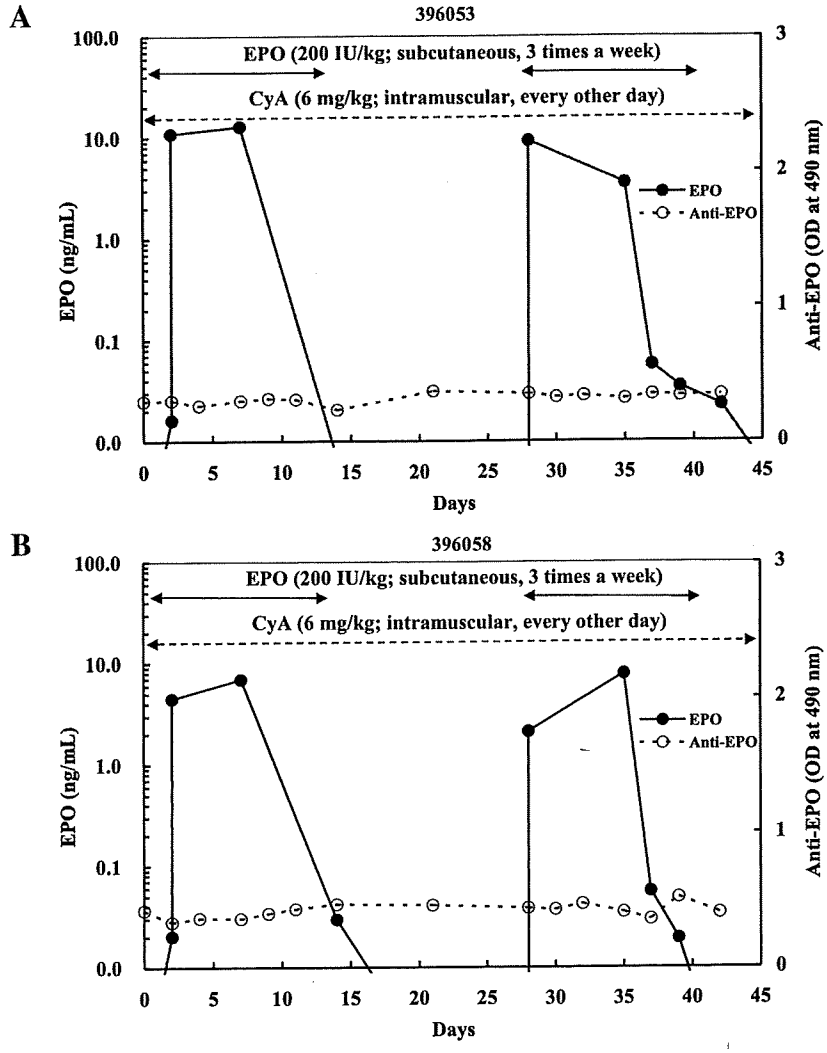

Fig. 2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by treatment with CyA in 2 cynomolgus monkeys (396053, 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of 200 to 400 *ng/ml*. Under the treatment with CyA, high serum levels of hEPO were obtained during hEPO administration. A second trial of hEPO administration resulted in a similar elevation of serum hEPO levels in 2 monkeys.

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20

Use of Simian Immunodeficiency Virus Vectors for Simian Embryonic Stem Cells

Takayuki Asano, Hiroaki Shibata, and Yutaka Hanazono

Summary

The ability to stably introduce genetic material into primate embryonic stem (ES) cells could allow broader application. In this chapter, we describe a method of gene transfer into simian (cynomolgus macaque) ES cells using a simian immunodeficiency virus-based lentivirus vector. When cynomolgus ES cells are transduced with a simian immunodeficiency virus vector encoding the green fluorescent protein (GFP) gene, a large fraction of cells (greater than 50%) fluoresce, and high levels of GFP expression persist for months as assessed by flow cytometry and real-time polymerase chain reaction. Thus, the use of GFP as a reporter gene allows direct and simple detection of successfully transduced ES cells and facilitates monitoring of ES cell proliferation and differentiation both in vitro and in vivo. In addition, this highly efficient gene transfer method allows faithful gene delivery to primate ES cells with potential for both research and therapeutic applications.

Key Words: Flow cytometry; gene transfer; green fluorescent protein; lentivirus vector; primate embryonic stem cells; real-time PCR; simian immunodeficiency virus vector.

1. Introduction

Nonhuman primate embryonic stem (ES) cells have remarkable similarities to human ES cells in all aspects, including morphology and surface marker expression. On the other hand, primate (both human and nonhuman) ES cells are quite distinct from mouse ES cells, for instance, in their growth velocity, feeder and leukemia inhibitory factor (LIF) dependency, and their morphology and surface marker expression. Therefore, experimental results using mouse ES cells may not be predictive of those in primates. These discrepancies stimulated us to use nonhuman primate (simian) ES cells as a predictive model to more closely reflect human ES cell characteristics and behavior *(1,2)*.

The lentivirus vector was first established from human immunodeficiency virus (HIV)-1 *(3)*. It can transduce quiescent cells such as neurons and hematopoietic stem cells *(3,4)*. Non-HIV lentivirus vectors have also been established by modifying feline

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immunodeficiency virus, equine infectious anemia virus, simian immunodeficiency virus (SIV), or bovine immunodeficiency virus *(5–9)*. Among primate lentivirus vectors, the merit of SIV vectors over HIV-1 vectors is safety. The sequence homology between HIV-1 and SIV is considerably low (approx 50%) *(10)*. The generation of replication-competent virus by recombination between SIV vectors and HIV-1 in human subjects is therefore highly unlikely. This provides a great advantage in safety over HIV vectors, especially when target cells are already infected with HIV or permissive to HIV infection.

HIV-1-based lentivirus vectors can efficiently transduce human cells but not those of Old World monkeys *(11)*. A species-specific cytoplasmic component confers the innate postentry restriction to HIV-1 infection in simian cells *(12)*. Unlike HIV-1 vectors, SIV vectors can efficiently transduce simian embryonic and hematopoietic stem cells *(13,14)*. In this chapter, we describe a method to use a SIV-based lentivirus vector for efficient gene transfer into simian (cynomolgus macaque) ES cells.

