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Use of genome-wide RNAi screens to identify regulators of embryonic stem cell self-renewal and pluripotency

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Summary

Embryonic stem cells (ESCs) are characterized by two defining features: pluripotency and self-renewal. They hold tremendous promise for both basic research and regenerative medicine. To fully realize their potentials, it is important to understand the molecular mechanisms regulating ESC pluripotency and self-renewal. The development of RNA interference (RNAi) technology has revolutionized functional genetic studies in mammalian cells. In recent years, genome-wide RNAi screens have been adopted to systematically study ESC pluripotency and self-renewal, and have uncovered many previously unknown regulators, including transcription factors, chromatin remodelers, and post-transcriptional modulators. Here, we describe a method for the identification of regulators of ESC pluripotency and self-renewal using RNAi screens, as well as assays for further validation and characterization of the identified candidates. With modifications, this method can also be adapted to study the fate specification events during ESC differentiation.

Keywords

Embryonic Stem cell; Self-Renewal; Pluripotency; RNAi; Genome-wide; Genetic Screen; Reporter Assay

1. Introduction

ESCs are derived from the inner cell mass of the blastocyst stage embryos. They have two distinctive characteristics: the ability to differentiate into any cell type of the three germ layers, known as pluripotency, and the ability to proliferate while maintaining the pluripotent state, known as self-renewal. Because of these properties, ESCs present a unique opportunity to advance many aspects of biology and medicine, such as mammalian development, disease modeling, drug screening, and stem cell therapies (1–3). To successfully use ESCs for these research and clinical applications, it is critical to understand the mechanisms that control their pluripotency and self-renewal. A mechanistic model of pluripotency and self-renewal will help to elucidate the molecular pathways involved in early embryonic development. It will also facilitate the efficient derivation, maintenance, and expansion of pluripotent stem cells, and provide guidance to the generation of desired cell types for therapeutic purposes. It is known that ESC pluripotency and self-renewal is

governed by the combination of signal-transduction pathways, transcription factors, epigenetic modifiers, and post-translational regulators (4). However, until recently, most of the current knowledge came from early studies that relied on candidate approaches or bootstrapping strategies, and novel classes of important self-renewal genes are continued to be discovered. Recent advances in RNAi technologies made it possible to interrogate gene function by loss-of-function screens on a genome-wide scale. To systematically study pluripotency and self-renewal, we and others have carried out large-scale RNAi screens in ESCs using various readouts from cell morphology, proliferation, to reporter assays (5–11). These screens identified many novel genes that play critical roles in self-renewal, as well as several protein complexes such as the Ccr4-Not, Tip60-p400, Paf1, and cohesion-mediator complexes (6–8, 11, 12). The success of these screens illustrates the power of RNAi and forward genetics in the study of pluripotency and self-renewal, and paves a path for functional genetic studies of ESC fate-specification in the future.

Here, we describe the method we employed for the genome-wide RNAi screen in ESCs. In the initial screen, a fluorescence reporter assay based on the Oct4GiP cells is used to determine the consequence of silencing individual genes on self-renewal. The Oct4GiP cells express the enhanced green fluorescent protein (EGFP) under the control of the Oct4 gene promoter. Oct4 is highly expressed in ESCs and quickly down-regulated during differentiation. As a result, EGFP expression in the Oct4GiP cells faithfully correlates with the ESC state, and fluorescence-activated cell sorting (FACS) analysis can be used to determine the self-renewal status of the cells at the single cell level. With this assay, the function of each gene in self-renewal and pluripotency can be quickly assessed by RNAi using a genome-wide siRNA library. Genes whose silencing leads to the loss of the reporter expression are likely to have important roles in ESCs and can be quickly identified. Finally, additional assays such as the alkaline-phosphatase (AP) staining and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) of lineage markers are used to further confirm and characterize the screen hits. The method described here can be carried out either manually or with automation by the liquid-handling instruments for increased throughput. With modifications, such as using different reporter cell lines and culture conditions, it can also be adapted to screen for genes involved in other aspects of ESC fate specification.

