concentration as that obtained by muscle- or liver-mediated gene transfer in our previous study (Mochizuki *et al.*, 2004). At a dose of 6×10^{10} VG/body, which was the standard dose for muscle- and liver-mediated gene transfer, the Epo concentration was less prominent; the plasma Epo concentration became comparable at a dose of 2×10^{11} VG/body. Therefore, even after the addition of F88, transduction efficiency was still low in adipose tissue. Whether there are any better methods to augment the efficiency of transduction, including the use of a higher vector dose or other serotype-derived vectors, needs to be investigated further.

In our series of experiments, all the transduced mice became polycythemic; therefore, transgene-derived Epo was functional (data not shown). Although the Epo concentration was augmented by the addition of Pluronic F88, there was no significant difference in blood hemoglobin levels or red blood cell counts among the groups. This is because the Epo concentrations in the transduced animals were far beyond the physiological dose–response window (Mochizuki *et al.*, 2004), and even modest Epo expression after injecting the vector without Pluronic F88 could result in polycythemic conditions. It is generally difficult to eliminate the possibility that the use of this excipient may alter the tropism of the vector and promote gene transfer to certain remote organ(s). Nonetheless, because removal of the transduced adipose tissue resulted in the elimination of the Epo (Fig. 4), we can exclude this possibility. Whether the tissue specificity of expression is common to all serotypes of AAV is yet to be confirmed. To test the tissue specificity, *db/db* mice are useful because they develop rich adipose tissues and a specific lobe can be completely removed by standard surgical procedures. On the other hand, the limitation of this model lies in the difficulty of long-term transgene expression: these animals were naturally diabetic and susceptible to thromboembolic events when they became polycythemic and eventually lost their lives after 4 weeks (Table 1). In this series of experiments, no clear threshold of Epo level on mortality was recognized, although all the "operated" animals attained long-term survival with normalized values of Epo and blood parameters. Therefore, in order to demonstrate long-term expression, a different transgene needs to be used.

Transducing adipose tissue may have another advantage with respect to immunology. Although the distribution and density of antigen-presenting cells within the adipose tissue remain unknown, it is possible that these cells are relatively scarce in the adipose tissue than in "standard" tissues such as muscle or liver. Therefore, the immune response against transgene product, which is a current hurdle in the field of gene therapy (Zaiss and Muruve, 2005), can partly be overcome by targeting adipose tissue. In our series of experiments, we did not observe any immunological responses to the transgene products or to the transduced adipose tissues. To test this hypothesis, a transgene product that is highly immunogenic to mice should be chosen and the outcome needs to be evaluated.

Adipose tissue is usually abundant in the body, can be easily transduced by simple vector injection, and can be removed safely. For these reasons, it is a potential depot organ for gene transfer. In this sense, there may be a wide range of applications of this method in supplemental gene therapy.

ACKNOWLEDGMENTS

The authors are grateful to Asahi Denka (Tokyo, Japan) for supplying samples of Pluronics and providing valuable suggestions concerning the use of these compounds. They also thank Miyoko Mitsu and Fumino Muroi for excellent technical assistance. This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labor, and Welfare, Japan; the High-Technology Research Center Project for Private Universities: a matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology, 2003–2007; and the 21st Century Centers of Excellence Program from the Ministry of Education, Culture, Sports, Science, and Technology.

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Received for publication March 23, 2006; accepted after revision July 20, 2006.

Published online: August 7, 2006.

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Improved Safety of Hematopoietic Transplantation with Monkey Embryonic Stem Cells in the Allogeneic Setting

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Key Words. Cynomolgus monkey • Hematopoiesis • Embryonic stem cell • In utero transplantation • Teratoma • Purging Tumor prevention

ABSTRACT

Cynomolgus monkey embryonic stem cell (cyESC)-derived in vivo hematopoiesis was examined in an allogeneic transplantation model. cyESCs were induced to differentiate into the putative hematopoietic precursors in vitro, and the cells were transplanted into the fetal cynomolgus liver at approximately the end of the first trimester (n = 3). Although cyESC-derived hematopoietic colony-forming cells were detected in the newborns (4.1%–4.7%), a teratoma developed in all newborns. The risk of tumor formation was high in this allogeneic transplantation model, given that tumors were hardly observed in immunodeficient mice or fetal sheep that had been xeno-transplanted with the same cyESC derivatives. It turned out that the cyESC-derived donor cells included a residual undifferentiated fraction positive for stage-specific embryonic antigen (SSEA)-4 (38.2% ± 10.3%) despite the rigorous differentiation culture. When an SSEA-4-negative fraction was transplanted (n = 6), the teratoma was no longer observed, whereas the cyESC-derived hematopoietic engraftment was unperturbed (2.3%–5.0%). SSEA-4 is therefore a clinically relevant pluripotency marker of primate embryonic stem cells (ESCs). Purging pluripotent cells with this surface marker would be a promising method of producing clinical progenitor cell preparations using human ESCs. STEM CELLS *2006;24:1450–1457*