2. Materials

2.1. Cells

1. Simian (rhesus or cynomolgus) ES cells *(1,2)*.
2. Mouse embryonic fibroblasts (MEFs) from CD-1 (also referred to as ICR [Institute of Cancer Research]) (Charles River, Wilmington, MA) or BALB/c mice (Charles River).
3. 293T human embryonic kidney cell line (ATCC, Manassas, VA; cat. no. 11268).

2.2. Culture Media and Reagents

1. Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO; cat. no. D-6429).
2. DMEM nutrient mixture F-12 1:1 mixture (DMEM/F12) (Invitrogen, Carlsbad, CA; cat. no. 11330-032).
3. ES cell-qualified fetal bovine serum (FBS; Invitrogen, cat. no. 10439-024).
4. 10,000 IU/mL penicillin-10,000 µg/mL streptomycin (100X; Invitrogen, cat. no. 15070-063).
5. 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-081).
6. 2-Mercaptoethanol (Sigma, cat. no. M3148).
7. FBS (Sigma, cat. no. F-2442).
8. Phosphate-buffered saline (PBS) (Invitrogen, cat. no. 10010-023).
9. Hanks balanced salt solution (HBSS) (Invitrogen, cat. no. 14025-092).
10. 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen, cat. no. 25200-056).
11. 2.5% trypsin (Invitrogen, cat. no. 15090-046).
12. Polybrene (Sigma, cat. no. S2667).
13. Culture medium for primate ES cells: DMEM/F12 containing 15% ES cell-qualified FBS, 2 m*M* L-glutamine, 100 IU/mL penicillin-100 µg/mL streptomycin, and 0.1 m*M* 2-mercaptoethanol.
14. Culture medium for 293T cells: DMEM containing 10% FBS and 100 IU/mL penicillin-100 µg/mL streptomycin.
15. Post-transfection medium: DMEM containing 20% FBS.

2.3. SIV Vectors

1. pVSV-G (sold as a part of the pantropic retroviral expression system; BD Biosciences Clontech, San Jose, CA; cat. no. 631512 and 631530).

2. SIV packaging plasmid and SIV gene transfer plasmid (for plasmid construction, *see* **ref. 7**).
3. Lipofectamine reagent (Invitrogen, cat. no. 18324-111).
4. Plus reagent (Invitrogen, cat. no. 11514-015).
5. Opti-MEM (Invitrogen, cat. no. 11058-021).
6. Stericup filters (Millipore, Billerica, MA; cat. no. SCHV U01RE).

2.4. Flow Cytometry

1. A flow cytometer equipped with an argon-ion laser (Becton Dickinson FACScan, FACS Caliber, or an equivalent).
2. Cell strainers (BD Falcon, San Jose, CA; cat. no. 352350).
3. Round-bottom test tubes with cell strainer caps (BD Falcon, cat. no. 352235).
4. Fluorescent-activated cell sorting (FACS) medium: 2% FBS and 0.1% NaN_3 (Wako, Osaka, Japan; cat. no. 197-11091) in PBS.
5. Fixing medium: 1% paraformaldehyde (Wako, cat. no. 064-00406) in PBS.
6. Phycoerythrin (PE)-conjugated antimouse-H-$2K^d$ monoclonal antibody (BD PharMingen, San Jose, CA; cat. no. 553566).

2.5. Real-Time Polymerase Chain Reaction

1. A real-time thermal cycler (ABI-PRISM 7000 sequence detection system or an equivalent).
2. A QIAamp DNA minikit (Qiagen, Hilden, Germany; cat. no. 51104).
3. A Quantitect SYBR green polymerase chain reaction (PCR) kit (Qiagen, cat. no. 204143).
4. MicroAmp optical 96-well reaction plates (Applied Biosystems, Foster City, CA; cat. no. N801-0560) and MicroAmp caps (Applied Biosystems, cat. no. N801-0535).
5. A spectrophotometer (Beckman Coulter DU 7500 or an equivalent).

3. Methods

3.1. Construction of SIV Vector

We have used the SIV vector derived from SIV African green monkey (SIVagm) *(7)* to transduce simian ES cells. SIV vectors can transduce simian ES cells more efficiently than adenovirus, adeno-associated virus, or oncoretrovirus vectors *(13)*. In addition, SIV vectors can efficiently transduce nondividing cells, for instance, the ocular tissue and adipocytes *(15,16)*.

Instead of depending on specific SIV entry via CD4 and other co-receptors, the vesicular stomatitis virus (VSV)-G envelope has generally been used to pseudotype SIV vectors. Because the cellular receptors for VSV-G, including phosphatidylserine, phosphatidylinositol, and GM3 ganglioside, appear to be very abundant and ubiquitous membrane components of most mammalian cells, VSV-G-enveloped viruses can infect a wide variety of cells and tissues. In addition to the broader range, VSV-G-pseudotyped viruses are physically more stable than naturally occurring lentiviruses and can be concentrated by centrifugation (*see* **Subheading 3.1.2.**).

3.1.1. Transfection

1. Dissociate exponentially growing 293T cells with 0.25% trypsin-ethylenediaminetetraacetic acid solution and plate 5×10^6 293T cells in a 100-mm plate (60–80% confluent) 1 d prior to transfection (*see* **Note 1**).
2. On the day of transfection, mix 4.5 µg of the gene transfer plasmid, 1.3 µg of the packaging plasmid, and 0.5 µg of the envelope plasmid (pVSV-G) in 750 µL of Opti-MEM.

3. Prepare the Plus reagent just prior to use and add 20 µL Plus reagent to the DNA solution (from **step 2**). Vortex gently and incubate the mixture at room temperature for 15 min.
4. Dilute 30 µL of the Lipofectamine reagent into 750 µL of OptiMEM in a separate tube.
5. Mix the DNA/Plus solution (770 µL; from **step 3**) and the Lipofectamine solution (780 µL; from **step 4**) followed by incubation at room temperature for 15 min.
6. During the incubation, replace the medium of 293T cells with 6.5 mL OptiMEM.
7. After the incubation, evenly add the DNA/Plus/Lipofectamine solution (1.55 mL total; from **step 5**) onto 293T cells and incubate the plate at 37°C, 5% CO_2. At 4 h after the transfection, add 8 mL DMEM containing 20% FBS.

