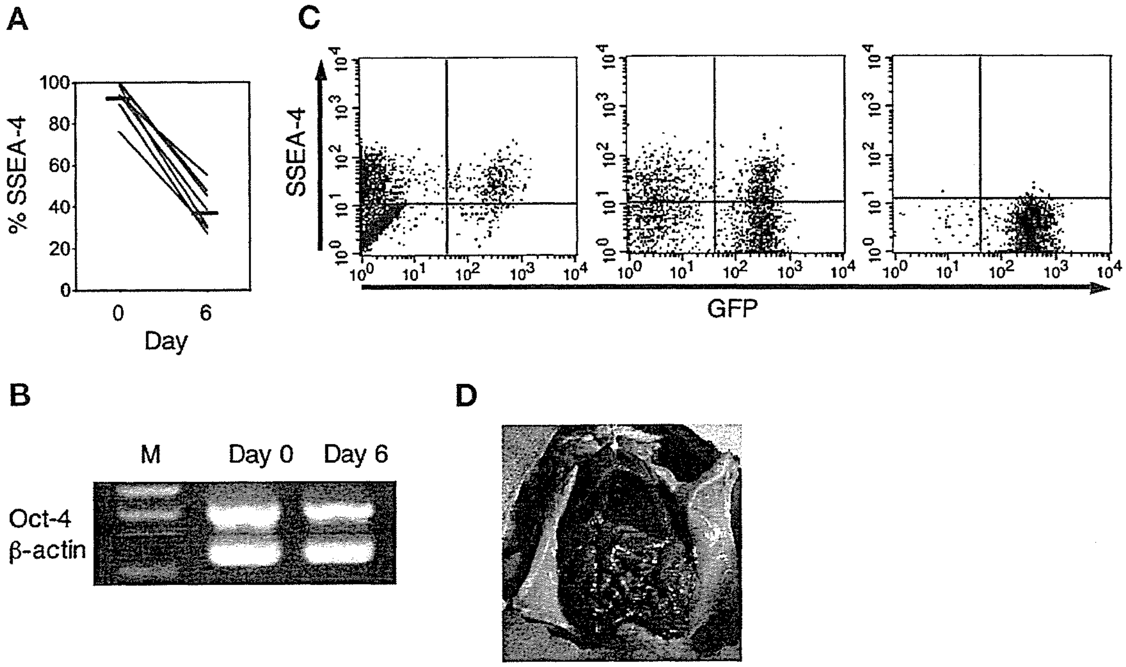

Figure 4. Purging SSEA-4$^+$ cells from among cyESC-derived progenitor cells. **(A):** Undifferentiated cyESCs (day 0) and cyESC-derivatives (day 6) were stained with anti-SSEA-4. The SSEA-4 expression (percentage of total) at day 0 and day 6 is shown (n = 8). **(B):** The Oct-4 expression at days 0 and 6 was also examined by RNA polymerase chain reaction. **(C):** Flow cytometric dot-plot profiles are shown for the SSEA-4 versus GFP expression at day 0 (left), at day 6 before the purge (middle), and at day 6 after the purge (right). Six independent experiments were conducted, and similar results were obtained. **(D):** No tumors were detected in any monkey after the transplantation of SSEA-4-negative day-6 cyESC derivatives (a representative monkey, no. 0981). Abbreviations: cyESC, cynomolgus embryonic stem cell; GFP, green fluorescent protein; M, molecular weight marker; SSEA, stage-specific embryonic antigen.

the fraction was not spoiled (2.3%–5.0%; Table 1), although the removed SSEA-4$^+$ fraction included some CD34$^+$ cells (data not shown).

DISCUSSION

We have previously described a method for hematopoietic engraftment from cyESCs [13]. cyESCs were first cultured for 6 days in vitro, and the day-6 cyESC-derived putative hematopoietic precursors were transplanted in vivo into fetal sheep liver after the first trimester, generating sheep with cynomolgus hematopoiesis. We transplanted the day-6 cells because the CD34 expression level was highest at this time point (Fig. 1C). We transplanted the cells into the liver because the liver is the major hematopoietic organ at this stage of gestation in sheep [34]. In the present study, we tested this method in a cynomolgus monkey allogeneic transplantation model and successfully detected cyESC-derived hematopoietic cells in cynomolgus recipients, albeit at low levels. cyESC-derived chimerism was, however, higher in the primate allogeneic transplantation model (2.3%–5.0%) than in our recently reported sheep xeno-transplantation model (1.1%–1.6%; [13]) (Table 1). To enhance ESC-derived hematopoiesis, further consideration is required of the in vitro culture conditions (i.e., the cytokine milieu, coculture- or embryoid body-associated cellular microenvironment, culture period, and genetic manipulation) and the in utero transplantation conditions (i.e., the preconditioning, route, and timing).

