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Chapter 34

Using Endogenous MicroRNA Expression Patterns to Visualize Neural Differentiation of Human Pluripotent Stem Cells

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Abstract

Many existing protocols for neuronal differentiation of human pluripotent cells result in heterogeneous cell populations and unsynchronized differentiation, necessitating the development of methods for labeling specific cell populations. Here we describe how microRNA-regulated lentiviral vectors can be used to visualize specific cell populations by exploiting endogenous microRNA expression patterns. This strategy provides a useful tool for visualization and identification of neural progeny derived from human pluripotent stem cells. We provide detailed protocols for lentiviral transduction, neural differentiation, and subsequent analysis of human embryonic stem cells.

Key words: Embryonic stem cell, Induced pluripotent stem cell, MicroRNA, Neuronal differentiation, FACS, Immunohistochemistry, Lentiviral vector

1. Introduction

During the last decade, a number of protocols that enable efficient differentiation of human pluripotent stem cells into neurons have been developed (1–3). Given this, human pluripotent stem cells, such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have become a unique cell source for studying early human brain development. They may also serve as an unlimited source of therapeutically active cells for regenerative medicine. However, current protocols for deriving neurons from human pluripotent stem cells give rise to heterogeneous cell populations both in regard to the temporal aspects and the cellular composition. This complicates molecular analysis of specific cell populations and may be a large hurdle when using cells for transplantation since

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contaminating undifferentiated cells may give rise to unwanted proliferation and tumors upon transplantation (4).

To track differentiating cell populations, reporter cell lines generated by homologous recombination (knock-in) or *via* BAC transgenes have been widely used in mouse cells (5–8). These strategies provide a robust way to visualize and isolate specific cell populations of differentiated pluripotent stem cells. Although possible, these strategies are often complicated to transfer to human cells due to technical issues (9), and only a few successful cases have been described (10, 11). To circumvent these difficulties, we have employed an alternative strategy that exploits the cells' endogenous microRNA (miRNA) machinery (12). Our approach is simple to use and offers a reporter system that can be as reliable as BAC transgenesis or knock-in technology.

MicroRNAs are small noncoding RNAs of 21–23 nucleotides that negatively regulate gene expression by binding complementary mRNAs and inhibit subsequent protein expression (13). Our system is based on a lentiviral vector that encodes a fluorescent reporter gene (in this case GFP) and tandem target sites for an miRNA (14, 15). When a microRNA is present in the cell, it binds to the target sites and downregulates GFP expression, while in cells that do not express the microRNA GFP, it is expressed. Based on this strategy, we used miR-292. It is specifically expressed in pluripotent cells, therefore only allowing GFP expression in differentiated cells (12). However, the versatility of the system allows the use of any microRNA of choice, including neuron-specific microRNAs (14).

Here we describe how this system can be used for human embryonic stem cells. We provide detailed protocols for the generation of lentiviral vectors, the transduction of hES-cells, and their differentiation into neurons. Finally, we provide protocols for analyzing these cells through flow cytometry and immunocytochemistry.

2. Materials

All catalog numbers are European.

2.1. Lentiviral Production and Titering

1. Cell culture lab certified for lentiviral vector production and use.
2. Virkon and tissue culture hood with UV-light.
3. 15-cm tissue culture-treated plastic dishes.
4. Ultracentrifugation tubes (Beckman L 60).
5. 293T cells – must never be grown to confluency.
6. 0.05% Trypsin (diluted from Invitrogen, cat. no. 15090-046).

7. *293T growth medium*:

   DMEM (Invitrogen, cat. no. 61965)

   10% FBS (Invitrogen, cat. no. 26140-095)

   1% Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

8. *IMDM virus production medium*:

   IMDM (Invitrogen, cat. no. 31980)

   10% FBS (Invitrogen, cat. no. 26140-095)

   1% Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

9. 0.1× TE buffer (1 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). Sterile filter and store at 4°C.

10. 2.5 M $CaCl_2$ solution (Sigma-Aldrich, cat. no. C7902. Sterile filter and store at −20°C).

11. 2× HBS buffer (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.12). Sterile filter and store in aliquots at −20°C.

12. Plasmids for packaging of third-generation viral vectors (16):

    (a) pMD2.G envelope plasmid.

    (b) pMDL Gag-Pol plasmid.