INTRODUCTION

Human embryonic stem cells (hESCs) hold great potential in the treatment of a variety of diseases and injuries because embryonic stem cells (ESCs) have the ability to proliferate indefinitely in culture and to differentiate into any cell type [1, 2]. Because ESCs are able to form teratomas when transplanted into immunodeficient mice, safety concerns would be raised against the clinical application of hESCs [3, 4]. It will be necessary to test the safety of these cells in animal transplantation models before clinical application. Nonhuman primate transplantation models would be desirable for this purpose; however, there have been only a few reports on these models [5–7]. The successful engraftment of transplanted cells in primates will not be achieved unless the immune rejection of transplanted cells is circumvented (e.g., through immunosuppressive treatment) [6]. The early gestational fetus may be a good recipient with which to circumvent immune rejection because the immune system is premature [8]. In addition, in the animal fetus, transplanted cells would engraft without conditioning of recipients such as irradiation or immunosuppressive treatment [9–12]. We have previously established a system for allogeneic transplantation of cynomolgus ESCs (cyESCs) using preimmune fetal monkeys as recipients [5].

We have also reported a novel method for hematopoietic engraftment from cyESCs in sheep [13]. The method is a combination of three steps: (a) differentiation in vitro to generate the putative hematopoietic precursors [14]; (b) transplantation of the cells in utero [15]; and (c) development into hematopoietic cells in vivo using the hematopoietic microenvironment of the fetal liver [16]. In the present study,

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we have examined the safety as well as the efficacy of hematopoietic engraftment of cells derived from cyESCs in the allogeneic transplantation model.

Materials and Methods

Animals

Pregnant cynomolgus monkeys (16–22 years old) were obtained by mating and were reared at the Tsukuba Primate Research Center in accordance with Rules for Animals Care and Management set forth by the Research Center and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan. Experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. The animals were free of intestinal parasites and were seronegative for herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus.

Cell Preparation

A cyESC line (CMK6G) stably expressing green fluorescent protein (GFP) was established after transfection of the parental cyESC line (CMK6) with the enhanced GFP gene (Clontech, Palo Alto, CA, http://www.clontech.com) [17]. cyESCs were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, http://www.kyowa.co.jp)-treated mouse (ICR or BALB/c; Clea Japan, Tokyo, http://www.clea-japan.com) embryonic fibroblasts as previously described [18]. The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% fetal calf serum (FCS; Invitrogen) [19].

cyESCs were induced to differentiate into the putative hematopoietic precursors as previously described [13]. Briefly, undifferentiated cyESCs were transferred onto mitomycin C-treated confluent OP9 cells and cultured for 6 days in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8% FCS, 8% horse serum (Invitrogen), 5×10^{-6} M hydrocortisone (Sigma, St. Louis, http://www.sigmaaldrich.com), and multiple cytokines, including 20 ng/ml recombinant human (rh) bone morphogenetic protein-4 (R&D Systems, Minneapolis, http://www.rndsystems.com), 20 ng/ml rh stem cell factor (Biosource, Camarillo, CA, http://www.biosource.com), 20 ng/ml rh vascular endothelial growth factor (VEGF; R&D Systems), 20 ng/ml rh Flt-3 ligand (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), 20 ng/ml rh interleukin-3 (PeproTech), 10 ng/ml rh interleukin-6 (PeproTech), 20 ng/ml rh granulocyte colony-stimulating factor (PeproTech), and 2 IU/ml rh erythropoietin (Roche, Basel, Switzerland, http://www.roche.com). The cells were resuspended in 0.1% human serum albumin (Sigma)/Hanks' balanced saline solution (Sigma) for transplantation.

Flow Cytometry

Primary antibodies (Abs) used in the present study were anti-human CD34 monoclonal Ab (mAb; BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), anti-human CD31 mAb (Pharmingen), anti-human CD45 mAb (Pharmingen), anti-human vascular endothelial (VE) cadherin mAb (Pharmingen), rabbit anti-human VEGF receptor (VEGFR)-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), and anti-stage-specific embryonic antigen (SSEA)-4 mAb (Chemicon, Temecula, CA, http://www.chemicon.com). All of them cross-reacted to cynomolgus counterparts as previously demonstrated [18, 20–22]. Secondary Abs were phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (Ig) Ab (DakoCytomation, Glostrup, Denmark, http://www.dako.com) and Alexa Fluor 647-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Cells stained with unlabeled primary Abs were incubated with fluorescence-labeled secondary Abs. Cells were incubated with either primary or secondary Ab for 20–60 minutes at 4°C. Regarding staining with the anti-VEGFR-2 Ab, the cells were incubated with biotin-conjugated goat anti-rabbit IgG Ab (Beckman Coulter, Miami, http://www.beckmancoulter.com), followed by PE-conjugated streptavidin (Beckman Coulter). Fluorescence-labeled cells were analyzed with a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Data analysis was performed using the CellQuest software (Becton, Dickinson and Company). Isotype-matched, irrelevant mAbs (DakoCytomation or Beckman Coulter) served as negative controls. Nonviable cells were excluded from analysis by propidium iodide (Sigma) costaining.

Cell Sorting

Cell sorting was performed to purge SSEA-4^+ cells from among the cultured cyESCs in vitro. Cells were incubated with the anti-SSEA-4 mAb for 1 hour at 4°C and washed twice with Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were then incubated with the PE-conjugated anti-mouse Ig Ab for 1 hour at 4°C and washed twice again. GFP-positive and SSEA-4-negative cells were sorted using an Epics Elite cell sorter (Beckman Coulter). Data acquisition was performed using the Expo2 software (Beckman Coulter).