3.1.2. Harvest and Concentration of Vector

1. Incubate the plate (from **Subheading 3.1.1.**) overnight and replace medium with 10 mL regular 293T growth medium.
2. At 24 h after media replacement, harvest the supernatant (which contains the vector) and filter it though a 0.45-µm pore membrane. The titer of vector will be 10^5–10^6 transducing units (TU) per milliliter (*see* **Note 2**).
3. Concentrate the vector supernatant at 42,500*g* for 2 h with a high-speed centrifuge.
4. After centrifugation, carefully discard the supernatant and resolve the pellet with PBS containing 5% FBS. The suspension volume should be 1/1000 to 1/100 of the initial volume. The final titer of vector will be 10^8–10^9 TU/mL (*see* **Note 3**).

3.2. Transduction

1. Plate 1.5×10^5 ES cells on an MEF (5×10^5 cells) feeder layer in a 35-mm dish and incubate the dish at 37°C, 5% CO_2, for 12–24 h.
2. Gently wash ES cells with HBSS and add 1 mL (half of the regular volume) of the growth medium.
3. Thaw a viral stock without foaming in a water bath at 37°C and add it to the culture (*see* **Note 4**).
4. After 10 h, aspirate the medium, gently wash ES cells once with HBSS, and replace with 2 mL fresh medium.
5. At 2–3 d after transduction, evaluate the transduction efficiency (*see* **Subheading 3.3.** and **Note 5**).

3.3. Assessment of Transduction Efficiency

After transduction, it is important to assess the transduction efficiency, usually 2–3 d after exposure to the vector. If a marker gene such as green fluorescent protein (GFP) is included in the vector, then you can assess the transduction efficiency by examining the marker gene expression. GFP expression can be easily monitored under a fluorescent microscope or by flow cytometry (*see* **Subheading 3.3.1.**). Another method to assess the transduction efficiency is to examine the SIV-provirus (vector integrated into the host genome) by real-time DNA-PCR (*see* **Subheading 3.3.2.**). It is particularly useful when marker genes are not available or marker gene expression levels are not high enough.

When cynomolgus ES cells are transduced once or twice with an SIV vector encoding the GFP gene, more than 50% of cells fluoresce, and the GFP expression persists for months. In addition, high levels of GFP expression are observed during embryoid body formation *(13)*. On the other hand, transduction of cynomolgus ES cells with an

oncoretrovirus vector results in lower gene transfer rates (less than 20%), suggesting that simian lentivirus vectors can transduce simian ES cells more efficiently than oncoretrovirus vectors ***(13)***.

3.3.1. Flow Cytometry

1. Aspirate old medium from the culture and rinse cells with HBSS (from **Subheading 3.2., step 5**). Add 2 mL 0.25% trypsin-HBSS to the dish and incubate for 5 min at 37°C. Detach ES cell colonies from the bottom by tapping with your fingers. Add 3 mL ES medium to the dish, disperse the cells into single cells using a 1-mL tip, and transfer the cell suspension to a 15-mL conical tube.
2. Spin cells in a centrifuge at 140*g* for 4–5 min. Aspirate the medium and resuspend the pellet in FACS medium. Pass the cell suspension through a cell strainer to remove cell clusters (*see* **Note 6**). Count a cell number and adjust it at $1–2 \times 10^6$ cells/mL.
3. Transfer 100 µL cell suspension ($1–2 \times 10^5$ cells) into a 1.5-mL tube. Add 0.1 µg (1 µL) of PE-conjugated antimouse H-2K^d monoclonal antibody solution to the tube and incubate it for 30–60 min on ice.
4. After incubation, add 1 mL FACS medium to the tube and spin cells at 800*g* for 5 min at 4°C. Aspirate medium and wash the pellet with FACS medium. Spin the cell suspension at 800*g* for 5 min at 4°C again.
5. Resuspend the pellet with 200–500 µL fixing medium. The cell suspension can be left at 4°C overnight until flow cytometric analysis.
6. Transfer the cell suspension to a round test tube through a strainer cap.
7. Perform flow cytometric analysis using a flow cytometer with excitation at 488 nm. The fluorescence data of GFP and PE can be obtained via FL1 and FL2 parameters, respectively. **Figure 1** shows a typical profile of cynomolgus ES cells transduced with an SIV vector expressing GFP. Cynomolgus ES cells are negative for antimouse H-2K^d, but co-cultured MEF feeder cells (derived from BALB/c mice) are positive for it; thus, you can distinguish both ES and MEF cells.

3.3.2. Real-Time PCR

1. Extract DNA from a culture pellet (containing both ES and MEF cells from **Subheading 3.2., step 5**) using a QIAamp DNA minikit (*see* **Note 7**). Assess the purity of DNA by checking a 260/280-nm absorbance ratio with a spectrophotometer. Preferably, it is higher than 1.75. Adjust the concentration of DNA stocks (dilute with DNase-free water) to 50 µg/mL.
2. Prepare a master mix for real-time PCR as shown in **Table 1** (*see* **Note 8**). Dispense 45 µL into each well of a MicroAmp optical 96-well reaction plate.
3. Add 5 µL (250 ng) template DNA to each well and seal the plate with MicroAmp caps.
4. Place the plate in a real-time thermal cycler and start a PCR program.
5. Analyze data according your software package (*see* **Note 9**).

4. Notes

1. Because 293T cells were established from 293 cells after transfection with the SV40 large T antigen and neomycin resistance genes, it is recommended to treat 293T cells with 800 µg/mL (active) of G418 for 1 wk once a month so the transgenes are not lost. It is, however, important to passage 293T cells several times without G418 before virus production to avoid contamination of G418 in the viral supernatant.