    (c) pRSV-Rev reverse transcriptase plasmid.

    (d) Transfer vector plasmid with reporter gene (i.e., GFP) under control of a constitutively active promoter (i.e., PGK) containing microRNA target sequences in the 3′UTR (see Note 11 and Fig. 1).

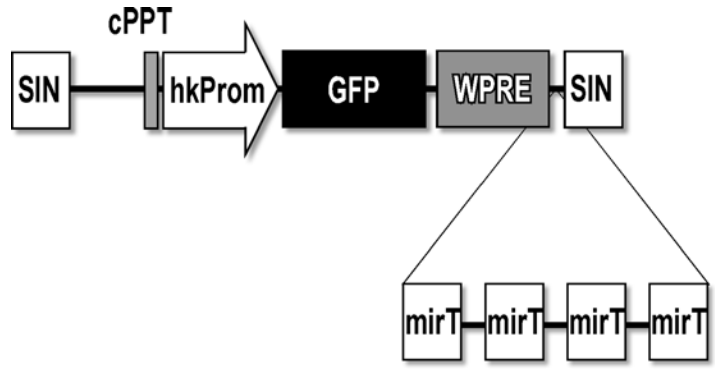

Fig. 1. *Design of microRNA-regulated reporter construct*. Schematic drawing of the integrated form of a typical microRNA-regulated lentiviral vector. A housekeeping promoter drives expression of GFP. The transgene expression is then regulated by four tandem copies of exact complementary sequences of a mature microRNA of choice. *SIN* self-inactivating LTR, *cPPT* central polypurine tract; *hkProm* housekeeping promoter; *GFP* enhanced green fluorescent protein; *WPRE* woodchuck hepatitis post regulatory element; *miRT* microRNA target sequence.

13. Ultracentrifuge (Beckman L60).
14. Rotor for ultracentrifuge (Beckman Coulter, SW32).
15. FACS cytometer instrument for flow cytometric analysis of GFP expression.

2.2. Culturing and Transduction of hESCs

1. Human ESC line (i.e., H9, HuES10).
2. Tissue culture-treated plasticware (i.e., 6-cm dishes, 6-well plates).
3. Irradiated or mitomycin-C-treated mouse embryonic fibroblasts (MEFs).
4. Matrigel Basement Membrane Matrix (BD Biosciences; cat. no. 354230):

   Thaw the frozen vial of matrigel on ice over night in the refrigerator. Dilute the thawed matrigel 1:20 in ice-cold DMEM with cooled pipettes. Prepare 5–15 mL aliquots in cooled 15 mL tubes and freeze at –20°C.
5. *Procedure for coating dishes with Matrigel*:

   Thaw the aliquots overnight on ice and dilute the aliquots 1:2 in ice-cold DMEM to yield a final dilution of 1:40. Cover dishes or plates with the diluted Matrigel solution and let them stand for 1 h at 37°C (i.e. 0.2 mL/cm^2). Aspirate the Matrigel solution and rinse dishes once with DMEM prior to plating cells. It is important that the Matrigel solution never reaches room temperature before coating, as this will lead to clumps of gelatinous precipitate on the dishes.
6. Gelatin (Sigma-Aldrich, cat. no. G2500). Dilute to 0.1% in PBS and sterilize by autoclaving. Sterilized solutions can be stored at 4°C for up to 1 year.
7. *Procedure for coating dishes with gelatin*:

   Incubate dishes with 0.1% gelatin solution for 30 min to 24 h at 37°C. Aspirate the gelatin solution and wash once in PBS prior to plating cells.
8. *MEF medium*:

   DMEM (Invitrogen, cat. no. 11965-118)

   10% FBS (Invitrogen, cat. no. 26140-095)

   1% L-glutamine (200 mM, Invitrogen, cat. no. 25030-081)

   1% Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
9. *hESC medium*:

   DMEM/F12 (Invitrogen, cat. no. 31330)

   20% knockout serum replacement (Invitrogen, cat. no. 10828-028)

   0.5% L-glutamine (200 mM, Invitrogen, cat. no. 25030-081)

0.1% 2-mercaptoethanol (Invitrogen, cat. no. 31350-010)

0.5% penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

1% MEM nonessential amino acids (Invitrogen, cat. no. 11140-035)

10 ng/mL FGF2 (R&D Systems, cat. no. 233-FB)