Teratomas developed in all animals, even after the transplantation of ESC-derived progenitor cells that had been cultured for 6 days in the differentiation medium. The risk of tumor formation was high, given that we could hardly detect tumors in immunodeficient mice or fetal sheep that had been transplanted with the same day-6 cyESC derivatives ([13] and our unpublished data). Innate immune responses against cynomolgus-derived tumors might be more rigorous in xeno-transplanted mice and sheep than in allo-transplanted monkeys, resulting in a failure to detect tumorigenesis in the xeno-transplantation models. Similarly, Erdo et al. reported that tumors developed after ESC-derived progenitor cell transplantation in the mouse-to-mouse setting, but not in the mouse-to-rat setting [35]. Our monkey allogeneic transplantation setting would therefore allow the strict evaluation of the in vivo safety of transplantation therapies using ESCs. However, given that teratomas indeed form when undifferentiated cyESCs alone are xeno-transplanted into immunodeficient mice, it is unclear why residual undifferentiated cells included among the day-6 cyESC derivatives did not form teratomas in immunodeficient mice or fetal sheep.

SSEAs that are developmentally regulated during early embryogenesis are widely used as markers to monitor the differentiation of both mouse and human embryos and ESCs [36–38]. Undifferentiated ESCs of both human and cynomolgus origin are characterized by the expression of SSEA-4 and by a lack of SSEA-1 [1, 2, 18]. We have therefore used SSEA-4 as a marker for the negative selection of an undifferentiated fraction. As a result of this negative selection, tumors were no longer detected in the monkeys after transplantation. On the other hand, Bieberich et al. recently developed a method for selective apoptosis of residual pluripotent stem cells using the transcription factor Oct-4 as a pluripotency marker to prevent teratoma formation [39]. They found that the expression of Oct-4 is colocalized with that of prostate apoptosis response-4, a protein mediating ceramide-induced apoptosis. Treatment of ESC-derived neural precursors with ceramide resulted in selective elimination of residual Oct-4-positive pluripotent cells. Our method, however, uses a cell surface marker to purge pluripotent cells. With this method, one can see the purging efficiency in real-time. This would be meritorious for clinical applications. Although we used a cell sorter to obtain the SSEA-4^- fraction in the present study, selection with beads would be easier and more appropriate for clinical applications.

To generalize the use of SSEA-4 for eliminating undifferentiated cells from among donor cells, we differentiated cyESCs into neural stem cells. After the culture, approximately 10% of cells were still positive for SSEA-4. When all the cells were transplanted into the striatum of Parkinson's cynomolgus monkeys, teratomas developed. We then transplanted cyESC-derived neural stem cells without an SSEA-4^+ fraction into the cynomolgus striatum and successfully detected the engraftment without tumor formation (our unpublished data). The removal of SSEA-4^+ cells is useful at least for hematopoietic and neural lineages.

CONCLUSION

We are now able to prevent the formation of tumors in nonhuman primate recipients by purging SSEA-4^+ cells from among ESC-derived progenitor cells without spoiling the engraftment. SSEA-4 is therefore a clinically relevant pluripotency marker of primate ESCs. Purging pluripotent cells with this marker would be a promising method for producing clinical progenitor cell preparations using hESCs to improve safety in vivo.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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NOTE *Laboratory Animal Science*

Prevention of Immune Responses to Human Erythropoietin in Cynomolgus Monkeys (*Macaca fascicularis*)

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ABSTRACT. Genes and proteins of human origin are often administered to monkeys for research purposes, however, it can be difficult to obtain sufficient levels of the products *in vivo* due to immunological clearance. In this study, we showed that human erythropoietin (hEPO) induces generation of anti-hEPO antibody in cynomolgus macaques (n=2), although 92% of amino acid residues are common between the human and macaque EPO. The administered hEPO was thus eliminated from the animals. On the other hand, when an immunosuppressant, cyclosporin A (CyA), was administered (6 mg/kg) intramuscularly every other day in combination with hEPO (n=2), no anti-hEPO antibody was generated and high serum levels of hEPO were obtained during administration of hEPO, resulting in an increase in serum hemoglobin levels. No adverse effects associated with CyA were observed. Thus, CyA treatment is useful for prevention of immune responses associated with the administration of human proteins in monkeys.