Transplantation and Delivery

Transplant procedures were previously described [5]. Briefly, animals were anesthetized via an intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, http://www.sankyo.co.jp) and received 0.5%–1.0% isoflurane by inhalation by means of an endotracheal tube. Cells ($0.16–46 \times 10^6$ cells per fetus; Table 1) were injected into the fetal liver through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester. The fetuses were delivered by cesarean section at 2–3 months after transplant (gestation 120–157 days, full term 165 days).

Colony Polymerase Chain Reaction

Cynomolgus clonogenic hematopoietic colonies were produced as previously described [20]. After cells were cultured in methylcellulose medium for 10–14 days, well-separated individual colonies were plucked into 50 μl of distilled water and digested with 20 μg/ml proteinase K (Takara, Shiga, Japan, http://www.takara-bio.com) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μl) was used for a nested polymerase chain reaction (PCR) to detect the *GFP* gene sequence. The outer primer set was 5′-AAGGACGACGGCAACTACAA-3′ and 5′-ACTGGGTGCTCAGGTAGTGG-3′, and the inner primer set was 5′-GCATCGACTTCAAGGAGGAC-3′ and 5′-GTTGTGGCGGATCTTGAAGT-3′. Amplification conditions for both the outer and inner PCR were 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The

Table 1. ESC-derived hematopoiesis and tumor formation

Animals	Animal no.	Transplanted cells	Purging SSEA-4$^+$ cells	Cell number per fetus ($\times 10^6$)	Donor-derived CFU in recipients[a] at birth (donor/total colony number)	Tumor formation	Observation period (months)
Monkeys	0031	Undifferentiated	−	3.90	n.d.	+	3
	2311	ESCs	−	0.16	n.d., Dead	+	2
	0321		−	0.21	n.d., Dead	+	2
	0841	Day-6 ESC-	−	10	4.1% (2/49)	+	3
	1551	derived cells	−	46	n.d., Dead	+	2.5
	0021		−	46	4.7% (4/85)	+	3
	0691	Day-6 ESC-	+	0.16	3.2% (2/62)	−	3
	0381	derived cells	+	1.40	5.0% (4/80)	−	3
	0022		+	0.17	2.3% (2/86)	−	3
	0981		+	0.31	4.1% (3/73)	−	3
	0051		+	0.31	n.d., Dead[b]	−	3
	1552		+	0.75	4.4% (2/45)	−	4
Sheep[c]	57	Day-6 ESC-	−	50	1.1% (1/91)	−	18
	55	derived cells	−	50	1.1% (1/91)	−	26
	141		−	78	1.1% (1/91)	−	26
	182		−	14	1.6% (1/63)	−	21

[a]Percentage of donor-derived CFU was calculated by dividing the number of CFU positive for the green fluorescent protein gene sequence by the number of CFU positive for the β-actin gene sequence. Donor-derived CFU were analyzed at delivery.
[b]Death due to ablation of placentae. Other deaths were presumably tumor-related.
[c]As published by Sasaki et al. [13].
Abbreviations: CFU, colony-forming units; ESC, embryonic stem cell; n.d., not done; SSEA, stage-specific embryonic antigen.

outer PCR products were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA, http://www.qiagen.com). Simultaneous PCR for the β-actin sequence was also performed to ensure DNA amplification of the sample in each colony. The primer set for β-actin was 5′-CATTGTCATGGACTCTGGCGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGGGC-3′. Amplification conditions for β-actin PCR were 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplified GFP (131 bp) and β-actin (234 bp) products were resolved on 2% agarose gel (Sigma) and visualized by ethidium bromide (Invitrogen) staining.

RNA PCR

Total RNA was extracted from cells of interest using the EZ1 RNA universal tissue kit (Qiagen). RNA was reverse-transcribed at 50°C for 30 minutes using the RNA LA PCR kit (Takara) with oligo dT primer. The resulting cDNA was then subjected to PCR. Regarding PCR for Oct-4, the primer set was 5′-GGACACCTGGCTTCGGATT-3′ and 5′-TTCGCTTTCTCTTTCGGGC-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 1.5 minutes. Regarding PCR for Scl, the primer set was 5′-GGGCGGAAAGCTGTTTGCGATT-3′ and 5′-TCGCTGAGAGGCCTGCAGTT-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 63°C for 1 minute, and 72°C for 1 minute. A simultaneous PCR for β-actin was also conducted on each cDNA sample as an internal control as described above. Amplified Oct-4 (697 bp), Scl (201 bp), and β-actin (234 bp) products were resolved on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

