

5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3'. The polymerase chain reaction (PCR) mixture consisted of 420 μ M dATP, 420 μ M dCTP, 420 μ M dGTP, 378 μ M dTTP, 42 μ M digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μ M of each GFP primer, 4.5 mM MgCl₂, 1 \times PCR buffer (Mg²⁺ free), and 4 U of Takara Taq DNA polymerase (Takara, Kyote). Sections were prepared with a Takara slide frame (Takara) from the infarct, peri-infarct, and nonischemic regions at 2 weeks after transplant. PCR was performed using a PTC100 thermal cycler (MJ Research, Watertown, MA) with the following conditions: 94°C for 1 minute and 57°C for 1 minute with 10 cycles. The digoxigenin-incorporated DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (DakoCytomation). The sections were then stained for HRP using a Vector SG substrate kit (Vector). Finally, the sections were counterstained with a Kernectrot solution (Muto, Tokyo) that stains nucleotides, mounted in glycerol, and examined under a light microscope.

ELISA

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) levels in tissue lysate or medium were assessed by ELISA (R&D Systems, Minneapolis) according to the manufacturer's instructions. Tissue lysate was obtained from the peri-infarct region (three samples from each monkey) at 2 weeks after transplant.

Briefly, tissue was homogenized and suspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, and protease inhibitor cocktail tablets (Complete Mini, Roche). The suspension was rocked at 4°C for 20 minutes and centrifuged at 16,000g and 4°C for 30 minutes. The supernatant was used for ELISA. The protein concentration of lysate was determined with DC Protein Assay (Bio-Rad, Hercules, CA).

RESULTS

Lentiviral Marking

The CD34⁺ fraction of autologous bone marrow cells was used for transplantation in our study (Table 1). Before transplantation, CD34⁺ cells were genetically marked with GFP using an SIV-based lentivirus vector. The ex vivo transduction results are summarized in Table 1. The transduced cells were frozen until transplantation. An aliquot of the transduced cells was examined in vitro for the endothelial differentiation ability. After the differentiation culture, a vessel-like structure was observed (Fig. 1A). The ability of cells to take up DiI-acetylated LDL and the expression of CD31, vWF, VE-cadherin, and VEGFR-2 suggested the endothelial lineage (Fig. 1B). We and others have already confirmed the ability of hematopoietic differentiation of the cells [20, 21]. Taken together, the SIV-mediated GFP gene transfer does not spoil the differentiation abilities of CD34⁺ cells. In addition, on average, 41% of cells fluoresced 48 hours after transduction, and 56% of

Table 1. Summary of ex vivo transduction and transplantation

	Sex	Age (y)	Body weight (kg)	Harvested bone marrow cell number	Isolated CD34 ⁺ cell number	Transplanted cell number	% GFP expression	
							Before ^a	After ^b
Saline group								
CTR01061 ^c	M	3	4.1			NA		
CTR99056	M	3	3.4					
CTR96116	F	5	3.2					
CTR99051	M	5	5.9					
CD34⁺ cell group								
BM01052	M	3	3.9	213 $\times 10^6$	1.00 $\times 10^6$	0.47 $\times 10^6$	49	87
BM01051 ^d	M	3	4.1	396 $\times 10^6$	5.14 $\times 10^6$	2.20 $\times 10^6$	51	54
BM97080 ^e	M	5	3.9	330 $\times 10^6$	2.35 $\times 10^6$	1.04 $\times 10^6$	49	67
BM90047	M	13	5.8	343 $\times 10^6$	3.10 $\times 10^6$	1.07 $\times 10^6$	16	14
Average		5	4.3	321 $\times 10^6$	2.90 $\times 10^6$	1.20 $\times 10^6$	41	56

^aBefore endothelial differentiation of GFP-transduced CD34⁺ cells.

^bAfter the in vitro endothelial differentiation.

^cCTR01061 died of heart failure 5 days after myocardial infarction.

^dBM01051 developed a ventricular aneurysm after myocardial infarction.

^eBM97080 was killed 12 weeks after the treatment. All other animals were killed 2 weeks after the treatment.

Abbreviations: GFP, green fluorescent protein; NA, not applicable.

endothelial cells still fluoresced after *in vitro* differentiation (Table 1), showing that the GFP expression is stable during the *in vitro* differentiation to endothelial cells. Thus, GFP was expected to serve as a good genetic tag after transplantation.

Acute Myocardial Infarction and Autologous Transplantation

Cynomolgus acute myocardial infarction was generated by ligating the left anterior descending artery. One to two hours after the ligation, GFP-transduced, autologous CD34⁺ cells were injected in the peri-ischemic zone at 10 sites (total, $1.20 \pm 0.73 \times 10^6$ cells; $n = 4$). In the control group, saline was injected in the same way ($n = 4$). We conducted contrast echocardiography immediately after the coronary ligation and found no significant differences in the blood flow defect size (percent blood flow defect compared with the total) between the cell-treated and saline-treated groups ($13.0 \pm 2.1\%$ versus $12.3 \pm 3.5\%$, $p = .75$), suggesting that the initial risk of infarction did not differ between the two groups. In addition, we tried to assess the cardiac isozyme of serum creatine kinase (CK) to evaluate the infarct size; however, either the immuno-inhibition assay or chemical luminescence immunoassay did not work well for cynomolgus monkey samples. We were at least able to show that total CK values at 24 hours after the ligation did not significantly differ between the two groups ($p = .83$).

One of the control monkeys (CTR01061) died of heart failure 5 days after myocardial infarction, and the other control monkeys showed a decrease in %FS at 2 weeks after infarction (Fig. 2). Thus, all four control animals showed the deteriorated cardiac function. In the cell-treated group, one monkey (*, BM01051) underwent ventricular fibrillation immediately after the ligation and survived after cardiopulmonary resuscitation but eventually developed a ventricular aneurysm. Only this animal showed a decrease in %FS despite CD34⁺ cell treatment; the other animals receiving CD34⁺ cells showed an increase in %FS (Fig. 2). CD34⁺ cell treatment may not be able to rescue such a heavily impaired heart but otherwise had a significant effect on cardiac function. Even an old monkey (BM90047, Table 1) showed improved %FS.

The relative blood flow in the peri-infarct to nonischemic control region was also significantly ameliorated in the CD34⁺ cell-treated monkeys compared with the saline-treated ones, as assessed using contrast echocardiography (Fig. 3A) and colored microspheres (Fig. 3B). An excellent correlation was found between the two methods (Fig. 3C; correlation coefficient = 0.93). Two groups (CD34⁺ cell-treated and saline-treated) were well separated on the panel, showing an obvious positive effect of CD34⁺ cell injection on the blood flow in the peri-infarct zone after acute myocar-

dial infarction. In fact, the average myocardial blood flow in the peri-infarct region in the absolute value was 0.988 ml/g per minute and 0.383 ml/g per minute for the cell-treated and saline-treated groups, respectively. Of note, the blood flow in the peri-infarct zone was ameliorated even in the animal with a ventricular aneurysm. On the other hand, the relative blood flow in the infarct to nonischemic region did not show

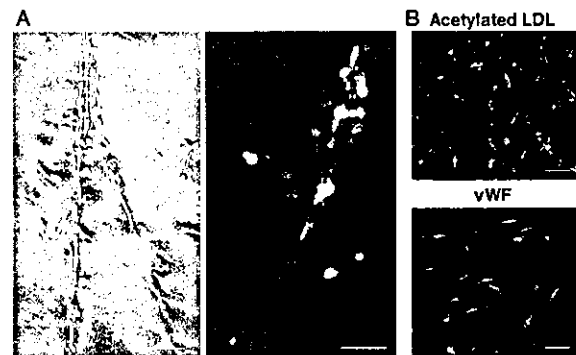


Figure 1. *In vitro* endothelial differentiation of cynomolgus CD34⁺ cells lentivirally transduced with GFP. The transduced CD34⁺ cells were differentiated to endothelial cells after 7 days in culture. (A): Representative vessel-like structure derived from CD34⁺ cells observed under a phase-contrast microscope (left) and a fluorescent microscope (right). (B): The transduced CD34⁺ cells differentiated into fluorescent cells (green) positive for the cellular intake of acetylated LDL and immunostaining for von Willebrand factor (vWF) (stained in red). Bar = 100 μ m. Abbreviations: GFP, green fluorescent protein; LDL, low-density lipoprotein.

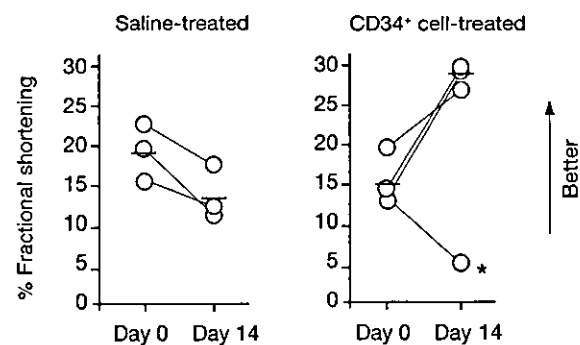


Figure 2. Improved cardiac function after CD34⁺ cell transplantation. Cardiac function was assessed by echocardiography in terms of percent fractional shortening (%FS) before and 2 weeks after treatment. One monkey in the saline-treated group (CTR01061) died of heart failure 5 days after myocardial infarction and is not included in the figure. One monkey in the CD34⁺ cell-treated group (*, BM01051) developed a left ventricular aneurysm after myocardial infarction. If this animal was excluded from the statistical analysis, the cardiac function was significantly improved in the CD34⁺ cell-treated compared with the saline-treated group in terms of the ratio of %FS at day 14 versus day 0 after transplant ($p = .017$).

a significant difference between the CD34⁺ cell-treated and saline-treated groups. The peri-infarct region was the injection site, and thus the highest degree of change would be expected there.

All monkeys except one CD34⁺ cell-treated monkey (BM97080) were examined for cardiac function and blood flow at 2 weeks after transplantation, and their tissue sections were finally prepared at this time point (see below). BM97080 was examined at 12 weeks, at which time the cardiac function was still improved compared with immediately after infarction (data not shown) and the blood flow data were in a position similar to the cell-treated group at 2 weeks (Fig. 3C).

In Vivo Tracking of Transplanted Cells

Two weeks after the transplantation, tissue sections were prepared from the infarct, peri-infarct, and nonischemic regions. Immunostaining of an endothelial marker CD31 demonstrated more vessels in the peri-infarct region of the CD34⁺ cell-treated than saline-treated myocardium (Fig. 4A). In fact, the capillary density of the peri-infarct region was significantly better preserved in the cell-treated than

saline-treated group, although there was no significant difference in the capillary density of the nonischemic control regions between the two groups (Fig. 4B).

Double immunostaining with anti-CD31 and anti-GFP showed that some cells in vessels were positive for both CD31 and GFP in the peri-infarct region (Fig. 5A). The result clearly indicates that at least some transplanted CD34⁺ cells gave rise to endothelial cells. However, we found that the transplanted cell progeny accounted for only a small fraction of endothelial cells after examining more than 100 sections of the peri-infarct region. In situ PCR for proviral GFP sequences also showed that few CD31-positive endothelial cells contained the GFP-provirus (Fig. 5B). There were no GFP-positive cardiomyocytes in more than 100 sections. Most of the transplanted cell progeny were found not incorporated in vessels (Fig. 5C). Hematoxylin-eosin staining did not show any noncardiac tissue regeneration in the myocardium.