KEY WORDS: cyclosporin A, cynomolgus monkey, erythropoietin.

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Macaque monkeys are widely used for preclinical testing of genes and proteins of human origin, taking advantage of their close phylogenetic relationship to humans [5, 11, 21]. Despite the genetic similarity between the two species, human gene products or proteins are often immunogenic to monkeys. An example is erythropoietin (EPO). EPO is a hematopoietic growth factor that stimulates the proliferation and differentiation of erythroid progenitor cells [10]. Recombinant human EPO (hEPO) has a variety of clinical uses [4, 6, 17, 22]. Although 92% of amino acid residues (142/166) are common between human and macaque EPO [12, 20], we showed here that hEPO induces potent immune responses in macaque monkeys, precluding its administration to monkeys.

Therefore, it is necessary to develop a method to prevent such immune responses following administration of hEPO. Among many immunosupressants available, cyclosporin A (CyA) is widely used to suppress detrimental immune reactions associated with allogenic bone marrow and organ transplantation [1–3, 19]. CyA is a calcineurin inhibitor that inhibits nuclear factor of activated T cells (NFAT) activity and induces immunosuppression [9, 13]. In this study, we showed that hEPO can be successfully administered to cynomolgus monkeys (*Macaca fascicularis*) without immunological clearance by using CyA.

Four cynomolgus monkeys (4–6 years old, 2.5–5.5 kg) bred in the Tsukuba Primate Research Center (Ibaraki, Japan) were used in this study (Table 1). The animals were free of intestinal parasites, herpes-B, simian type-D retrovirus, and simian varicella virus. This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [8] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [14]. The protocols of the experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

First, we administered hEPO (Chugai, Tokyo, Japan) subcutaneously to a cynomolgus monkey (099054) at a dose of 3,000 IU/kg three times a week and assessed the hEPO concentrations in the serum by enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany). Low levels (< 1.0 *ng*/m*l*) of hEPO were detected for the first 3 weeks, but thereafter the levels decreased to the lowest limit of detection (0.01 *ng*/m*l*) despite continued administration of hEPO (Fig. 1A). Assessment by ELIZA revealed that anti-hEPO antibody was being generated [7] (Fig. 1A), and the hEPO was cleared from the serum. A second cynomolgus monkey (001051) was intravenously (instead of subcutaneously) given a much lower dose of hEPO (200 IU/kg, three times a week). During administration, very low levels (< 0.1 *ng*/m*l*) of hEPO were detected with the exception of one time point (1.0 *ng*/m*l* at day 28), and the levels eventually decreased to zero (Fig. 1B). Despite the lower dose, anti-hEPO antibody was generated again (Fig. 1B), leading to clearance of hEPO from the serum. Although we did not try subcutaneous administration of 200 IU/kg hEPO in the present study (Table 1), we assumed that subcutaneous administration of 200 IU/kg hEPO would also result in anti-hEPO antibody generation

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Table 1. Characteristics of cynomolgus monkeys subjected to hEPO administration

	Animal (Sex)	Age (years)	Body Weight (kg)	hEPO		CyA		Hemoglobin Levels (g/dl)		Complication
				Dose (IU/kg)	Administration Route and Frequency	Dose (*n*g/kg)	Administration Route and Frequency	Day 0	Day 35	
hEPO Only	099054 (Male)	5	5.5	3000	Subcutaneous (3 times a week)	–	–	12.7	12.6	Antibody production
	001051 (Female)	4	2.5	200	Intravenous (3 times a week)	–	–	12.4	12.4	Antibody production
	Average	4.5	4.0	–	–	–	–	12.6	12.5	–
hEPO and CyA	396053 (Female)	6	3.2	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	10.9	11.6	None
	396058 (Female)	6	4.0	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	11.1	12.0	None
	Average	5.5	3.6	–	–	–	–	11.0	11.8	–

given that intravenous administration of the same dose of hEPO produced this result. The reason for this was subcutaneous administration is known to induce a stronger immune response than intravenous administration [16]. The hemoglobin levels did not increase in either animal (Table 1). Despite the genetic similarity of EPO between humans and macaques [12, 20], hEPO is a potent immunogen in macaque monkeys. This is the first report on the immune responses in monkeys following administration of hEPO.