In Utero Transplantation and Delivery

cyESCs stably expressing GFP were used in this study [17]. In the setting of allogeneic transplantation, GFP was used as a genetic tag to track transplanted cell progeny. We employed the OP9 stromal cell coculture method instead of the embryoid body formation method to facilitate the hematopoietic differentiation [19, 23, 24] (Fig. 1A, 1B). According to the flow cytometric analysis, CD34, CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]), CD144 (VE-cadherin), and VEGFR-2 (Flk-1) were all upregulated on day 6 but decreased thereafter (Fig. 1C–1E, 1G). Among the markers examined, CD34 is a widely used surface marker of hematopoietic stem cells in both human and monkey subjects [25–27]. The others are key markers of hemangioblasts (which generate endothelial and hematopoietic lineages) in both mice and humans [14, 28]. Cells positive for both VEGFR-2 and VE-cadherin emerged on day 6 (Fig. 1H). CD45, however, was not detected until day 12 (Fig. 1F). Despite the hemangioblast marker expression on day 6, the hematopoietic *Scl* gene was upregulated at this time point as assessed by RNA PCR (Fig. 1I), implying that the hematopoietic commitment might have already occurred on day 6 [29, 30]. We therefore designated the day 6 cyESC-derived progenitor cells as putative hematopoietic precursors. The time course profiles presented here were similar to those of hESCs [14, 24]. The GFP expression was stable during the 6-day culture (Fig. 1A, 1B) and afterward (data not shown).