2. Materials

2.1 Mouse ESC culture

1. Water-jacketed CO₂ tissue culture incubator (Thermo)
2. Tissue culture hood (biological safety cabinet)
3. Tissue culture plates or flasks
4. 0.1% gelatin (Sigma) in water
5. PBS without Ca or Mg
6. 0.05% trypsin
7. Hemocytometer

8. M15 medium: DMEM (Invitrogen) supplemented with 15% ES-qualified fetal bovine serum (FBS), 1000 U/ml ESGRO (Millipore), 1× Non-essential amino acids (Invitrogen), 1× EmbryoMax Nucleosides (Millipore), and 10 μ M β -mercaptoethanol.

2.2. Genome-wide RNAi Screen in ESCs

2.2.1. siRNA transfection

1. 384-well PCR plates (Thermo Scientific)
2. 384-well pipette tips (Thermo Scientific)
3. Flat-bottom 384-well tissue culture plates (Corning)
4. 2–10, 5–50ul, 50–300ul Multi-channel pipette (Thermo Scientific)
5. Multi-channel aspirator (Corning) or vacuum wand (VP-Scientific).
6. Microplate dispenser (Thermo Scientific Wellmate microplate dispenser or equivalent)
7. Liquid handling system (Agilent Velocity 11 or equivalent)
8. OptiMEM (Invitrogen)
9. Lipofectamine 2000 (Invitrogen)
10. Genome-wide siRNA library (Thermo Scientific mouse siGenome library or equivalent)
11. M15 medium

2.2.2 FACS analysis

1. Multi-channel aspirator (Corning) or vacuum wand (VP-Scientific).
2. 2–10, 5–50ul, 50–300ul Multi-channel pipette (Thermo Scientific)
3. BD LSRII FACS analyzer with the HTS unit or other similarly equipped FACS analyzer
4. Reagent reservoirs (Thermo Scientific)
5. 0.25% trypsin
6. PBS + 10% FBS

2.3. Hit validation

2.3.1. Alkaline phosphatase staining

1. Zeiss Axiovert 40 CFL with AxioCam MRC Camera or equivalently equipped microscope
2. 96-well and 24-well tissue culture plates (Corning)
3. AP staining kit II (STEMGENT)

4. PBST: 1× PBS, 0.05% Tween 20

2.3.2. RT-qPCR analysis

1. CFX384 real-time thermal cycler (Bio-Rad or equivalent)
2. 96-well and 24-well tissue culture plates (Corning)
3. 96 wells or 384 wells PCR plate (Bio-Rad)
4. Aurum Total RNA Mini Kit (Bio-Rad or other RNA extraction kits)
5. iScript cDNA synthesis kit (Bio-Rad or other reverse transcription kits)
6. SsoFast EvaGreen Supermix (Bio-Rad or other SybrGreen qPCR mix)

3. Methods

The screen is carried out in three phases: the primary screen, the secondary screen, and hit validation (Fig. 1A). In the primary screen, the Oct4GiP ESCs are transfected with the SMARTpool siRNAs from the mouse siGenome library in 384-well plates (Fig. 1B). The effect of individual gene silencing is examined by the Oct4GiP reporter assay to identify candidate genes that are important for pluripotency and self-renewal. In the secondary screen, the four individual siRNAs of the SMARTpools against the primary hits are re-screened with the reporter assay, and only genes that score again are considered positive hits. Finally, in hit validation, the gene silencing efficiency for the siRNAs identified in the secondary screen is verified, and the positive hits are tested with two additional assays, the AP staining and the lineage marker expression analysis, to further confirm their roles in ESCs.

In all the steps described below, follow the general guidelines for good cell culture practice to avoid contaminating the cells.