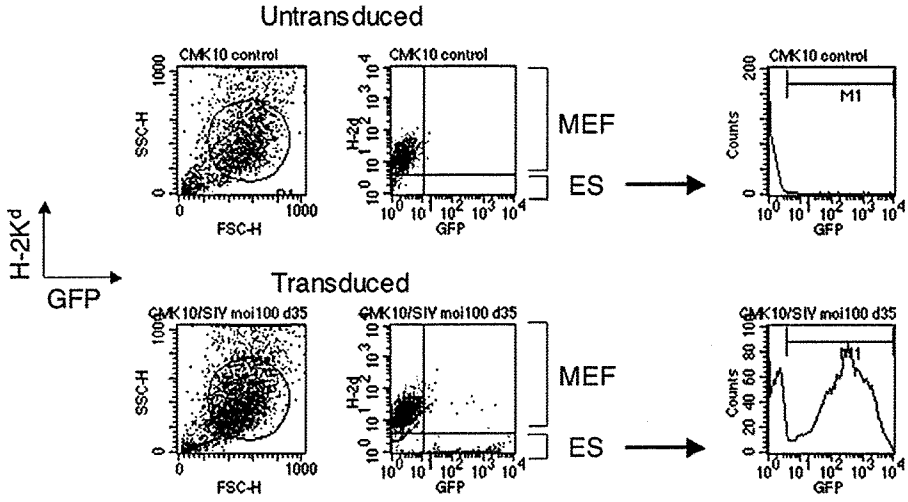

Fig. 1. Assessment of transduction efficiency by flow cytometry. The transgene green fluorescent protein (GFP) expression was analyzed on a FACScan using *CellQuest* software 2–3 d after transduction with a GFP-expressing simian immunodeficiency virus vector. The co-cultured BALB/c-derived feeder cells could be distinguished from the cynomolgus embryonic stem cells using PE-conjugated mouse antimouse H-2K^d monoclonal antibody, which does not react to cynomolgus cells but does react to BALB/c cells.

2. The titer (transducing units, TU) of vector is defined as the ability to transduce target cells. For instance, 10^5 TU/mL indicates that 1 mL vector solution is able to transduce 10^5 cells. We usually use 293T cells as targets to assess the titer. The titer of virus can also be assessed in terms of genomic copies (often designated gc). Genomic copy number of SIV vector can be evaluated by RNA dot-blot or quantitative RNA-PCR.
3. The vector solution can be stored at −80°C at least for several months. The titer will decrease even at −20°C. Frozen stocks should be thawed quickly in a water bath at 37°C just prior to use. Avoid repeated freezing and thawing, or the titer will decrease.
4. The passage of ES cells before and after lentiviral transduction is the same as the routine passage; 30–100 cells per clump is the best. You do not have to disperse clumps for transduction. Vectors are added at 10–50 TU per target cell. We sometimes add polybrene (final concentration 4–8 μg/mL) in the transduction culture and other times do not add it. It does not seem that polybrene improves the transduction efficiency with SIV vectors unlike the case with oncoretrovirus vectors. It is suggested that ES cell exposure to lentivirus solution is no longer than 12 h. Longer exposure may result in a large decrease in ES cell number, presumably because of the toxicity of the pseudotyped envelope VSV-G protein. Serum may greatly hamper lentiviral transduction. If you do not obtain good gene transfer efficiency, then it is suggested to remove the serum from your transduction culture.
5. The transgene expression in ES cells can be enhanced by changing the promoter or adding *cis*-acting elements in the vector. The *cis*-acting sequences include the central polypurine and termination tract (cPPT) to facilitate nuclear import of the viral complex and the woodchuck posttranscriptional regulatory element (WPRE) to increase transgene expression *(17)*. **Figure 2** shows variable GFP expression in cynomolgus ES cells transduced with SIV vectors containing various promoters and cPPT/WPRE sequences.

Table 1
Real-Time PCR Reaction Mixture

Master mix	Volume per reaction	Final concentration
2X QuantiTect SYBR green PCR master mix	25 µL	1X
Forward primer (10 µ*M*)	2.5 µL	0.5 µ*M*
Reverse primer (10 µ*M*)	2.5 µL	0.5 µ*M*
Water	15 µL	NA
Total volume of master mix	45 µL	NA
Template DNA sample (50 µg/mL)	5 µL (250 ng DNA)	5 µg/mL
Total volume of reaction mixture	50 µL	NA

GFP sequence primer set: 5′- CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′- GGT CTT TGC TCA GGG CGG ACT-3′. Internal control cynomolgus β-actin sequence primer set: 5′-CAT TGT CAT GGA CTC TGG CGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG GGC-3′. NA, not applicable.

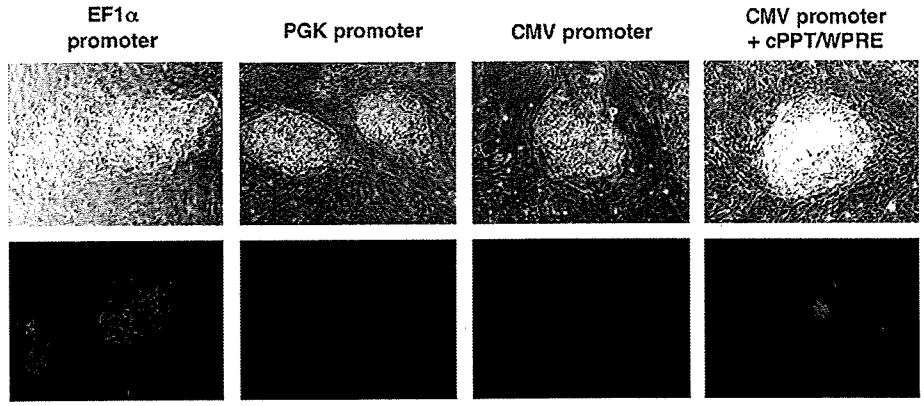

Fig. 2. Promoters and *cis*-acting sequences in simian immunodeficiency virus (SIV) vectors affect transgene expression. Cynomolgus embryonic stem (ES) cells were transduced with green fluorescent protein (GFP)-expressing SIV vectors at 30 TU per target cell. The vectors contain the elongation factor (EF) 1α, phosphoglycerate kinase (PGK), or cytomegalovirus promoter (CMV). The transduced ES cells were observed at d 5 with a fluorescent microscope under a bright field (upper) or dark field (lower). In this cynomolgus ES cell line (CMK6), the usage of the EF1α promoter resulted in the highest GFP expression. In addition, the GFP expression could be enhanced by the inclusion of two *cis*-acting sequences, the central polypurine and termination tract (cPPT) and the woodchuck posttranscriptional regulatory element (WPRE) (rightmost panel).