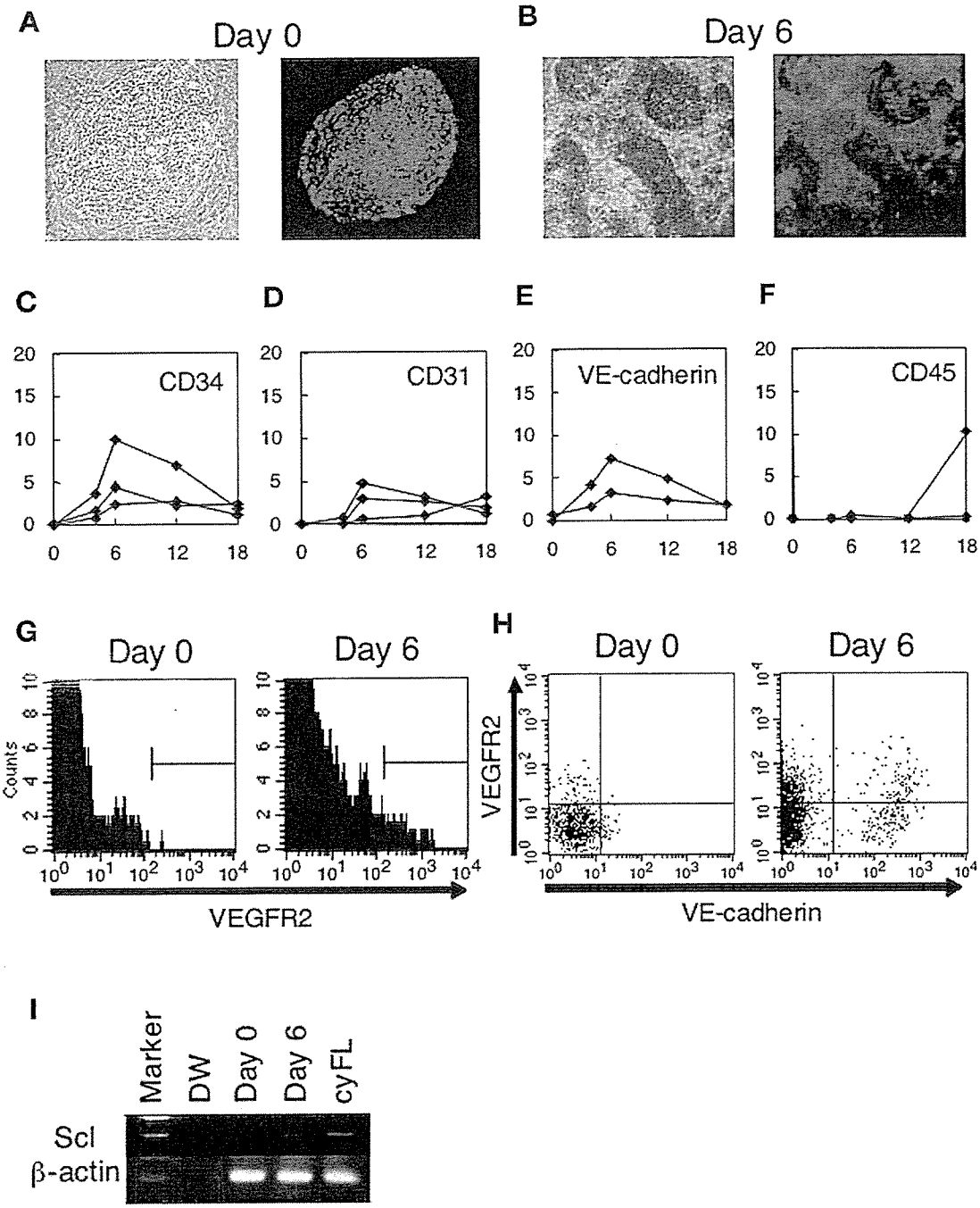

Figure 1. Flow cytometric analysis during the in vitro differentiation of cyESCs. Undifferentiated cyESCs expressing green fluorescent protein were cultured on OP9 cells with multiple cytokines (see Materials and Methods). **(A):** Cells on day 0 are shown in bright (left) and dark (right) fields. **(B):** Cells on day 6 are shown in bright (left) and dark (right) fields. **(C):** Cells on days 0, 4, 6, 12, and 18 were stained for CD34. **(D):** Cells on days 0, 4, 6, 12, and 18 were stained for CD31. **(E):** Cells on days 0, 4, 6, 12, and 18 were stained for VE-cadherin. **(F):** Cells on days 0, 4, 6, 12, and 18 were stained for CD45. The vertical axis shows the fraction (percentage) of cells that were stained positive. **(C–F):** Results of two or three independent experiments are shown. **(G):** Although cells on day 0 already express low levels of VEGFR-2, a VEGFR-2^{high} population did not emerge until day 6. **(H):** Dot-plot profiles for VEGFR-2 and VE-cadherin expression indicate that cells positive for both VEGFR-2 and VE-cadherin emerged until day 6. **(G, H):** Representative results from three independent experiments are shown. **(I):** The *Scl* gene expression was upregulated on day 6 to a level similar to that in the cynomolgus fetal liver as assessed by RNA polymerase chain reaction. Day-6 cells (putative hematopoietic precursors) were used for transplantation. Abbreviations: cyESC, cynomolgus embryonic stem cell; cyFL, cynomolgus fetal liver; DW, distilled water; VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor.

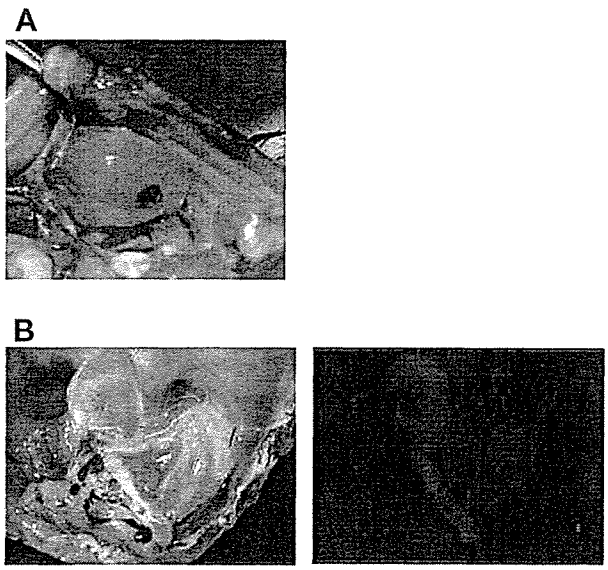

Figure 2. Tumor formation after the transplantation of cynomolgus embryonic stem cell (cyESC)-derived progenitor cells. Tumors formed in all three monkey fetuses transplanted with the day-6 cyESC-derived progenitor cells (putative hematopoietic precursors). **(A):** A representative tumor in the thoracic cavity at 3 months after transplantation (monkey no. 0841). **(B):** The tumor was observed in bright (left) and dark (right) fields under a fluorescence microscope.

Teratoma Formation

The undifferentiated cyESCs ($n = 3$) or cyESC-derived putative hematopoietic precursors ($n = 3$) were transplanted in utero into allogeneic fetuses in the liver under ultrasound guidance at approximately the end of the first trimester (49–66 days, full term 165 days) (Table 1). Regardless of whether the undifferentiated cyESCs or putative hematopoietic precursors were transplanted, tumors were found in the thoracic or abdominal cavities in all the six animals at 2–3 months after transplant (Table 1; Fig. 2A). The tumors fluoresced (Fig. 2B) and consisted of three germ layer cells. Thus, they were teratomas derived from transplanted cells. However, tumors were hardly observed in fetal sheep (1/10; [13] and our unpublished data) (Table 1) and immunodeficient (nonobese diabetic/severe combined immunodeficient) mice (3/10; our unpublished data) after the same putative hematopoietic precursors were transplanted.

In Vivo cyESC-Derived Hematopoiesis

Regarding the newborn monkeys that had been transplanted with the putative hematopoietic precursors, we harvested cells from the femur, cord blood, and liver and plated the cells in methylcellulose medium to produce clonogenic hematopoietic colonies (colony-forming units [CFU]) (Fig. 3A). The monkey cells generated colonies of clear hematopoietic morphology in this assay (Fig. 3B). To detect transplanted cell-derived, GFP-positive colonies, we tried to observe GFP fluorescence of colonies under a fluorescent microscope but were hampered by the high autofluorescence. We then conducted PCR for the *GFP* gene sequence in DNA isolated from each colony (colony PCR) (Fig. 3C). The transplanted cell-derived CFU were clearly detected in the animals (4.1% and 4.7%; Table 1). We repeated the colony PCR and confirmed that the results were reproducible.