On the other hand, we found that in vitro conditioned medium of CD34⁺ cell culture for endothelial differentiation contained high levels of VEGF, whereas unconditioned medium did not contain detectable VEGF, as assessed

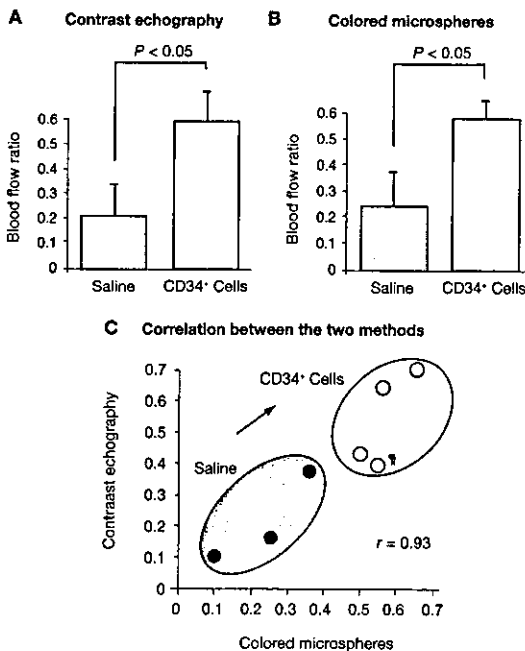


Figure 3. Improved regional blood flow after CD34⁺ cell transplantation. Myocardial contrast echocardiography (A) and colored microspheres (B) showed a significantly ameliorated blood flow ratio (the peri-infarct to nonischemic control region) in the CD34⁺ cell-treated monkeys ($n = 3$) compared with the saline-treated monkeys ($n = 3$) at 2 weeks after treatment. (C): An excellent correlation was found between the two methods. A CD34⁺ cell-treated monkey (♂, BM97080) that was examined at 12 weeks after transplant is included in the panel (C) but excluded from the statistical analysis in (A) and (B).

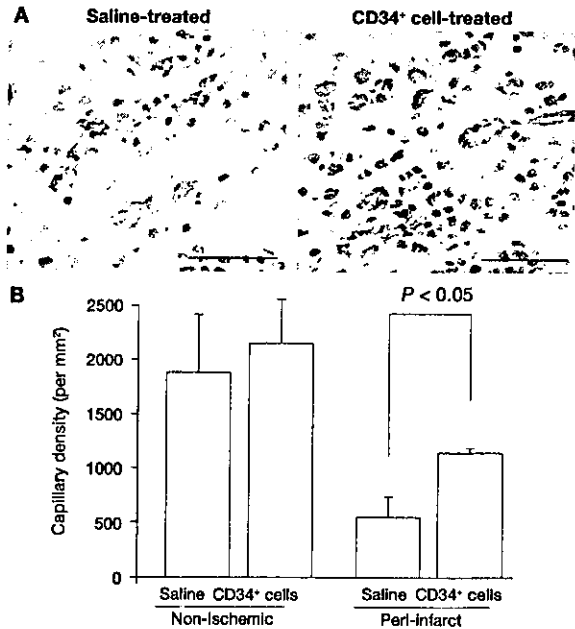


Figure 4. Neoangiogenesis in the ischemic myocardium. Tissue sections were prepared at 2 weeks after the treatment. (A): Representative results of immunostaining with anti-CD31 (stained in brown) in the peri-infarct region of the saline-treated and CD34⁺ cell-treated myocardium. Bar = 50 μm . (B): The density of CD31-positive capillaries in the peri-infarct and control nonischemic regions in the saline-treated and CD34⁺ cell-treated groups. Five fields for each section were randomly selected ($n = 3$ for the saline injection, $n = 3$ for the CD34⁺ cell injection), and the number of CD31-positive capillaries was counted (average \pm standard deviation).

by ELISA (Fig. 6A). In addition, in vivo VEGF levels in the peri-infarct tissue were significantly higher in the CD34⁺ cell-treated than saline-treated group (Fig. 6B, left), although in vivo levels of bFGF differed little between the two groups (Fig. 6B, right).

DISCUSSION

Although gained with small numbers of cynomolgus monkeys, our data suggest that the direct transplantation of bone marrow CD34⁺ cells, even without coronary bypass grafts or percutaneous coronary intervention, results in improved regional blood flow and cardiac function after myocardial infarction in nonhuman primates. Furthermore, we have tried to see the contribution of transplanted CD34⁺ cells to the

repair of ischemic myocardium. To this end, we genetically marked CD34⁺ cells with GFP using an SIV-based lentiviral vector before transplantation. Lentiviral vectors can transduce nondividing cells unlike oncoretroviral vectors, and thus the ex vivo culture period with multiple cytokines to allow cell cycling can be reduced to 1 day or less [20, 22, 23]. This is the great advantage of using lentiviral vectors over oncoretroviral vectors for transduction of multipotent stem cells, given that extended ex vivo culture of stem cells may result in loss of multilineage differentiation and engraftment abilities [24]. Human immunodeficiency virus (HIV)-1-based lentiviral vectors can efficiently transduce human cells, but not Old World monkey cells [25]. According to a recent report, a species-specific cytoplasmic component confers the innate

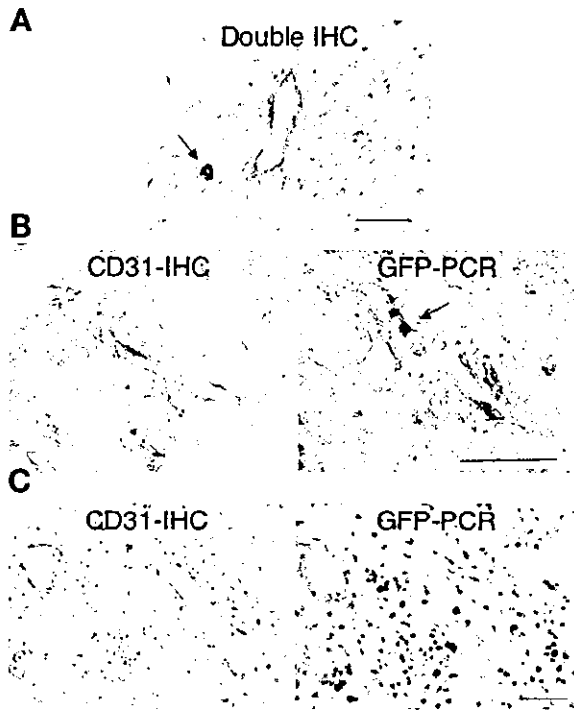


Figure 5. In vivo fate of transplanted cells. Cardiac sections were prepared at 2 weeks after transplantation. (A): Double immunohistochemistry (IHC) with anti-CD31 and anti-green fluorescent protein (GFP) in the peri-infarct region of the CD34⁺ cell-treated myocardium. Some cells (arrow) were positive for both CD31 (stained in brown) and GFP (stained in black), but such cells were rare. (B, C): Serial sections from the peri-infarct region of the CD34⁺ cell-treated myocardium. One section (left) was stained with anti-CD31 (stained in brown), and the other (right) was assessed by in situ polymerase chain reaction (PCR) for proviral GFP sequences (stained in black). (B): Some CD31-positive endothelial cells contained the GFP-provirus (arrow, right panel), but such cells were rare. (C): Transplanted cell progeny (cells positive for GFP-provirus in the right panel) were not incorporated in vessels (cells positive for CD31 in the left panel). Bar = 50 μm.

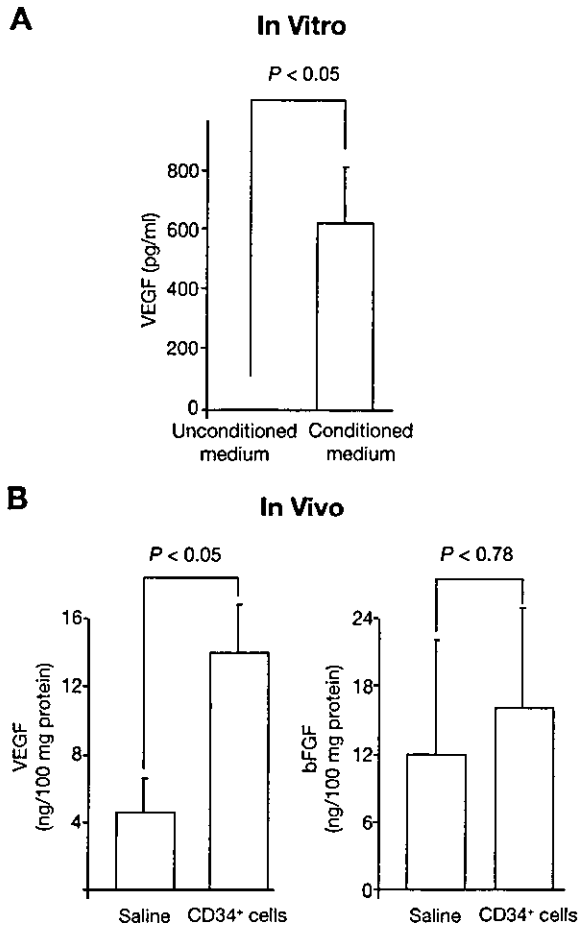


Figure 6. VEGF is implicated in the neoangiogenesis. (A): Unconditioned and conditioned media of in vitro CD34⁺ cell cultures for endothelial differentiation were examined for VEGF by ELISA. The average ± standard error of six culture dishes is shown. (B): Lysates (three samples per monkey) from the peri-infarct region of the CD34⁺ cell-treated (monkey, *n* = 3) and saline-treated (monkey, *n* = 3) myocardium were prepared and examined for VEGF and basic fibroblast growth factor (bFGF) by ELISA. Data are shown as the average ± standard error. Abbreviation: VEGF, vascular endothelial growth factor.

postentry restriction to HIV-1 infection in simian cells [26]. Unlike HIV-1-based lentiviral vectors, SIV-based ones can efficiently transduce simian hematopoietic stem/progenitor cells [21]. In this study, we also used an SIV-based lentiviral vector and achieved the efficient gene transfer into simian CD34⁺ cells.

As a result of this marking study, we found only a few GFP-positive cells incorporated into the vascular structure in the ischemic myocardium at 2 weeks after transplantation. GFP-positive cardiomyocytes were not detectable. The existence of GFP-positive endothelial cells can be explained by fusion events [27, 28]. However, if that is the case, GFP-positive cardiomyocytes should have also been detected, given that cardiomyocytes are even easier targets of fusion than endothelial cells [11, 29]. Whether fusion occurred or not, only a few transplanted cells gave rise to nonhematopoietic cells in our primate model.

There are several possible explanations for the very low prevalence of transplanted cell-derived endothelial cells or cardiomyocytes in the ischemic myocardium. First, 2 weeks was too short or the number of transplanted cells was too small to see the nonhematopoietic differentiation. However, the cardiac function and regional blood flow were ameliorated by this time point and with this number of transplanted cells. Thus, if transplanted cell-derived, nonhematopoietic differentiation was a reason for the improvement, transplanted cells at this number should have given rise to such cells by this time point. In fact, Orlic et al. [8] observed transplanted cell-derived endothelial cells and cardiomyocytes within 11 days after transplant in mice. In addition, we observed the endothelial differentiation from CD34⁺ cells within 7 days *in vitro* (Fig. 1). However, we cannot formally rule out a possibility that inflammatory responses after generation of infarction might have negative effects on engraftment of transplanted cells. Second, the SIV vector failed to transduce stem or progenitor cells that might be responsible for nonhematopoietic differentiation. Even if the transduction was successful, the cytokine treatment during the transduction or GFP expression in the cells spoiled the differentiation abilities. However, we have shown that the SIV vector successfully transduced cells that were capable of differentiating into GFP-expressing endothelial cells (Fig. 1). We have not examined the differentiation ability to cardiomyocytes, because the method to differentiate CD34⁺ cells to cardiomyocytes *in vitro* has not been well established. Thus, we cannot formally rule out the possibility that the *ex vivo* culture spoiled the ability to differentiate to cardiomyocytes or reduced the ability to differentiate to endothelial cells. Third, cells expressing xenogeneic GFP were rejected via immune responses.