6. As an ES cell number considerably decreases after passing cells through a strainer before flow cytometry, start experiments with a sufficient number of cells.
7. MEF cells are cotransduced with SIV vector together with ES cells. Therefore, it is suggested to passage transduced ES cells onto untransduced MEF cells several times before DNA

extraction to avoid contamination of transduced MEF cells. In addition, because ES cells are cultured on MEF cells, it is difficult to extract DNA separately from ES or MEF cells. Thus, it is important to know the fraction (percent) of ES cells in total cultured cells (ES plus MEF cells) before DNA extraction in order to calculate the transduction efficiency of ES cells. The fraction (ES vs total cells) can be assessed by flow cytometry (*see* **Subheading 3.3.1.** and **Fig. 1**).

8. We usually use a SYBR green method (Qiagen Quantitect SYBR green PCR kit) rather than a probe method. The former is easier. For the SYBR green method, you do not have to develop specific primers or a probe; rather, regular primer sets are used. It is, however, important to confirm that the PCR does not generate nonspecific bands on an agarose gel because the SYBR green method quantifies all PCR products, including nonspecific ones, if any.
9. The positive control is genomic DNA extracted from cells that contain a known copy number of the target sequence per cell. Dilute the DNA with genomic DNA from naive control monkeys to make a series of diluted positive controls (100, 10, 1, 0.1, 0.01%). The quantitative PCR should be certified each time to yield linear amplifications in the range of the intensity of positive control series (0.01–100%, correlation coefficient >0.98). To certify equal amounts of loaded sample DNA, an internal control sequence (for instance, β-actin) in the same sample should be subjected to real-time PCR. Calculated transduction efficiency (percent) indicates a fraction of cells successfully transduced with SIV vector given that each vector-positive cell contains one copy of the provirus.

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In Vivo Tumor Formation From Primate Embryonic Stem Cells

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Summary

To achieve human embryonic stem (ES) cell-based transplantation therapies, allogeneic transplantation models of nonhuman primates would be particularly useful. In this chapter, we describe an example of this model. We prepared cynomolgus ES cells genetically marked with the green fluorescent protein. The cells were transplanted into the allogeneic fetus because the fetus is immunologically premature and does not induce immune responses to transplanted cells. In addition, fetal tissue compartments are rapidly expanding, presumably providing space for engraftment. At 3 mo posttransplantation, a fluorescent teratoma, obviously derived from transplanted ES cells, was found in the fetus. However, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction. Transplanted cynomolgus ES cells can engraft in allogeneic fetuses. The cells will, however, form a tumor if they "leak" into an improper space, such as the thoracic cavity.

Key Words: Allogeneic transplantation; genetic marking; green fluorescent protein; immunological tolerance; *in situ* PCR; *in utero* transplantation; primate embryonic stem cells; teratoma.

1. Introduction

Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue types ***(1,2)***, human ES cell-based transplantation therapies are considered to hold a great potential in the treatment of a variety of diseases and injuries. To address the safety and efficacy of these therapies, allogeneic transplantation models of large animals, especially nonhuman primates, would be useful. However, it has been difficult to transplant primate ES cells or their derivatives into allogeneic hosts. There are two major reasons for this. First, the efficient and stable marking of primate ES cells has been difficult. It is necessary to distinguish transplanted allogeneic ES cell progeny from surrounding host cells. Second, the immune

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rejection of transplanted cells must be circumvented for a sustained engraftment. The cells would otherwise be cleared by immune responses.

We have previously reported highly efficient gene transfer into cynomolgus ES cells using a lentivirus vector derived from the simian immunodeficiency virus *(3)*. Lentiviral transgene expression in ES cells is stable, with minimal levels of transcriptional silencing *(4,5)*. In addition, cynomolgus ES cell sublines stably expressing green fluorescent protein (GFP) were established after electroporation of a GFP-expressing plasmid *(6)*. By using such cynomolgus ES cells genetically modified to express GFP, it is now possible to distinguish transplanted allogeneic ES cell progeny from surrounding host cells as GFP will serve as a good genetic tag.

The early gestational fetus is a good recipient with which to circumvent immune rejection because the immune system is premature *(7,8)*. Furthermore, in the animal fetus, "space" would be relatively available for engraftment as compared to the adult because of the rapid expansion of fetal tissue compartments. Thus, transplanted cells could engraft without conditioning of recipients, such as by irradiation or immunosuppressive treatment.

In this chapter, we show a method to transplant nonhuman primate (cynomolgus macaque) ES cells *(9)* into xenogeneic immunodeficient mice to form teratoma. In addition, we show methods to transplant nonhuman primate (cynomolgus macaque) ES cells stably expressing GFP *(3,6)* into the allogeneic fetus *in utero* and to examine the in vivo fate of transplanted cells using GFP as a genetic tag. At 3 mo after the allogeneic *in utero* transplantation, a fluorescent tumor, obviously derived from transplanted ES cells, was found in the thoracic or abdominal cavity. Notably, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction (PCR). Thus, transplanted cynomolgus ES cells can engraft in allogeneic fetuses. However, the cells will form a tumor if they "leak" into an improper space, such as the thoracic and abdominal cavities *(10)*.

2. Materials

2.1. Cells

1. Cynomolgus ES cells stably expressing GFP (*see* Chapters 20 and 21, this volume).
2. Mouse embryonic fibroblasts from CD-1 (also referred to as ICR) (Charles River, Wilmington, MA) or BALB/c mice (Charles River).