On the other hand, two cynomolgus monkeys (396053, 396058) were given 6 mg/kg of CyA (Sandimmun; Novartis Pharma, Basel, Switzerland) intramuscularly every other day in combination with subcutaneous hEPO administration (200 IU/kg, three times a week) (Table 1). CyA concentrations in the plasma were assessed by radioimmunoassay according to a previously reported method [15], and it was found that the concentrations were maintained within an effective range of 200 to 400 *n*g/m*l*. As a result, no anti-hEPO antibody was generated in either monkey and high serum levels (around 10 *n*g/m*l*) of hEPO were obtained during administration of hEPO (Figs. 2A and 2B). A second trial of hEPO resulted in a similar elevation of the serum levels of hEPO (Figs. 2A and 2B). The hemoglobin levels apparently increased in response to administration of hEPO (Table 1), suggesting that the hEPO trial was effective when CyA was administered together. Blood biochemistry tests revealed no adverse effects associated with the CyA and hEPO treatment.

We have thus established a method to prevent immune responses to hEPO in cynomolgus monkeys using CyA. In fact, this method has successfully been applied to our preclinical monkey testing, and the long-term (around 1 year) efficacy and safety of CyA administration has been well demonstrated [18]. CyA administration will be useful in preventing immune responses when human proteins are administered to monkeys for research purposes.

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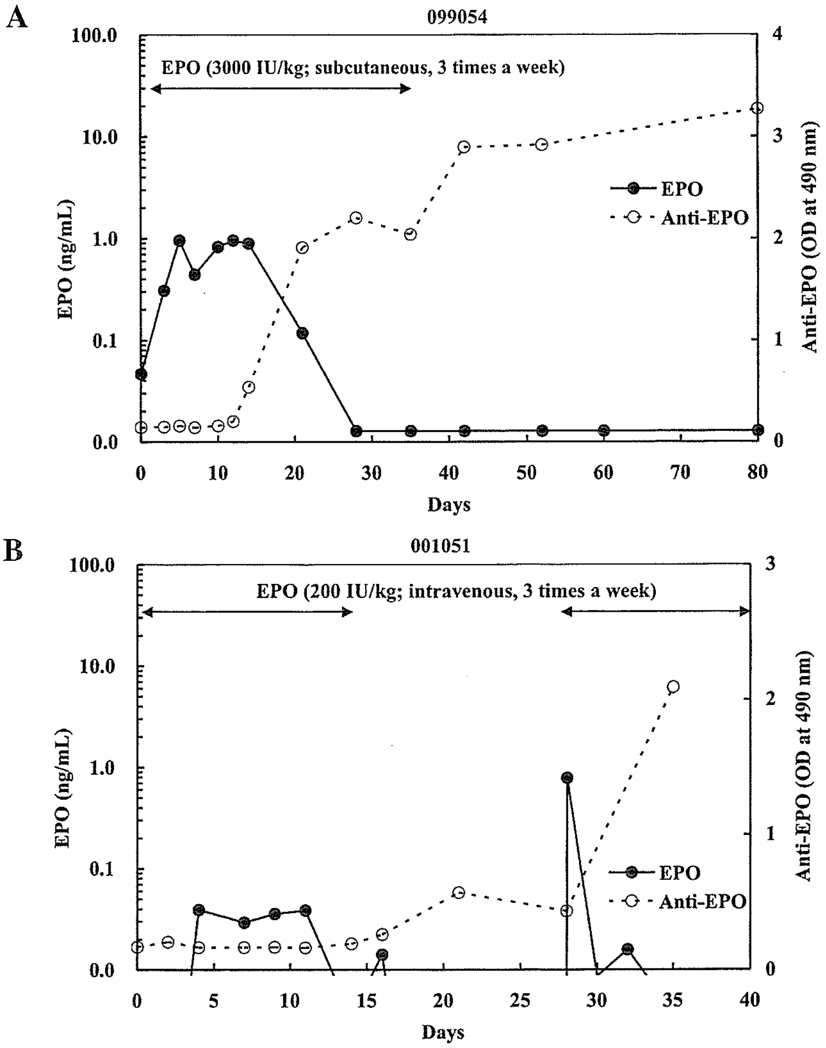