10. *Preparation of conditioned hESC medium*:
    (a) Plate irradiated or mitomycin-C-treated MEFs at a high density (30–50,000 cells/cm^2) on gelatin-coated dishes in MEF medium and let them attach overnight.
    (b) After 24 h, wash cells once in PBS and add hESC medium (0.3–0.4 mL medium/cm^2).
    (c) Harvest the medium 24 h later and store at 4°C till use. The cells can be used for generating CM for up to 2 weeks with harvest every day.
    (d) Filter the conditioned medium with a 0.22 μm filter and add additional 10 ng/mL of FGF2 before use.
11. *hESC freezing medium*:

    50% hESC medium

    43% FBS (Invitrogen, cat. no. 26140-095)

    7% DMSO
12. Accutase (Invitrogen, cat. no. A11105-01). Aliquot and store at –20°C. Stable for 2 weeks at 4°C.
13. Dispase (STEMCELL Technologies, cat. no. 07913). Aliquot and store at –20°C. Stable for 1 week at 4°C.
14. ROCK inhibitor (Y-27632 from Tocris Bioscience). Resuspend in H_2O to 10 mM and store aliquots at –20°C. Aliquots are stable at 4°C for at least 2 weeks.

2.3. Neural Differentiation of hESCs

1. *KSR medium*:

   KnockOut DMEM (Invitrogen, cat. no. 10829-018)

   15% KnockOut Serum Replacement (Invitrogen, cat. no. 10828-028)

   1% L-glutamine (200 mM, Invitrogen, cat. no. 25030-081)

   0.1% 2-mercaptoethanol (Invitrogen, cat. no. 31350-010)

   1% penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

   1% MEM non-essential amino acids (Invitrogen, cat. no. 11140-035)
2. *Modified N2 medium*:

   DMEM:F12 (Invitrogen, cat. no. 32500-035)

   8.6 mM glucose

   24 mM $NaHCO_3$

20 nM progesterone

30 nM sodium selenite

60 µM putrescine

25 µg/mL insulin (Sigma-Aldrich, cat. no. 16634). Dissolve stock in 0.1 M HCl.

0.1 mg/mL apotransferrin

1% penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

3. SB 431542 (Tocris Bioscience cat. no. 1614). Dissolve to 20 mM in DMSO and store at –20°C for up to 3 months. Do not dissolve in EtOH since this will destabilize the compound.
4. Recombinant Human Noggin/Fc Chimera (R&D Systems, cat. no. 3344-NG-050).
5. Recombinant Human BDNF (R&D Systems, cat. no. 248-BD).
6. Ascorbic acid (Sigma-Aldrich, cat. no. A5960). Make stock solutions of 200 mM in H_2O.
7. Matrigel Basement Membrane Matrix (BD Bioscience; cat. no. 354230, see Sect. 2.2.4).
8. Polyornithine (Sigma-Aldrich, cat. no. P3655). Make sterile filtered stock solutions of 1.5 mg/mL in H_2O and store at –20°C. Working solutions (15 µg/mL in H_2O) can be stored at 4°C for up to a week.
9. Laminin (Invitrogen, cat. no. 23017-015). Make stock solutions of 1 mg/mL in PBS and store at –80°C.
10. Fibronectin (Invitrogen, cat. no. 33010-018). Make stock solutions of 1 mg/mL in PBS and store at –20°C. Aliquots can be stored at 4°C for up to 2 weeks.
11. *Procedure for coating dishes with polyornithine, laminin and fibronectin*

    Dilute PO stock solutions 1:100 in H_2O to yield a final concentration of 15 µg/mL. Add the solution to wells and incubate at 37°C overnight (i.e. 0.2 mL/cm^2). Aspirate the PO solution and wash three times in H_2O. Prepare FN+lam solution by adding 1:200 of FN and 1:200 of laminin to PBS to yield a final concentration of 5 µg/mL for each. Add the FN+lam solution to the PO-coated wells at 0.2 mL/cm^2 and incubate at 37°C for 24–72 h. Wash the plates once in PBS prior to plating the cells.

