAG-21 bp antisense-TTTTTG-3′) and a reverse oligo (5′-AATTCAAAAA-21 bp sense-CTCGAC-21 bp antisense-3′). The CTCGAG sequence, an *Xho*I restriction enzyme site, is designed to form a loop structure between the sense and antisense strand of the shRNA.
2. Order oligos from any commercial vendor and resuspend them in RNase/DNase-free molecular grade water (MediaTech Inc., Cat. no. 46-000-CM) at a concentration of 20 μM. Prepare oligo duplex mixture by mixing of 5 μl forward oligo with 5 μl of reverse oligo, 5 μl 10× NEB buffer 2, and 35 μl ddH_2O.
3. Incubate oligo duplex mixture in a beaker of boiling water for 5–10 min and then remove the breaker from the heating plate. Allow water to cool down to room temperate slowly in order to allow for the oligos to anneal. It will take several hours to cool down. Alternatively, annealing and cooling can be performed using a Thermocycler, using the cycle ramp step. Use a decline of 1 °C/cycle until you reach 25 °C with a cycle time of 1 min.
4. Digest pLKO.pig vector with *Age*I and *Eco*RI. Usually, we use 6 μg pLKO.pig plasmid, 7 μl of 10× NEB buffer 1, 6 μl of *Age*I (NEB,

Cat. no. R0522L, 5000 U/ml), 4 μl of *Eco*RI (NEB; Cat. no. R0101L, 20,000 U/ml), and add ddH_2O to 70 μl. Incubate the digestion reaction at 37 °C for 1 h 40 min. (*Note*: A longer incubation time will cause overdigestion and result in lower ligation efficiency.)

5. Run the digested pLKO.pig vector (~8 kb) on a 0.8% agarose gel, purify plasmid by QIAquick gel extraction kit (Qiagen; Cat. no. 28706), and resuspend extracted plasmid in 30 μl elution buffer, which is provided in the QIAquick gel extraction kit.
6. Ligate annealed oligos with digested vector by using 2 μl of annealed oligos, 1 μl of digested pLKO.pig plasmid, 2 μl of 10× NEB T4 DNA ligase buffer, 2 μl of NEB T4 DNA ligase (NEB, Cat. no. M0202L, 400,000 cohesive end U/ml), and 13 μl of ddH_2O. Incubate the ligation product at room temperate for 2 h.
7. Transform 5 μl of ligation product into 50 μl of DH5α competent cells and plate bacteria on LB agar containing 50 μg/ml ampicillin.
8. Pick up the clones and isolate plasmids by QIAprep spin miniprep kit (Qiagen; Cat. no. 27106).
9. Check plasmid by digesting with *Eco*RI and *Nco*I restriction enzyme. Clones containing a correct pLKO.pig-shRNA should have three fragments of 5059, 1980, and 1307 bp.
10. Select positive plasmids for DNA sequencing. The sequence primer used for pLKO.pig is 5′-CAAGGCTGTTAGAGAGATAATTGGA-3′, which is the same sequencing primer used for pLKO.puro. (*Note*: It is important to sequence your shRNA insert using a sequencing protocol that takes the hairpin configuration into consideration. We routinely sequence our shRNA plasmid using the services provided by Genewiz Inc.)

4.3. Generation and concentration of lentiviral particles

Generation of lentiviral particles should be done using appropriate protective gear/clothing (lab coat gloves) in a biohazard hood and in accordance with Federal Institutional Biohazard Committee guidelines. All material and reagents that have been in contact with viral particles ought to be soaked in 10% bleach for a minimum of 20 min before being discarded in biohazard bags and autoclaved.

1. Sixteen to 24 h before transfection, plate 2×10^6 human embryonic kidney (HEK) 293T cells in a 10-cm tissue culture plate using 10 ml regular culture medium without antibiotics (DMEM high glucose supplemented with 10% FBS, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate).
2. On the day of transfection, carefully aspirate medium and add 3 ml fresh Opti-MEM I reduced-serum medium (Opti-MEM; Invitrogen;

Cat. no. 11058-021) gently. (*Note*: In order to prevent detaching or washing away of 293T cells, do not wash 293T cell with DPBS.)

3. Mix 8 μg pLKO.pig, 6 μg packaging plasmid pCMV-dR8.2 dvpr (Addgene plasmid #8455), and 2 μg VSV-G encoding plasmid pCMV-VSV-G (Addgene plasmid #8454) in 300 μl Opti-MEM. Vortex briefly and incubate DNA mixture at room temperature for 15 min. In the meantime, dilute 22 μl of SuperFect Transfection Reagent (Qiagen; Cat. no. 301305) in 300 μl Opti-MEM and incubate at room temperature for 15 min.
4. Add DNA mixture to SuperFect and incubate at room temperature for 30 min.
5. Add DNA–SuperFect mixture dropwise to culture plate and incubate at 37 °C in a humidified environment and 5% CO_2.
6. After 3 h add 10 ml of fresh regular culture medium with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).
7. After an additional 12–16 h, replace medium with 10 ml fresh ESC culture medium without LIF and grow cells at 37 °C, 5% CO_2 in a humidified environment for an additional 36–48 h. During this time, cells will produce and secrete lentiviral particles.
8. Collect viral supernatant and remove cell debris by filtration through a 0.45 μm low protein binding filter (Corning Life Sciences, Cat. no. 0976155).
9. Filtered viral particles can be used immediately or concentrated. For concentration, add viral supernatant to the top portion of an Amicon Ultra-15 Centrifuge filter units with ultracel membrane (Millipore; Cat. no. UFC903024) and concentrate viral volume to 0.5 ml by spinning at 1600×*g* at 4 °C for 15–20 min.
10. Use viral particles either right away or store at −80 °C until use.