Fig. 1. Administration of only hEPO in cynomolgus monkeys. After subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero (A). Anti-hEPO antibody was also generated in another monkey (001051) receiving hEPO intravenously at a lower dose (200 IU/kg), leading to clearance of hEPO from the serum (B).

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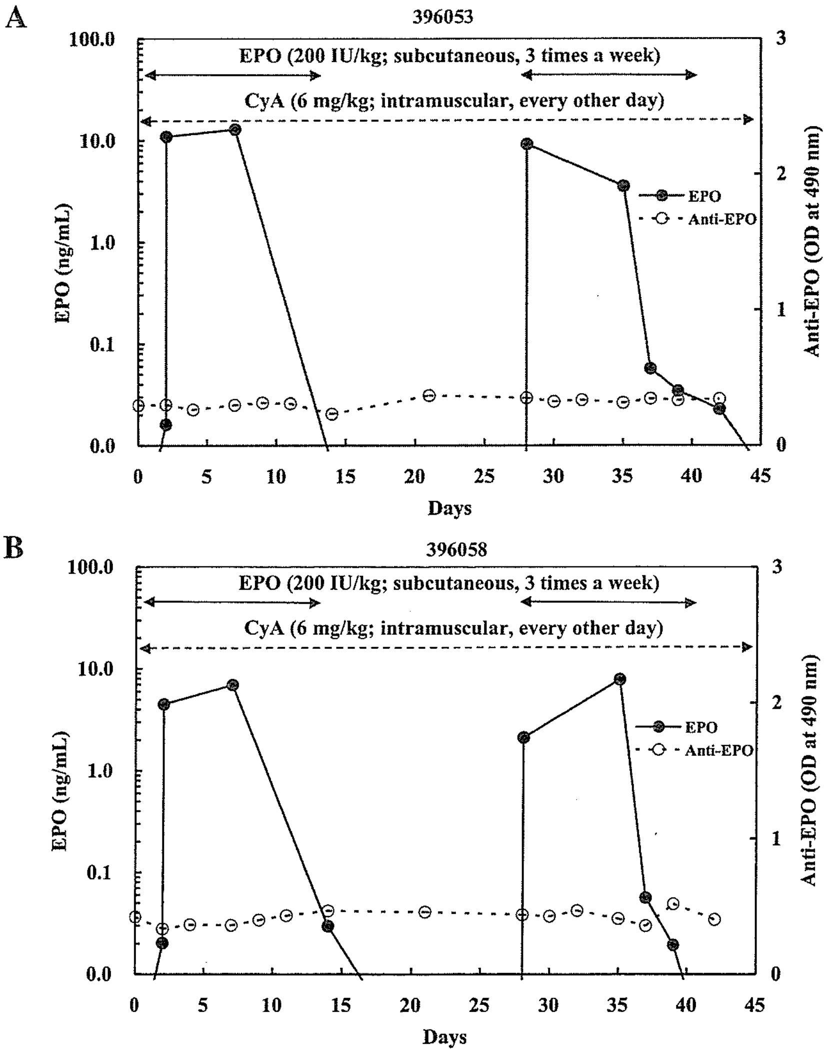

Fig. 2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by treatment with CyA in 2 cynomolgus monkeys (396053, 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of 200 to 400 *ng*/m*l*. 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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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.