2.4. Monitoring of Reporter Gene Expression

1. Inverted fluorescence microscope.
2. FACS cytometer instrument for flow cytometric analysis of GFP expression.
3. 4% paraformaldehyde solution for fixation.
4. *Blocking buffer*:

PBS

5% serum (use serum from host species of secondary antibodies)

0.1% Triton-X100

5. Primary antibodies for staining:
   (a) Oct-3/4 (Santa Cruz Biotechnology, Inc., cat. no. sc-5279, use 1:200)
   (b) Pax6 (Developmental Studies Hybridoma Bank, use 1:100)
   (c) βIII-tubulin (Promega G7121, use 1:1,000)
6. RNA isolation kit (RNeasy mini kit, QIAGEN, cat. no. 74104).
7. SuperScript III reverse transcriptase (Invitrogen, cat. no. 18080-044).
8. Random primers (Invitrogen, cat. no. 48190-011).
9. Primers for real-time PCR of cDNA from differentiating cells
   (a) Primers for pluripotency-associated genes (i.e., Oct4, Nanog)
   (b) Primers for neural progenitor markers (i.e., Pax6, Sox1, Ngn2)
   (c) Primers for neuronal markers (i.e., MAP2, Tau)
   (d) Primers for virus-specific sequences (i.e., GFP or WPRE)
10. SYBR green master mix (Roche Applied Science, cat. no. 04887352001).
11. Real-time PCR instrument for quantitative PCR (i.e., LightCycler 480).

3. Methods

3.1. Lentiviral Vector Production and Titering

Caution: All plasticware which has been in contact with virus and with virus-transduced cells for up to 7 days after transduction should be thoroughly decontaminated with Virkon before disposal. The following lentiviral vector production protocol is based on a calcium phosphate transfection method using a third-generation lentiviral vector system (14, 16, 17). This protocol can be scaled up as needed.

1. 24 h before transfection, plate 9×10^6 293T cells in growth medium in a 15-cm tissue culture dish.
2. 2 h before transfection, change medium to 22.5 mL IMDM medium.

3. For transfection, mix plasmids: 9 μg pMDG + 12.5 μg pMDL + 6.25 μg pRSV-Rev + 32 μg transfer vector (microRNA-regulated reporter plasmid). Bring the plasmid mix to a final volume of 1,125 μL with 0.1× TE buffer.
4. Add 125 μL 2.5 M $CaCl_2$ solution to the DNA/TE mix and leave at room temperature for 5 min.
5. While vortexing, add 1,250 μL 2× HBS solution dropwise to the DNA/TE/$CaCl_2$ mix to form DNA/calcium phosphate precipitate.
6. Add the total precipitate to the 15-cm dish with 293T cells and rock the dish back and forth to distribute the precipitate evenly across the dish.
7. After 12–14 h, change medium to 16 mL fresh IMDM medium.
8. 30–36 h after changing the medium, harvest the virus-containing supernatant.
9. Spin down the supernatant at 800 × *g* for 10 min to remove cellular debris and subsequently filter the supernatant with a 0.2-μm filter.
10. Ultracentrifuge the filtered supernatant at 19,500 RPM for 2 h at 4°C.
11. After ultracentrifugation, decant the supernatant and add 100–200 μL PBS to the pellet without pipetting. Leave the pellet in PBS for at least 2 h at 4°C to dissolve the virus particles.
12. Mix and aliquot the viral suspension and store at −80°C.
13. To determine the viral titer, plate 1×10^5 293T cells in a 6-well plate and transduce on the same day with various dilutions of virus suspension (i.e. 0.01–3 μL virus suspension) (see Note 1).
14. 3 days later, dissociate the transduced 293T cells with trypsin and spin down in growth medium.
15. Resuspend the cells in 500 μL growth medium with 2% PFA and leave at room temperature for 15 min before FACS analysis (see Note 2).
16. Calculate the concentration of transducing units (TU) per mL, i.e., if 0.1 μL virus suspension results in 7% GFP+ cells of the total population, then 0.01 μL virus suspension contains $0.07 \times 100{,}000 = 7{,}000$ TU → this corresponds to 7×10^8 TU/mL (see Note 3).

3.2. Culturing and Transduction of hESCs

hESCs are cultured on irradiated or mitomycin-C-treated mouse embryonic fibroblasts (MEFs) in hESC medium. Colonies should be routinely passaged every 7 days with dispase onto new MEFs. MEFs should be plated no later than 48 h and no earlier than 8 h

before plating of hESCs. This protocol does not describe routine culturing of hESCs, but focuses on how to generate efficient lentiviral transduction of hESCs. The addition of ROCK inhibitor (Y-27632) will improve survival of the cells after Accutase passage and during transduction.