However, 2 weeks is too short to allow immune elimination of GFP-expressing cells in monkeys [30, 31]. Fourth, the GFP expression was shut down because of transcriptional silencing *in vivo*, resulting in negative immunostaining with anti-GFP. To examine this possibility, we tried to detect the provirus (vector integrated into genome) in the cardiac tissue by *in situ* PCR and found again that only a few CD31-positive endothelial cells contained the GFP-provirus (Fig. 5B), thus arguing against transcriptional silencing-based negative immunostaining with anti-GFP. Taken together, we concluded that most transplanted cell progeny were not incorporated into the repaired, nonhematopoietic tissues.

Our results are in agreement with recent reports that transplanted hematopoietic cells are unable to transdifferentiate into nonhematopoietic cells in ischemic myocardium in mice [9–11]. Our studies confirm and extend these findings in a couple of ways. First, we show that the cardiac function can be indeed significantly improved after injection of hematopoietic cells in a nonhuman primate model, although the above studies used murine myocardial infarction models and did not address the potential beneficial effects of hematopoietic cell injection. Second, the improvement is unlikely to be the result of generation of transplanted cell-derived endothelial cells or cardiomyocytes. Finally, we have found that cultured CD34⁺ cells secrete VEGF and that the CD34⁺ cell-treated myocardium contains a significantly higher level of VEGF than the saline-treated myocardium. This observation raises a possibility that some angiogenic cytokines secreted from transplanted cells (paracrine effects) potentiate angiogenic activity of endogenous cells. VEGF would be a candidate. Despite this, the delivery of a single agent (VEGF) failed in clinical trials for cardiac ischemia [32]. *In situ* multiple cytokine production and coordinated action may be essential for clinical benefits [33, 34]. It will be important to explore and identify cytokines responsible for the paracrine effect. If transplanted cells serve as cytokine factories rather than stem cells in ischemic tissues, it is not surprising that not only stem cells but other types of cells may also work [35]. The concept of stem cell therapeutics for ischemic diseases needs additional consideration.

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REFERENCES

- 1 Assmus B, Schachinger V, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 2002;106:3009–3017.
- 2 Strauer BE, Brehm M, Zeus T et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002;106:1913–1918.
- 3 Tse HF, Kwong YL, Chan JK et al. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 2003;361:47–49.
- 4 Perin EC, Dohmann HF, Borojevic R et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation* 2003;107:2294–2302.
- 5 Stamm C, Westphal B, Kleine HD et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003;361:45–46.
- 6 Wollert KC, Meyer GP, Lotz J et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* 2004;364:141–148.
- 7 Asahara T, Murohara T, Sullivan A et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–967.
- 8 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001;410:701–705.
- 9 Murry CE, Soonpaa MH, Reinecke H et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;428:664–668.
- 10 Balsam LB, Wagers AJ, Christensen JL et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004;428:668–673.
- 11 Nygren JM, Jovinge S, Breitbach M et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat Med* 2004;10:494–501.
- 12 Hanazono Y, Terao K, Ozawa K. Gene transfer into non-human primate hematopoietic stem cells: implications for gene therapy. *STEM CELLS* 2001;19:12–23.
- 13 Shibata H, Hanazono Y, Ageyama N et al. Collection and analysis of hematopoietic progenitor cells from cynomolgus macaques (*Macaca fascicularis*): assessment of cross-reacting monoclonal antibodies. *Am J Primatol* 2003;61:3–12.
- 14 Nakajima T, Nakamaru K, Ido E et al. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000;11:1863–1874.
- 15 VandenDriessche T, Thorrez L, Naldini L et al. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood* 2002;100:813–822.
- 16 Kobayashi N, Yasu T, Yamada S et al. Influence of contrast ultrasonography with perflutren lipid microspheres on microvessel injury. *Circ J* 2003;67:630–636.
- 17 Wei K, Jayaweera AR, Firoozan S et al. Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. *Circulation* 1998;97:473–483.
- 18 Hale SL, Alker KJ, Kloner RA. Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow in dogs. *Circulation* 1988;78:428–434.
- 19 Haase AT, Retzel EF, Staskus KA. Amplification and detection of lentiviral DNA inside cells. *Proc Natl Acad Sci U S A* 1990;87:4971–4975.
- 20 Hanazono Y, Asano T, Ueda Y et al. Genetic manipulation of primate embryonic and hematopoietic stem cells with simian lentivirus vectors. *Trends Cardiovasc Med* 2003;13:106–110.
- 21 Hanawa H, Hematti P, Keyvanfar K et al. Efficient gene transfer into rhesus repopulating hematopoietic stem cells using a simian immunodeficiency virus-based lentiviral vector system. *Blood* 2004;103:4062–4069.
- 22 Miyoshi H, Smith KA, Mosier DE et al. Transduction of human CD34⁺ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 1999;283:682–686.
- 23 Horn PA, Keyser KA, Peterson LJ et al. Efficient lentiviral gene transfer to canine repopulating cells using an overnight transduction protocol. *Blood* 2004;103:3710–3716.
- 24 Dunbar CE, Takatoku M, Donahue RE. The impact of ex vivo cytokine stimulation on engraftment of primitive hematopoietic cells in a non-human primate model. *Ann N Y Acad Sci* 2001;938:236–245.
- 25 Owens CM, Yang PC, Gottlinger H et al. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol* 2003;77:726–731.
- 26 Stremmlau M, Owens CM, Perron MJ et al. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* 2004;427:848–853.
- 27 Terada N, Hamazaki T, Oka M et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 2002;416:542–545.
- 28 Ying QL, Nichols J, Evans EP et al. Changing potency by spontaneous fusion. *Nature* 2002;416:545–548.
- 29 Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;425:968–973.
- 30 Rosenzweig M, Connole M, Glickman R et al. Induction of cytotoxic T lymphocyte and antibody responses to

- enhanced green fluorescent protein following transplantation of transduced CD34(+) hematopoietic cells. *Blood* 2001;97:1951–1959.
- 31 Heim DA, Hanazono Y, Giri N et al. Introduction of a xenogeneic gene via hematopoietic stem cells leads to specific tolerance in a rhesus monkey model. *Mol Ther* 2000;1:533–544.
- 32 Henry TD, Annex BH, McKendall GR et al. The VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis. *Circulation* 2003;107:1359–1365.
- 33 Cao R, Brakenhielm E, Pawliuk R et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med* 2003;9:604–613.
- 34 Ziegelhoeffer T, Fernandez B, Kostin S et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res* 2004;94:230–238.
- 35 Kinnaird T, Stabile E, Burnett MS et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 2004;109:1543–1549.

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Hematopoietic Microchimerism in Sheep After In Utero Transplantation of Cultured Cynomolgus Embryonic Stem Cells

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Background. Although directed differentiation of human embryonic stem (ES) cells would enable a ready supply of cells and tissues required for transplantation therapy, the methodology is limited. We have developed a novel method for hematopoietic development from primate ES cells. We first cultured cynomolgus monkey ES cells in vitro and transplanted the cells in vivo into fetal sheep liver, generating sheep with cynomolgus hematopoiesis.

Methods. Cynomolgus ES cells were induced to mesodermal cells on murine stromal OP9 cells with multiple cytokines for 6 days. The cells (average 4.8×10^7 cells) were transplanted into fetal sheep in the liver ($n=4$) after the first trimester (day 55–73, full term 147 days). The animals were delivered at full term, and two of them were intraperitoneally administered with human stem-cell factor (SCF).

Results. Cynomolgus hematopoietic progenitor cells were detected in bone marrow at a level of 1% to 2% in all four sheep up to 17 months posttransplant. No teratoma was found in the lambs. After SCF administration, the fractions of cynomolgus hematopoiesis increased by several-fold (up to 13%). Cynomolgus cells were also detected in the circulation, albeit at low levels (<0.1%).

Conclusions. Long-term hematopoietic microchimerism from primate ES cells was observed after in vitro differentiation to mesodermal cells, followed by in vivo introduction into the fetal liver microenvironment. The mechanism of such directed differentiation of ES cells remains to be elucidated, but this procedure should allow further investigation.

Keywords: Primate embryonic stem cells, In utero transplantation, Hematopoietic microchimerism, Sheep.

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A major barrier for most tissue or cellular transplantation therapies is the shortage of donors. Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue cells (1, 2), directed differentiation of human ES cells into functionally defined tissue types is a goal in providing an inexhaustible and potentially customized supply of transplantable cells or tissues. Clearly directed differentiation of ES cells is still in its infancy, and the methodology is quite limited. Many researchers have studied in vitro specific differentiation programs through manipulation of the cytokine milieu, cellular microenvironment, and conditional activation of specific gene expression (3–5). On the other hand, we and other groups have shown a line of evidence that undifferentiated ES cells respond to local cues after transplantation and differentiate into site-specific cells in rodent and nonhuman pri-

mate allogeneic transplantation models (6, 7). These studies have highlighted the importance of the in vivo local microenvironment for directed differentiation of ES cells. ES cells can be induced to differentiate into specific cells if exposed to the proper microenvironment. In this study, we have tried to use the in vivo fetal sheep liver microenvironment for hematopoietic development from primate ES cells.

Sheep in utero transplantation has been used as an assay system for human hematopoiesis (8). The fetal sheep is immunologically tolerant of allogeneic skin grafts or xenogeneic human hematopoietic cells before 75 days of gestation, which allows avoidance of the immunologic barriers present in postnatal models (8–10). In this model, long-term human/sheep hematopoietic chimeras have been established after the transplantation of human hematopoietic stem cells into the fetal sheep at a pre-immune stage (8). It has also been reported that human mesenchymal stem cells engraft and show site-specific differentiation after in utero transplantation in sheep (11).

We have used nonhuman primate (cynomolgus monkey) ES cells (12) because this is the most faithful model for human ES cells for generating hematopoietic chimera in sheep. We first cultured cynomolgus ES cells in vitro to differentiate into mesodermal cells and introduced the cells into fetal sheep liver after the first trimester. Fetal liver is a hematopoietic organ at this stage of fetuses. We then examined the in vivo fate of transplanted cell progeny long term.

MATERIALS AND METHODS

Cell Preparation

Cynomolgus macaque ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan) treated mouse (BALB/c, Charles River Japan,

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Kanagawa, Japan) embryonic fibroblasts, as described previously (12). The mouse bone-marrow stromal-cell line OP9 was maintained in α -minimum essential medium (Gibco, Rockville, MD) supplemented with 20% fetal calf serum, as previously described (13).