4.4. Infection of mouse ESCs

The same safety rules described above apply here.

1. Seed 5×10^4 CCE cells into a well of the gelatin-precoated 12-well tissue culture plate and grow for 12–16 h in a humidified environment at 37 °C/5% CO_2.
2. Cells should reach approximately 50–60% confluency in the 12–16 h of grow. Only then aspirate medium and replace with 0.5 ml fresh ESC culture media containing LIF and 16 μg/ml polybrene (officially known as hexadimethrine bromide, Sigma; Cat. no. H9268).
3. Infect cells by addition of 0.5 ml of concentrated viruses. The final polybrene concentration is 8 μg/ml.
4. Twenty-four to 26 h postinfection, replace media with 1 ml fresh ESC culture medium.

5. The next day (day 2 postinfection), split cells into 1-well of a gelatin-precoated 6-well plate in 2 ml of total ESC medium and continue culture for 2 days. Change medium daily.
6. On day 4 postinfection, split cells at a ratio of 1:10 into 1-well of a 6-well plate and culture for an additional 2 days with daily medium changes.
7. Six days after infection, select lentivirus-infected cells by replacing medium with ESC medium supplemented with 2 μg/ml puromycin (Sigma; Cat. no. P8833) (Fig. 18.3). (*Note*: The optimal puromycin concentration needs to be predetermined for each cell type/line. We find that 2 μg/ml puromycin kills all CCE and NG4 mouse ESCs within 2–3 days.)
8. Replace medium with fresh puromycin-containing medium for the next 2 days. Ideally, all surviving cells should be GFP positive, which also indicates that every selected cells harbor the shRNA expression cassette.

4.5. Quantification of shRNA-mediated gene silencing efficiency

It is important to determine the efficiency of the lentivirus-mediated mRNA knockdown in order to evaluate the physiological effect of silencing of your gene of interest. Several distinct methods can be applied to verify knockdown efficiency. These include real-time RT-PCR, Northern blotting, and Western blotting analyses. In order to exclude possible off-target effects of the designed shRNA, one may design two or more different shRNAs targeting a single target. Similar phenotypic changes by these individual shRNAs targeting the same gene, including similar changes in gene-expression profiles, reduce the likelihood of an off-target effect. The best way to exclude possible off-target effects is to reexpress an shRNA-immune version of the target gene which should rescue gene expression and

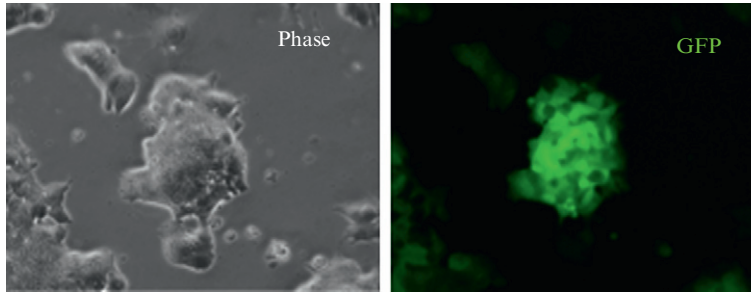

Figure 18.3 ESCs infected with shRNA-expressing pLKO.pig lentiviruses. Mouse CCE ESCs were infected with pLKO.pig lentiviral particles and selected with puromycin (2 μg/ml). After puromycin selection, almost 100% of cells express GFP.

the phenotypic changes (Ivanova *et al.*, 2006). The straightforward rescue strategy of overexpressing the coding sequence (CDS) can be applied if the shRNA targets untranslated regions in the target mRNA. Should the shRNA however target the CDS, it will be necessary to generate a rescue clone containing silent mutations in the region of the CDS targeted by the shRNA.

5. Concluding Remarks

The last few years have revealed many novel insights into the regulation of ESC self-renewal and pluripotency using high-throughput technologies, including global mRNA expression analysis, protein abundance measurements using mass spectrometry, genome-wide mapping of protein–DNA interaction and histone modifications. The wealth of this information provides us with a ''parts list'' or signature of pluripotent stem cells. With the discovery of RNAi and the continued development of improved RNAi systems, the functional analysis of genes has become relatively easy. RNAi also enables us to perform for the first time, unbiased screens targeting gene families or the entire genome in a short period of time. Finally, the use of siRNA or esiRNA, and shRNAs should be viewed as complementary approaches in the application of RNAi as a functional genetic tool in mammalian cells.

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