1. For lentiviral transduction, use hESC cultures of good quality with high density (high hESC/MEF ratio). Before using the cells, remove differentiated colonies from the dish with a vacuum-connected Pasteur pipette.
2. On the day of transduction, coat 6-well plates with matrigel (see Sects. 2.2.4 and 2.2.5, coat 1× well for each virus condition).
3. Wash hESCs once in PBS, and add Accutase to dissociate cells (500 µL to a 6-well). Leave cells with Accutase in the incubator for 5–10 min.
4. Use a 1-mL pipette to triturate cells to single cells, and spin down cells in 10 mL hESC medium at 200 × *g* for 5 min.
5. Wash cells once again in 10 mL hESC medium and spin down.
6. Resuspend the cells in conditioned hESC medium with 10 µM Y-27632. Count the cells and plate 5×10^5 cells/well in 6-well plates in 2 mL conditioned medium with 10 µM Y-27632.
7. 2 h after plating, add virus to the cells for a final MOI of 20–30 (i.e., $1–1.5 \times 10^7$ transducing units for 5×10^5 cells) (see Note 4).
8. 1, 2, and 3 days after transduction, change medium to conditioned hESC medium + 10 µM Y-27632.
9. 4 days after transduction, passage transduced cells with Accutase: Wash cells once in PBS and add 500 µL accutase/well. Leave for 5–10 min in the incubator until all cells have loosened from the dish. Use a 1 mL pipette to triturate cells to single cells, and spin down cells in 10 mL hESC medium at 200 × *g* for 5 min.
10. Wash cells once again in 10 mL hESC medium and spin down.
11. Count and plate the transduced hESCs on new MEFs at a concentration of 4×10^5 cells/well in 6-well plates in hESC medium with 10 µM Y-27632. Save an aliquot of the cell suspension in media for FACS analysis (i.e., 300 µL cell suspension with a concentration of $\geq 5 \times 10^5$ cells/mL) (see Notes 2 and 5).
12. Change medium and expand cells with dispase until sufficient number of cells is obtained for freezing and for performing experiments (see Note 6).

3.3. Neural Differentiation of hESCs

The differentiation protocol described here is based on a previously published protocol for neural induction (1). The protocol can be scaled up as needed. If specific subtypes of neural cells are desired, additional factor can be added to the protocol during differentiation. For the GFP-mir292T construct, GFP will start to turn on at day 4 of differentiation, and it will increase up to day 20 (see Fig. 2b).

1. 6–8 days before differentiation, passage hESCs with dispase as normally, and plate colonies onto Matrigel-coated plates in conditioned hESC medium (see Sects. 2.2.4 and 2.2.5). Change medium every day to conditioned hESC medium until the colonies are ready to be passaged again. This passage on Matrigel will eliminate the presence of MEFs before starting differentiation.
2. 1–3 days before the start of differentiation, prepare Matrigel-coated 48-well plates and passage colonies from Sect. 3.3.1 with Accutase to yield single cells (see Sects. 2.2.4, 2.2.5, and 3.2.3). Spin down and wash cells × 2 in hESC medium.
3. Plate single cells onto the Matrigel-coated 48-well plates at a density of 150–200,000 cells/well in conditioned hESC medium + 10 μM Y-27632.
4. When the cells are 80–90% confluent (1–2 days after plating), start differentiation (see Note 7).
5. To initiate differentiation (= d0), aspirate medium and replace with KSR medium + 10 μM SB431542 and 200 ng/mL noggin (see Note 8).
6. Replace this medium on d2 and d4.
7. On d5 of differentiation, replace medium with 75% KSR medium + 25% N2 medium + 10 μM SB431542 and 200 ng/mL noggin.
8. On d7 of differentiation, replace medium with 50% KSR medium + 50% N2 medium + 10 μM SB431542, 200 ng/mL noggin, and 100 ng/mL FGF8a.
9. On d9 of differentiation, replace medium with 25% KSR medium + 75% N2 medium + 100 ng/mL FGF8a, 20 ng/mL BDNF, and 200 μM ascorbic acid.
10. On d10 of differentiation, aspirate differentiation media and add 100 μL Accutase to coat the well. Incubate at 37°C for 15 min or until all cells are rendered to single cells.
11. Triturate the cells in the dish using a 1-mL pipette with additional N2 medium until the cells are in a single cell suspension.