2.2. Teratoma Formation in Immunodeficient Mice

1. 6- to 8-wk-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) (*see* **Note 1**).
2. Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA; cat. no.14025-092).
3. Dulbecco's modified Eagle's medium/nutrient mixture F-12 1:1 mixture (DMEM/F12) (Invitrogen, cat. no. 11330-032).
4. ES cell-qualified fetal bovine serum (Invitrogen, cat. no. 10439-024).
5. 10,000 IU/mL penicillin-10,000 µg/mL streptomycin (100X; Invitrogen, cat. no. 15070-063).
6. 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-081).
7. 2-Mercaptoethanol (Sigma, St. Louis, MO; cat. no. M3148).

8. Culture medium for primate ES cells: DMEM/F12 containing 15% ES cell-qualified fetal bovine serum, 2 m*M* L-glutamine, 100 IU/mL penicillin-100 µg/mL streptomycin, and 0.1 m*M* 2-mercaptoethanol.
9. 0.25% trypsin in HBSS (2.5% trypsin 10X liquid; Invitrogen, cat. no. 15090-046).
10. 1% bovine serum albumin (BSA fraction V; Sigma, cat. no. A4503) in HBSS.

2.3. Teratoma Formation in Allogeneic Fetuses

1. Anesthetic and surgical facilities for primates (including ultrasound and inhalation anesthesia equipment) *(11)*.
2. A time-dated pregnant cynomolgus monkey of 50- to 70-d gestation (*see* **Note 2**) *(12)*.
3. Ketamine hydrochloride (Ketalar® 50; Sankyo, Tokyo, Japan).
4. Isoflurane (Forane®; Dainippon Pharmaceutical, Osaka, Japan).
5. A percutaneous transhepatic cholangiography (PTC) needle (22-gage, Sonoguide PTC needle type B; Hakko Medical, Nagano, Japan; cat. no. 22412210).
6. A 1-mL syringe (Terumo, Tokyo, Japan; cat. no. SS-01T) filled with graft cells (10^5–10^7 cells in 200–500 µL).
7. A 1-mL syringe (Terumo, cat. no. SS-01T) filled with normal saline (for flushing).

2.4. Sample Preparation

1. 4% paraformaldehyde (Wako, Osaka, Japan; cat. no. 169-18432) and 8% sucrose (Wako, cat. no. 192-00012) in phosphate-buffered saline (PBS; Invitrogen, cat. no. 10010-023).
2. OCT compound (Tissue Tek series; Sakura, Zoeterwoude, Netherlands; cat. no. 4583) containing 10% sucrose.

2.5. In Situ *PCR*

1. A PTC100 Peltier thermal cycler (MJ Research, Waltham, MA).
2. 20 µg/mL proteinase K (Sigma, cat. no. 39450-01-6) in PBS.
3. 0.1% Triton X-100 (Sigma, cat. no. T8787) in PBS.
4. A slide frame for *in situ* PCR (slide seal; Takara, Shiga, Japan; cat. no. 9066 [25 µL] or cat. no. 9067 [65 µL]).
5. 50 µL Digoxigenin dNTP labeling mix (Roche, Basel, Switzerland; cat. no. 1277065).
6. Rabbit anti-Digoxigenin polyclonal antibody, horseradish peroxidase labeled (Dako, Glostrup, Denmark; cat. no. P5104) diluted (1:100) in 2% BSA and 5% horse serum (Invitrogen, cat. no. 16050-130) in PBS.
7. A Vector SG substrate kit (Vector, Burlingame, CA; cat. no. SK-4700).
8. Kernechtrot solution (0.1% Kernechtrot in aluminum sulfate; Muto, Tokyo, Japan; cat. no. 4087).

3. Methods

3.1. Teratoma Formation in Immunodeficient Mice

1. Wash ES cells with HBSS twice and add 0.25% trypsin to the dish at 37°C for 3 min. Neutralize trypsin with ES culture medium and make a suspension of ES cell clumps.
2. Transfer the cell suspension into a 50-mL conical tube, centrifuge it at 140*g* for 4 min, and resuspend the pellet with 20 mL 1% BSA/HBSS.
3. Centrifuge the cell suspension again at 140*g* for 4 min and resuspend the pellet with an appropriate volume of 1% BSA/HBSS (10^6 cells in 150–200 µL per injection site).
4. Aspirate the ES cell suspension into a 1-mL syringe with a 23-gage needle and inject the suspension into NOD/SCID mice subcutaneously (*see* **Note 3**).

5. Resulting tumors will be palpable at 8–13 wk after the injection. Expose, observe, and excise tumors.
6. Fix tumor samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh frozen samples, embed samples (5 × 5 × 3 mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.2. Teratoma Formation in Allogeneic Fetuses

3.2.1. Anesthesia

1. Prepare a pregnant monkey around the end of first trimester (50–70 d; full term 165 d) (*see* **Note 2**).
2. Give the monkey 10 mg/kg ketamine hydrochloride intramuscularly. Secure the monkey on a table and monitor maternal heart rate by electrocardiography (*see* **Note 4**).
3. Induce and maintain anesthesia by inhalation of isoflurane (1.5–2%) mixed with 100% oxygen via a mask.

3.2.2. In Utero *Transplantation*

1. Shave whole abdomen and sterilize the surface with iodine solution (from **Subheading 3.2.1., step 3**).
2. Determine fetal position by transabdominal ultrasound with a 7.5-MHz convex probe (*see* **Note 5**).
3. Let an assistant secure the other side of the uterus while an operator holds the transducer parallel to the intended course of the needle.
4. Select an optimal entry site into the uterine cavity, avoiding the placental tissue.
5. Insert a 23-gage PTC needle through the maternal skin and uterine wall into the amniotic cavity and then into the desired site (e.g., peritoneal cavity, brain, or liver) under continuous ultrasound guidance (*see* **Note 6** and **Fig. 1**). A small push of an injector can visualize a tip of the needle on echocardiography.
6. Let an assistant gently inject the cells (200–500 µL) and flush the needle with 100 µL normal saline. The operator should focus on keeping the tip of the needle in an appropriate position.
7. Confirm adequate heart beats after the procedure (*see* **Note 7**).