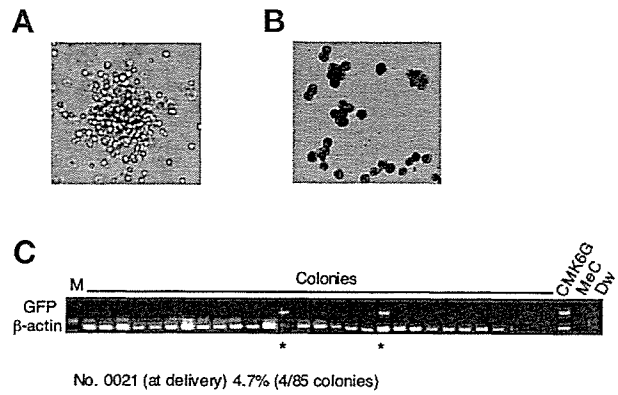

Figure 3. cyESC-derived hematopoiesis in vivo. **(A):** Bone marrow, cord blood, and liver cells were harvested from newborn monkeys and placed in methylcellulose medium to produce clonogenic hematopoietic colonies. **(B):** A cytospin specimen (stained with the May-Giemsa method) of plucked colonies reveals mature neutrophils. To identify cyESC-derived colonies, well-separated individual colonies were plucked and examined for the GFP sequence by PCR. Plucked MeC alone (not containing colonies) served as a negative control. PCR of the β-actin sequence in the same colonies was simultaneously performed as an internal control. Colony PCR was repeated at least twice. **(C):** Representative colony PCR results for monkey no. 0021. Asterisk indicates bands positive for the GFP sequence. Abbreviations: CMK6G, positive control green fluorescent protein-expressing cynomolgus cells; cyESC, cynomolgus embryonic stem cell; DW, distilled water; GFP, green fluorescent protein; M, molecular weight marker; MeC, methylcellulose; PCR, polymerase chain reaction.

We detected both granulocytic and erythroid cynomolgus CFU. In the peripheral blood, however, we were not able to detect cells expressing GFP by flow cytometry. It turned out that, as assessed by quantitative PCR, the fractions of GFP-positive cells in the peripheral blood were very small (<0.1%). Low peripheral "chimerism" has been reported more than once in other in utero transplantations of ESCs or hematopoietic stem cells such as in mice, sheep, and pigs [13, 31–33].

Purging SSEA-4$^+$ Cells of the Putative Hematopoietic Precursors

We examined the expression of an undifferentiated primate ESC marker, SSEA-4, in the undifferentiated cyESCs (day 0) and putative hematopoietic precursors (day 6). The proportion of SSEA-4$^+$ cells was 93.4% ± 8.1% and 38.2% ± 10.3% among the day-0 and -6 cells, respectively (Fig. 4A). A substantial number of cells were still positive for SSEA-4 after the rigorous differentiation culture. In addition, a considerable number of cells expressing another undifferentiated marker, Oct-4, remained among the day-6 population as assessed by RNA-PCR (Fig. 4B). Those residual undifferentiated cells might be responsible for the formation of teratomas in the recipients.

To prevent teratomas from forming in recipients, we purged SSEA-4$^+$ cells of the putative hematopoietic precursors and transplanted the SSEA-4$^-$ population into the fetal monkey liver ($n = 6$) (Fig. 4C). At delivery, tumors were no longer observed in the six animals that had been transplanted with the sorted SSEA-4$^-$ cells (Fig. 4D). The transplanted cell-derived CFU were clearly detected in the newborns, and

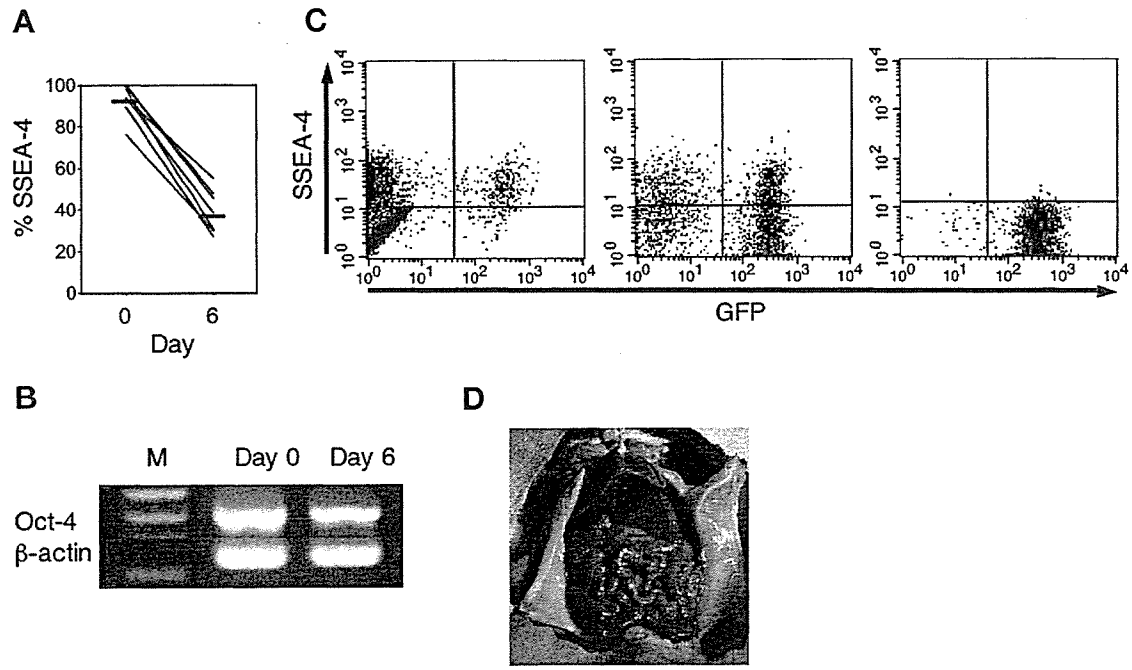

Figure 4. Purging SSEA-4$^+$ cells from among cyESC-derived progenitor cells. **(A):** Undifferentiated cyESCs (day 0) and cyESC-derivatives (day 6) were stained with anti-SSEA-4. The SSEA-4 expression (percentage of total) at day 0 and day 6 is shown (n = 8). **(B):** The Oct-4 expression at days 0 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.