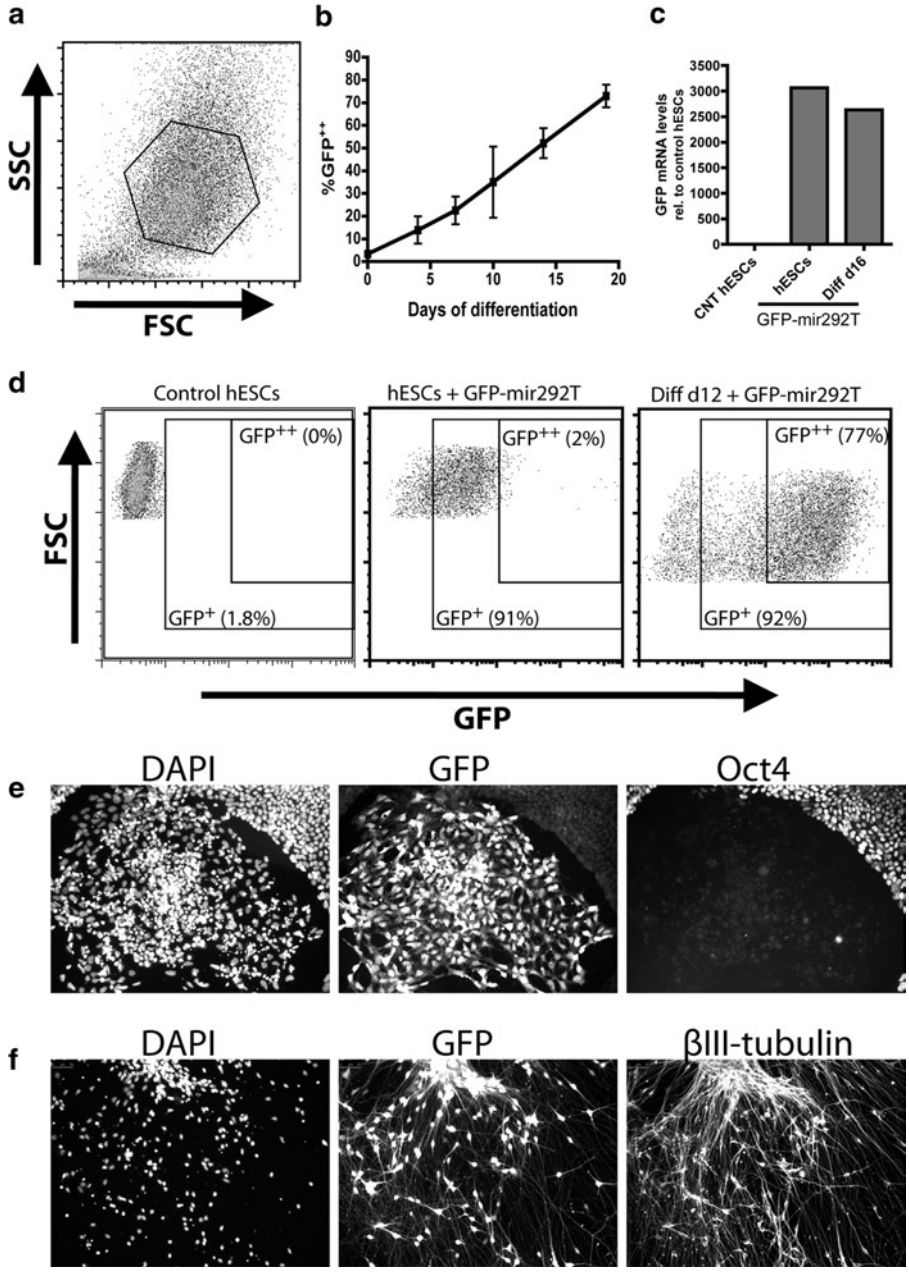

Fig. 2. *Monitoring reporter gene expression in differentiating hESCs.* Analysis of the GFP-mir292T reporter vector in differentiating hESCs by flow cytometry, qRT-PCR analysis, and staining. (**a**) FACS plot showing gating settings for the live cell population. (**b**) Time course of GFP expression analyzed by flow cytometry in neural differentiation of GFP-mir292T hESCs. (**c**) Analysis of vector GFP mRNA expression in hESCs before (hESCs) and after (diff d16) differentiation for 16 days. (**d**) Representative FACS plots of untransduced hESCs (control) and GFP-mir292T hESCs before and after neural differentiation. The middle panel shows the baseline leakiness of the GFP-mir292T construct in undifferentiated hESCs. (**e**) Staining for Oct4 and DAPI at an early timepoint during differentiation shows localization of GFP expression exclusively in the GFP-negative population. (**f**) Staining for βIII-tubulin and DAPI after terminal neuronal differentiation shows bright GFP expression in the neuronal cell population.

12. Wash and centrifuge cells twice in N2 media.
13. Resuspend the cells in N2 media, and determine the cell concentration using a hemocytometer.
14. Adjust the cell concentration to 1×10^7/mL.
15. Plate the cells in droplets onto dried wells coated with polyornithine, fibronectin, and laminin. Spot one or several droplets of 5 μL of the cell suspension in each well and let the cells attach for 15 min in the incubator before adding N2 containing BDNF (20 ng/mL), FGF8a (100 ng/mL), and ascorbic acid (200 μM).
16. Culture cells in this medium for 4–10 days or until mature neuronal cells are visible.

3.4. Monitoring of Reporter Gene Expression

At different timepoints during differentiation, the microRNA-regulated GFP levels can be monitored at the protein level either by fluorescence microscopy or flow cytometry on live cells or by staining of fixed cells. For an accurate and quantitative measure of the time course of GFP protein regulation, flow cytometry is the preferable method. Note that some microRNAs regulate their target vector through degradation of the viral mRNA, whereas others exert translational control of reporter protein synthesis. To analyze whether your target vector is regulated at the mRNA or the protein level, a flow cytometric time course analysis of GFP expression must be complemented by a qRT-PCR analysis of viral mRNA levels. For the GFP-mir292T vector, GFP expression in human cells is exclusively regulated at the protein level. Thus, high levels of vector-derived mRNA can be detected both in the pluripotent and the differentiated state, but GFP fluorescence is only detected after differentiation (see Fig. 2b, c)