3.2.3. Caesarian Section

1. Prepare the pregnant monkey after transplantation as described in **Subheading 3.2.1.** *In utero* transplantation is usually done around the end of the first trimester (50–60 d) (*see* **Subheading 3.2.2.**). The full term is 165 d; therefore, the *in utero* incubation time of transplanted ES cells is about 3 mo.
2. Expose the gravid uterus through a midline incision and deliver the fetus through a low transverse hysterotomy (*see* **Note 8**).
3. Clamp and divide the cord. Remove the placenta and cord. Close the uterus and abdomen with absorbable sutures.
4. Insert a small catheter (24-gage intravenous catheter) into the umbilical vein and irrigate the newborn with normal saline to completely wash out fetal blood for mercy killing. Open the chest and abdomen, observe the whole body, and excise tumors (*see* **Fig. 2A–C**). Collect tissues.
5. Fix tissue samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh

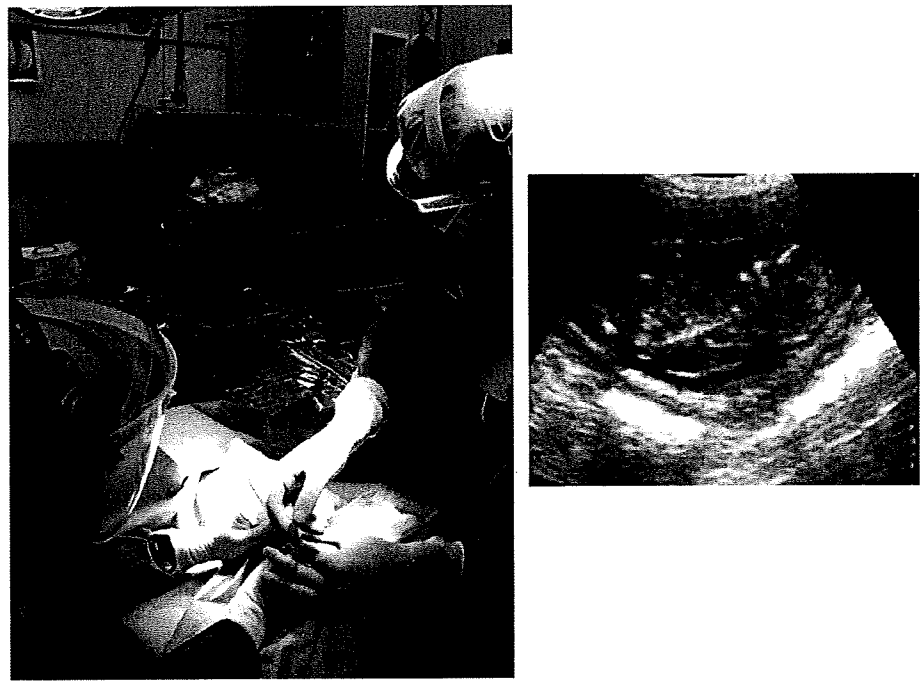

Fig. 1. *In utero* transplantation. Pregnant monkeys were anesthetized by intramuscular administration of ketamine hydrochloride (Ketalar). Cynomolgus ES cells genetically modified to express GFP (10^6 cells/fetus) were injected into the fetal abdominal cavity or liver through a 23-gage needle using an ultrasound-guided technique around the end of the first trimester (**left**). The full term is 165 d. The weight of the fetus at the time of transplantation was estimated at 20 g, which is equivalent to that of an adult mouse (**right**).

frozen samples, embed samples ($5 \times 5 \times 3$ mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.3. In Situ *Detection of Transplanted Cell Progeny*

You may examine tissue sections for in vivo fate of transplanted cell progeny by *in situ* PCR, which amplifies marker (GFP) sequences ***(10,13)***. It is especially useful when it is difficult to identify cells by staining specific surface markers, when GFP fluorescence is hampered by the high autofluorescence of tissue samples, or when the transgene expression is shut down ("silenced") in vivo.

3.3.1. Cell Wall Permeabilization

1. (Optional) If a tissue section is embedded in paraffin, then dewax it by dipping the slide in xylene three times, each for 10 min, and then in 100% ethanol three times, each for 10 min. Air-dry the slide.
2. Soak the slide in 20 µg/mL proteinase K/PBS and incubate it at 37°C for 10 min (*see* **Note 9**).