For the *in vitro* differentiation (induction of hematopoietic differentiation), ES cells were seeded onto mitomycin-C-treated confluent OP9 cell layers in culture dishes in Iscove's modified Dulbecco's medium (Gibco) supplemented with 8% horse serum (Gibco), 8% fetal calf serum, 5×10^{-6} M hydrocortisone (Sigma, St. Louis, MO), and multiple cytokines including 20 ng/mL recombinant human (rh) bone morphogenetic protein-4 (BMP-4; R&D, Minneapolis, MN), 20 ng/mL rh stem-cell factor (SCF, Amgen, Thousand Oaks, CA), 20 ng/mL rh interleukin-3 (Research Diagnostics, Flanders, NJ), 20 ng/mL rh interleukin-6 (Ajinomoto, Osaka, Japan), 20 ng/mL rh vascular endothelial growth factor (VEGF, R&D), 20 ng/mL rh granulocyte colony-stimulating factor (Chugai, Tokyo, Japan), 10 ng/mL rh Flt-3 ligand (Research Diagnostics), and 2 IU/mL rh erythropoietin (Chugai). During differentiation, media were changed every 2 to 3 days. After 6 days of culture, cells were dissociated with 0.25% trypsin (Gibco), collected with a cell scraper, washed with Hanks' balanced salt solution (HBSS, Gibco), resuspended in 0.4 mL of 0.1% bovine serum albumin/HBSS, and used for transplantation. Human cord-blood CD34⁺ cells used in the present study were obtained at Jichi Medical School Hospital with informed consent.

Transplant Procedures

Pregnant Suffolk ewes (Japan Lamb, Hiroshima, Japan) were bred at the Utsunomiya University Farm. Fetal sheep at 55 to 79 days of gestation (full term 147 days) were used. Before transplantation, ewes were sedated with ketamine (10 mg/kg intramuscularly) and received a 0.5% to 1.0% halothane-oxygen mixture by inhalation by way of an endotracheal tube. The uterus was exposed through a midline laparotomy incision. Donor cells were injected into the fetus in the liver through the uterine wall using a 25-gauge needle under ultrasound guidance. After closure of the abdominal wall, penicillin and streptomycin were administered. The fetus was allowed to come to term. After birth, some lambs were intraperitoneally administered rhSCF at a dosage of 60 μ g/kg once a day for 18 or 5 consecutive days.

Hematopoietic Progenitor Assay

To assess cynomolgus hematopoiesis in sheep, clonogenic hematopoietic colonies were produced by growing bone-marrow cells in methylcellulose with defined rh cytokines (Methocult GF+ and MegaCult-C, Stem Cell Technologies, Vancouver, Canada). Bone-marrow cells were aspirated from the iliac bone. From harvested bone-marrow cells, a leukocyte cell fraction was obtained after red-blood-cell lysis with ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA; Wako, Osaka, Japan). Cells were plated in triplicate at 1 to 5×10^4 cells per 35-mm plate. After 14 days, individual colonies were plucked into 50 μ L of distilled water and digested with 20 μ g/mL proteinase K (Takara, Shiga, Japan) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μ L) was used for polymerase chain reaction (PCR) amplification to detect cynomolgus-specific β 2-mi-

croglobulin gene sequences. Nested PCR was performed. The outer primer set was 5'-GTC TGG ATT TCA TCC ATCTG-3' and 5'-GGC TGT GAC AAA GTC ACA TGG-3', and the inner primer set was 5'-GTCTGG ATT TCA TCC ATCTG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Amplification conditions for both outer and inner PCR were 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Amplified products (135 bp) were resolved on 2% agarose gel and visualized by ethidium bromide staining.

PCR Southern Blotting

Cellular DNA was extracted from peripheral blood and bone-marrow cells after birth and subjected to the cynomolgus-specific β 2-microglobulin PCR as described above. The PCR products were resolved on 2% agarose gel and transferred to Hybond-N+ (Amersham, Cleveland, OH). The membrane was hybridized with a radiolabeled cynomolgus-specific β 2-microglobulin probe generated by PCR using the following primers: 5'-GTC TGG ATT TCA TCC ATCTG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Radiolabeling of a probe was performed using a DNA labeling kit (Amersham).

RESULTS

In Vitro Culture and In Utero Transplantation

Cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. They form colonies on feeder cells (Fig. 1a). For induction of hematopoietic differentiation, undifferentiated ES cells were placed on murine stromal OP9 cells (13) in the presence of multiple cytokines including human BMP-4 and VEGF (14). The cell number increased by 10-fold at day 6, and cobblestone-like cells emerged at this time point (Fig. 1b). These cells were negative or only weakly positive for CD34 (hematopoietic) and CD31 (endothelial) markers as assessed by flow cytometry and had little clonogenic hematopoietic colony forming ability in culture (data not shown). These cells likely represent a differentiated population of mesodermal cells. We then transplanted the day-6 ES derivatives (average 4.8×10^7 cells, Table 1) into fetal sheep (n=4) by way of direct injection into the liver under ultrasound guidance after the first trimester (Fig. 1c).

Hematopoietic Microchimerism after Birth

The animals were delivered at full term. Marrow cells were harvested from the iliac bone and plated in methylcellulose. Clonogenic hematopoietic colonies (colony-forming units [CFU]) were thus produced to examine hematopoietic chimerism in the sheep (Fig. 2). Sheep bone-marrow cells generated colonies of clear hematopoietic morphology in this assay. Cynomolgus and sheep cells were equivalent in their colony-formation ability in this culture, and each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. Therefore, the ratio of cynomolgus to sheep colony number is considered a chimeric fraction. To distinguish cynomolgus versus sheep colonies, we tried to immunostain colonies with antihuman class I, and more directly hematopoietic-specific antihuman CD45, but they reacted to sheep counterparts or generated a considerable nonspecific staining. We then conducted PCR for monkey-specific β 2-microglobu-

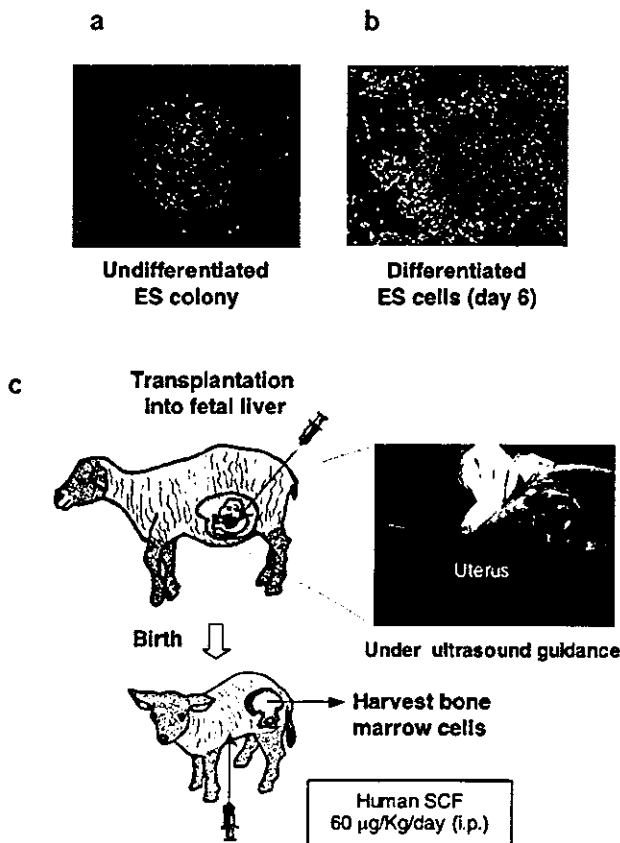


FIGURE 1. In utero transplantation of day 6 cynomolgus embryonic stem (ES)-derived cells. (a) Undifferentiated cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. (b) For hematopoietic induction, undifferentiated ES cells were placed on murine stromal OP9 cells in the presence of multiple cytokines. Cobblestone-like cells emerged at day 6. (c) The day 6 ES derivatives were transplanted directly into the fetal liver after the first trimester. After birth, marrow cells were harvested from the iliac bone and examined for cynomolgus versus sheep hematopoiesis. Some lambs were intraperitoneally (i.p.) administered with human stem-cell factor (SCF) to specifically stimulate cynomolgus hematopoiesis.

lin DNA sequences on DNA isolated from each colony (Fig. 2) (colony PCR). Cynomolgus CFU were clearly detected at a level of 1% to 2% in the bone marrow of all four sheep (Table 1). We repeated colony PCR and confirmed that data were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. Cynomolgus megakaryocytic colonies were not detected, but it was no wonder considering the low frequency of cynomolgus megakaryocytic colony formation in vitro (15). The longest follow-up was 17 months after transplantation (14 months after birth), and cynomolgus CFU were still detectable. No lamb developed a teratoma.

Selective Expansion of Cynomolgus Hematopoiesis

To selectively stimulate cynomolgus compared with endogenous hematopoiesis in the sheep, we administered two

animals with human SCF, a cytokine that does not cross-react to stimulate sheep hematopoietic progenitors (16). A time-course profile of the two animals (No. 141 and No. 182) is shown in Figure 3. In both sheep, in response to the human SCF administration for 18 days, the fractions of cynomolgus CFU increased several-fold (up to 13.2% at day 174 and 4.7% at day 112 posttransplantation, respectively). After cessation of SCF administration, the fractions fell to the original levels. Resumption of human SCF at the same dose but for 5 days produced a similar elevation in the chimeric fractions (up to 4.4% at day 364 and 8.8% at day 187 posttransplantation, respectively). No adverse effects associated with human SCF administration were observed. We did not examine antibody responses to human SCF in the sheep, but this was unlikely to occur because second trials of human SCF administration did work (i.e., increased the chimeric fractions) in both sheep.

Cynomolgus cells were also detected in the circulation, although the fraction was very low (<0.1%), even after human SCF administration as assessed by PCR Southern blotting (Fig. 4). The low levels of cynomolgus cells hampered the lineage analysis by flow cytometry. To detect cynomolgus T lymphocytes in the sheep, we collected the peripheral blood and tried to selectively expand cynomolgus T lymphocytes in the culture with human interleukin-2, antimonkey CD3 (FN-18), and antihuman CD28 (Kolt-2, cross-reacting to monkey CD28) (17). However, sheep lymphocytes were also stimulated to expand, and we failed to detect cynomolgus T lymphocytes.

Comparative Study of Repopulating Ability

Next, we transplanted human cord-blood hematopoietic stem cells ($CD34^+$ cells, average 1.8×10^6 cells) into fetal sheep ($n=4$) instead of day-6 cynomolgus ES derivatives. We used a $CD34^+$ fraction because it was widely used both in sheep in utero transplantation (18, 19) and in clinical transplantation of hematopoietic stem cells (20), although some investigators used a human $CD34^-$ fraction for sheep in utero transplantation and obtained hematopoietic chimera as well or even better in serial transplantation experiments (21). For the present, however, we considered that the $CD34^+$ fraction was an appropriate standard with which to compare the repopulating ability of day-6 cynomolgus ES-derived cells as well. The chimeric fraction in bone-marrow CFU after in utero transplantation of $CD34^+$ cells was 1% to 9% (average 3.7%). Fractions of donor-derived cells in the peripheral blood were also very low (<0.1%) after the transplantation of $CD34^+$ cells. The average cell numbers necessary to achieve 1% chimerism in CFU after birth were estimated to be 4.3×10^7 and 6.0×10^5 for day-6 cynomolgus ES-derived cells and human cord-blood $CD34^+$ cells, respectively.

