Video Article

Oct4GiP Reporter Assay to Study Genes that Regulate Mouse Embryonic Stem Cell Maintenance and Self-renewal

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Abstract

Pluripotency and self-renewal are two defining characteristics of embryonic stem cells (ES cells). Understanding the underlying molecular mechanism will greatly facilitate the use of ES cells for developmental biology studies, disease modeling, drug discovery, and regenerative medicine (reviewed in [1,2]).

To expedite the identification and characterization of novel regulators of ES cell maintenance and self-renewal, we developed a fluorescence reporter-based assay to quantitatively measure the self-renewal status in mouse ES cells using the Oct4GiP cells [3]. The Oct4GiP cells express the green fluorescent protein (GFP) under the control of the Oct4 gene promoter region [4,5]. Oct4 is required for ES cell self-renewal, and is highly expressed in ES cells and quickly down-regulated during differentiation [6,7]. As a result, GFP expression and fluorescence in the reporter cells correlates faithfully with the ES cell identity [5], and fluorescence-activated cell sorting (FACS) analysis can be used to closely monitor the self-renewal status of the cells at the single cell level [3,8].

Coupled with RNAi, the Oct4GiP reporter assay can be used to quickly identify and study regulators of ES cell maintenance and self-renewal [3,8]. Compared to other methods for assaying self-renewal, it is more convenient, sensitive, quantitative, and of lower cost. It can be carried out in 96- or 384-well plates for large-scale studies such as high-throughput screens or genetic epistasis analysis. Finally, by using other lineage-specific reporter ES cell lines, the assay we describe here can also be modified to study fate specification during ES cell differentiation.

Video Link

The video component of this article can be found at https://www.jove.com/video/3987/

Protocol

1. Oct4GiP Mouse ES Cell Maintenance

Oct4GiP cells were kindly provided by Dr. Austin Smith. They were derived from the 129/Ola mice carrying an Oct4-GFPiresPac transgene [4,5] . They are maintained in gelatin-coated tissue culture plates in the ESGRO complete plus clonal grade medium (Millipore), or in the M15 medium: DMEM (Invitrogen) supplemented with 15% FBS, 1000 U/ml ESGRO (Millipore), 1x Non-essential amino acids (Invitrogen), 1x EmbryoMax Nucleasides (Millipore), and 10 μM β-mercaptoethanol.

1. Coat plates with 0.1% gelatin (Sigma) at room temperature for 30 minutes (0.1 ml gelatin solution/cm^2).
2. Plate Oct4GiP cells in gelatin-coated plates at ~2 x $10^4/cm^2$.
3. Split the cells every two days with 0.05% trypsin.

2. siRNA Transfection in Oct4GiP Cells

The Oct4GiP reporter assay is most conveniently carried out in gelatin-coated flat bottom 96- or 384-well plates (Corning or BD) in the M15 medium. The overall procedure is outlined in **Figure 1**.

1. siRNA-lipid complexes assembly:
    1. For transfection in 96-well plates, assemble siRNA-lipid complexes in U-bottom 96-well plates.
    2. In each well, mix 10 μl OptiMEM (Invitrogen) and 0.3 μl Lipofectamine 2000 (Invitrogen) and incubate at room temperature for 5 min.
    3. Add 5 pmol siRNA to the lipid-OptiMEM mixture and incubate for another 15 minutes. The siRNA : lipid ratio is 100 pmol : 6ul.
    4. Transfer the siRNA-lipid complexes in OptiMEM from the U-bottom plate to the gelatin-coated flat-bottom plate with a multi-channel pipette.

5. For transfection in 384-well plates, prepare the siRNA-lipid complexes using 0.1 µl Lipofectamine 2000 and 2 pmol siRNA in 10 µl OptiMEM.

Note: The final siRNA concentration in the transfections is 50 nM. Carry out 3-4 biological replicates for each siRNA transfection. Set up master mixtures of the siRNA-lipid complexes in the U-bottom plate and aliquot into the flat-bottom gelatin-coated plate.

2. Oct4GiP cell plating and transfection:
    1. For transfection in 96-well plates, collect the Oct4GiP cells and resuspend them in fresh M15 medium to 9 x 10^4 cells/ml.
    2. Add 100 µl of the cell suspension to each well using a multi-channel pipette.
    3. Mix the cell suspension with the pre-aliquoted siRNA-lipids complexes in the well by pipeting up and down 3-5 times. Change tips for different siRNA transfections.
    4. For Transfection in 384-well plates, resuspend Oct4GiP cells to 6 x 10^4 cells/ml and aliquot 30 µl cell suspension to each well.

Note: The optimal cell plating density is usually 3 x 10^5 cells/cm^2, but it may require further optimization for siRNAs that dramatically affect cell growth or viability. Low plating density will lead to poor cell survival during transfection. High plating density will lead to high background due to high cell confluence induced differentiation. If necessary, test plating density between 2-4 x 10^5 cells/cm^2.