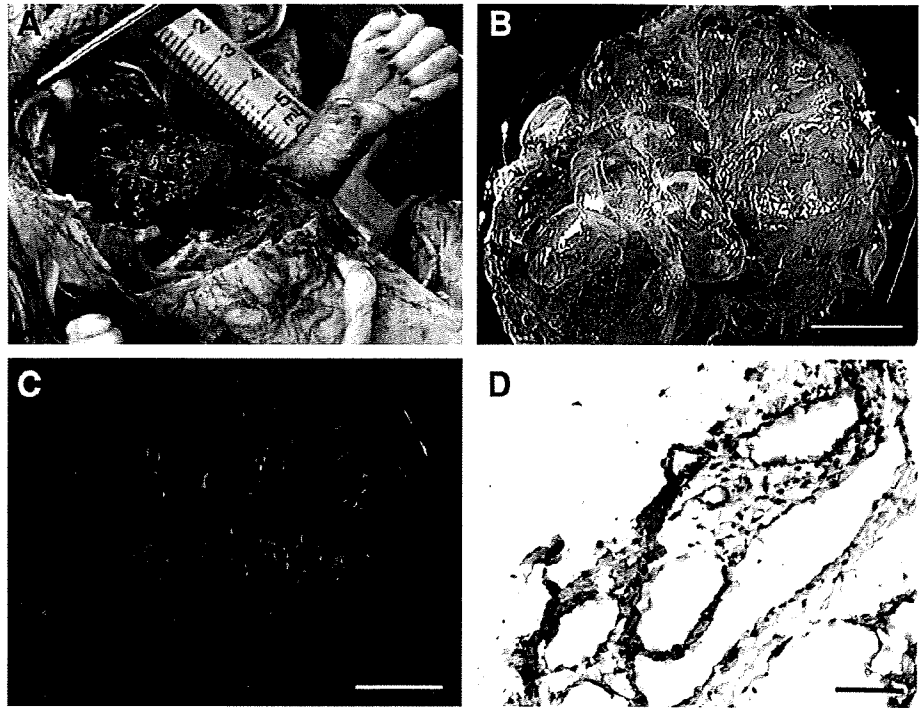

Fig. 2. Teratoma formation in a cynomolgus fetus after transplantation of allogeneic ES cells. (**A**) A tumor (4 × 3 × 2.5 cm) was detected in the thoracic cavity 3 mo after allogeneic transplantation of ES cells expressing GFP. The tumor was observed in (**B**) a bright field and (**C**) a dark field (**C**) under a fluorescence stereomicroscope. GFP was expressed in the tumor, clearly indicating that the tumor was derived from transplanted ES cells. (**D**) The GFP gene was also detected in the tumor cells by *in situ* PCR (stained black). Bar in **B** and **C** = 1 cm. Bar in **D** = 50 μm.

3. Soak the slide in 0.1% Triton X-100/PBS for 5 min and wash it with PBS twice, each for 5 min.
4. Soak the slide in 95% ethanol for 10 min and then in 100% ethanol twice, each for 10 min, to remove proteins and air-dry it.

3.3.2. In Situ *PCR*

1. Attach a slide frame to the slide (from **Subheading 3.3.1., step 4**) and incubate it at 95°C for 5 min.
2. Apply a master mix of *in situ* PCR to the slide at room temperature (*see* **Note 10** and **Table 1**).
3. Cover the slide with a film (*see* **Note 11**).
4. Place the slide upside down in a PTC100 Peltier thermal cycler and start a cycling program (i.e., 94°C for 1 min and 55°C for 2 min with 15 cycles; *see* **Note 12**).

3.3.3. Detection

1. Remove the slide frame gently after PCR (from **Subheading 3.3.2., step 4**) and soak the slide in two changes of PBS for 5 min each.
2 Dropwise add an horseradish peroxidase-labeled anti-Digoxigenin solution (diluted 1:100 with 2% BSA and 5% horse serum in PBS) onto the slide and incubate it at 37°C for 2 h.

Table 1
***In Situ* PCR Reaction Mixture**

Master mix	Volume per reaction	Final concentration
10X PCR buffer (Mg^{2+} free)	2.5 µL	1X
25 m*M* $MgCl_2$	4.5 µL	4.5 m*M*
dNTPs mixture (2.5 m*M* each)	3 µL	420 µ*M* dATP
Digoxigenin (DIG) DNA labeling mix (Roche)	3 µL	420 µ*M* dCTP
		420 µ*M* dGTP
		378 µ*M* dTTP
		42 µ*M* DIG-dUTP
Forward primer (10 µ*M*)	2 µL	0.8 µ*M*
Reverse primer (10 µ*M*)	2 µL	0.8 µ*M*
Takara Taq polymerase (5 U/µL)	0.8 µL	0.16 U/µL
Water	7.2 µL	NA
Total volume of master mix	25 µL	NA

NA, not applicable.

Primer set for the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′.

3. Soak the slide in two changes of PBS for 5 min each.
4. Dropwise add a Vector SG solution onto the slide, stain it for 3–10 min, and gently wash it with water for 10 min.
5. Dropwise add a Kernechtrot solution and incubate the slide at room temperature for 1–2 min to stain nucleotides and gently wash it with water for 10 min.
6. Mount the slide with glycerol and observe it under a light microscope (*see* **Note 13** and **Fig. 2D**).

4. Notes

1. Although we use NOD/SCID mice (lack of B and T lymphocytes but presence of natural killer cells), SCID mice are usually used in many other laboratories to form teratomas from ES cells. NOD/SCID mice are more highly immunodeficient than SCID mice; thus, NOD/SCID mice may be better in the setting of xenotransplantation.
2. Cynomolgus or rhesus monkeys are the most appropriate to work with because of ES cell availability and their size. In the monkey fetus, "the window of opportunity" for successful tolerance induction may be earlier and narrower than thought *(14)*. To avoid immune responses, transplantation at earlier days (around 40–50 d) may be better.
3. It is not necessary to disperse ES cell clumps to single cells when transplanting ES cells into mice (or other animals). We transplanted about 1×10^6 ES cells (corresponding to two confluent 60-mm dishes) per site in mice. There is, however, considerable variation among reports: from 10–15 clumps (200 cells) per site *(2)* to 5×10^6 cells per site *(15)*. ES cells are usually transplanted subcutaneously into the hind leg muscle, testis capsule, or abdominal cavity. In our experiments, a teratoma was formed in any site. It is recommended to choose injection sites you can observe easily from the outside and from which you can easily excise tumors.
4. For ultrasound-guided transplantation operations, endotracheal intubation is not necessary.
5. We prefer a small convex transducer rather than a big linear transducer because of the small size of the monkey fetus. Although a needle adapter is available, we prefer the freehand technique.

6. You may puncture transplacentally when the placenta is located anteriorly. Bleeding from the placenta usually stops spontaneously. However, we recommend every effort to avoid this approach by manipulation.
7. The survival rate with this *in utero* transplantation technique is currently 100%, excluding those fetuses that died from massive teratoma formation.
8. Uterine atony requiring oxytocin administration is quite rare in primates.
9. The treatment with proteinase K may need longer time depending on samples.
10. The amount of master mix per slide is 25 μL for Takara cat. no. 9066 and 65 μL for cat. no. 9067.
11. Slides are attached to the Takara slide seal kit. Be careful not to trap air under films.
12. The PCR conditions should be optimized for each *in situ* PCR.
13. The results should be observed within the same day. On the following day, the tissue would peel off, making examination difficult.

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