1. *Staining of transduced cells during differentiation:*
   (a) Perform differentiations in 48-well plates, and at different timepoints during differentiation, fix the differentiating cells in 4% PFA for 15 min at 37°C.
   (b) Wash cells three times in PBS and incubate cells in blocking buffer at room temperature for 1 h.
   (c) Dilute primary antibodies in blocking buffer and incubate on cells overnight at 4°C or room temperature.
   (d) Wash three times in PBS and add secondary fluorescent antibodies diluted in blocking buffer together with DAPI or another nuclear dye.
   (e) Wash three times in PBS and evaluate colabeling of GFP with pluripotency and differentiation markers. (See Note 9 and Fig. 2e, f).

2. *Flow cytometric analysis on live transduced cells*
   (a) Perform differentiations in 48-well plates, and at different timepoints during differentiation, analyze cells by flow cytometry.
   (b) On the day of analysis, remove media from the plate, and wash twice in PBS to remove any dead or floating cells.
   (c) Add Accutase (100 µL/well) and incubate at 37°C for 10–15 min.
   (d) Triturate cells with a 100 µL pipette until a single cell suspension is achieved. It is important that all cells in the suspension are single cells, since doublets will give biased results on the FACS. If cells are not yet completely dissociated, incubate the cell suspension in Accutase for 10–15 min more.
   (e) Add 400 µL N2 medium to the cells and transfer the cell suspension to a FACS tube for analysis. If cell clumps or stringy fibers of cells are present in the suspension due to DNA leakage, the cell suspension should be filtered through a 40 µm cell strainer before analysis.
   (f) Run the sample through the FACS machine and gate for live cells in the FSC/SSC channels (see Note 2 and Fig. 2a).
   (g) Analyze GFP fluorescence intensity for the live cell population in the FL1 channel (see Note 5 and Fig. 2d).
3. *Quantitative RT-PCR analysis of viral mRNA levels:*
   (a) Perform differentiations in 48-well plates, and at different timepoints during differentiation, lyse cells in 350 µL RLT buffer + β-mercaptoethanol, to extract total RNA following the RNeasy kit manual.
   (b) Perform cDNA synthesis on 1–5 µg of total RNA, using SuperScript III and random primers following the producer's manual.
   (c) Perform real-time quantitative PCR on cDNA using primers for detection of viral mRNA as well as primers for pluripotency and differentiation-associated genes following the Sybr Green manual (see Note 10).