ACKNOWLEDGMENTS

We thank Norio Nakatsuji (Kyoto University, Kyoto, Japan) and Yasushi Kondo (Tanabe Seiyaku Co., Ltd., Osaka, Japan) for providing cyESCs; Toru Nakano (Osaka University, Osaka, Japan) for providing OP9 cells; and Naomi Terao and Naomi Takino for technical assistance. This study was supported by grants (JMS 21st Century COE program, High-tech Research Center program, and Creation of Innovations) from the Ministry of Education, Culture, Sports, Science and Technology of Japan as well as grants (KAKENHI) from the Ministry of Health, Labor and Welfare of Japan.

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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(Received 13 September 2005/Accepted 17 January 2006)

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.

J. Vet. Med. Sci. 68(5): 507–510, 2006

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/ml*) of hEPO were detected for the first 3 weeks, but thereafter the levels decreased to the lowest limit of detection (0.01 *ng/ml*) 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/ml*) of hEPO were detected with the exception of one time point (1.0 *ng/ml* 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 (*ng*/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 *ng*/ml. As a result, no anti-hEPO antibody was generated in either monkey and high serum levels (around 10 *ng*/ml) 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.

ACKOWLEDGEMENTS. We thank Hayato Narita, Hiromi Ogawa, and Katsuhiko Komatsuzaki for the handling and care of the monkeys. The hEPO was kindly supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). This study was supported by grants (KAKENHI, HAITEKU, JMS 21st Century COE Program, and Creation of Innovations) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and grants (KAKENHI) from the Ministry of Health, Labour and Welfare of Japan.

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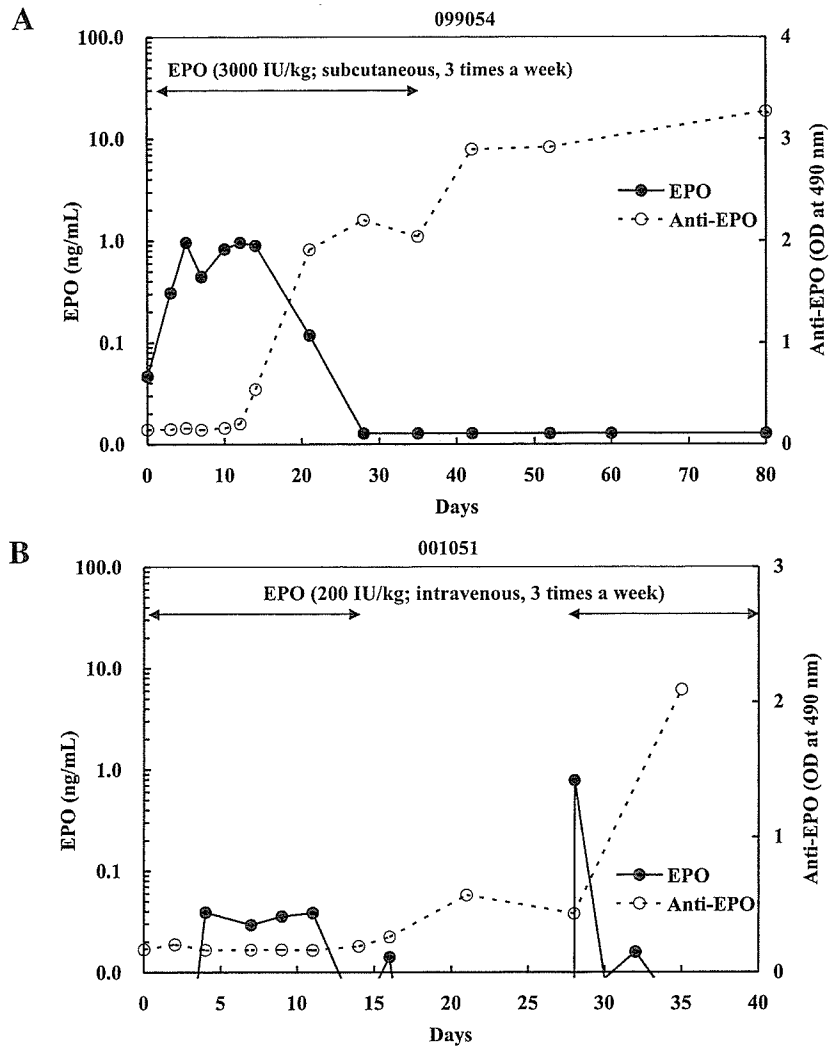

Fig. 1. Administration of only hEPO in cynomolgus monkeys. After subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero (A). Anti-hEPO antibody was also generated in another monkey (001051) receiving hEPO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