3. Medium change:
    1. Remove old medium using multi-channel aspirator (Corning) or vacuum wand (VP-Scientific). Be careful not to scratch the cells at the bottom of the plates.
    2. Feed the cells with fresh ES cell medium (100 µl for 96-well plate and 30 µl for 384-well plate) using multi-channel pipette every day.

3. FACS Analysis

1. Cell dissociation:
    1. Four days after transfection, remove medium using multi-channel aspirator (Corning) or vacuum wand (VP-Scientific).
    2. For 96-well plates, rinse cells once with 100µl PBS.
    3. Add 25 µl 0.25% trypsin to each well using a multi-channel pipette.
    4. Incubate at room temperature for 5minutes with occasional agitation, and visually inspect to ensure complete detachment of the cells.
    5. Add 90 µl of PBS with 10% FBS to inactivate the trypsin and dissociate cells into a single cell suspension by repeated pipetting.
    6. For 384-well plates, disassociate cells with 10 µl trypsin and quench with 30 µl PBS with 10% FBS.

2. FACS analysis:
    1. Analyze the GFP fluorescence on the BD LSRII FACS analyzer equipped with the HTS unit or other similarly equipped FACS analyzer (such as Accuri or Intellicyt).
    2. Use the high-throughput mode on the HTS and analyze 10 µl of cell suspension. Set the count threshold to 1.0 x 10^4 cells.
    3. Adjust PMT voltage and threshold to capture the cells in the forward vs. side-scatter plot. Gate for the live cell population in the forward vs. side-scatter plot.
    4. Create a histogram plot for the GFP channel. Set the gate in the GFP channel so that ~10% of the cells appear to be GFP-negative in the mock or control-siRNA transfected cells.
    5. Determine % GFP-negative cells from each treatment: % Differentiated cells = % GFP-negative cells. Compare the % Differentiated cells between experimental- and control-siRNA transfections using 2-tailed t-test, and score the positive hits with p-values < 0.01.

4. Representative Results

Oct4, Nanog, and Sox2 are three genes that play critical roles in the maintenance of ES cell self-renewal [6,7,9-11]. **Figure 2** shows that the Oct4GiP reporter assay can readily detect the differentiation caused by silencing these factors in the Oct4GiP ES cells.

Figure 2A shows the Oct4GiP ES cells are GFP-positive when maintained as ES cells. **Figure 2B** shows the forward vs. side scatter plot and the histogram of the GFP channel of the Oct4GiP cells transfected with the control- or Oct4-siRNAs. It is necessary to gate for live cells in the forward vs. side scatter plot, as the dead cells and debris are GFP-negative and will increase background. On the other hand, doublet discrimination to exclude possible cell clumps is not always needed. In the control-siRNA transfected cells, the vast majority of the cells should be GFP-positive. If obvious GFP-negative populations are present in the control-siRNA transfected wells, the starting Oct4GiP cells or the transfection procedure may have been compromised and the result may not be interpretable.

Figure 2C shows the bar graph of % Differentiated cells (% Differentiated cells = % GFP-negative cells) from control-, Oct4-, Nanog-, and Sox2-siRNA transfected cells.

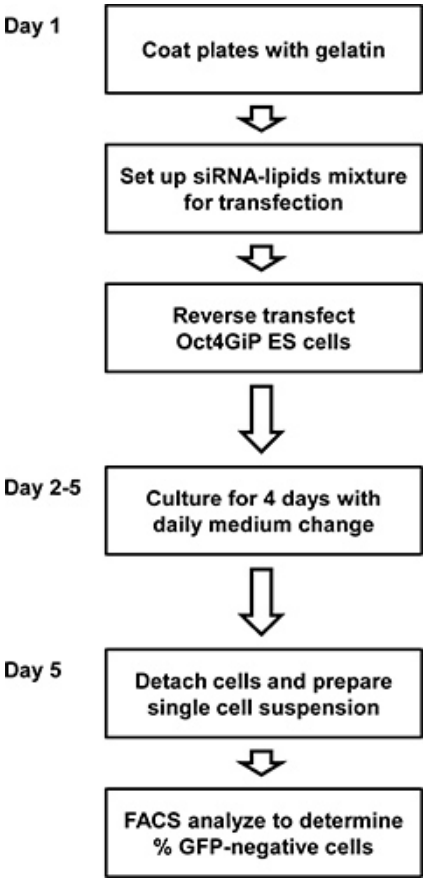

Figure 1. Outline of the Oct4GiP reporter assay.