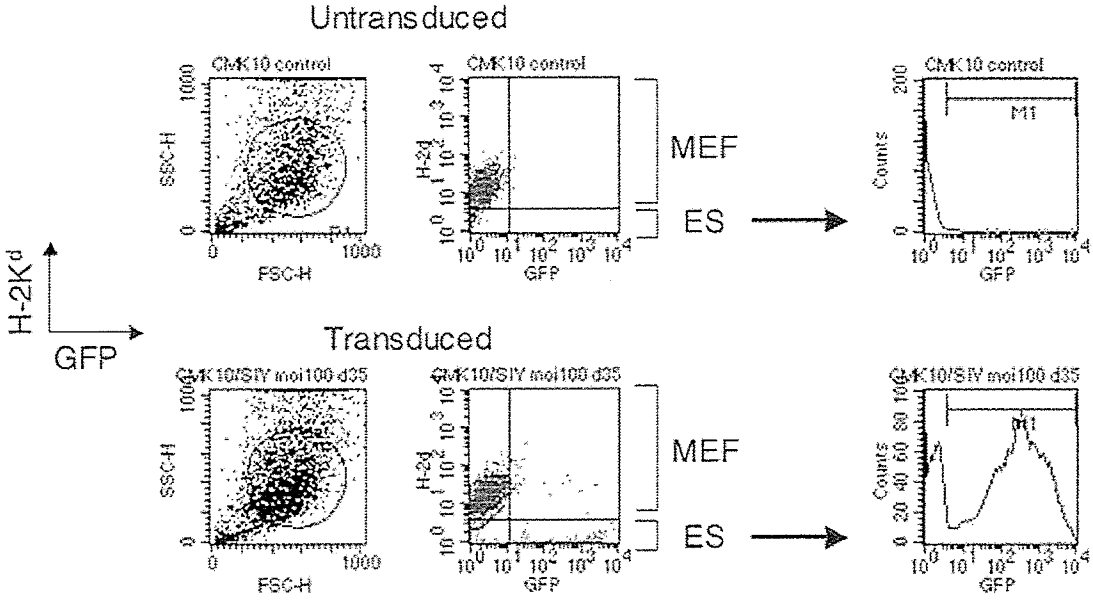

Fig. 1. Assessment of transduction efficiency by flow cytometry. The transgene green fluorescent protein (GFP) expression was analyzed on a FACScan using *CellQuest* software 2–3 d after transduction with a GFP-expressing simian immunodeficiency virus vector. The co-cultured BALB/c-derived feeder cells 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.

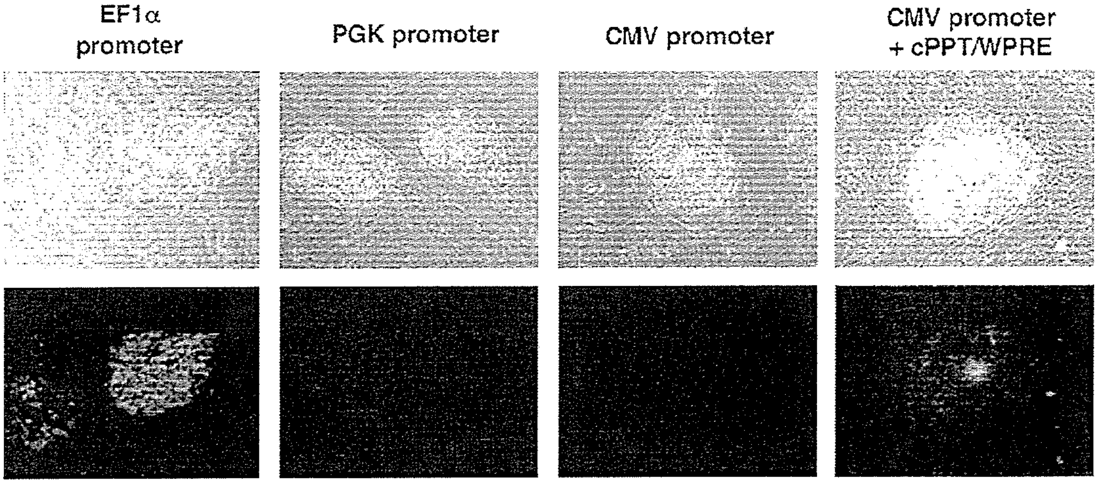

Fig. 2. Promoters and *cis*-acting sequences in simian immunodeficiency virus (SIV) vectors affect transgene expression. Cynomolgus embryonic stem (ES) cells were transduced with green fluorescent protein (GFP)-expressing SIV vectors at 30 TU per target cell. The vectors contain the elongation factor (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