3.1 Oct4GiP ESC culture

1. Coat tissue culture plates with 0.1% gelatin: Add the 0.1% gelatin solution (0.1 ml gelatin solution/cm²) to 10-cm tissue culture plates or other tissue culture vessels of choice. Incubate at room temperature for 30 minutes or longer.
2. Plate Oct4GiP ESCs: Remove the gelatin solution and plate $\sim 2 \times 10^4$ /cm² Oct4GiP cells in 10 ml of the M15 medium in each 10-cm plate. Culture the cells in a tissue culture incubator at 37 °C, 5% CO₂. This step can be scaled up or down based on the number of cells needed.
3. Change the medium every day.
4. Split the cells every two-three days (*see* Note 1): Remove medium. Rinse cells with PBS, and dissociate the cells with 1 ml 0.05% trypsin for each 10-cm plate at room temperature for ~ 5 min. Neutralize the trypsin by adding fresh M15 medium, 5 mls for each 10-cm plate, and dissociate cells into single cell suspension by repeated

¹Do not let ESC culture become over-confluent, as over-confluency can result in increased ESC differentiation.

pipetting (*see* Note 2). Collect cells in a 15-ml conical tube by centrifugation at 1000 rpm for 5 min. Remove supernatant and resuspend the cells in fresh M15 medium. Count the cells on a hemocytometer or with an automated cell counting device, and plate in 10-cm plates as described above.

3.2 Genome-wide RNAi Screen in Oct4GiP ESCs

3.2.1 siRNA transfection

1. Transfection mixture assembly: Prepare a master mix of OptiMEM and Lipofectamine 2000 at a ratio of 80:1 (vol/vol). Aliquot 10 μ l of the master mix to each well of the 384-well PCR plates and incubate at room temperature for 5 min. Add 2 pmol siRNA from the siRNA library working stock to the OptiMEM-Lipid mixture in each well (*see* Note 3, ⁴), and incubate for another 15 minutes to allow the formation of the siRNA-Lipid complexes. This step can be carried out either manually or with robotic automation depending on the scale of the screen (*see* Note 5, ⁶).
2. Transfection of the Oct4GiP cells: Coat 384-well tissue culture plates with 0.1% gelatin before assembling the transfection mixture. Harvest the Oct4GiP cells and resuspend in fresh M15 medium to 7×10^4 cells/ml. Remove gelatin from the 384-well plates, and aliquot 30 μ l of the cell suspension to each well (*see* Note 7). Transfer the siRNA-Lipid mixture to the cell suspension in each well and mix by pipetting up and down 3–5 times. Use new pipette tips for each plate of transfections. Move the plates to the tissue culture incubator.
3. Medium change: Change medium every 24 hrs. Remove medium using the multi-channel aspirator or vacuum wand, and add 40 μ l fresh M15 medium. Be careful not to scratch the cells at the bottom of the plates. Return the plates to the incubator.

3.2.2. FACS analysis

1. Cell dissociation: Remove medium and rinse the cells with PBS. Add 10 μ l 0.25% trypsin to each well in 384-well plate and incubate at room temperature for ~5 min with occasional agitation. Visually inspect to ensure complete detachment of the cells. Add 30 μ l of PBS with 10% FBS to neutralize the trypsin and dissociate cells into a single cell suspension by repeated pipetting. Prepare one plate at a time to avoid cell aggregation and keep the plate on ice until ready for FACS analysis.

²Do not over-trypsinize ESCs to avoid clumping and loss of cell viability. Completely dissociate the cells into single cell suspension before plating to avoid heterogeneity in colony size and quality during subsequent cultures.

³To reduce the edge effect, fill the edge wells in the 384-well plates with PBS and do not use them for the screen.

⁴In each 384-well plate, assign designated wells for the following controls (two wells for each control): lipids-only, non-targeting siRNA, *Oct4* siRNA, *Plk1* siRNA. The lipids-only and non-targeting siRNA wells serve as negative controls. The *Plk1* well serves as a positive control for the transfection, as *Plk1* is essential and its down-regulation causes cell death that can be easily detected. The *Oct4* well serves as a positive control for the screen, as *Oct4* is required for ESC pluripotency and self-renewal.