4. Notes

1. With this virus production protocol, it is expected to achieve a total of 1×10^7 to 1×10^8 TU from one 15 cm dish.
2. cytometry on live cells should only be done >7 days after transduction since this involves transfer of transduced cells outside

the viral vector certified cell culture laboratory. If analysis is done less than 7 days after transduction, cells should be fixed in a medium solution containing 2% PFA solution for 15 min before analysis.

3. The calculations of viral titer should be based on transductions resulting in <15% GFP positive cells. Higher transduction efficiencies will underestimate the viral load due to the presence of cells containing more than one integrated vector.
4. If high-titer virus is a limiting factor, transductions can be done in smaller wells and scaled down to fewer cells and fewer viruses. This will only mean that it will take longer to expand the transduced cells in large quantities.
5. Most miR-regulated reporter constructs will have some degree of background leakiness, which can be hard to see in the microscope, but which is clearly visualized by flow cytometry. Thus, for the GFP-mir292T construct, the efficiency of transduction can be visualized in the hESC population by flow cytometry. Ideally, >90% of the cells should show a shift to the right in the FL1 channel if the transduction efficiency is high. If the efficiency of transduction is not satisfactory in the first round, the cells can be transduced once again by taking them back to Step 1 in Sect. 3.2 and if necessary, by adjusting the amount of virus added to the cells in Step 7 in Sect. 3.2.
6. Ideally, a transduced cell population should be stable over time; however, we observe some instability of the transgene expression with repeated passaging. This can be due to either silencing of the transgene or selective growth of nontransduced cells in the culture over time. Thus, we recommend that transduced cell populations are passaged no more than six times. After this, a new round of transduction should be performed.
7. The confluency of the cells at the start of differentiation is crucial for the efficiency of differentiation, so it is important that the differentiation is not started before the cells are 80–90% confluent. For H9 cells, if 180,000 cells/well are plated and the quality and survival of the cells are good, then the cells should be ready to start differentiation on the next day. If you wish to start differentiation after 2 days, 130,000–150,000 cells can be plated in stead. To start 3 days later, 100,000 cells can be plated. Note that the survival of the cells will drop exponentially with lower plating densities. The optimal plating densities will depend on the cell line used.
8. When differentiating the cells, the medium volume in the well should be high since the cell density is extremely high and medium is only changed every other day (i.e., use 600–800 µL medium/well for a 48-well plate)

9. In the neural differentiation paradigm, it is relevant to follow the regulation of GFP in parallel with the loss of pluripotency and the acquisition of neural fate. For this purpose, we recommend staining for Oct-3/4 as a marker of pluripotency, Pax6 as a marker of early neural progenitors and βIII-tubulin as a marker of neuronal cells (see Sect. 2.4.5 for antibodies and dilutions). The EGFP reporter protein maintains fluorescent properties after PFA fixation, so it is not normally necessary to stain for GFP.
10. When performing qRT-PCR on viral sequences from transduced cells, it is important to keep in mind that any contamination with genomic DNA or viral plasmid will give rise to a signal which is indistinguishable from that of the viral vector cDNA. Thus, for transduced cells, we recommend that DNase treatment is always included in the RNA purification protocol and that a parallel control lacking SuperScript enzyme is included in the PCR reaction. This control will reveal whether contamination with plasmids or genomic DNA is present in your samples.
11. The optimal design of the microRNA-regulated vector ultimately depends on the cell type and the experimental paradigm. Examples of variations include the choice of promoter, miRNA-target sequence, and reporter gene. We refer previous papers where it is extensively described how microRNA-regulated lentiviral vectors can be optimized for various purposes (14, 15). It should be noted that most microRNA-regulated vectors can be transferred between species due to the high degree of conservation among microRNAs. The miR-292T vector used this chapter is based on the sequence of the murine mmu-miR-292 but also works efficiently in human cells due to presence of several close homologues, including hsa-miR-371.

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