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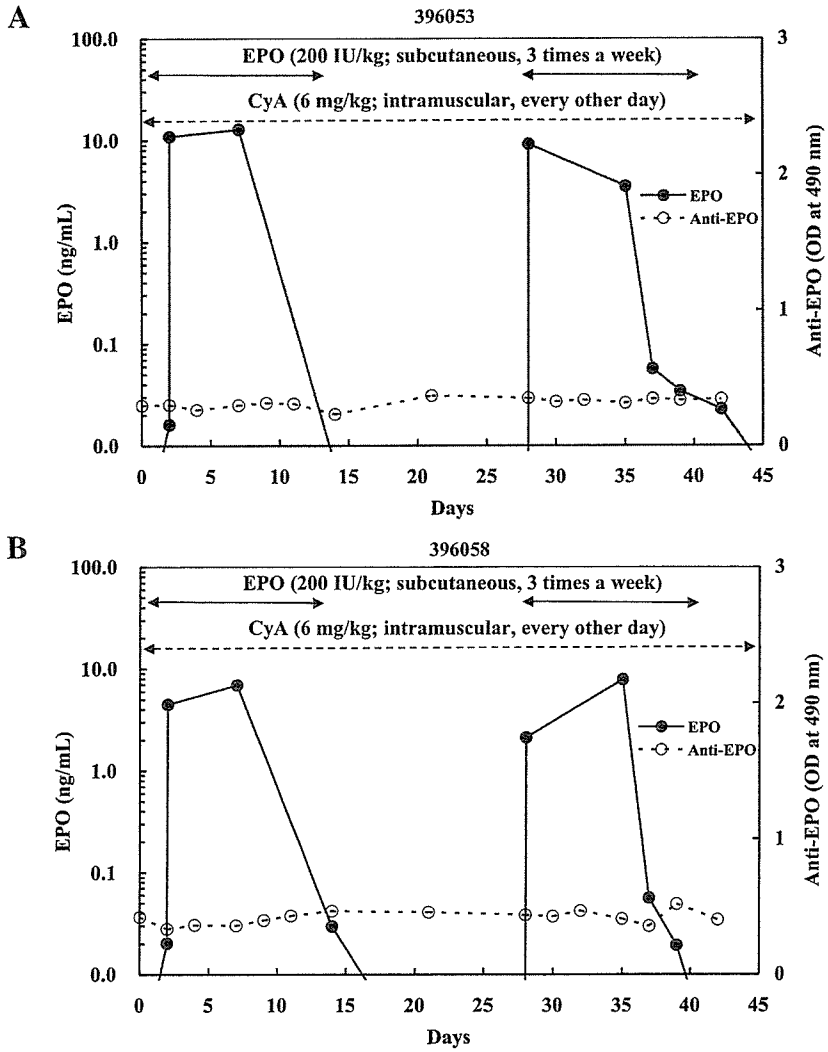

Fig. 2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by treatment with CyA in 2 cynomolgus monkeys (396053, 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of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

From: *Methods in Molecular Biology, vol. 329: Embryonic Stem Cell Protocols: Second Edition: Volume 1*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

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.

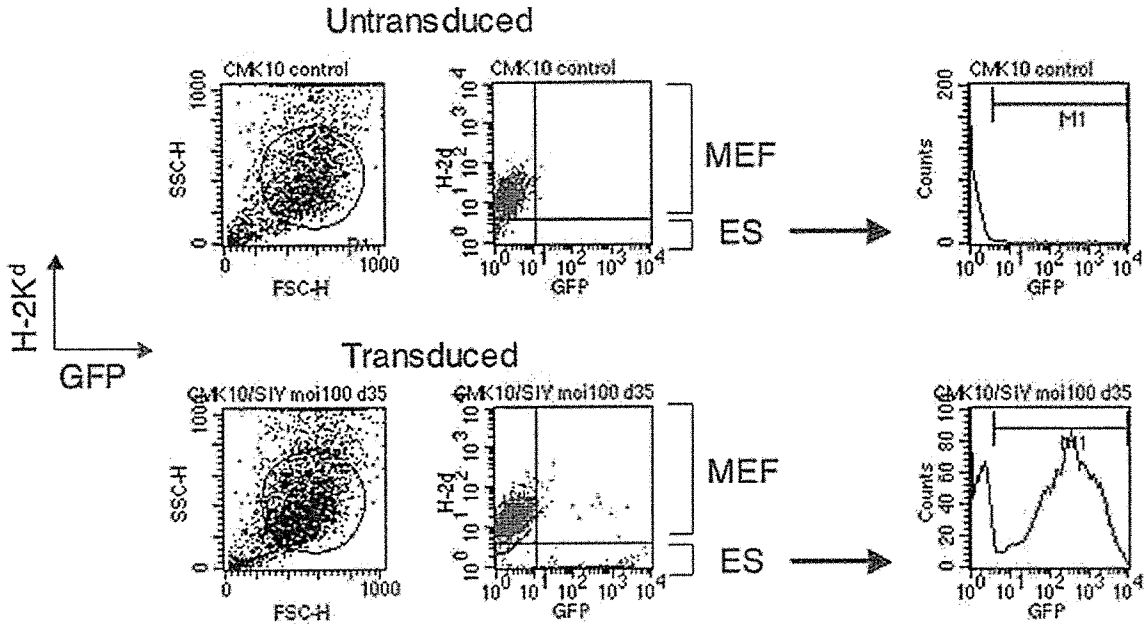

Fig. 1. Assessment of transduction efficiency by flow cytometry. The transgene green fluorescent protein (GFP) expression was analyzed on a FACScan using *CellQuest* software 2–3 d after transduction with a GFP-expressing simian immunodeficiency virus vector. The co-cultured BALB/c-derived feeder cells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