⁵For large-scale screens, the transfection step is usually carried out with liquid handling systems and dispersers such as the Velocity 11 and the Wellmate. For small to medium scale screens, it can be performed by manual pipetting with multi-channel pipettes.

⁶It is recommended that the siRNA screen is carried out in duplicate or triplicate to reduce the false-positive rate.

⁷For siRNA transfections, the optimal cell plating density is $\sim 2 \times 10^4$ cells/cm² in general. But this number may require additional optimization by titration. Low plating density usually leads to poor cell survival during transfection, and high plating density causes high background due to increased spontaneous differentiation.

2. FACS analysis: Analyze the cells on the BD LSRII FACS analyzer using the HTS unit. Adjust PMT voltage and threshold to correctly capture the cells in the forward vs. side-scatter plot. Set the HTS unit in high-throughput mode and analyze 10 μ l of cell suspension from each well (*see* Note 8). Prepare the next plate during each run.
3. Data analysis: Use the lipid-only or control-siRNA transfected cells as controls to set the gates for the analysis. First, gate for the live cell population in the forward vs. side-scatter plot. Next, gate for the GFP-negative cells in the GFP channel: set the gate so that ~10% of the cells appear to be GFP-negative in the controls (Fig. 2A). Determine the percentage of GFP-negative cells in each well (Fig. 2B). In the primary screen, genes are scored as positive hits if the corresponding Smartpool siRNA increased the percentage of GFP-negative cells by two standard deviations from the plate average (Fig. 1B). In the secondary screen, genes are scored as positive hits if any of the individual siRNAs increased the percentage of GFP-negative cells by two standard deviations from the average of the control wells (*see* Note 9).

3.3 Hit validation

3.3.1. Analysis of the gene silencing efficiency

1. siRNA transfection: Coat 24-well tissue culture plates with 0.1% Gelatin. Assemble the siRNA-Lipid complexes similarly as described above, by mixing 50 μ l of OptiMEM, 1.5 μ l Lipofectamine 2000, and 25 pmol siRNA (siRNAs against the positive hits from the secondary screen) in 96-well U-bottom plates. Include lipids-only and non-targeting siRNAs as controls. Aliquot 0.5 ml Oct4GiP cells at 2×10^5 cells/ml in M15 medium in each well of the gelatin-coated 24-well plates, and add the siRNA-Lipid mixture to the cells. Mix well and transfer the plates to the incubator. Change medium the next day.
2. RNA extraction and reverse transcription: 48 hrs after transfection, remove the medium from the cells, and lyse cells directly with the lysis buffer from the Aurum Total RNA Mini Kit. Extract total RNAs from the transfected cells according to the instructions of the kit, and use 1 μ g of the total RNA to set up reverse transcription with the iScript cDNA synthesis kit.
3. Real-time quantitative polymerase chain reaction (RT-qPCR): Design RT-qPCR primers for the target genes using online tools such as the Primer3Plus. Add the cDNAs, primers, and the Ssofast Evagreen supermix in 384-well RT-PCR plates and perform PCR in the real time thermocycler. Use housekeeping genes such as β -*Actin* or *Gapdh* for normalization, and determine the gene silencing efficiency by comparing the relative expression of the target genes in cells transfected with lipids-only or non-targeting siRNAs to those transfected with gene-specific siRNAs. Effective siRNAs should lead to greater than 60% reduction in target gene expression (Fig. 3A).

⁸It takes about 1 hour to complete the FACS analysis for each 384-well plate.

⁹Reporter assays based on other reporter ESCs, such as the Nanog-GFP cells, may be used after the secondary screen to quickly validate and further narrow down the positive hits.