DISCUSSION

We have generated long-term hematopoietic microchimerism in sheep derived from ES cells. Achieving hematopoietic reconstitution from ES cells has been an enormous challenge. The difficulty is attributable to the developmental immaturity of ES-derived cells, which most closely resemble primitive embryonic yolk-sac hematopoietic progenitors (22). The processes governing embryonic versus adult blood formation are distinct, and mouse ES cells do not contribute

TABLE 1. In utero transplantation and donor-cell engraftment in sheep

Transplanted cells	Animals (sex)	In utero transplantation		Donor cell-derived CFU in bone marrow* (months posttransplant)		
		Transplanted cell number per fetus	Gestational day at transplantation (Full term 147 days)	After birth	After human SCF administration	Average cell number necessary for 1% chimerism
Day 6 ES-derived cells	No. 57 (male)	5.0×10^7	67	1.1% (1/91) at 3.5 months	ND	4.3×10^7
	No. 55 (female)	5.0×10^7	55	1.1% (1/91) at 5 months	ND	
	No. 141 (male)	7.8×10^7	73	1.1% (1/91) at 3 months	13.2% (12/91) at 6 months	
	No. 182 (male)	1.4×10^7	66	1.6% (1/63) at 3 months	8.8% (8/91) at 6 months	
Human cord blood CD34 ⁺ cells	No. 71-1 (male)	2.0×10^6	69	8.8% (8/91) at 1 months	ND	6.0×10^5
	No. 71-2 (female)	2.0×10^6	69	4.4% (4/91) at 3.5 months	ND	
	No. 99-1 (male)	1.5×10^6	79	1.1% (1/91) at 1 months	ND	
	No. 99-2 (female)	1.5×10^6	79	4.4% (4/91) at 2 months	ND	

* Percent cynomolgus CFU was calculated by dividing the number of CFU positive for the cynomolgus-specific $\beta 2$ -microglobulin gene sequence by the total number of CFU analyzed.

CFU, colony-forming unit; ND, not done; ES, embryonic stem.

to hematopoietic reconstitution in irradiated mice, unlike stem cells isolated from adult bone marrow (23, 24). An effective approach to this obstacle has recently been reported. Genetically engineering mouse ES cells to express a specific transcription factor (HoxB4) or signaling molecule (STAT5) during a specific developmental window has resulted in hematopoietic reconstitution from ES cells in irradiated mice (5, 25). However, the requirement for artificial over-expression of those genes is undesirable for clinical applications. Our method to develop hematopoietic engraftment from primate ES cells does not require genetic manipulation, and it is a combination of two steps: in vitro differentiation into mesodermal cells followed by in vivo development into hematopoietic cells in the proper microenvironment of fetal sheep liver.

To examine whether transplanted day-6 ES-derived cells engraft in other tissues, we did one more in utero transplantation experiment using same cultured cynomolgus ES cells (mesodermal cells at day 6) and delivered a fetus at 1 month after transplantation. Although cynomolgus CFU were detected in fetal liver and cord blood, cynomolgus cells were not detectable in any other tissues by a sensitive PCR after the fetal blood was completely washed out (data not shown). Therefore, our method appears to direct the fate of primate ES cells to the hematopoietic lineage.

We assumed that the initial in vitro culture of ES cells into mesodermal cells is crucial for successful engraftment in the fetal liver. In fact, when we transplanted undifferentiated (day 0) cynomolgus ES cells into fetal sheep ($n=2$), we failed to detect cynomolgus CFU or other nonhematopoietic cells in any fetal tissue at 1 month posttransplantation as assessed

by a sensitive PCR (data not shown). Therefore, undifferentiated ES cells do not appear to engraft in fetal sheep, unlike adult mesenchymal or hematopoietic stem cells (8, 11), but day-6 ES derivatives (mesodermal cells) can engraft and are susceptible to hematopoietic specification in the fetal-liver microenvironment. For the successful initial in vitro culture of ES cells, there are some points to be noted. First, we used stromal OP9 cells as a feeder. Second, we included BMP-4 and VEGF in the culture medium. The coculture with OP9 and inclusion of BMP-4 and VEGF promote hematopoietic differentiation of ES cells (13, 14, 26–28). Finally, we cultured cells in vitro for a relatively short period (6 days) to avoid over-maturation of cells (14). The xenogeneic fetal liver is able to provide such cultured ES cells with an adequate microenvironment for support of hematopoietic development (29). Factors present in the fetal liver responsible for the development remain to be elucidated.

On the other hand, several issues remain to be further investigated. Although the chimeric fractions increased by several-fold (up to 13%) after SCF administration, the increase was transient. To stably enhance the ES-derived chimeric fraction, an enrichment of cells responsible for the engraftment will be needed before transplantation, as suggested from Table 1 (see average cell numbers necessary to achieve 1% chimerism). Another issue is a very low level of donor-derived cells in the peripheral blood. Ours is quite different from previous human-to-fetal sheep experiments that demonstrated easily detectable peripheral blood chimerism (16, 19). This has hampered the lineage analysis of cynomolgus cells in our sheep to obtain further evidence to support hematopoietic differentiation from ES cells. Low levels of pe-

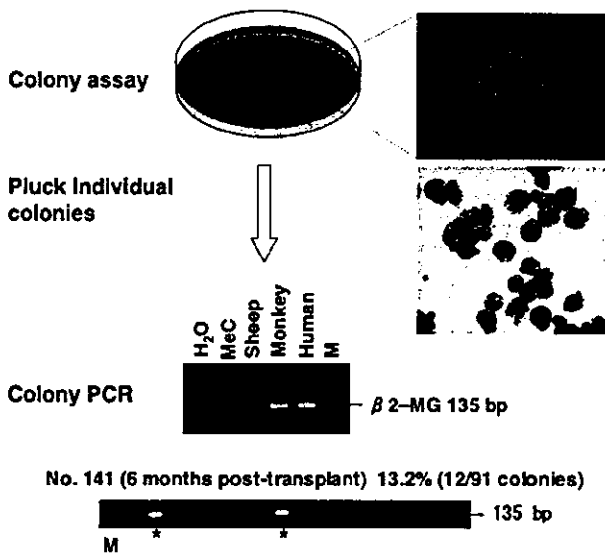


FIGURE 2. Assessment of cynomolgus hematopoiesis in sheep after birth. Bone-marrow cells were harvested from lambs and placed in methylcellulose. Hematopoietic colonies were thus formed. A cytospin specimen (stained with the Wright-Giemsa method) of plucked myeloid colonies demonstrated mature neutrophils. Each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. To detect cynomolgus colonies, individual colonies were plucked and examined for cynomolgus-specific $\beta 2$ -microglobulin ($\beta 2$ -MG) sequences by polymerase chain reaction (PCR). PCR products were analyzed on 2% agarose gel. Plucked methylcellulose (MeC) alone (not containing colonies) and sheep colonies generated no bands by the PCR. Colony PCR was repeated at least twice. Representative colony PCR results of sheep No. 141 shown. *Bands positive for the cynomolgus-specific sequence. M, molecular weight marker.

ripheral chimerism were, however, also found after human CD34⁺ cells were transplanted in our study. Therefore, the issue is not specific to transplanted ES cells, but is likely to be attributable to the experimental system. A possible explanation may be the different sheep species used in our study (Suffolk versus Dorset Merino). Another possible explanation is immune responses caused by relatively later gestational ages (in the second trimester, day 55–79) at transplantation in our study compared with other recent studies (day 40–45) (19). The immune response may have cleared xenogeneic cells from the circulation. The existence of microchimerism does not necessarily guarantee or predict tolerance in other systems (30, 31). “The window of opportunity” for successful tolerance induction may be earlier and narrower. To avoid sensitization, transplantation at earlier days may be more efficacious.

In conclusion, long-term hematopoietic microchimerism from primate ES cells is possible after in vitro differentiation to mesodermal cells, followed by in vivo transplantation into the fetal-liver microenvironment. We have used nonhuman primate ES cells in the current study, but if human ES cells are similarly used, human blood cells can be generated in sheep. This procedure should allow for further investigation.

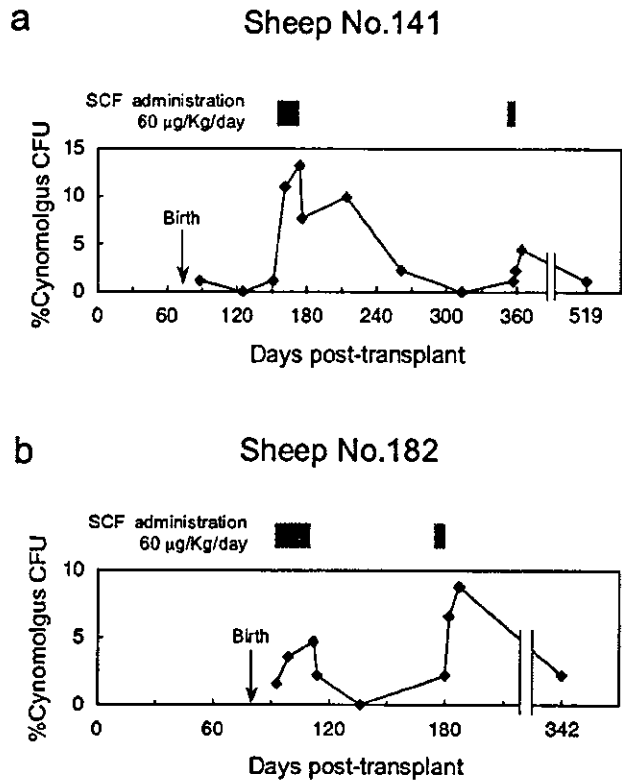


FIGURE 3. Time course of hematopoietic chimerism in the sheep receiving human SCF. (a) In sheep No. 141, human SCF was intraperitoneally administered at 60 µg/kg once a day from day 156 posttransplantation for 18 days. SCF administration was then stopped and tried again from day 352 for 5 days. (b) In sheep No. 182, human SCF was similarly administered from day 94 posttransplantation for 18 days, followed by a second administration at the same dose from day 175 for 5 days. Horizontal axis indicates days after transplantation. Vertical axis shows cynomolgus/sheep chimerism (a ratio of cynomolgus vs. sheep CFU in the bone marrow). Period of human SCF administration (gray bars).

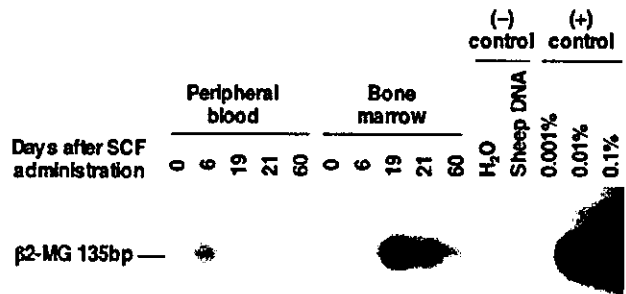


FIGURE 4. Detection of cynomolgus cells in the circulation. DNA was extracted from whole peripheral blood or bone-marrow nucleated cells after birth and subjected to cynomolgus-specific $\beta 2$ -microglobulin ($\beta 2$ -MG) PCR, and Southern blot analysis. Data before and after SCF administration shown. Positive controls show 0.001, 0.01, and 0.1% chimerism (cynomolgus to sheep). Cynomolgus cells were detectable after SCF administration, albeit at low levels (<0.1%).