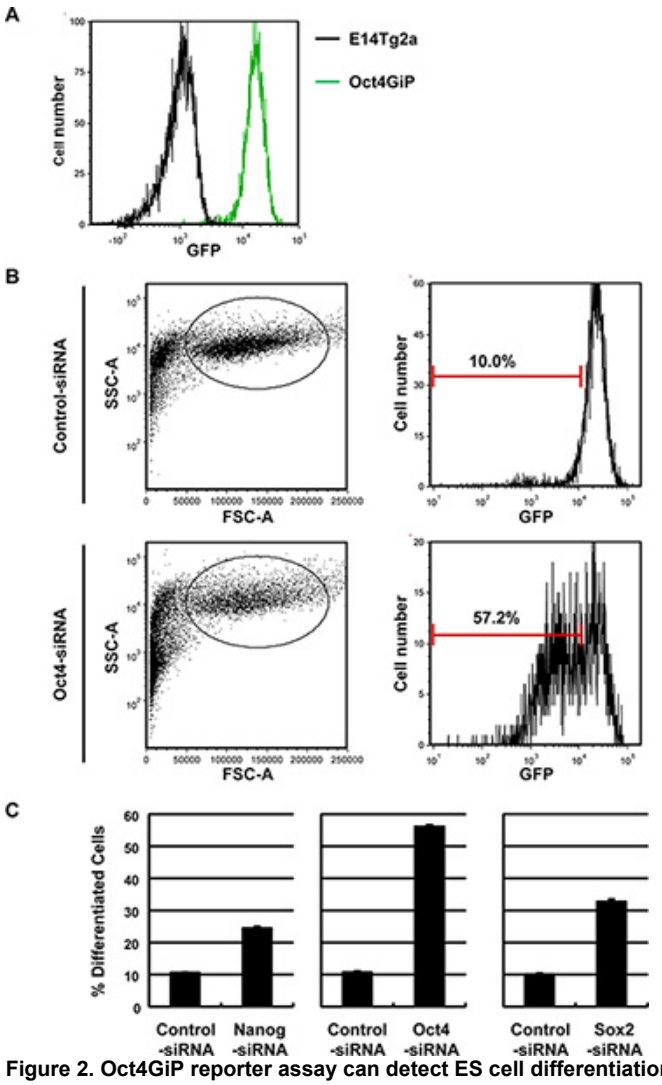

Figure 2. Oct4GiP reporter assay can detect ES cell differentiation caused by Nanog, Oct4, or Sox2 silencing. A) E14Tg2a (wild-type, black line) and Oct4GiP (green line) cells were analyzed by FACS. Histogram of the GFP-channel shows that Oct4GiP cells are GFP-positive. **B)** Oct4GiP cells were transfected in 96-well plates with the Control- or Oct4-siRNA and FACS analyzed 4 days after transfection. Forward vs. side scatter plots and histograms of the GFP-channel of the transfected cells are shown. **C)** Oct4GiP cells were transfected with the Control-, Nanog-, Oct4-, or Sox2-siRNA. The % Differentiated Cells was determined from the % GFP-negative cells 4 days after transfection, and was plotted as mean +/- standard error of the mean (n = 4). Click here to view larger figure.

Discussion

The Oct4GiP reporter assay we describe above can quantitatively measure the extent of self-renewal vs. differentiation. Compared to other available methods, such as the morphology-based [12] and proliferation/viability-based assays, it offers higher sensitivity and throughput, as well as a more direct measurement of the ES cell state. It is therefore well suited for large-scale screens and genetic epistasis analysis. Indeed, we and others have successfully used the Oct4GiP reporter assay for genome-wide RNAi screens [3,8]. Like all assays, however, it also has limitations. It can only be used to study genes that directly or indirectly regulate Oct4 promoter activity, and it may falsely identify genes that affect GFP expression, stability, or function post-transcriptionally. Therefore, additional assays or secondary screens, such as alkaline phosphatase staining [13] (Millipore, SCR004) and immunofluorescence staining or quantitative RT-PCR of pluripotency markers [3,8,12,14] are required to confirm results obtained from this method.

The Oct4GiP reporter assay relies on efficient gene silencing. Effective siRNAs and efficient siRNA transfections are key to the success of the assay. Although we only described use of the reporter assay with siRNA transfections, the assay can also be performed with DNA transfections to silence or overexpress genes of interest. DNA transfection efficiency is usually lower than that of siRNAs and may reduce the strength of the phenotype.

Besides what was described in this protocol, the Oct4GiP reporter assay can be modified for identifying and characterizing genes that negatively regulate self-renewal as well. In that case, the cells can be transfected with siRNAs and cultured in differentiation conditions. Silencing of negative regulators of self-renewal will enhance self-renewal and sustain GFP expression, which can be detected by FACS similarly as described in the current protocol.

Besides the Oct4GiP reporter cells, other reporter cell lines using ES cell specific promoters, such as the Nanog-GFP [15-18] (Millipore, SCR089) and Rex1-GFP [19] cells, have also been generated. They can also be used to study ES cell self-renewal and maintenance using the same strategy. However, because there is noticeable difference in the activity and specificity of these ES cell marker gene promoters, these reporter cell lines behave differently in terms of background level and sensitivity and will thus complement each other. Finally, by using other lineage-specific reporter ES cell lines, our strategy can be modified to study fate-specification of ES cells and facilitate the use of ES cell as an *in vitro* model for mammalian early development.

Disclosures

No conflicts of interest declared.

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