3.3.2. AP staining and lineage marker expression analysis

1. siRNA transfection: Coat 96-well tissue culture plates with gelatin. Assemble the siRNA-Lipid complexes similarly as described above using 10 ul of OptiMEM, 0.6 ul Lipofectamine 2000, and 10 pmol siRNA in 96-well U-bottom plates, and include lipids-only and non-targeting siRNAs as controls. Aliquot 100 ul Oct4GiP cells at 4×10^5 cells/ml in M15 medium in each well of the gelatin-coated 96-well plates, and add the siRNA-Lipid mixture to the cells. Mix well and transfer the plates to the incubator.
2. Re-plating the cells: The next day, remove the medium and rinse the cells in the 96-well plates with PBS. Add 25 ul 0.25% trypsin to each well and incubate at room temperature for ~ 5 min. Neutralize trypsin with 100 ul M15 medium in each well, and dissociate cells into single cell suspension with repeated pipetting. From each well, transfer 60 ul of the cell suspension to one well of a gelatin-coated 24-well plate, and transfer the other 60 ul to another 24-well plate, generating two replicates from each transfection (*see* Note 10). Add 0.5 ml/well M15 medium to the 24-well plates. Mix well and transfer the plates to the incubator. Culture the cells for a total of 3 days from the day of re-plating in the M15 medium with medium change every day.
3. AP staining: Four days after transfection, use one set of the replicate 24-well plates for AP staining. Carry out the staining with the AP staining kit II from STEMGENT. Take pictures of the stained cells using the Zeiss Axiovert 40 CFL microscope equipped with the AxioCam MRC Camera. Cells transfected with lipids-only or non-targeting siRNA should show typical ESC morphology as compact and dome-like colonies with strong AP-staining. Those transfected with siRNAs against pluripotency genes should differentiate and appear flat and dispersed with much reduced staining (Fig. 3B).
4. Analysis of lineage marker expression: Extract total RNA from the cells in the other set of the replicate 24-well plates using the Aurum Total RNA Mini Kit. Prepare cDNAs from 1 ug total RNA with the iScript cDNA synthesis kit. Carry out RT-qPCR for pluripotency markers such as *Oct4*, *Nanog*, *Sox2* and differentiation markers such as *Cdx2*, *Gata3*, and *Brachyury*, using housekeeping genes such as β -*Actin* or *Gapdh* for normalization. Cells transfected with siRNAs against pluripotency genes should show reduced expression of the pluripotency markers and/or increased expression of the differentiation markers (Fig. 3C).

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¹⁰For genes that dramatically affect cell growth or viability, set up the initial transfections in multiple wells and pool them for re-plating to compensate for cell loss.

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Figure 1. The overall procedure for the genome-wide RNAi screen in ESCs

(A) In the primary screen, the Oct4GiP ESCs are transfected with the siGenome SMARTpool siRNAs in 384-well plates. Four days after transfection, the primary hits are identified by FACS using the Oct4GiP reporter assay. In the secondary screen, the cells are transfected with individual siRNAs against the primary hits, and genes corresponding to the siRNAs scored again are considered confirmed hits. Additional assays such as knockdown efficiency verification, alkaline phosphatase staining, and lineage marker expression analysis are carried out to further validate the hits. (B) A representative result of the Oct4GiP reporter assay for one 384-well plate from the primary screen. Red: primary hits; Green: negative-controls included on the plate (non-targeting siRNAs); Grey: other genes on the plate. Red line: two standard deviations from the plate mean.

Figure 2. The Oct4GiP reporter assay

Oct4GiP ESCs are transfected with the Control-, Cnot1-, Cnot2-, or Cnot3-siRNAs in 96-well plates and FACS analyzed 4 days after transfection. (A) Live cells are first gated in the forward vs. side scatter plots, and % GFP-negative cells are then determined from the histograms of the GFP-channel. (B) % GFP-negative cells from independent biological replicates are plotted as mean $\pm$ SEM ($n = 6$).

Figure 3. Validation of the screen hits

(A) Oct4GiP cells are transfected with the indicated siRNAs and Cnot1, Cnot2, and Cnot3 mRNA expression are determined by RT-qPCR 2 days after transfection. (B) Oct4GiP cells are transfected with the indicated siRNAs and re-plated the next day. Cells are stained by AP staining 4 days after transfection (C), and the expression of lineage markers are determined RT-qPCRs (D).