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REFERENCES

- Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; 282: 1145.
- Reubinoff BE, Pera MF, Fong C, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 2000; 18: 399.
- Schuldiner M, Yanuka O, Itskovitz-Eldor J, et al. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2000; 97: 11307.
- Carpenter MK, Inokuma MS, Denham J, et al. Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* 2001; 172: 383.
- Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 2002; 109: 29.
- Bjorklund LM, Sanchez-Pernate R, Chung S, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 2002; 99: 2344.
- Asano T, Ageyama N, Takeuchi K, et al. Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. *Transplantation* 2003; 76: 1061.
- Zanjani ED, Flake AW, Rice H, et al. Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. *J Clin Invest* 1994; 93: 1051.
- Silverstein AM, Prendergast RA, Kranek KL. Fetal response to antigenic stimulus. IV. Rejection of skin homografts by the fetal lamb. *J Exp Med* 1964; 119: 955.
- Flake AW, Harrison MR, Adzick NS, et al. Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras. *Science* 1986; 233: 776.
- Liechty KW, MacKenzie TC, Shaaban AF, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat Med* 2000; 6: 1282.
- Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev Dyn* 2001; 222: 273.
- Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 1994; 265: 1098.
- Chadwick K, Wang L, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* 2003; 102: 906.
- Shibata H, Hanazono Y, Ageyama N, et al. Collection and analysis of hematopoietic progenitor cells from cynomolgus macaques (*Macaca fascicularis*): assessment of cross-reacting monoclonal antibodies. *Am J Primatol* 2003; 61: 3.
- Flake AW, Hendrick MH, Rice HE, et al. Enhancement of human hematopoiesis by mast cell growth factor in human-sheep chimeras created by the in utero transplantation of human fetal hematopoietic cells. *Exp Hematol* 1995; 23: 252.
- Zhang D, Murakami A, Johnson RP, et al. Optimization of ex vivo activation and expansion of macaque primary CD4-enriched peripheral blood mononuclear cells for use in anti-HIV immunotherapy and gene therapy strategies. *J Acquir Immune Defic Syndr* 2003; 32: 245.
- Young AJ, Holzgreve W, Dudler L, et al. Engraftment of human cord blood-derived stem cells in preimmune ovine fetuses after ultrasound-guided in utero transplantation. *Am J Obstet Gynecol* 2003; 189: 698.
- Noia G, Pierelli L, Bonanno G, et al. The intracoelomic route: a new approach for in utero human cord blood stem cell transplantation. *Fetal Diagn Ther* 2004; 19: 13.
- Berenson RJ, Bensinger WI, Hill RS, et al. Engraftment after infusion of CD34⁺ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 1991; 77: 1717.
- Zanjani ED, Almeida-Porada G, Livingston AG, et al. Reversible expression of CD34 by adult human bone marrow long-term engrafting hematopoietic stem cells. *Exp Hematol* 2003; 31: 406.
- Lu S-J, Li F, Vida L, et al. CD34⁺CD38⁻ hematopoietic precursors derived from human embryonic stem cells exhibit an embryonic gene expression pattern. *Blood* 2004; 103: 4134.
- Muller AM, Dzierzak EA. ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. *Development* 1993; 118: 1343.
- Hole N, Graham GJ, Menzel U, et al. A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonic stem cell differentiation in vitro. *Blood* 1996; 88: 1266.
- Kyba M, Perlingeiro RC, Hoover RR, et al. Enhanced hematopoietic differentiation of embryonic stem cells conditionally expressing Stat5. *Proc Natl Acad Sci U S A* 2003; 100(Suppl 1): 11904.
- Kauffman DS, Hanson ET, Lewis RL, et al. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2001; 98: 10716.
- Li F, Lu S, Vida L, et al. Bone morphogenetic protein 4 induces efficient hematopoietic differentiation of rhesus monkey embryonic stem cells in vitro. *Blood* 2001; 98: 335.
- Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* 1997; 89: 981.
- Zhang CC, Lodish HF. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood* 2004; 103: 2513.
- Anderson CC, Matzinger P. Immunity or tolerance: opposite outcomes of microchimerism from skin grafts. *Nat Med* 2001; 7: 80.
- Gleit ZL, Fuchimoto Y, Yamada K, et al. Variable relationship between chimerism and tolerance after hematopoietic cell transplantation without myelosuppressive conditioning. *Transplantation* 2002; 74: 1535.

RESEARCH ARTICLE

Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved

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Efficient gene transfer and regulated transgene expression in primate embryonic stem (ES) cells are highly desirable for future applications of the cells. In the present study, we have examined using the nonintegrating Sendai virus (SeV) vector to introduce the green fluorescent protein (GFP) gene into non-human primate cynomolgus ES cells. The GFP gene was vigorously and stably expressed in the cynomolgus ES cells for a year. The cells were able to form fluorescent teratomas when transplanted into immunodeficient mice. They were also

able to differentiate into fluorescent embryoid bodies, neurons, and mature blood cells. In addition, the GFP expression levels were reduced dose-dependently by the addition of an anti-RNA virus drug, ribavirin, to the culture. Thus, SeV vector will be a useful tool for efficient gene transfer into primate ES cells and the method of using antiviral drugs should allow further investigation for regulated SeV-mediated gene expression. Gene Therapy (2005) 12, 203–210. doi:10.1038/sj.gt.3302409 Published online 14 October 2004

Keywords: primate embryonic stem cell; Sendai virus vector; gene transfer; green fluorescent protein; pluripotency; ribavirin

Introduction

Since human embryonic stem (ES) cell lines have the ability to both proliferate indefinitely and differentiate into multiple tissue cells,^{1,2} they are expected to have clinical applications as well as to serve as models for basic research and drug development. Although efficient and stable gene transfer into primate ES cells would be useful for such purposes, it has been difficult and only lentiviral vectors have been successful in achieving it.^{3–5} We have previously developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase.⁶ SeV vectors can efficiently introduce foreign genes without toxicity into airway epithelial cells,⁷ vascular tissue,⁸ skeletal muscle,⁹ synovial cells,¹⁰ retinal tissue,¹¹ and hematopoietic progenitor cells.¹² Here we report that the SeV-mediated gene transfer into primate ES cells is very efficient and stable even after the terminal differentiation of the cells. In addition, we show that SeV-mediated transgene expression levels can be reduced by the addition of a ribonucleoside analog, ribavirin, to the culture. Ribavirin is a mutagen and inhibitor of viral RNA polymerase.^{13,14} It shows antiviral activity against a variety of RNA viruses and is used to treat infections of hepatitis C virus in combination with interferon- α ^{15,16} and of lassa

fever virus.¹⁷ The method of using antiviral drugs might offer a novel approach for regulated SeV-mediated gene expression in primate ES cells.

Results

SeV-mediated gene transfer into ES cells

In this study, we have used an SeV vector, which is capable of self-replication but incapable of transmitting to other cells.⁶ The vector does not encode the fusion (F) protein (Figure 1a), which is essential for viral entry into cells. It can be propagated only in a packaging cell line expressing the F protein. The green fluorescent protein (GFP) gene was introduced after the leader sequence of the vector genome. Cynomolgus ES cells¹⁸ were exposed to the SeV vector for 24 h. Flow cytometric analysis at 2 days after infection showed that 15, 38, and 61% of cells fluoresced at 2, 10, and 50 transducing units (TU) per cell, respectively (Figure 1b). The gene transfer efficiency of about 60% is comparable to or even better than that for lentiviral vectors.³ We confirmed that the undifferentiated cell fractions remained unchanged after the infection with SeV vector, as assessed by the expression of undifferentiated markers, alkaline phosphatase and SSEA-4 (data not shown). The GFP expression after infection was stable at least for a month. On the other hand, the GFP gene transfer to cynomolgus ES cells with adenovirus- and adeno-associated virus (AAV)-based vectors resulted in much lower expression levels (<20% by flow cytometry) and the levels declined to zero within a week after infection (Figure 1c).

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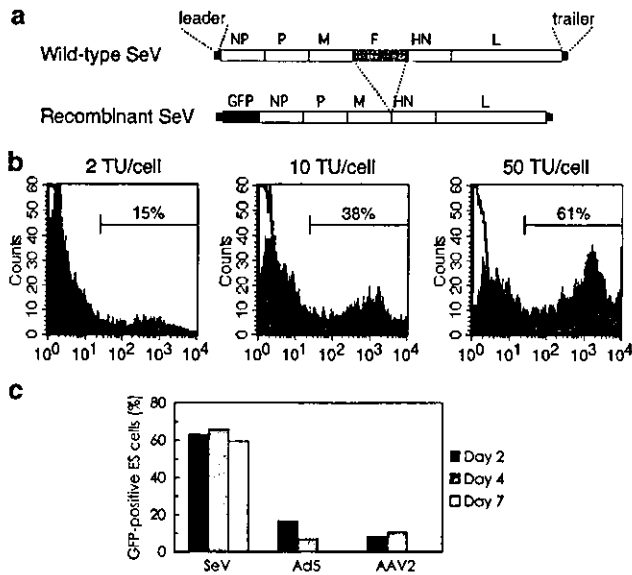


Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, and L) are in order from 3' to 5' in the negative-strand RNA. In the SeV vector, the entire fusion (F) gene was removed and the GFP gene was introduced at a unique NotI site between the leader sequence and NP gene. (b) The GFP expression by the SeV vector in cynomolgus ES cells. Cynomolgus ES cells were infected with the SeV vector at 2, 10, and 50 TU/cell. The flow cytometric profiles at day-2 postinfection are shown in gray. The white areas indicate uninfected ES cells. The fractions of GFP-positive cells are indicated. (c) The GFP expression levels in cynomolgus ES cells infected with the SeV (50 TU/cell), adenovirus serotype 5 (Ad5, 3.4×10^2 g.c./cell), and AAV serotype 2 (AAV2, 2.4×10^4 g.c./cell) vectors. The fractions of GFP-positive cells were examined by flow cytometry at 2, 4, and 7 days postinfection.

We plucked fluorescent ES cell colonies under a fluorescent microscope once at 1 month after infection and propagated them. After this selection procedure, approximately 90% of the ES cells expressed GFP (Figure 2a and b) and the high-level expression was stable for a year as assessed by flow cytometry (Figure 2c, upper). The mean fluorescence intensity per cell was also stable (Figure 2c, lower), indicating that the replicating vector genome was almost equally delivered to each cell of all progeny. The self-replication of the SeV vector in infected cells was confirmed by RNA-PCR that amplified the viral RNA genomic sequence (Figure 3a). The GFP cDNA sequence, however, could not be detected by DNA-PCR in the infected cells (Figure 3b), indicating that no DNA phase was involved in the GFP expression.

Pluripotency of infected ES cells

The SeV-infected, fluorescent cynomolgus ES cells were able to form fluorescent tumors when transplanted into immunodeficient mice (Figure 4a–c). The fluorescence was observed uniformly by fluorescent microscopy (Figure 4d and e). The tumors consisted of all three embryonic germ layer cells (Figure 4f–i). Thus, the SeV-infected ES cells were capable of forming teratomas and the SeV infection did not spoil the pluripo-

tency of ES cells. The infected, fluorescent cynomolgus ES cells were also able to generate fluorescent embryoid bodies (Figure 5a and b), MAP-2-positive neurons (Figure 5c), clonogenic hematopoietic colonies (Figure 5d and e), and mature functional (NBT test-positive) neutrophils (Figure 5f and g), all of which fluoresced. In addition, the GFP expression levels were not decreased during the teratoma formation or differentiation, indicating that no ‘silencing’ of the transgene occurred.

Drug-inducible reduction of transgene expression

Next, we examined whether ribavirin inhibits the replication and transcription of the SeV vector resulting in a reduction of transgene expression. We first used a rhesus monkey kidney cell line (LLC-MK2) to test the effect of ribavirin on the replication and transcription of the SeV vector. LLC-MK2 is a standard control cell line for SeV infection. Ribavirin was added at various concentrations 2 days after the infection. The formation of viral particles quantified by the hemagglutination assay decreased drastically upon the addition of ribavirin (Figure 6a). The decrease was dependent on the dose of ribavirin. The GFP expression was also depressed dose-dependently (Figure 6b). Thus, ribavirin dose-dependently inhibits the replication and transcription of the SeV vector in LLC-MK2 cells. The toxicity associated with ribavirin was not observed in LLC-MK2 cells.

We then examined the effect of ribavirin on SeV-infected, fluorescent cynomolgus ES cells. The addition of ribavirin also resulted in a dose-dependent reduction of GFP expression in the cells (Figure 6c). Although the GFP expression was almost completely inhibited after a 3-day exposure with 4 mM of ribavirin, the cells could not be propagated thereafter. Ribavirin at high concentrations (>1 mM) hampered the proliferation of cynomolgus ES cells. With lower concentrations (0.5–0.75 mM) of ribavirin, the GFP expression level decreased by half. After the discontinuation of ribavirin treatment, the cells could be propagated and nearly regained the original level of GFP expression. The undifferentiated cell fractions were unchanged after the discontinuation as assessed by alkaline phosphatase and SSEA-4 staining (Figure 6d).

Discussion

There are several advantages in using SeV vectors over other vectors. (i) SeV vectors can infect nondividing, quiescent cells as well as dividing cells unlike oncoretroviral vectors.^{7–11} Thus, they can be used to infect cells that are terminally differentiated as well as at various stages of differentiation, whether they are dividing or not. (ii) SeV vector-mediated gene transfer does not require a DNA phase. Thus, there is no concern about the unwanted integration of foreign sequences into the host genome unlike with oncoretroviral or lentiviral vectors. (iii) Transgene expression is stable even in dividing cells since the SeV vector replicates by itself in the cytoplasm of host cells. On the other hand, gene transfer using nonreplicating adenoviral and AAV vectors resulted in decreased levels of transgene expression in dividing cells over time, since the non-replicating transgene was

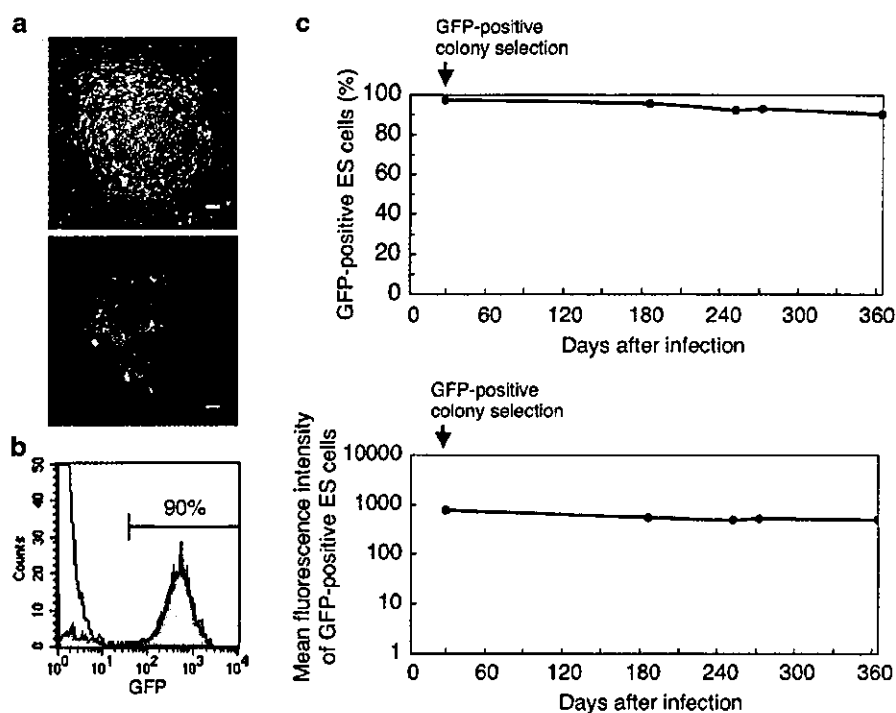


Figure 2 Stable SeV-mediated transgene expression in cynomolgus ES cells. Fluorescent ES cell colonies were plucked under a fluorescent microscope once at 1 month after infection and the cells were further propagated. (a) Phase-contrast (upper) and fluorescence (lower) images of a cynomolgus ES cell colony at day 370 after infection. Bar = 100 μ m. (b) Flow cytometric analysis of SeV-infected cynomolgus ES cells at day 370 after infection (shown in green). The percentage of GFP-positive cells is indicated. Uninfected, parental cynomolgus ES cells are indicated by another line (white area). (c) The percentage of GFP-positive cells (upper) and mean fluorescence intensity per GFP-positive cell (lower) after infection with the SeV vector at 10 TU/cell are shown as a function of time (days).

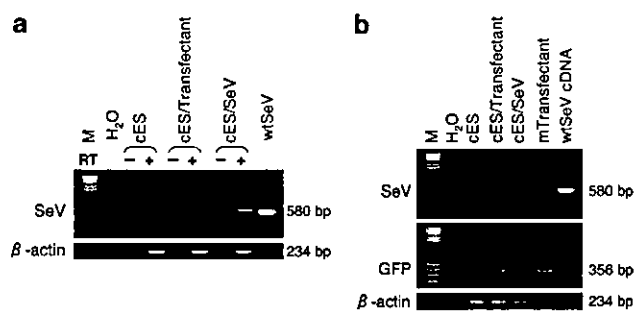


Figure 3 DNA-independent replication and transcription of SeV vector. Total cellular RNA and DNA were extracted from cynomolgus ES cells at day 284 after infection with the SeV vector. RNA-PCR (a) and DNA-PCR (b) for the SeV RNA genome or GFP sequence were conducted. The cynomolgus β -actin sequence was used as an internal control. In the RNA-PCR (a), negative results obtained without reverse transcriptase (designated RT-) confirmed that the amplified products were not derived from cellular DNA. M, 100-kb DNA ladder; cES, naive cynomolgus ES cells; cES/Transfectant, cynomolgus ES cells stably expressing the GFP gene after transfection;³³ cES/SeV, cynomolgus ES cells infected with the SeV vector; wtSeV, wild-type SeV genome; mTransfectant, a GFP-positive mouse cell line after transfection.

diluted out. (iv) The SeV vector is much less unlikely to generate wild-type virus *in vitro* or *in vivo* than oncoretroviral and lentiviral vectors, since homologous recombination between RNA genomes is very rare indeed in negative-strand RNA viruses.¹⁹ (v) The SeV genome is not subject to cellular epigenetic modifications

such as methylation, and thus it is unlikely that methylation-based silencing of transgene expression occurs.

No cytotoxic or differentiating effect on ES cells associated with the SeV infection was observed in our study. However, the wild-type SeV contains immunogenic surface proteins, hemagglutinin-neuraminidase (HN) and F proteins, which potentially induce antibody responses.^{20,21} For future clinical applications, it would be desired that as many viral genes as possible are deleted from the vector backbone to permit reapplication, improve the safety, and lessen the possible toxicity of SeV vectors. To this end, we have developed a series of attenuated SeV vectors that are F gene-deleted,⁶ F gene-deleted with preferable mutations,²² M gene-deleted,²³ or have deletions of both F and M genes.²⁴ The modified vectors would be safer for *in vivo* use.

Ribavirin at high concentrations seems toxic to ES cells; presumably, it directly hampers viability and proliferation potential of ES cells. However, we cannot tell whether the observed toxicity is simply due to its toxicity to ES cells, as feeder cells are more highly sensitive to ribavirin than ES cells. In fact, while feeder cells died at 1 mM of ribavirin, cocultured ES cells were alive at this concentration for some time. Cynomolgus ES cells lose pluripotency and proliferation potential without feeder cells. Thus, the observed toxicity to ES cells may also be a secondary event following the injury of feeder cells. Whether the cytotoxicity is primary or secondary, it will be necessary to find modified compounds of less cytotoxicity.

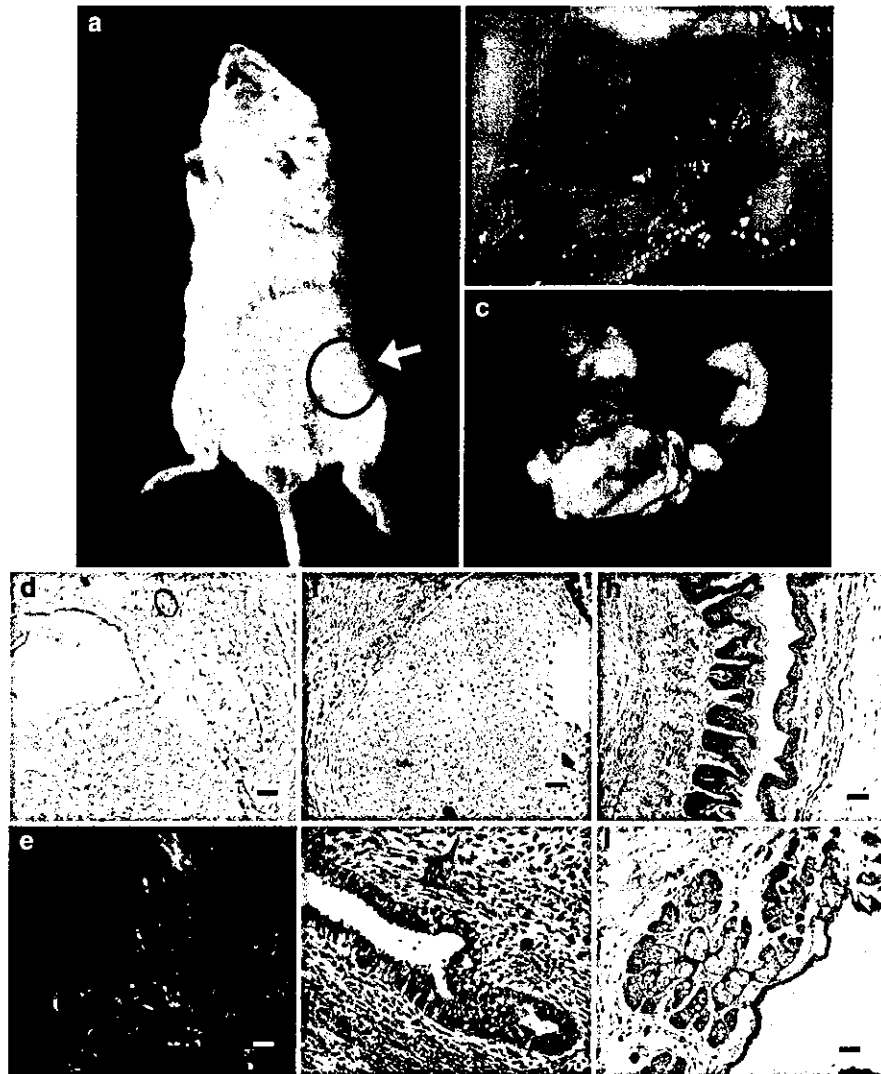


Figure 4 Pluripotency of SeV-infected cynomolgus ES cells. Tumors formed in NOD-SCID mice after inoculation of the SeV-infected cynomolgus ES cells (a). The tumor was fluorescing ((b), bright field; (c), dark field). Fluorescence was observed uniformly in the tumor under a fluorescent microscope ((d), bright field; (e), dark field). The tumor contained all three embryonic germ layer cells; cartilage (f), ciliated columnar epithelium (g), skin (h), and sebaceous gland (i) (stained with hematoxylin and eosin). Bar = 100 μ m.

Materials and methods

Cell culture

Cynomolgus ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c) embryonic fibroblasts as described previously.¹⁸ The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 15% ES cell-qualified fetal calf serum (FCS; Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin, Irvine Scientific, Santa Ana, CA, USA). The ES cell colonies were routinely passaged every 3–4 days after dissociation with a combined approach of 0.25% trypsin (Invitrogen) digestion and mechanical cutting. Alkaline phosphatase staining was conducted with an Alkaline Phosphatase Chromogen Kit

(Biomedex, Foster City, CA, USA). Embryoid bodies were produced by culturing ES cell aggregates in Petri dishes. LLC-MK2 cells (1×10^6) were grown in six-well plates and cultured in Eagle's minimal essential medium (Invitrogen) supplemented with 10% FCS.

Vectors

The F-defective SeV vector carrying the GFP gene was constructed as previously described.⁶ The vector titer was 1.8×10^9 TU/ml determined by counting fluorescent cells after the infection of LLC-MK2 cells. Gene transfer was conducted by adding various concentrations of the SeV vector solution to culture media. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added. In some experiments, ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Sigma) was added at various concentrations to the culture media after infection. The

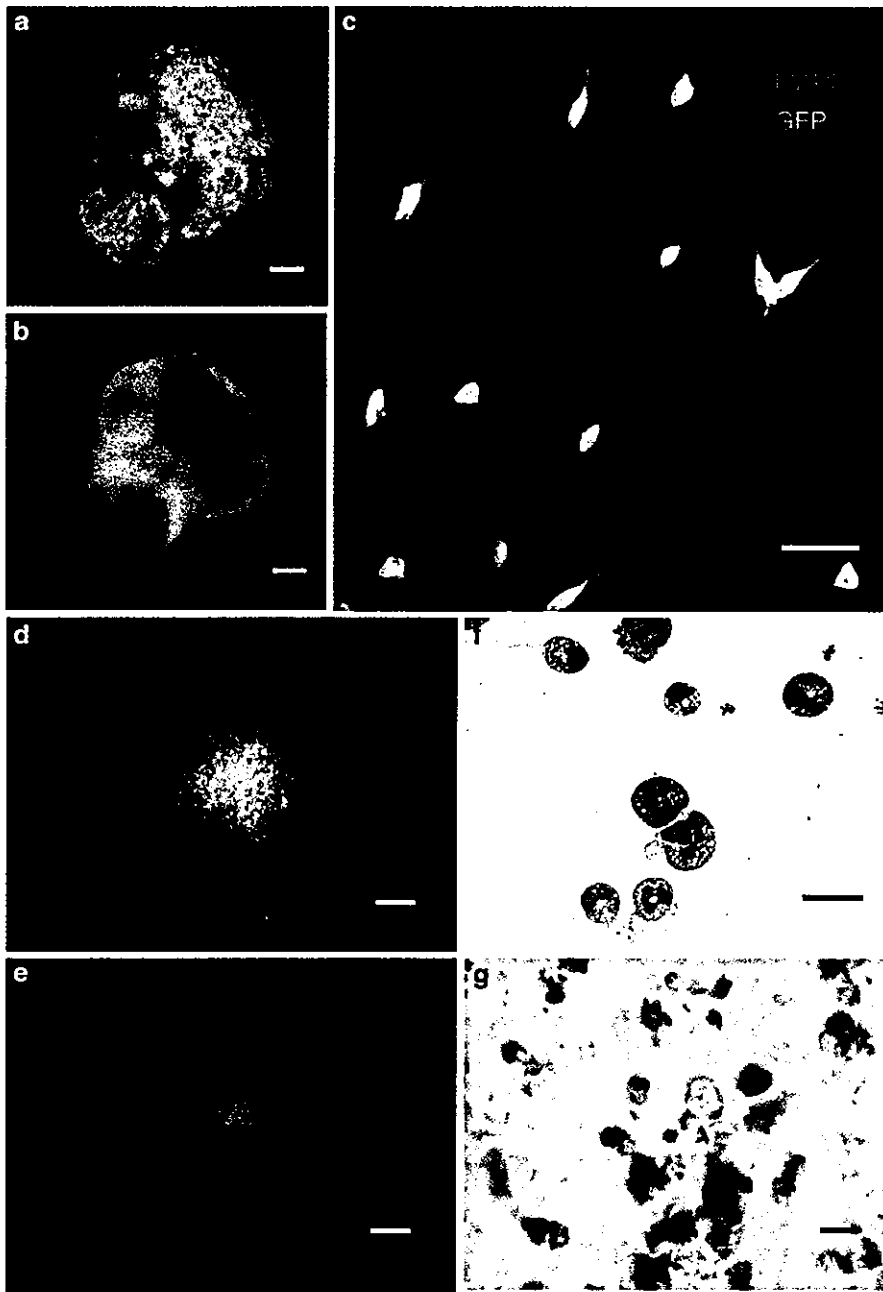


Figure 5 Stable transgene expression during differentiation. A day-20 cystic embryoid body was observed under a fluorescent phase-contrast microscope, confirming that the embryoid body was fluorescing ((a), bright field; (b), dark field). After infection with the SeV vector, fluorescent cynomolgus ES cells differentiated into neural cells. Double immunostaining with anti-GFP (green) and anti-MAP-2 (red) confirmed that differentiated neural cells expressed GFP (c). Yellow cells indicate GFP-expressing neurons. SeV-infected, fluorescent cynomolgus ES cells also differentiated into fluorescent hematopoietic cells. A clonogenic hematopoietic colony was fluorescing ((d) bright field; (e), dark field). A cytospin specimen of hematopoietic colony cells (Wright-Giemsa staining) showed that the cells were mature granulocytes (f). The infected ES cell-derived, fluorescent neutrophils were positive for NBT (stained in black (g)). Bar = 100 μ m (a, b, g); 50 μ m (c, f); 500 μ m (d, e).

viral particles in infected cells were quantified by a hemagglutination assay as described previously.²⁵

An adenovirus serotype 5-based vector carrying the GFP gene was constructed as reported.²⁶ It contained the cytomegalovirus (CMV) promoter, simian virus (SV)-40 intron, and SV-40 polyadenylation signal. An AAV serotype 2-based vector expressing the GFP gene under the control of the chicken β -actin promoter with the CMV immediate-early enhancer (a gift from Dr J Miyazaki)

was prepared as described previously.²⁷ Gene transfer experiments were performed using 3.4×10^2 genome copies (g.c.)/cell of the adenoviral vector or 2.4×10^4 g.c./cell of the AAV vector. The period of exposure was 48 h.

Flow cytometry

GFP and SSEA-4 expression was analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) using the

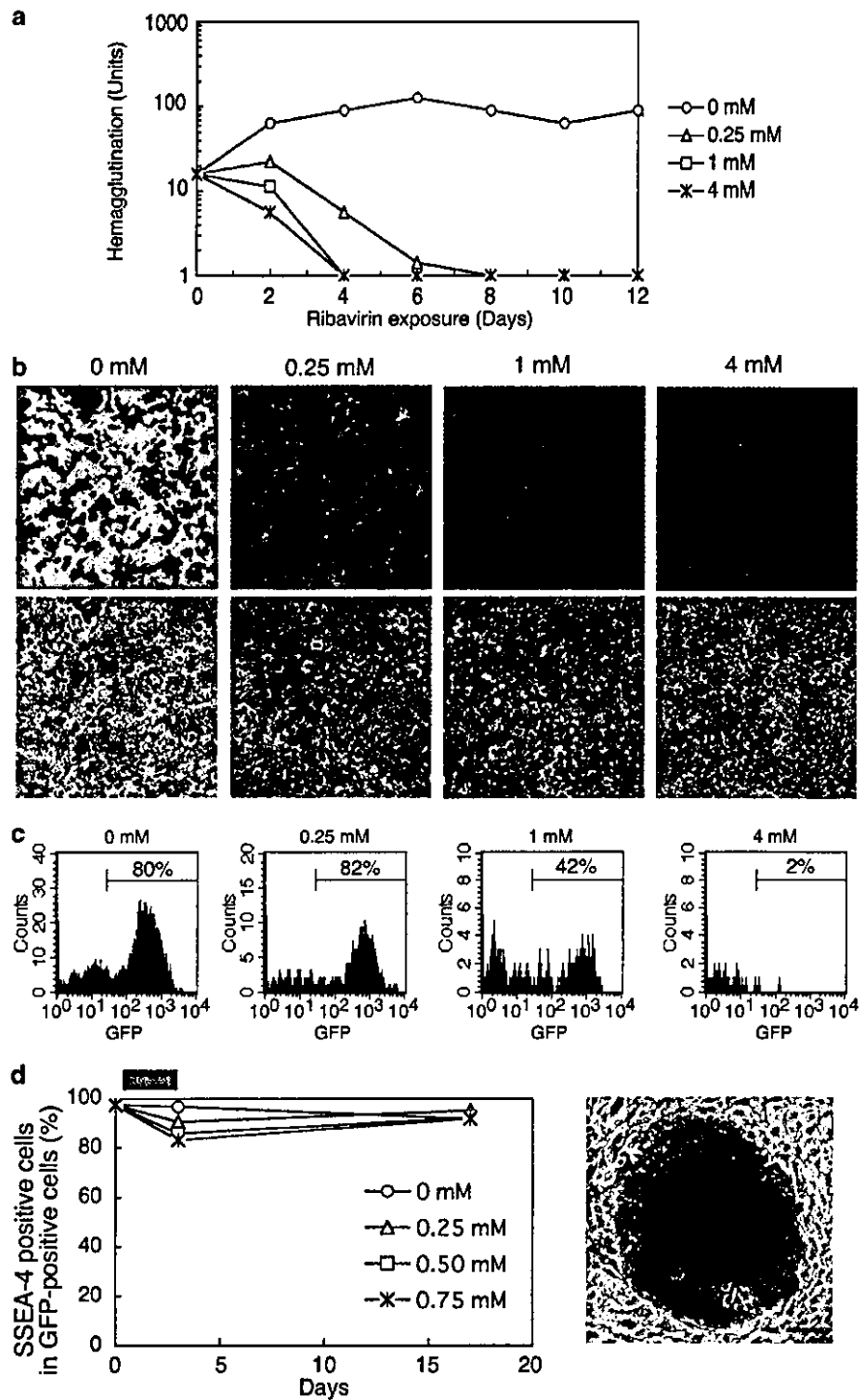


Figure 6 Ribavirin-regulated transgene expression. (a) A rhesus kidney cell line (LLC-MK2) was infected with the SeV vector at 3 TU/cell. Ribavirin was started at various concentrations on day 2 after the infection. The formation of viral particles in the infected LLC-MK2 cells was examined by the hemagglutination assay. (b) The ribavirin-treated LLC-MK2 cells were observed under a fluorescent microscope after an 8-day exposure of ribavirin (upper, dark field; lower, bright field). (c) Ribavirin was added at various concentrations to the SeV-infected, fluorescent cynomolgus ES cells. The GFP expression was assessed by flow cytometry after a 3-day exposure of ribavirin. (d) The fractions of SSEA-4-positive ES cells were assessed by flow cytometry with anti-SSEA-4 before and after a 3-day exposure of ribavirin and are shown as a function of time (days) in the left panel. A gray bar indicates ribavirin treatment. ES cells were stained for alkaline phosphatase (in red) at day 21 after a 3-day exposure of 0.75 mM ribavirin and are shown in the right panel. Bar = 100 μ m.

CellQuest software (Becton Dickinson). For SSEA-4 staining, cells were incubated with a primary antibody, anti-SSEA-4 (MC-813-70; Chemicon, Temecula, CA, USA), and then a secondary antibody, PE-conjugated

F(ab')₂ fragment of rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark). Cocultured BALB/c feeder cells could be distinguished from cynomolgus ES cells by using PE-conjugated anti-mouse